Canine Coronaviruses: Epidemiology, Evolution and Pathobiology

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ISBN: 978-90-393-5055-3

Cover design by V. Tarallo

Printed by Grafica 080, Bari - Italy

Canine Coronaviruses:

Epidemiology, evolution and pathobiology

Coronavirussen bij de hond

Epidemiologie, evolutie en pathobiologie

(met een samenvatting in het Nederlands)
(con abstract in italiano)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
donderdag 4 juni 2009 des middags te 4.15 uur

door

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geboren op 11 november 1973 te Bari, Italië

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The work described in this thesis was financed by grants from the Italian Ministry of University (PRIN 2002, 2005, 2006) and from the Italian Ministry of Health (Ricerca corrente 2003, Ricerca finalizzata 2007). Printing of this thesis was financially supported by grants from the Italian Ministry of Health, Ricerca finalizzata 2007, project "Mammal coronaviruses: molecular epidemiology, vaccine development and implications for animal and human health".

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General introduction

Adapted from **An update on canine coronaviruses: viral evolution and pathobiology**

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Veterinary Microbiology, 2008; 132, 221-234

CORONAVIRUS TAXONOMY, GENOMIC ORGANIZATION AND REPLICATION CYCLE

Coronaviruses (family *Coronaviridae*, order *Nidovirales*) are enveloped, positive-strand RNA viruses that are associated with enteric and/or respiratory disease in mammals and birds (18). On the basis of phylogenetic analysis (22, 23), three major CoV groups can be distinguished. Group 1 CoVs include canine coronavirus (CCoV), feline coronaviruses (FCoVs) type I and type II, transmissible gastroenteritis virus (TGEV) of swine, porcine respiratory coronavirus (PRCoV), porcine epidemic diarrhea virus (PEDV) and human coronaviruses 229E (HCoV-229E) and NL63 (HCoV-NL63). Recently, a ferret coronavirus was identified as a new member (53). Currently, group 2 CoVs are organized into bovine-coronavirus-like (subgroup 2a) and severe acute respiratory syndrome (SARS)-like (subgroup 2b) viruses. Members of subgroup 2a are bovine coronavirus (BCoV), mouse hepatitis virus (MHV), rat coronaviruses, porcine haemagglutinating encephalomyelitis virus (PHEV), human coronavirus (HCoV) OC43, human enteric coronavirus (HECV) 4408 (18), equine coronavirus (ECoV) (25), HCoV-HKU1 (54) and canine respiratory coronavirus (CRCoV) (19). SARS-CoV has been placed within group 2 CoVs in a subgroup 2b, together with SARS-like animal CoVs (24, 52). To accommodate recently identified CoVs of bats and wild carnivores (16, 47) subgroups 2c and d have been proposed. Group 3 comprises avian bronchitis virus, some coronaviruses of turkey and Beluga whale coronavirus (35).

The Coronavirus Study Group of the International Committee of Taxonomy of Viruses recently proposed to revise the family *Coronaviridae* to include the corona- and toroviruses as subfamilies (*Corona-* and *Torovirinae*) and to convert the coronavirus phylogroups 1, 2 and 3 into genera (Alpha-, Beta- and Gamma-coronavirus, respectively) (9, Fig. 1). The proposal is based upon rooted phylogeny and quantitative pair-wise sequence comparison (22, 23) and includes a clear definition of CoV species demarcation in accordance with that used in other virus families. Given their close genetic relatedness (more than 96% amino acid identity in the key replicase 1ab domains), TGEV, CCoV and FCoV should be considered not as separate viruses but rather as host range variants of the same species, a view already expressed by Marian Horzinek and coworkers many years ago (29).

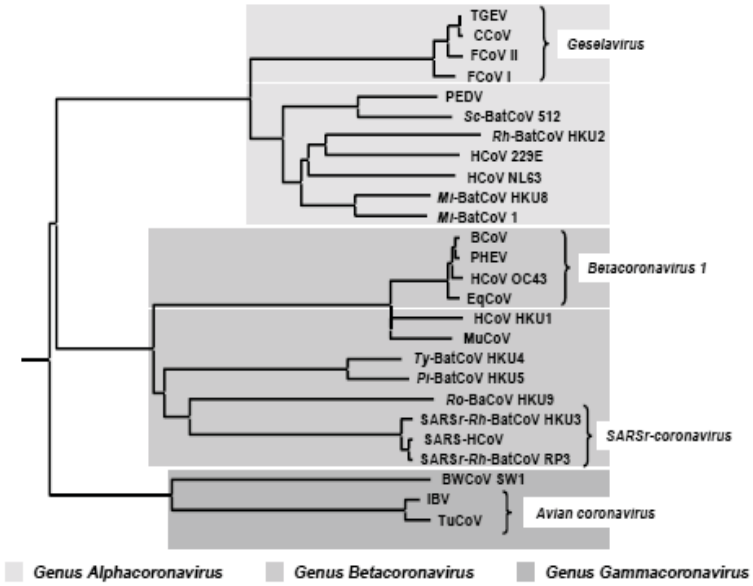

Fig. 1. Rooted neighbour-joining tree inferred from multiple amino acid alignments of the RdRp, illustrating the relationships between the proposed coronavirus genera and between the viruses lumped in the new species *Geselavirus*, *Betacoronavirus 1*, *SARS-related coronavirus* and *Avian Coronavirus* (from reference 9).

Among RNA viruses, CoVs possess the largest genome, 27.6 to 31 kb in size. The 5′-most two-thirds of the genome comprises the replicase gene, which consists of two overlapping open reading frames, ORF 1a and 1b. Located downstream of ORF1b are up to 11 ORFs that code for the four common structural proteins (spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins) and a variable set of accessory proteins. The S protein mediates viral attachment to specific cell receptors and fusion between the envelope and plasma membrane and it is the main inducer of virus-neutralising antibodies. (18). The small membrane (E) protein plays an important role in viral envelope assembly (50), but it is not essential for virus propagation (15, 31, 39). The membrane (M) protein, the most abundant structural component, is a type III glycoprotein consisting of a short amino-terminal ectodomain, a triple-spanning transmembrane domain, and a long carboxyl-terminal inner domain (42). Antibodies to the M protein of MHV can neutralise viral infectivity, but only in the presence of complement (6). The nucleocapsid (N) protein is a highly basic phosphoprotein that in addition to its function in the virion also modulates viral RNA synthesis (18). Group 2a CoVs possess an additional structural protein, the hemagglutinin-esterase (HE), closely related to the hemagglutinin-esterase fusion protein of influenza C virus (7, 58). Additional ORFs encoding non-structural accessory proteins have been recognized in CoV genomes and their number, nucleotide sequence and gene order can vary remarkably among different CoVs (4, 8, 17, 27, 32). The function of the accessory proteins is in most cases unknown and as a rule they are not essential for virus replication. They do play an important role, however, in virus host interactions as they are generally maintained during natural infection (28) and their loss -either through spontaneous mutation or reversed genetics- results in reduced virulence (26, 38, 46, 55, 57).

CoVs attach to specific cellular receptors via the S protein. Whereas CCoV-II employs aminopeptidase N as its receptor, the CCoV-I receptor remains to be identified. The spike then mediates the fusion between the viral and cell membranes, which results in the release of the nucleocapsid into the cell. Upon uncoating, the viral genome becomes translated to yield two polyproteins, pp1a and pp1ab; the synthesis of the latter product requires a ribosomal frameshift. Papain-like and picornavirus 3C-like protease domains in pp1a process pp1a and pp1ab to yield the mature replicase proteins, which include an RNA-dependent RNA-polymerase (RdRp), a helicase, a 3'-to-5' exonuclease, a poly(U)-specific endoribonuclease, and an *S*-adenosylmethionine-dependent ribose 2'-*O*-

methyltransferase.. These products are involved in the coronavirus RNA replication and/or interference with the host cell metabolism (59). Replication of the CoV RNA involves the synthesis of a full-length negative-strand RNA that is present at low concentrations and serves as a template for the synthesis of full-length genomic RNA. The genes downstream of ORF1b are expressed through a 3'-coterminal nested set of subgenomic (sg) mRNAs. According to the generally accepted model for CoV transcription, sg minus-strand RNAs are produced via a discontinuous 3'-extension step which is regulated by transcription regulating sequences (TRSs) that are present upstream of (most) ORFs and also at the 5'-end of the genome. The minus-strand RNAs in turn serve as templates for the synthesis of complementary sg mRNAs, of which only the 5' end is generally translated (44).

