

Genotype-specific fluorogenic RT-PCR assays for the detection and quantitation of canine coronavirus type I and type II RNA in faecal samples of dogs

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Abstract

Two genotype-specific fluorogenic RT-PCR assays were developed for the detection and quantitation of canine coronavirus (CCoV) type I and type II RNA in the faeces of dogs with diarrhoea. Both the fluorogenic assays showed high specificity, sensitivity and reproducibility, allowing a precise quantitation of CCoV type I and type II RNA over a linear range of about eight orders of magnitude (from 10^1 to 10^8 copies of standard RNA). Comparison with genotype-specific gel-based RT-PCR assays revealed that the fluorogenic assays were more sensitive and more rapid than conventional amplifications, with a large increase in throughput. The genotype-specific fluorogenic assays were then used to detect and measure viral loads in the faecal samples collected from dogs naturally or experimentally infected with type I, type II, or both genotypes. Of 174 samples collected from naturally infected dogs, 77 were positive for CCoV type I and 46 for CCoV type II. Thirty-eight dogs were found to be infected naturally by both genotypes, with viral RNA titres generally higher for type I in comparison to type II. At the same time, dogs infected experimentally shed type I RNA with higher titres with respect to type II.

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1. Introduction

Canine coronavirus (CCoV) is an enveloped, single-stranded RNA virus, belonging to group I of the family *Coronaviridae*, together with transmissible gastroenteritis virus (TGEV) of swine, porcine respiratory coronavirus (PRCoV), porcine epidemic diarrhoea virus (PEDV), human coronavirus 229E (HCoV 229E), and feline coronaviruses (FCoVs). The CCoV genome is composed for about two-thirds of 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 spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins encoded by ORF2, ORF4, ORF5 and ORF6, respectively (Luytjes, 1995; Enjuanes et al., 2000).

CCoV replicates primarily in enterocytes on the villus tips of the small intestine and also in the epithelium of the large intestine of dogs, causing mild to severe enteritis; long-term viral shedding, up to several months, has been observed in the faeces of infected pups (Pratelli et al., 2001a, 2002c).

Since the virus is difficult to isolate in cell cultures (Tennant et al., 1994; Pratelli et al., 2000), a nested PCR assay, targeting the M gene, was developed for the diagnosis of CCoV infection, with high sensitivity and specificity (Pratelli et al., 1999). FCoV RNA is quantifiable by means of a fluorogenic RT-PCR assay (Gut et al., 1999). Analogously,

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a TaqMan RT-PCR assay for the specific detection and quantitation of CCoV RNA in the faeces of infected dogs has been established. This method is highly sensitive and reproducible, allowing several samples to be processed in a few hours, with a large increase in throughput. By using the established fluorogenic RT-PCR assay, it was possible to trace the course of natural and experimental infections in dogs (Decaro et al., 2004).

Recently, two CCoV genotypes have been identified in the faeces of pups with enteritis, which have been referred to as CCoV type I and type II (Pratelli et al., 2003b, 2004). Type I and type II are distinguishable by means of molecular methods, which are able to amplify selectively fragments of the ORF2 (S gene) and ORF5 (M gene) (Pratelli et al., 2002a, 2004), but no quantitative techniques have been developed which could differentiate the two genotypes. In addition, the established fluorogenic assay was able to detect both genotypes, although it tended to underestimate the viral load in dogs infected with type I, probably due to the presence of mismatches in the binding region of primers and probe (unpublished results). The simultaneous detection of both type I and type II in the faeces of pups with diarrhoea (Pratelli et al., 2004) has highlighted the need to establish genotype-specific fluorogenic RT-PCR assays, in order to obtain an exact quantitation of the two genotypes present in the same samples. Such a method would be helpful for understanding the pathogenetic mechanisms of the newly recognized CCoV type I, since this virus has not yet been adapted to grow in vitro (Pratelli et al., 2003b, 2004).