CANINE ENTERIC CORONAVIRUS (CCoV)

History and pathobiology

The first report on CCoV infection appeared in 1971, when Binn and colleagues isolated a coronavirus (strain 1-71) from dogs with acute enteritis in a canine military unit in Germany (3). The disease could be reproduced in young dogs by experimental infection with the purified virus thus ful-filling Koch's postulates (30). Since then, several CCoV outbreaks have been reported worldwide, showing that CCoV is an important enteropathogen of the dog. Serological and virological investigations demonstrated that CCoV is widespread in the dog population, and the virus is highly prevalent in kennels and animal shelters (1, 5, 36, 37, 41, 45, 49, 56). CCoV infection is characterized by high morbidity and low mortality. The virus is shed at high titres in the faeces and transmitted via the faecal-oral route (48). CCoV infection is generally restricted to the alimentary tract, leading to the onset of clinical signs typical of gastroenteris including loss of appetite, vomiting, fluid diarrhea dehydration and, only very rarely, death. Although, in general, CCoVs do not cause systemic disease, the virus has been isolated from several tissues (tonsils, lungs and liver) of experimentally-infected pups (48). Fatal disease commonly occurs as a consequence of mixed infections of CCoV with canine parvovirus type 2 (CPV-2) (12, 14), with canine adenovirus type 1 (13) or with canine distemper virus (10).

CCoV genotypes

Currently, two genotypes of CCoV are known, that have been designated CCoV types I (CCoV-I) and II (CCoV-II) (2, 11, 40, 51, 56). These genotypes differ mainly in their spike proteins that share only 54% identity (40). Moreover, CCoV-I strains possess a unique ORF, 624 nt in length, that is completely absent in FCoV-I strains and of which only remnants remain in the genomes of CCoV-II and TGEV (33, Fig. 2). Computer-aided analysis of this additional ORF, designated ORF3, showed that its product is a soluble 24 kDa glycoprotein, which may either function intracellularly or become secreted from the infected cells.

In China, both CCoV-I and II were detected in the faeces of healthy foxes and raccoon dogs. A limited comparative sequence analysis, restricted the M genes, suggested that these viruses were very closely related to Italian CCoV-I and -II strains (34, 51).

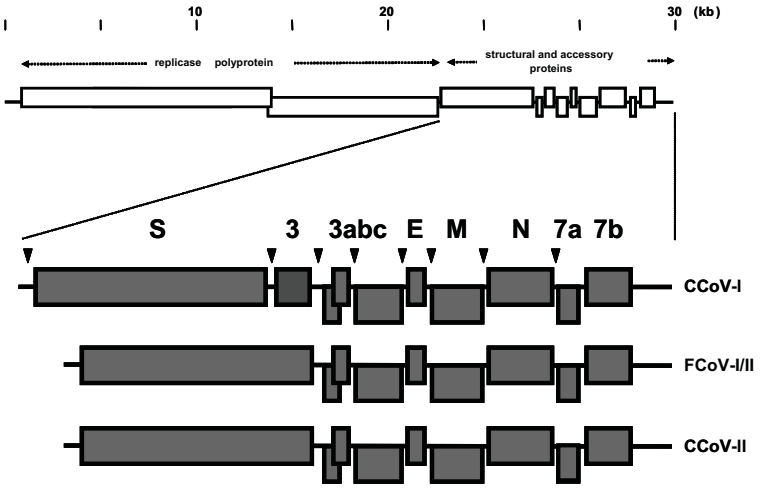

Fig. 2. Schematic representation of the genomes of CCoVs and FCoVs depicting the genetic differences among the CCoV genotypes. Genes encoding for structural and non-structural proteins are shown in grey and white, respectively. ORF sizes are not drawn to scale. The arrows indicate the transcription regulating sequences preceding each CoV gene (courtesy Raoul J. de Groot).

Virulent strains

In general, CCoV infections are mild and often asymptomatic (48). However, in recent years several virulent CCoV strains were described.

A hypervirulent variant of CCoV type II caused an outbreak of severe gastroenteritis in a beagle colony in the United Kingdom (43). In the U.S., two cases of fatal CoV disease in pups without evidence of co-infection by CPV-2 were reported by Evermann et al. (21). CCoV infection was demonstrated by immunohistochemistry in the gut and by electron microscopical detection of virions in the intestinal contents. Histopathology showed moderate depletion and necrosis of lymphoid tissues, including thymus, spleen, lymph nodes and gut-associated lymphoid tissues, in both pups. Unfortunately, the authors did not genetically characterise the CCoV strains involved.

Finally, high virulence CCoV variants were associated with an outbreak of fatal canine gastroenteritis in Sweden (20). Importantly, some Swedish strains possessed S genes with 5' and 3' ends corresponding to those of CCoV type I and type II, respectively, thus indicating their possible origin from recombination events between the two CCoV genotypes.

SCOPE OF THE THESIS

In this thesis, I have investigated the epidemiology, molecular biology and pathobiology of CCoV, with particular emphasis on CCoV-II.

In chapter 2, the molecular epidemiology of the two CCoV genotypes was investigated by using conventional type-specific RT-PCR assays targeting the spike and membrane protein genes. The results showed that simultaneous infections caused by both genotypes are common and in fact account for about 75% of all CCoV infections.

In chapters 3a and 3b, real-time TaqMan RT-PCR assays were established for sensitive detection, quantification and rapid genotyping of CCoV in clinical samples.

Chapters 4 and 5 focus on the identification and molecular characterization of a hypervirulent CCoV-II variant with unique pathobiological properties; this virus, designated CB/05 caused an outbreak of fatal, systemic disease in dogs.

The results of experimental infections with strain CB/05 are discussed in chapter 6, confirming that it spreads systemically, causing lymphopenia and severe clinical signs in infected dogs. However, the outcome of the infection appears to be dependent upon age as the disease tends to run a more severe course in younger pups.

In chapter 7, the isolation, genomic and biological characterization of four CCoV-II strains with a potential recombinant origin in the spike-protein gene with transmissible gastroenteritis virus of swine is reported.

Finally, in the last chapter (chapter 8), the obtained results are discussed in the context of a more general view on the current evolution of animal CoVs through the emergence of new genotypes, biotypes or host variants.

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Two genotypes of canine coronavirus simultaneously detected in the fecal samples of dogs with diarrhea

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Journal of Clinical Microbiology, 2004; 42, 1797-1799

ABSTRACT

Sixty-nine fecal samples from diarrheic pups were examined by reverse trnascription-PCR assays for the M and the S genes of canine coronaviruses (CCoVs). The isolates in ten samples were recognized as CCoV type I, and the isolates in 6 samples were recognized as as CCoV type II, while isolates if 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 (9, 11). 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 (8, 13). 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 (14).

The results of a virological investigation by reverse trnascription-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 (6).

Feline cell whole foetus (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 on 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 on the cryolysates of the third cell culture passage 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.

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	GTGCTTCCTCTTGAAGGTACA	+	6900-6920[c]	239
CCoV2[b]			TCTGTTGAGTAATCACCAGCT	-	7118-7138[c]	
EL1F	S	I	CAAGTTGACCGTCTTATTACTGGTAG	+	2611-2636[d]	346
EL1R			TCATATACGTACCATTATAGCTGAAGA	-	2930-2956[d]	
Can1F	M	II	TAACATTGCTCTCAGGGAATTTG	+	6937-6959[c]	202
CCoV2[b]			TCTGTTGAGTAATCACCAGCT	-	7118-7138[c]	
S5	S	II	TGCATTTGTGTCTCAGACTT	+	3991-4010[c]	694
S6			CCAAGGCCATTTTACATAAG	-	4665-4684[c]	

[a] Data from reference 12. [b] Data from reference 6.
[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).

The RNA was reverse transcribed with random hexamers by using MuLV reverse transcriptase (Applied Biosystems, Rome, Italy) and then amplified with AmpliTaq DNA polymerase (Applied Biosystems). For amplification of CCoVs type I, the primer pairs CCoV1a-CCoV2, which amplified a fragment of the M gene (12) and EL1F-EL1R, the sequence for which was selected from a relatively conserved region of the Elmo/02 CCoV type I spike gene (14), were used.

For amplification of CCoV type II, the 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 (13). 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 NCBI and EMBL were used for sequence comparison. Phylogenetic and molecular evolutionary analyses were performed with MEGA software, version 2.1 (4). Maximum-parsimony trees were processed by using a heuristic algorithm with bootstrapping of over 100 replicates.

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

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

Strain	Genotype	Accession number	Reference
Insavc	CCoV type II	D13096	3
K 378	CCoV type II	- [a]	
UCD1	FCoV type I	AB086902	5
Black	FCoV type I	AB086903	5
79-1146	FCoV type II	X56496	16
79-1683	FCoV type II	AB086904	5
259/01	CCoV type I	AF502583	13

[a] The strain was kindly supplied by L.E. Carmichael (J.Baker Institute, Ithaca, NY) and the nucleotide sequence of the M gene was obtained by the authors.

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

Strain	Genotype	Accession number	Reference
Insavc	CCoV type II	D13096	3
K 378	CCoV type II	X77047	17
UCD1	FCoV type I	AB088222	5
Black	FCoV type I	AB088223	5
79-1146	FCoV type II	X06170	2
79-1683	FCoV type II	X80799	E. L. Lewis, direct submission
Elmo/02	CCoV type I	AY170345	14

Thirteen out 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 1b). 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 1d).

Serological investigations suggest that CCoV infection is widespread in pet and in kennel dogs (10, 15). In contrast, little evidence for CCoV-associated gastroenteritis in dogs has been collected, and only few strains have been adapted to growth in vitro (7).

Therefore, the development of PCR assays for the detection of CCoV (1, 6) 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 (8), 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 (11). Finally, an evident genetic divergence from the reference CCoV strains has been observed in the genome of CCoVs identified in the feces of puppies, strongly indicating that a new genotype of CCoV is widespread in dogs (13, 14).

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 this 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 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 pathogenetic role in dogs.

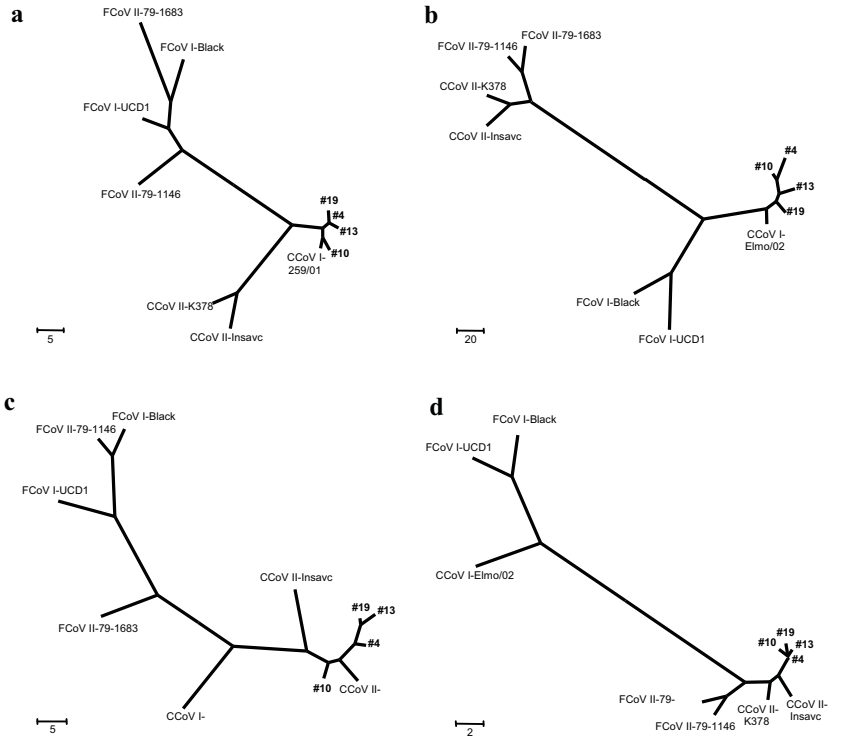

Fig. 1. Maximum-parsimony trees constructed with different genome fragments of canine and feline coronaviruses. (**a)** Fragment of the M gene obtained with primer pair CCoV1a-CCoV2 (239 bp). (**b)** Fragment of the S gene obtained with primer pair EL1F-EL1R (346 bp). (**c)** Fragment of the M gene obtained with primer pair Can1F-CCoV2 (202 bp). (**d)** Fragment of the S gene obtained with primer pair S5-S6 (694 bp). The trees are unrouted 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.

ACKNOWLEDGEMENTS

This study was supported by grants from Ministry of University (2002) of Italy (project: Enteriti virali del cane) and from CEGBA (Centro di Eccellenza di Genomica in Campo Biomedico e Agrario).

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

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Journal of Virological Methods, 2004; 119, 145-150

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 10^1 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 simultaneously analyzed 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 resulted 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 developed CCoV fluorogenic RT-PCR assay, combined with its wide dynamic range and high throughput, make this method especially suitable in the efficacy trials on CCoV vaccines.

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 diarrhea 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), ORF1*a* and ORF1*b*, 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 (7).

In young pups, sometimes in combination with other pathogens, CCoV infection may cause severe diarrhea, vomiting, dehydration, inappetence, and, occasionally, death. CCoV shedding in faeces occurs for 6 to 9 days post-infection (12). Nevertheless, a long-term viral shedding has been detected by PCR in CCoV infected pups (21, 23).

Traditionally, diagnosis of CCoV infection was made using virus isolation or electronic microscopy, but these methods have been demonstrated 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 been identified as the gold standard technique for the CCoV diagnosis (18, 19). Several RT-PCR based methods have been developed for detecting CCoV RNA in the faeces of dogs, but none of these were designed to be quantitative (3, 17, 18, 24). 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 TaqMan RT-PCR enables a reproducible, sensitive and specific quantitation of viral RNA (2, 4, 6 8, 14, 28-30).

In the present study we describe 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'→ 3' exonuclease activity of *Taq* polymerase, resulting in an increase

of the reporter dye's fluorescence released in the course of the PCR amplification (9, 10). The analytical performance of the CCoV fluorogenic RT-PCR was evaluated in comparison to that of a conventional qualitative RT-PCR assay. Then, the established fluorogenic assay was 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.

MATERIALS AND METHODS

Samples

A total of 78 faecal samples, collected from diarrhoeic pups in different geographic areas of Italy, were processed in order to detect CCoV and quantify viral RNA amounts in the faeces. In addition, one conventional dog, 3 months of age, which had been tested negative for CCoV antigen in the faeces by RT-PCR (18) and for CCoV antibodies by ELISA (22), was administered 4 ml (2 ml intranasally and 2 ml orally) of a cell culture medium containing 10^5 $TCID_{50}$/50 µl of a CCoV field strain, as previously described (26, 27). Faecal samples of the infected dog were daily collected for 28 days and subjected to both CCoV conventional RT-PCR and real-time analysis.

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°C until their use.

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 (5) 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® RNA Easy 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°C and used only once.

Primer and probe design

The ORF5 nucleotide sequences of several CCoV strains (25) were aligned using the BioEdit software package (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.

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

Primer/probe	Sequence 5' to 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: D13096).

[b] Conventional RT-PCR (18) ; [c] Fluorogenic assay.

[d] FAM, 6-carboxyfluorescein ; [e] TAMRA, 6-carboxytetramethylrhodamine.

Reverse transcription

Triplicates of the standard dilutions and RNA templates were simultaneously subjected 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 1X (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°C for 30 min, followed by a denaturation step at 99°C for 5 min.

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 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 analyzed 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]).

Conventional RT-PCR

Conventional RT-PCR, amplifying a 409-bp fragment of the ORF5 of CCoV, was carried out as previously described (18). Briefly, PCR amplification was performed using 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.

RESULTS

Analytical performance of the CCoV fluorogenic RT-PCR assay

To compare the analytical sensitivity, ten-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, ten-fold dilutions in Dulbecco's Minimal Essential Medium (D-MEM) of the CCoV vaccinal strain 257/98-3c (27), starting from $10^{5.50}$ $TCID_{50}$/50 µl, were processed. Each standard or virus dilution was being quantified three times separately. As shown in Table 2, the detection limit of the TaqMan RT-PCR was 1-2 logs higher than that of conventional RT-PCR, ranging around 10^1 copies/µl and $10^{-1.50}$ $TCID_{50}$/50µl for standard RNA and CCoV strain, respectively, with a detection rate of 100% for each positive dilution.

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.

Serial ten-fold dilutions of standard RNA (from 10^1 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). 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.