In the present study, the development of genotype-specific TaqMan RT-PCR assays is described for the rapid screening of faecal samples from diarrhoeic dogs and for the quantitation of CCoV type I and type II RNA. The assays established were used to determine the type I and type II RNA amounts in the faeces of dogs and to follow the course of infection in dogs infected experimentally with CCoV type I, type II or both genotypes.

2. Materials and methods

2.1. Samples

A total of 174 faecal samples, collected from pups with diarrhoea in different geographical areas of Italy, were processed in order to detect and quantify CCoV type I and type II RNA.

In addition, three mix-bred dogs, 2 months of age, negative for CCoV RNA in the faeces by RT-PCR (Pratelli et al., 1999) and for specific antibodies by Elisa (Pratelli et al., 2002b), were infected experimentally with CCoV type I (one dog), type II (one dog) or both genotypes (one dog) by the administration of 5 ml (2.5 ml intranasally and 2.5 ml orally) of different faecal homogenates (10%, w/v) containing about 10^7 RNA copies/ml of type I, type II or both genotypes (Pratelli et al., 2004). Faecal samples of the dogs infected

experimentally with CCoV type I or type II were collected daily for 28 days, whereas the dog experimentally infected with both genotypes was sampled daily for 90 days. Totally, 146 faecal samples were collected from the three experimentally infected dogs.

The specimens from all the infected dogs were subjected both to genotype-specific conventional RT-PCR and real-time analysis.

2.2. RNA extraction

RNA was extracted from 10 mg of faeces with the QIAamp[®] RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol, eluted in 50 μ l of nuclease-free water and stored at -70°C until its use. One microliter per reaction was used as template.

2.3. Standard RNA preparation

In vitro-transcribed ORF5 genes of strains 259/03 (Pratelli et al., 2003a) and 45/93 (Buonavoglia et al., 1994) were used as standard RNAs for the CCoV type I- and type II-specific fluorogenic assays, respectively. The PCR-amplified gene of each strain 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, as previously described (Decaro et al., 2004). Transcripts were quantified by spectrophotometer and subjected to 10-fold dilutions, containing from 10^8 to 10^0 molecules/ μ l. Each dilution of type I and type II standard RNAs, made in TE (Tris-HCl, EDTA, pH 8.0) buffer containing 30 μ g carrier RNA (tRNA from *Escherichia coli*, Sigma-Aldrich Srl, Milan, Italy) per ml, was frozen at -70°C and used once only.

2.4. Hydrolysis probe and primer sets

The ORF5 nucleotide sequences of several strains of CCoV type I and type II (Pratelli et al., 2003a) were aligned using the BioEdit software package (<http://www.mbio.ncsu.edu/RnaseP/info/programs/BIOEDIT/bioedit.html>). Assay target region was first identified by visual inspection of sequence alignment, and then exact primer and probe sequences, specific for type I or type II, were selected by using a primer design software (Beacon Designer, Bio-Rad Laboratories Srl). The probe specific for type I and that specific for type II presented at the 3' end the nucleotide triplet ACA or TGT, which is unique to each genotype (Pratelli et al., 2001b, 2002c, 2003a), leading us to predict a correct differentiation between the two genotypes. Both probes were labelled with 6-carboxyfluorescein (FAM) at the 5'-end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. Oligonucleotide synthesis was made by Applera Italia (Applied Biosystems, Monza, Italy). The position and sequence of the primers and probe used for TaqMan RT-PCR amplification are reported in Table 1.

Table 1
Genotype-specific oligonucleotides used in CCoV fluorogenic and conventional RT-PCR assays