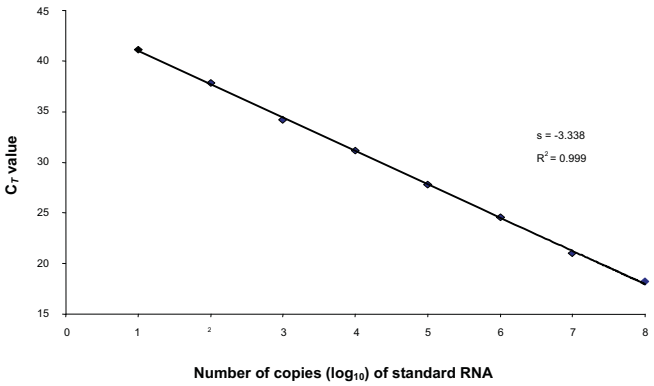

Fig. 1. Standard curve of the CCoV fluorogenic RT-PCR assay. Ten-fold dilutions of standard RNA prior to amplification were used, as indicated on the *x* axis, whereas the corresponding cycle threshold (C_T) values are presented on the *y* axis. Each dot represents the result of triplicate amplifications of each dilution. The coefficient of determination (R^2) and the slope value (*s*) of the regression curve were calculated and are indicated.

Analysis of the 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. 2a, 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). CCoV RNA loads assessed by real-time analysis on the faeces of the naturally infected dogs are schematically represented in Fig. 2b. The analyzed samples contained a wide range of CCoV RNA amounts, from 10^1 to 7.5 x 10^7/µl of template, with a median titre of about 10^3/µl of template.

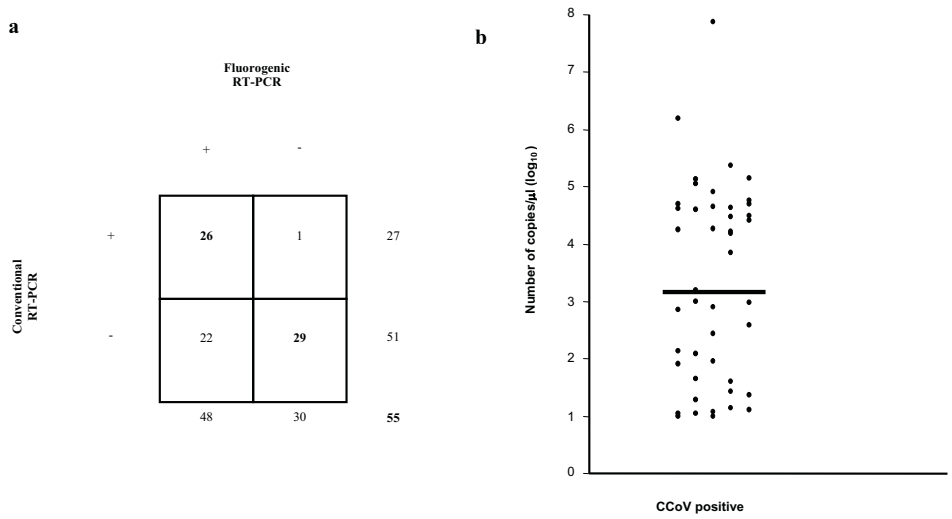

Fig. 2. Detection **(a)** and quantitation **(b)** of CCoV in faecal samples of naturally infected dogs. **(a)** Comparison between conventional and fluorogenic RT-PCR. Numbers indicate the samples positive (+) or negative (-) for CCoV. Results according to both techniques are shown in bold. **(b)** Logarithmic distribution of the number of genomic RNA copies per µl of template. Each dot represents a faecal sample; bar indicates the median value.

Analysis of the 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 x 10^5 RNA copies/µl of template).

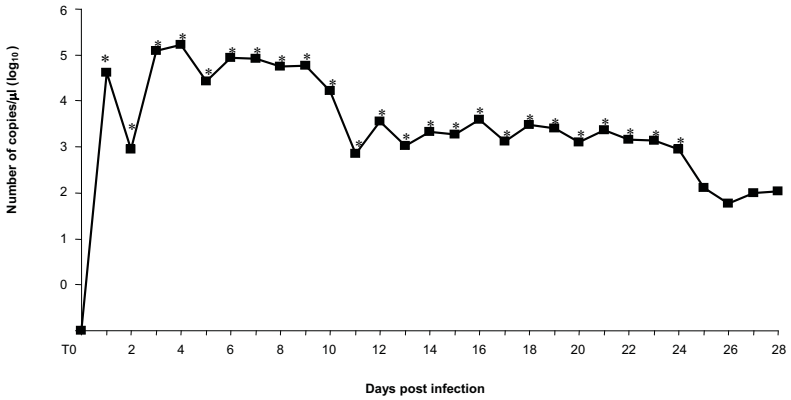

Fig. 3. Number of copies of CCoV genomic RNA in the faecal samples of the experimentally infected dog by fluorogenic RT-PCR. Asterisks indicate the CCoV positive samples by conventional RT-PCR.

DISCUSSION

We describe the development of a quantitative, simple, rapid and reproducible method for the detection and quantitation of CCoV RNA in faecal samples of dogs experimentally or naturally infected 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 analyzed. 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 to conventional RT-PCR, the fluorogenic assay presents many advantages. In addition to its greater sensitivity, this technique shows a considerable rapidity, allowing several samples to be processed in few hours, with a large increase in throughput. Then, 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 previously amplified products. 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 (16). In order to reduce the risk of contamination, we have strictly separated 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 (11, 13-15, 31). 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, 4 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 naturally infected dogs 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 (20, 23).

Finally, CCoV quantitation by real-time analysis could be an 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 only evaluated, as previously described (26, 27), 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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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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Journal of Virological Methods, 2004; 130, 72-78

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 diarrhea. 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.

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 diarrhea 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 (5, 9).

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 (14, 15).

Since the virus is difficult to isolate in cell cultures (12, 21), a nested PCR assay, targeting the M gene, was developed for the diagnosis of CCoV infection, with high sensitivity and specificity (11). FCoV RNA is quantifiable by means of a fluorogenic RT-PCR assay (8). Analogously, 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 (4).

Recently, two CCoV genotypes have been identified in the faeces of pups with enteritis, which have been referred to as CCoV type I and CCoV type II (19, 20). 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) (16, 20), 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 diarrhea (20) 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 (19, 20).

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.

MATERIALS AND METHODS

Samples

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

In addition, 3 mix-bred dogs, 2 months of age, negative for CCoV RNA in the faeces by RT-PCR (11) and for specific antibodies by Elisa (17), 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 (20). 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.

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 µl of nuclease-free water and stored at –70°C until its use. One microliter per reaction was used as template.

Standard RNA preparation

In vitro-transcribed ORF5 genes of strains 259/03 (18) and 45/93 (3) 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 (4). Transcripts were quantified by spectrophotometer and subjected to ten-fold dilutions, containing from 10^8 to 10^0 molecules per µl. Each dilution of type I and type II standard RNAs, made 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, was frozen at –70°C and used once only.

Hydrolysis probe and primer sets

The ORF5 nucleotide sequences of several strains of CCoV type I and type II (18) were aligned using the BioEdit software package (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 (14, 15, 18), 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.

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 1X (KCl 50 mM, Tris-HCl 10 mM, pH 8,3), $MgCl_2$ 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.

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

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

[a] Conventional RT-PCR (16, 20).

[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).

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

Fluorogenic RT-PCR assays

The fluorogenic genotype-specific RT-PCR assays were undertaken in an i-Cycler iQ™ 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 IQ™ 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.

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 (16, 20). 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, 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 RT-PCR products were detected by electrophoresis in 1.5% agarose gel and visualization under UV light.

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 5,000 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 (1). 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 SD 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 (CTTCTAACCGAGGTCGAAACGTA) and Flu-M179rev (GGATTGGTCTTGTCTTTAGCCA) 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 SD) were excluded from analysis.

RESULTS

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: 6 CCoV II culture-adapted strains (Insavc-1, S378, K378, SE, 257/98, 144/02), 5 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 CCoVs. 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, ten-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 the type I and the 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 x 10^4 RNA copies) to 31.88% (3.09 x 10^2 RNA copies), whereas the interassay CVs were comprised between 31.72% (1.74 x 10^3 RNa copies) and 47.04% (2.87 x RNA 10^2 copies). For the type II assay, the intra-assay and interassay CVs ranged from 12.47% (1.01 x 10^3 RNA copies) to 42.47% (3.32 x RNA 10^1 copies) and from 22.33% (2.83 x 10^6 RNA copies) to 53.95% (1.14 x 10^1 RNA copies), respectively.

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.

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

The faeces of 174 dogs with diarrhea, collected during years 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 5 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 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.