| Primer/probe | Sequence 5' to 3' | Sense | Position | Amplicon size (bp) | Specificity |
|------------------------|-------------------------------|-------|------------------------|--------------------|--------------|
| CCoV1a ^a | GTGCTTCCTCTTGAAGGTACA | + | 502–522 ^c | 239 | CCoV type I |
| CCoV2 ^a | TCTGTTGAGTAATCACCAGCT | – | 720–740 ^c | | |
| CCoVI-F ^b | CGTTAGTGCACCTTGAAGAAGCT | + | 478–499 ^c | 111 | |
| CCoVI-R ^b | ACCAGCCATTTTAAATCCTTCA | – | 567–588 ^c | | |
| CCoVI-Pb ^b | FAM-CCTCTTGAAGGTACACCAA-TAMRA | + | 508–526 ^c | | |
| Can1F ^a | TAACATTGCTCTCAGGGAATTTG | + | 6937–6959 ^d | 202 | CCoV type II |
| CCoV2 ^a | TCTGTTGAGTAATCACCAGCT | – | 7118–7138 ^d | | |
| CCoVII-F ^b | TAGTGCATTAGGAAGAAGCT | + | 6878–6897 ^d | 105 | |
| CCoVII-R ^b | AGCAATTTTGAACCCTTC | – | 6966–6982 ^d | | |
| CCoVII-Pb ^b | FAM-CCTCTTGAAGGTGTGCC-TAMRA | + | 6906–6922 ^d | | |

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

^a Conventional RT-PCR (Pratelli et al., 2002a, 2004).

^b Fluorogenic RT-PCR.

^c Oligonucleotide position is referred to the sequence of CCoV type I strain 259/01 (accession: AF502583).

^d Oligonucleotide position is referred to the sequence of CCoV type II strain Insavc-1 (accession: D13096).

2.5. Reverse transcription

Triplicates of the standard dilutions and RNA templates were reverse transcribed in a reaction volume of 20 µl containing 1 µl of RNA, PCR buffer 1× (KCl 50 mM, Tris-HCl 10 mM, pH 8.3), MgCl₂ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), random hexamers 2.5 U, RNase inhibitor 1 U, MuLV reverse transcriptase 2.5 U (Applied Biosystems). Synthesis of c-DNA was carried out at 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min.

2.6. Fluorogenic RT-PCR assays

The fluorogenic genotype-specific RT-PCR assays were undertaken in an i-Cycler iQTM Real-Time Detection System (Bio-Rad Laboratories Srl, Milan, Italy) and the data were analyzed with the appropriate sequence detector software (version 3.0). The 50 µl-reaction mixture for both genotype-specific assays contained 25 µl of iQTM Supermix (Bio-Rad Laboratories Srl), 600 nM of each primer, 200 nM of probe and 20 µl of c-DNA. The thermal profile consisted of activation of iTaq DNA polymerase at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 53 °C (type I-specific assay) or 48 °C (type II-specific assay) for 30 s and extension at 60 °C for 1 min.

2.7. Conventional gel-based RT-PCR assays

Genotype-specific amplifications were carried out using primer pairs targeting the M gene of CCoV type I and type II, as described previously (Pratelli et al., 2002a, 2004). The position and sequence of the primers used for the conventional RT-PCR assays are reported in Table 1.

Briefly, RT-PCR amplification was carried out using GeneAmp[®] RNA PCR (Applied Biosystems, Applied 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 RT-PCR products were detected by electrophoresis in 1.5% agarose gel and visualisation under UV light.

2.8. Internal control

In order to verify the absence of RNA losses during the extraction step and of PCR inhibitors in the RNA templates, an internal control (IC) was added to all the faecal samples prior to RNA extraction. The IC consisted of 5000 copies/ml of faecal suspension of RNA transcripts from plasmid pM-TOPO, used as standard RNA in a real-time RT-PCR assay for the detection of avian influenza virus (Bedini et al., 2004). The fixed amount of the IC added to each sample had been calculated to give a mean C_T value in the real-time RT-PCR assay of 35.33 with a S.D. of 0.79, as calculated by 30 separate runs. Real-time RT-PCR for IC detection was carried out in a separate run, using primers Flu-M32for (CTTCTAACCGAGGTC-GAAACGTA) and Flu-M179rev (GGATTGGTCTTGTCTT-TAGCCA) and minor groove binder probe 74F-matrix (FAM-CTCGGCTTTGAGGGGGCCTGA-MGB). Samples in which the C_T value for the IC was >36.91 (average plus 2 S.D.) were excluded from analysis.