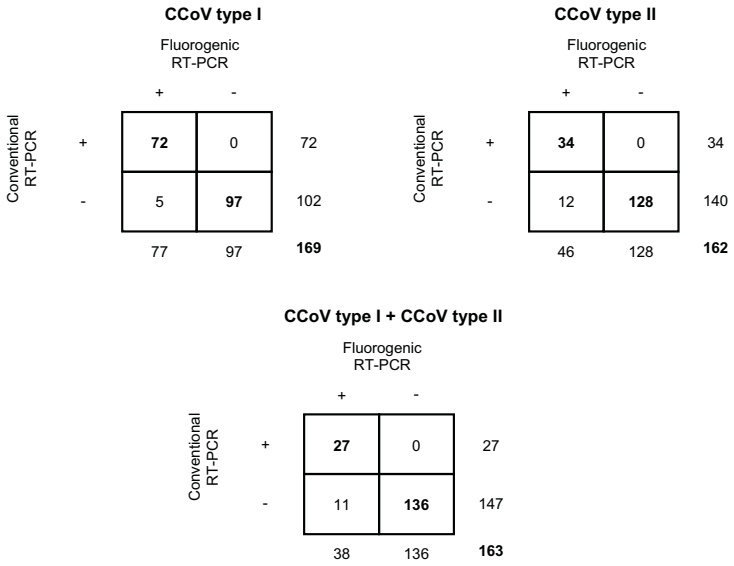

Fig. 1. Detection of CCoV type I and type II RNA in faecal samples of dogs infected naturally by conventional and fluorogenic genotype-specific RT-PCR assays. Numbers indicate the samples positive (+) or negative (-) for CCoV. Results according to both techniques are shown in bold.

Quantitation of viral RNA showed that faecal samples contained wide ranges of CCoV loads, from 3.93 x 10^2 to 5.80 x 10^6 molecules/µl of template for type I (median value = 9.14 x 10^4) and from 1.15 x 10^1 to 1.73 x 10^6 molecules/µl of template for type II (median value = 2.24 x 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).

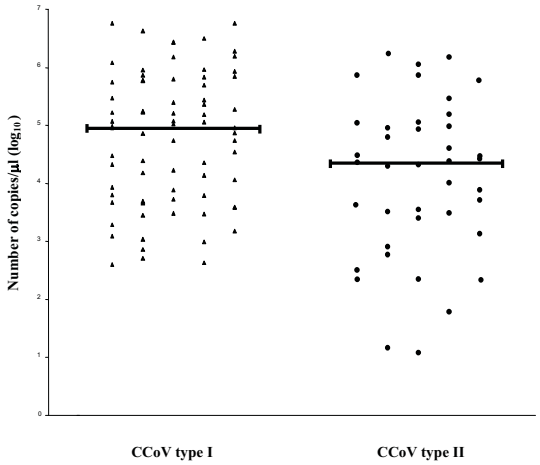

Fig. 2. Logarithmic distribution of the RNA amounts of CCoV type I and type II in the faeces of dogs infected naturally. Values are expressed as RNA copy numbers per µl of template. Each dot represents a faecal sample; bars indicate the median values.

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 x 10^6 RNA copies/µl of template (Fig. 3a), whereas the highest amount of type II was observed at dpi 4 (1.35 x 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 x 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 x 10^5 RNA copies/µl of template) (Fig. 3c).

As expected, the viral RNA titres detected by the fluorogenic RT-PCR assay developed previously (4) 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 diarrhea 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).

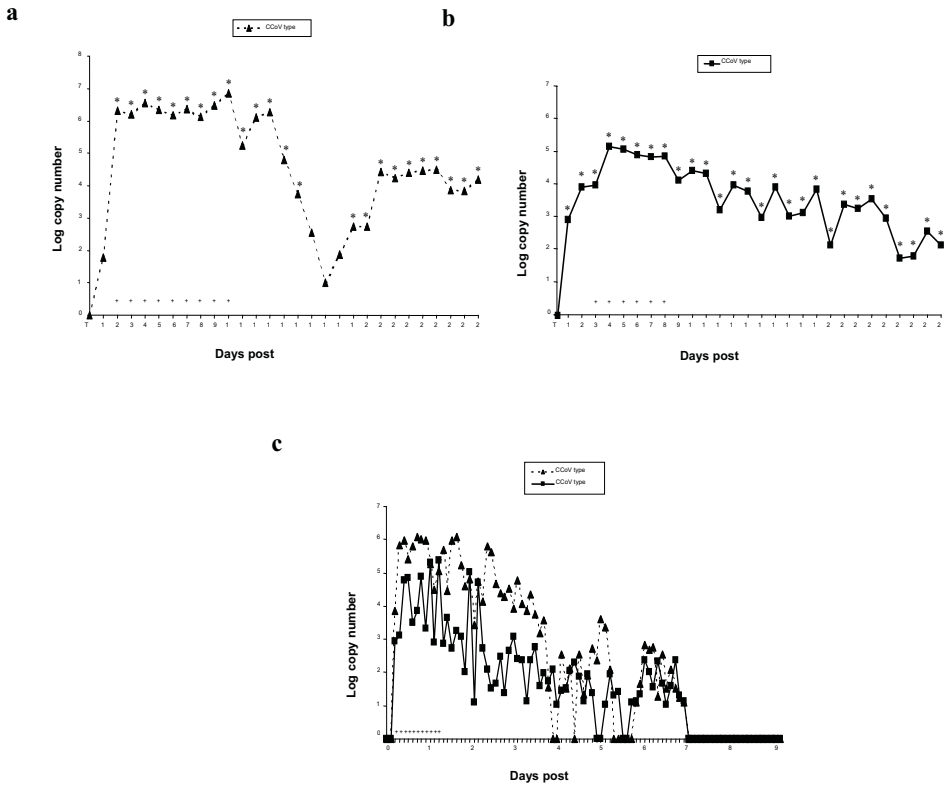

Fig. 3. Number of copies of viral RNA per µl of template detected in faecal samples of the dogs infected with CCoV type I **(a)**, type II **(b)**, or both genotypes **(c)** by the genotype-specific fluorogenic RT-PCR assays. Asterisks indicate the samples tested CCoV-positive by the conventional RT-PCR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

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 diarrhea. 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 (7), 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 (20). 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 (2, 6), although it grows poorly in cell cultures (10).

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 diarrhea 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 (19, 20). 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 (4) and, further, virus titration cannot be used for CCoV type I cannot be cultivated in cell culture.

ACKNOWLEDGEMENTS

This work was supported by grants from Ministero della Salute (Ricerca corrente 2003: "Studio del coronavirus del cane come modello animale per le ricombinazioni genetiche dei coronavirus").

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Canine coronavirus highly pathogenic for dogs

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Emerging Infectious Diseases, 2006; 12, 492-494

ABSTRACT

Canine coronavirus (CCoV) is usually responsible for mild, self-limiting infections restricted to the enteric tract. We report an outbreak of fatal disease in puppies caused by a pathogenic variant of CCoV that was isolated from organs with severe lesions.

Coronaviruses are large, enveloped, positive-stranded RNA viruses (7). Three different coronaviruses have been identified in dogs (2, 9). Canine coronavirus (CCoV) type I and type II are included in group 1 coronaviruses, and their evolution is related to that of feline coronavirus (FCoV) type I and type II. FCoV type II originated by heterologous recombination between CCoV type II and FCoV type I, while CCoV type I is genetically more similar to FCoV type I than to CCoV type II (9). In addition, two FCoV biotypes that differ in pathogenicity have been observed in cats.

The onset of acute fatal disease (feline infectious peritonitis) is caused by pantropic variants (able to disseminate throughout the organism) of enteric FCoVs with deletions or recombinations in the 3c and 7b genes at the 3′ end of the FCoV genome (13). Similarly, changes in tissue tropisms in porcine and murine coronaviruses (4, 5) and adaptation of the recently recognized severe acute respiratory syndrome–associated coronavirus (3) to humans have been related to mutations or deletions. A third canine coronavirus, CRCoV, detected in the respiratory tract, has ≤96.0% amino acid (aa) conservation in the spike (S) protein with bovine coronavirus within group 2 coronaviruses, which provides strong evidence for a recent host-species shift (2).

Coronavirus infection in dogs is usually restricted to the enteric tract. The infection is self-limiting and in general produces only mild or asymptomatic forms of enteritis (12). We report the identification of a pantropic, highly pathogenic variant of CCoV type II.

THE STUDY

In May 2005, a severe outbreak of fatal systemic disease occurred in a pet shop in Bari, Italy. Clinical symptoms were initially observed in 3 Miniature Pinschers (45 days of age) and 1 Cocker Spaniel (53 days of age) and consisted of fever (39.5°C–40°C), lethargy, inappetence, vomiting, hemorrhagic diarrhea, and neurologic signs (ataxia, seizures) with death after 2 days. The same symptoms were observed 3-4 days later in 2 other Miniature Pinschers (45 days of age) and 1 Pekinese (56 days of age). Necropsy of the dogs showed hemorrhagic enteritis, abundant serosanguineous fluid in the abdominal cavity, and severe lesions in the parenchymatous organs. The lungs had multiple, patchy, red areas of consolidation. Livers were yellow-brown and congested, with hemorrhages on their surfaces, and spleens were enlarged with subcapsular hemorrhages. Variable gross

changes in other organs included multifocal hemorrhagic renal cortical infarcts and petechial hemorrhages on lymph node surfaces.

Virologic and bacteriologic investigations on the parenchymatous organs did not detect common canine pathogens, notably canine parvovirus type 2, canine distemper virus, canine adenovirus type 1 and type 2. CCoV type I and type II were identified in the intestinal contents of all puppies by genotype-specific real-time reverse transcription–polymerase chain reaction (RT-PCR) assays (1). CCoV type II RNA was also detected in lungs (median $1.0^8 \times 10^6$ RNA copies/μL of template), spleen (median 4.46×10^6 RNA copies/μL of template), liver (median 9.02×10^4 RNA copies/μL of template), kidney (median 7.54×10^4 RNA copies/μL of template), and brain (median 5.23×10^3 RNA copies/μL of template). Virus-induced cytopathic effect was observed in A-72 cells, and CCoV type II strain (CB/05) was isolated from all tissues examined except brain tissue. Immunohistochemical analysis with a CCoV-specific monoclonal antibody detected CCoV antigen in the organs with gross lesions that were examined (lungs, kidneys, liver, spleen, gut, and lymph nodes) (Fig. 1).

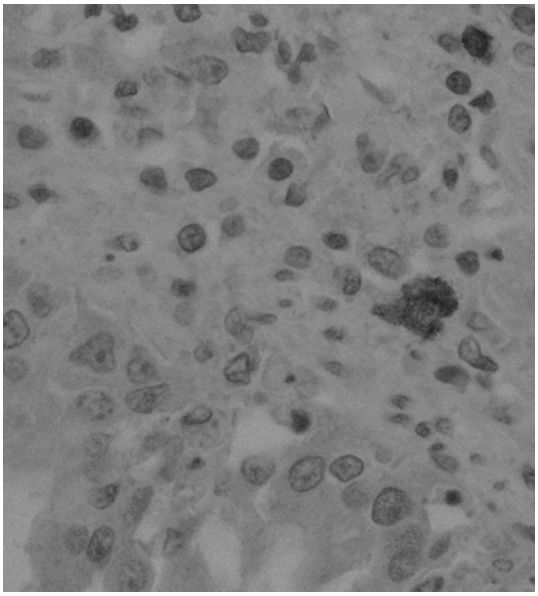

Fig. 1. Immunohistochemical detection of canine coronavirus antigen (brown staining) in canine lung tissue by a specific monoclonal antibody (magnification ×400).

The sequence of the 3′ end of the genome (8.8 kb) of the pantropic CCoV strain was determined by RT-PCR amplification and sequencing of overlapping fragments. The S, envelope, and membrane proteins and nucleoprotein showed a high degree of amino acid identity with the cognate open reading frames (ORFs) of CCoV type II. The S protein of strain CB/05 had the highest identity to FCoV type II strain 79-1146 (Fig. 2).

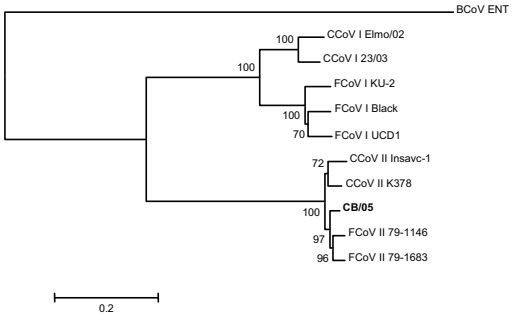

Fig. 2. Neighbor-joining tree of the spike protein of canine coronavirus (CCoV) and feline coronavirus (FCoV). The following reference strains were used for phylogenetic analysis: CCoV type I strains Elmo/02 (Genbank accession no. AY307020) and 23/03 (AY307021); CCoV type II strains Insavc-1 (D13096) and K378 (X77047); FCoV type I strains KU-2 (D32044), Black (AB088223) and UCD-1 (AB088222); FCoV type II strains 79-1146 (X06170) and 79-1683 (X80799); and bovine coronavirus (BCoV) strain ENT (NC_003045). The tree is rooted on BCoV-ENT and drawn to scale. A statistical support was provided by bootstrapping >100 replicates. The scale bar represents 20 substitutions per 100 sequence positions.

Comparison with strain CB/05 was possible only with CCoV type II strains Insavc-1 (5) and BGF (11) and CCoV type I strains Elmo/02 and 23/03 (9, 10) because of a lack of data on the 3′ end of the CCoV genome in the genes encoding for nonstructural proteins (NSPs) 3a, 3b, 3c, 7a, and 7b. NSPs 3a, 7a, and 7b were not altered. NSP 3b (22 aa) was 49 aa shorter than expected because of a 38-nucleotide deletion and a frame shift mutation in the downstream sequence that introduced an early stop codon. NSP 3c (244 aa) was 6 aa shorter and 79 aa longer than the cognate proteins of the enteropathogen strain BGF and the attenuated strain Insavc-1a, respectively.

To confirm the pathogenic potential of strain CB/05, we experimentally infected two 6-month-old dogs (authorization no. 67/2002-C released by Ministry of Health of

Italy). Two milliliters of cryolysate of a lung-derived first-passage virus in A-72 cells were administered intranasally to the dogs. The cell cryolysate tested negative for other common canine pathogens and had a 50% tissue culture infectious dose of $10^{5.50}$/50 µL on A-72 cells and 1.18×10^7 RNA copies/µL of template by real-time RT-PCR. The virus was reisolated from the experimentally infected dogs. Severe clinical symptoms characterized by pyrexia (temperature 39.8°C–40.1°C), anorexia, depression, vomiting, diarrhea, and leukopenia were observed that persisted 8–10 days. Despite the severe symptoms, the dogs slowly recovered from their illness.

CONCLUSIONS

Point mutations or deletions in the S protein and NSPs have been associated with changes in tropism and virulence of coronaviruses (3, 4, 6, 8, 13). CCoV strain CB/05 showed intact structural and nonstructural proteins, with an S protein closely related to that of other type II CCoVs. The only striking change was the truncated form of NSP 3b. Whether the deletion in the ORF of NSP 3b is involved in pathobiologic changes should be assessed with reverse genetic systems.

The present study describes for the first time the occurrence of fatal infections in dogs by coronaviruses. Experimental infection of dogs with the virus isolate resulted in a severe systemic disease that mimicked the clinical symptoms observed in the outbreak. However, the different ages at infection (6 months vs. <2 months) likely resulted in the disease being nonfatal. Accordingly, the appearance of pathogenic CCoV variants should always be regarded as a potential threat to domestic dogs and considered when unexplainable fatal disease outbreaks occur in puppies. Epidemiologic studies are required to determine whether the pantropic CCoV strain is a new coronavirus variant emerging in canine populations or a widespread infectious agent of dogs that usually goes undetected. Vaccination trials could also help determine whether the CCoV vaccines currently available are effective against the highly virulent CCoV strain.

The 2002–2003 SARS epidemic has demonstrated that the study of animal coronaviruses is paramount to understanding the ecology and evolution of human coronaviruses. The coronaviruses of carnivores provide a paradigmatic model of how

coronaviruses cross the species barriers, adapt to new host species, and change their pathogenicity.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Health of Italy (Ricerca corrente 2003: “Studio del coronavirus del cane come modello animale per le ricombinazioni genetiche dei coronavirus”).