3. Results

3.1. Analytical specificity, sensitivity and reproducibility of the CCoV genotype-specific fluorogenic assays

To verify that the genotype-specific fluorogenic assays can be used to differentiate and quantify the two CCoV genotypes, several strains were selected and tested, which had been previously characterized as CCoV type I or type II by conventional genotype-specific RT-PCR assays: six CCoV II

culture-adapted strains (Insavc-1, S378, K378, SE, 257/98, 144/01), five faecal samples positive for only type II, 11 faecal samples positive for only type I, and each dilution of the type I and type II standard RNAs. In addition, RNA preparations from various coronaviruses of group I, including TGEV, FCoV type I, FCoV type II, or sterile water were tested by conventional and fluorogenic amplifications. It was demonstrated that both conventional and fluorogenic assays were highly specific for the detection and differentiation of type I and type II CCoV. No amplification was obtained from most of the other coronaviruses of group I; however, TGEV was amplified successfully by the type II-specific assays (conventional and fluorogenic), according to the absence of significant mismatches encountered in the targeted region (data not shown).

To determine the detection limit of the genotype-specific conventional and fluorogenic assays, 10-fold dilutions of the type I and type II standard RNAs, ranging from 10^8 to 10^0 molecules, were tested by both methods. Each standard dilution was tested three times separately. The detection limits of the fluorogenic assays were 1–2 logs higher than those of conventional gel-based RT-PCR assays, since both TaqMan assays were able to detect even lower amounts of viral RNA (10^1 copies) compared to the conventional amplifications (10^3 copies for type I and 10^2 – 10^3 copies for type II).

The standard curves of the two genotype-specific fluorogenic RT-PCR assays span eight orders of magnitude and show linearity over the entire quantitation ranges, with slope values of -3.444 for the type I assay and of -3.378 for the type II assay. The coefficients of linear regression (R^2) were equal to 0.9991 and 0.9995 for type I and type II assay, respectively.

In order to mimic the natural conditions, standard RNAs were also spiked in CCoV-negative faeces prior to RNA extraction and real-time analysis. The performance of the fluorogenic assays was found not dissimilar to those obtained by diluting the standard RNA in TE, thus showing that no remarkable RNA losses occurred during nucleic acid extraction (data not shown).

To assess the reproducibility of the two fluorogenic methods, the interassay and intra-assay coefficient of variations (CVs) were calculated by testing in 10 consecutive runs (CV interassay) or 10 times in the same run (CV intra-assay) faecal samples containing different amounts of CCoV type I- or type II-RNA. For both the assays, satisfactory CVs between runs and within run were obtained. For the type I assay, the intra-assay CVs ranged from 4.95% (sample containing 9.58×10^4 RNA copies) to 31.88% (3.09×10^2 RNA copies), whereas the interassay CVs were comprised between 31.72% (1.74×10^3 RNA copies) and 47.04% (2.87×10^2 RNA copies). For the type II assay, the intra-assay and interassay CVs ranged from 12.47% (1.01×10^3 RNA copies) to 42.47% (3.32×10^1 RNA copies) and from 22.33% (2.83×10^6 RNA copies) to 53.95% (1.14×10^1 RNA copies), respectively.

3.2. Internal control detection

The IC was detected in all the examined samples, with C_T values below the threshold value of 36.91. Therefore, significant RNA losses and DNA polymerase inhibition did not occur during nucleic acid extraction and PCR amplification, respectively.

3.3. Analysis of faecal samples of dogs infected naturally with CCoV type I, type II or both genotypes

The faeces of 174 dogs with diarrhoea, collected during the year 2002–2003, were submitted to genotype-specific conventional and fluorogenic RT-PCR assays. By using conventional gel-based amplification, 72 samples were found to be positive for type I, and 34 samples for type II. In 27 samples both genotypes were detected. By using TaqMan amplification, 77 samples resulted positive for type I, and 46 samples for type II with a simultaneous detection of both genotypes in 38 samples. Totally, 169 samples were in accordance to conventional and fluorogenic type I amplifications (72 positive and 97 negative), whereas 162 samples were in agreement by conventional and fluorogenic type II amplifications (34 positive and 128 negative). No sample, which was found CCoV positive by conventional RT-PCR was found to be negative by real-time analysis. Conversely, the conventional RT-PCR assays were not able to amplify five samples positive to type I and 12 samples positive to type II, which were amplified successfully by the fluorogenic assays (Fig. 1). Sequence analysis of the fluorogenic RT-PCR products confirmed the

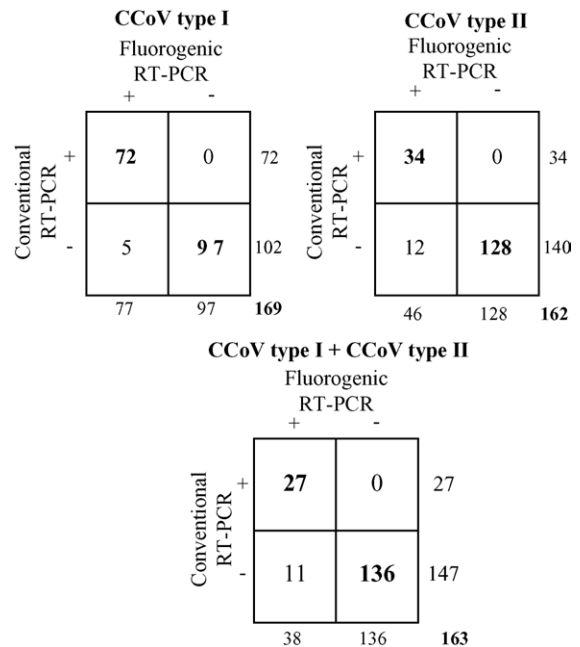


Fig. 1. Detection of CCoV type I and type II RNA in faecal samples of dogs infected naturally by conventional and fluorogenic genotype-specific RT-PCR assays. Numbers indicate the samples positive (+) or negative (-) for CCoV. Results according to both techniques are shown in bold.

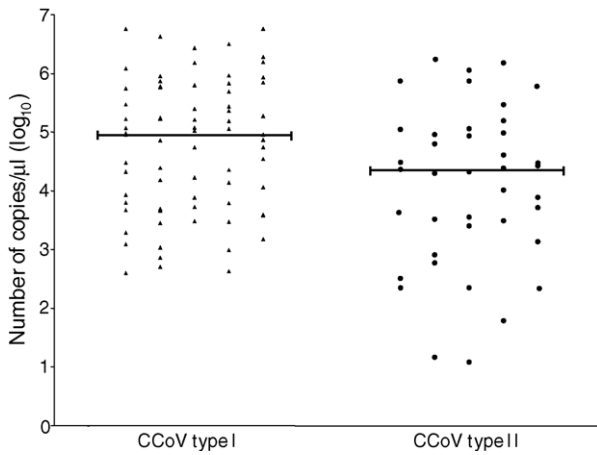


Fig. 2. Logarithmic distribution of the RNA amounts of CCoV type I and type II in the faeces of dogs infected naturally. Values are expressed as RNA copy numbers per μl of template. Each dot represents a faecal sample; bars indicate the median values.

specificity of the amplifications (data not shown). However, two samples which gave a positive signal in the type I fluorogenic assay and six samples detected by the type II fluorogenic assay were not quantifiable since they contained RNA amounts below the detection limit of their respective assays.

Quantitation of viral RNA showed that faecal samples contained wide ranges of CCoV loads, from 3.93×10^2 to 5.80×10^6 molecules/ μl of template for type I (median value = 9.14×10^4) and from 1.15×10^1 to 1.73×10^6 molecules/ μl of template for type II (median value = 2.24×10^4). Indeed, the logarithmic distribution of CCoV RNA amounts revealed that the type I titres were generally higher than those of type II (Fig. 2).

3.4. Analysis of faecal samples of dogs infected experimentally with CCoV type I, type II or both genotypes

The results of the analysis of the faecal samples collected from the dogs infected experimentally with CCoV type I, type II or both genotypes are reported in Fig. 3. The dog infected with type I and that infected with type II shed virus for the entire observation period (28 days). Shedding of type I reached a peak at day post infection (dpi) 10, with a titre of 7.00×10^6 RNA copies/ μl of template (Fig. 3a), whereas the highest amount of type II was observed at dpi 4 (1.35×10^5 RNA copies/ μl of template) (Fig. 3b).