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Molecular characterization of the virulent canine coronavirus CB/05 strain

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Virus Research, 2007; 125, 54-60

ABSTRACT

This paper characterizes a virulent strain (CB/05) of canine coronavirus (CCoV) isolated from the internal organs of pups that had died of a systemic disease without evidence of other common canine pathogens. High viral RNA titres were detected in the internal organs by a real-time RT-PCR assay specific for CCoV type II. Sequence analysis of the 3' end (8.7 kb) of the genomic RNA of strain CB/05 revealed conserved structural as well as non-structural proteins, with the exception of a truncated form of non-structural protein 3b. The exceptional form was due to a 38-nucleotide deletion and a frame shift in ORF3b that introduced an early stop codon. By phylogenetic analysis of the structural proteins, the spike (S) protein was found to cluster with feline coronavirus type II strain 79-1683, whereas the envelope (E), membrane (M) and nucleocapsid (N) proteins segregated together with the reference strain Purdue of transmissible gastroenteritis virus of swine.

INTRODUCTION

Canine coronavirus (CCoV) is an enveloped, single-stranded RNA virus responsible for mild to severe enteritis in dogs. CCoV belongs to group I coronaviruses within the family *Coronaviridae*, along with feline coronaviruses (FCoVs) types I and II, transmissible gastroenteritis virus (TGEV) of swine, porcine respiratory coronavirus (PRCoV), porcine epidemic diarrhea virus (PEDV) and human coronavirus 229E (HCoV-229E) (11). The main nonstructural protein of CCoV is the replicase, which is encoded by two large open, partially overlapping, reading frames (ORFs), ORF1a and ORF1b that encompass the 5' two-thirds of the genomic RNA. The structural proteins comprise the S, E, M and N proteins encoded by ORFs 2, 4, 5 and 6, respectively (11). The region between the S and M genes (ORFs 3a, 3b, 3c and 4) comprises four accessory genes that encode nonstructural (nsp3a, nsp3b, nsp3c) as well as structural (E) proteins. The very 3' end of the CCoV RNA is occupied by an untranslated region with a poly-A tail, preceded by two ORFs (7a and 7b) that encode two nonstructural proteins (3, 13, 24, 41, 43, 44).

To date, two different CCoV genotypes are known, which have been designated CCoVs type I and type II on the basis of their genetic relatedness to FCoVs type I and type II, respectively (34, 35). Molecular methods have been established to differentiate those two genotypes (8), which have been shown to cause enteric infections in dogs (36). CCoV type I has not been adapted to in-vitro growth and differs from CCoV type II with a higher genetic relatedness to FCoV type I. There also is an additional ORF, ORF3, located between ORFs 2 and 3a (N. Decaro, unpublished data).

CCoV is generally recognized as the aetiological agent of self-limiting infections of the small intestine, which can lead to clinical signs typical of gastroenteritis, i.e., inappetence, diarrhea and vomiting. Fatal disease commonly occurs as a consequence of mixed infections with CCoV together with canine parvovirus type 2 (CPV-2) (32), canine adenovirus type 1 (CAdV-1) (9, 33) or canine distemper virus (CDV) (5). Systemic disease has not previously been ascribed to CCoV infection alone, although the virus has been isolated previously from several tissues (tonsils, lungs and liver) of pups infected experimentally (40).

Recently, a highly virulent CCoV strain (CB/05) was described (2) and it was shown to have been responsible for an outbreak of fatal, systemic disease in pups. In the present study, we report the molecular characterization of CCoV strain CB/05.

METHODS

Clinical case

In May 2005, a severe outbreak of a fatal, systemic disease affected seven dogs housed in a pet shop in the Apulia region of Italy (2). Clinical signs were first observed in four pups that comprised three Miniature Pinschers and one Cocker Spaniel, 45 and 53 days of age, respectively. Signs in the index case consisted of fever (39.5-40°C), lethargy, inappetance, vomiting, haemorrhagic diarrhea, leukopenia (WBC counts < 3,000 cells/μl) and neurological signs (ataxia, seizures), followed by death within 2 days after the onset of the symptoms. Similar signs were observed a few days later in two additional Miniature Pinschers (117/05-A, 117/05-B) and in a 56 day-old Pekinese pup (117/05-C) that died rapidly. The bodies of those three pups were submitted for laboratory investigation. Necropsy examination revealed severe gross lesions in lungs, liver, spleen, and kidneys. Extensive subacute bronchopneumonia was evidenced both in the cranial and caudal lobes, while coalescing degeneration was present within the livers and spleens; also zones of haemorrhagic infarction were observed in the kidneys.

Screening for canine pathogens

Using standardized methods, samples of brain, lung, liver, spleen, kidney, mesenteric lymph nodes and intestinal contents were examined for the major canine bacterial and viral pathogens, e.g., *Bordetella bronchiseptica*, *Pasteurella multocida*, *Leptospira interrogans* (16), reoviruses (7, 28), rotaviruses (15), caliciviruses (21, 29), CPV-2 (6), canine adenoviruses (23), CDV (10), canid herpesvirus (39), CCoV (4).

Virus isolation

Intestinal contents and tissue samples were homogenized (10% w/v) in Dulbecco's minimal essential medium (D-MEM), together with antibiotics (penicillin 5000 IU/ml, streptomycin 2500 μg/ml, amphotericin B 10 μg/ml), and inoculated into cell cultures.

The following cell lines were used for virus isolation attempts: canine fibroma (A-72), Madin Darby canine kidney (MDCK), Walter Reed canine cells (WRCC), Crandell feline kidney (CrFK), and *felis catus* whole foetus (fcwf). The cells were grown in D-MEM supplemented with 10% foetal calf serum (FCS). When the monolayers were

confluent, the medium was removed and the cells were washed two times with FCS-free medium and inoculated with clarified tissue homogenates. After an adsorption period of 60 min at 37°C, the fluid portion of each inoculated cell culture was replaced with fresh serum-free medium. Infected cells were monitored daily for the occurrence of cytopathic effects (CPE) and, after 5 days of incubation, the inoculated cells were tested for CCoV antigen by an immunofluorescence (IF) assay using a monoclonal antibody targeting the N protein. Each sample was passaged three times prior to being judged negative for CCoV.

Typing and quantitation of CCoV

RNAs extracted with the commercial kits QIAamp® Viral RNA Mini Kit (Qiagen S.p.A., Milan, Italy) from the intestinal contents, and QIAamp® RNeasy Mini Kit (Qiagen S.p.A.) from the tissue samples, were subjected to differential real-time RT-PCR assays for identification and quantitation of CCoV type I and type II (8). Reverse transcription was carried out using GeneAmp® RNA PCR (Applied Biosystems, Applera Italia, Monza, Italy), following the manufacturer's recommendations. Real-time PCR for both genotype-specific assays was performed in a 50 µl-reaction mixture containing 25 µl of IQ™ Supermix (Bio-Rad Laboratories Srl), 600 nM of primers CCoVI-F (CGTTAGTGCACTTGGAAGAAGCT)/CCoVI-R (ACCAGCCATTTTAAATCCTTCA) or CCoVII-F (TAGTGCATTAGGAAGAAGCT)/CCoVII-R (AGCAATTTTGAACCCTTC), 200 nM of probe CCoVI-Pb (FAM - CCTCTTGAAGGTACACCAA-TAMRA) or CCoVII-Pb (FAM - CCTCTTGAAGGTGTGCC-TAMRA) 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 (CCoV type I-specific assay) or 48°C (CCoV type II-specific assay) for 30 s and extension at 60° C for 1 min.

Sequence and phylogenetic analyses of the virulent CCoV strain

The 3' end of the genome of strain CB/05 was amplified using SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies, Invitrogen. Milan, Italy), according to the manufacturer's instructions. Seven partially overlapping fragments encompassing the ORFs 2 (S gene), 3a, 3b, 3c, 4 (E gene), 5 (M gene), 6 (N gene) 7a and 7b were amplified using primer pairs El-Ins1 (GGATTACTAARGADKGGTAAGTTGC)/S2 (CAACTTCACTTGAAGCAACA), SIIF (GCATTAGTAGTTGAAAACACAGC)/SIIR

(TCAAGTCTGGCACCCATTG), S3 (GAGACTTTCAACCAATTAGC)/UCD6R (TTAATGAATGTGAACTTTTTCAATAGG), SM1F (CAGTTTGAAAGTTATGAACCTATTGA)/M6 (TCCCTGAGAGGACATTTAGA), MNIF (CAACAGATGCAAGAACTGAYAA)/MNIR (TTAGTTCGTTACCTCATCAATAATCTC), 7abF (TGATCCTAAGACTGGACAATTCC/7abR (AAATCTAGCATTGCCAAATCAAA).