The dog infected with both CCoV genotypes was monitored for 90 days, indicating the exact time duration of the shedding of type I and type II. This dog shed type I RNA for 67 days, from dpi 2 to 68, with peaks at dpi 7 and 16 (1.21×10^6 RNA copies/ μl of template), while shedding of type II was observed for 68 days, from dpi 2 to 69, reaching a peak at dpi 12 (2.41×10^5 RNA copies/ μl of template) (Fig. 3c).

As expected, the viral RNA titres detected by the fluorogenic RT-PCR assay developed previously (Decaro et al.,

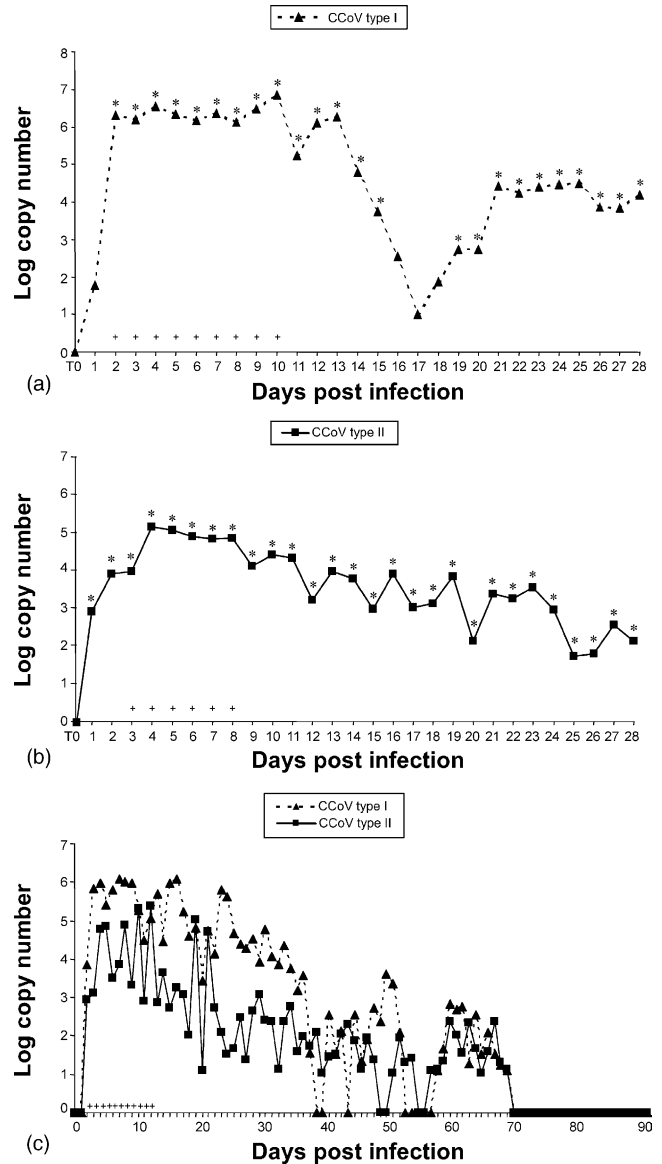


Fig. 3. Number of copies of viral RNA per μl of template detected in faecal samples of the dogs infected with CCoV type I (a), type II (b), or both genotypes (c) by the genotype-specific fluorogenic RT-PCR assays. Asterisks indicate the samples tested CCoV-positive by the conventional RT-PCR assays. Symbol (+) indicates days post infection in which diarrhoea was observed.

2004) were found approximately the same for type II and lower for type I in comparison to those calculated by the genotype-specific assays (data not shown).

All the infected dogs developed diarrhoea concomitantly with the detection of the highest CCoV RNA amounts in the faeces, which was more severe and long lasting in the dog inoculated with both type I and type II (Fig. 3).

4. Discussion

Two genotype-specific fluorogenic RT-PCR assays were developed for the detection and quantitation of CCoV type I

and type II RNA in the faeces of dogs with diarrhoea. Both fluorogenic assays were found to be highly specific, sensitive and reproducible, allowing an accurate viral RNA quantitation over a linear range of about eight logs (from 10^1 to 10^8 RNA copies). However, the assays should be considered semi-quantitative rather than quantitative methods, since it is not possible to exactly determine and compare viral loads in dogs using faeces as samples. In fact, the total volume of faeces (and thus the volume into which the viruses are secreted or diluted) can hardly be determined and will vary between different dogs and between different periods of a diarrhoeic episode of one dog. In contrast with the high specificity of the two assays, TGEV was amplified successfully by the type II-specific fluorogenic assay. However, this finding should not affect the detection of CCoV type II in the faeces of dogs, since TGEV, even if able to replicate in the canine host (Larson et al., 1979), has never been reported, isolated or detected from the faeces of dogs.

Theoretically, a duplex fluorogenic assay for the simultaneous detection and quantitation of both genotypes in the same reaction could be developed by using genotype-specific probes, labelled with different fluorophores. However, separate assays were chosen in order to avoid any interference between the probes in the same reaction, which might affect the precise quantitation of type I and type II RNA. Furthermore, since the two assays have different annealing temperatures, they cannot be run at the same time in the i-Cycler iQ™ real-time detection system.

The CCoV genotype-specific fluorogenic assays have several advantages over conventional gel-based RT-PCR assays. They enable simultaneous processing of a large number of samples, with a substantial increase in throughput and a considerable time saving. The only disadvantage related to real-time RT-PCR is represented by the higher costs, which consist of the cost of conventional RT-PCR plus the cost of the labelled probe. Nevertheless, the high throughput associated with the TaqMan assay compensates for this additional cost and permits processing of multiple samples with minimal labour time and very low risks of carry-over contamination. The performance of the IC added to the faecal samples demonstrated that the commercial kit used for nucleic acid extraction is highly efficient for the recover and purification of RNA.

By the genotype-specific fluorogenic RT-PCR assays, the faecal samples collected from dogs infected naturally were found to contain CCoV type I, type II, or both genotypes. Several dogs were infected simultaneously by both type I and type II, showing that dual CCoV infections are very frequent in the dog population, as reported previously (Pratelli et al., 2004). Interestingly, type I was found more widespread in the dog population with respect to type II. An analogous distribution has been also described for feline coronaviruses, since FCoV type I is more frequently detected in cats than FCoV type II (Hohdatsu et al., 1992; Benetka et al., 2004), although it grows poorly in cell cultures (Pedersen et al., 1984).

Furthermore, the CCoV type I titres in the faeces were generally higher than those of type II. These findings were confirmed by the analysis of the specimens of the dogs infected experimentally, in whose faeces shedding of type I was observed with higher titres in comparison to type II. Nevertheless, the onset of a more severe diarrhoea in the dog infected with both genotypes seems to confirm previous observations of increased severity of the clinical course of dual CCoV infections with respect to that noticed in single-genotype infections (unpublished results).

The genotype-specific fluorogenic RT-PCR assays may be useful for studying the pathogenesis of infections by CCoVs and particularly by type I, since attempts to adapt this genotype to *in vitro* growth were unsuccessful (Pratelli et al., 2003b, 2004). Finally, vaccine trials against CCoV infections could also take advantage of the newly established fluorogenic assays for the evaluation of the vaccine efficacy by determining CCoV shedding in the faeces of vaccinated dogs after challenge with a field strain of type I or type II. In fact, by conventional RT-PCR only, the time duration of viral shedding is valuable, while evaluation of the vaccine efficacy should also take into account the reduction in the titres of the challenge virus in the faeces. Theoretically, CCoV type II titres in the faeces of challenged dogs could be evaluated by titration in cell culture, but it has been noted that this method is poorly sensitive and inaccurate (Decaro et al., 2004) and, further, virus titration cannot be used for CCoV type I which cannot be cultivated in cell culture.

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