The PCR-amplified products were sequenced by Genome Express (Meylan, France) and the obtained sequences were assembled and analyzed using the BioEdit software package (20) and the NCBI's (htttp://www.ncbi.nlm.nih.gov) and EMBL's (http://www.ebi.ac.uk) analysis tools. GenBank accession number DQ112226 was assigned to the sequenced 8.7-kb fragment. ORFs contained in the amplified genomic region were determined either with the ORF Finder tool of NCBI or on the basis of the similarity to known coronavirus proteins. The ORFs identified in this manner were translated and the predicted amino acid (aa) sequences were saved as individual files for further analyses. Phylogenetic and molecular evolutionary analyses were conducted using Mega3 (27). Phylogenetic trees, based on the S, E, M and N proteins of CCoV strain CB/05 were elaborated using both parsimony and neighbor-joining methods, supplying a statistical support with bootstrapping over 100 replicates.

RESULTS

CCoV detection and isolation from tissue samples of dogs infected naturally

The bodies of the dead pups tested negative by all of the screening assays; however, real-time RT-PCR for CCoV detected viral RNA in the intestinal contents and, unexpectedly, in all the tissue samples examined. By genotype-specific real-time RT-PCR assays, both CCoV genotypes were detected in the intestinal contents. Viral loads ranged from 4.23×10^4 to 8.99×10^3 and from 9.24×10^4 to 6.12×10^5 RNA copies/µl of template for CCoV type I and type II, respectively. On the other hand, only CCoV type II was detected in the parenchymatous organs, with RNA titres ranging from 9.52×10^2 (pup

117/05-B, brain) to 6.87 x 10^6 RNA copy numbers/µl of template (pup 117/05-C, spleen) (Table 1). Sequence analysis showed a 100% nucleotide (nt) identity among the real-time RT-PCR products amplified from the samples of the three pups (data not shown).

CCoV type II was successfully isolated in cell cultures from all tissue samples, except the brain. Viral titres of the brain samples were found to be very low by type-specific real-time RT-PCR (Table 1). Only A-72 and fcwf cells were found to be permissive to viral infection**;** in contrast, VERO, MDCK and CrFK cells were negative by the IF test for CCoV after three passages. Virus isolation was achieved at the first passage from the spleen and lungs of pup 117/05-C (strain CB/05). The CPE was characterized by cell rounding and lysis of the infected monolayers. Viral titres reached at the 3rd passage on A-72 and fcwf cells were $10^{5.75}$ and $10^{3.25}$ $TCID_{50}$/50 µl of viral suspension, respectively.

Table 1. CCoV RNA titres in the organs of dogs infected with strain CB/05

	117/05-A		117/05-B		117/05-C	
Tissue/sample	RRT-PCR	VI	RRT-PCR	VI	RRT-PCR	VI
Faeces	6.12 x 10^5	+	9.24 x 10^4	+	3.17 x 10^5	+
Lung	1.08 x 10^6	+	2.74 x 10^4	+	2.32 x 10^6	+
Spleen	4.46 x 10^6	+	1.20 x 10^5	+	6.87 x 10^6	+
Liver	9.02 x 10^4	+	5.98 x 10^4	+	3.12 x 10^5	+
Kidney	7.54 x 10^5	+	1.40 x 10^4	+	2.53 x 10^5	+
Mesenteric lymph node	8.09 x 10^4	+	2.01 x 10^4	+	5.17 x 10^4	+
Brain	5.23 x 10^3	-	9.52 x 10^2	-	1.25 x 10^4	-

RRT-PCR, real-time RT-PCR. Results are expressed as CCoV type II RNA copy number/µl of template.
VI, virus isolation. Results are expressed as positive (+) or negative (-).

Sequence analysis and phylogeny

The nt sequence of a 8.7-kb genomic region of strain CB/05 was determined by amplification and sequencing of overlapping fragments, including the full length of the spike (S), 3a, 3b, 3c, envelope (E), membrane (M), nucleoprotein (N), 7a and 7b genes. The inferred amino acid (aa) sequences were compared to the analogous sequences available in the online databases. The spike protein was 1454 aa in length and**,** by analysis with the NetNglyc server (http://www.cbs.dtu.dk/services/NetNGlyc/)**,** was found to contain 31 potential N-glycosylation sites, matching the best identity (95%) to FCoV type II strain 79-1683 (Table 2).

Table 2. Amino acid identity in the structural proteins of group I CoVs reference strains to strain CB/05

Coronavirus strain	GenBank accession no.	Amino acid identity (%) to isolate CB/05			
		S	E	M	N
CCoV II-Insavc1	**D13096**	92	95	90	92
CCoV II-BGF10	**AY342160**	92	87	89	92
CCoV I-Elmo/02	**AY307020** (S), **AY426983** (E), ND (M, N)	44	85	87	89
CCoV I-23/03	**AY307021** (S), **AY426984** (E) **AY548235** (M, N)	44	86	86	89
FCoV II-79-1146	**NC_007025**	94	78	84	77
FCoV II-79-1683	**X80799** (S), **FCY13921** (E, M, N)	95	97	87	79
FCoV I-KU-2	**D32044** (S), **AAB47501** (M) **AB086881** (N)	43	NA	82	76
FCoV I-Black	**AB088223** (S), **AB086903** (M, N),	42	NA	82	77
FCoV I-UCD1	**AB088222** (S), **AB086902** (M, N)	43	NA	84	77
TGEV-Purdue	**NC_002306**	80	96	94	95

ND, sequence not deposited.

NA, sequence not available.

By phylogenetic analysis, two divergent CCoV clusters could be identified, with strain CB/05 segregating with type II CCoVs and FCoVs (Fig. 1a). Several residues encountered in the N-terminus of the S protein were unique to strain CB/05, including Ile-4, Val-8, Ala-18, Asp-34, Pro-73, Ala-116, Asn-125, His-129, Thr-143, Arg-154, His-197, Asp-255, and Gly-291, Ala-407, Thr-459. In order to verify whether the same substitutions were shared by other strains circulating in Italy, the 5' end of the S gene, encoding for the N-terminal 500 amino acids of the S protein, was amplified from five type II CCoV strains recovered from recent field outbreaks of enteritis in dogs from different regions of Italy. By sequence analysis, a 94-98% identity was found in the deduced amino acid sequences (data not shown). All of the N-terminus residues initially found unique to strain CB/05

were shared by the CCoV strains currently circulating in Italy**,** with the exception of residues Pro-73, Asn-125 and Ala-407 that were present only in strain CB/05. An aa substitution (Asp to His) at position 125 also was displayed by the virulent strain BGF10.

The envelope protein was 82 aa long and had the highest identity (97%) to FCoV type II strain 79-1683 (Table 2), in whose cluster it was found to fall (Fig. 1b). Due to a lack of data from the 3' end of the CCoV genome in the genes encoding for nonstructural proteins, comparison of strain CB/05 was possible only with CCoV type II strains Insavc-1 (24) and BGF10 (38) and with CCoV type I strains Elmo/02 and 23/03 (N. Decaro, unpublished data). While the nonstructural protein (nsp) 3a had the same length as the other type I and type II CCoVs (78 aa), the nsp3b was 22-aa long (49-aa shorter than expected) due to the presence of a 38-nt deletion at position 4704 and to a frame shift in the sequence downstream from the deletion that introduced an early stop codon (Fig. 2). Genetic analysis of enteric CCoV strains circulating in Italy revealed that the deletion in ORF3b was unique to CB/05 strain (data not shown). The nsp3c (244 aa) was 6-aa shorter and 79-aa longer than the cognate proteins of the enteropathogen strain BGF10 and the attenuated strain Insavc-1, respectively.

The membrane protein was 262 aa in length and contained 3 N-glycosylation sites. The highest identity (94%) was obtained against TGEV strain Purdue (Table 2), in whose cluster the virulent CCoV was found to fall by phylogeny (Fig. 1c). In the N protein, which was 383 aa long, strain CB/05 was found to be closely related to TGEV strain Purdue both by sequence analysis (95% of aa identity, Table 2) and phylogeny (Fig. 1d). Nsp7a was 101 aa long and had one N-glycosylation site, whereas nsp7b had a length of 213 aa and no N-glycosylation sites, unlike to other type I and type II CCoVs that displayed one N-glycosylation site. However, by sequence analysis, both nsp7a and nsp7b were found closely related to the analogous proteins of strains BGF (96% and 79% of aa identity, respectively) and Insavc-1 (95% and 79% of aa identity, respectively).

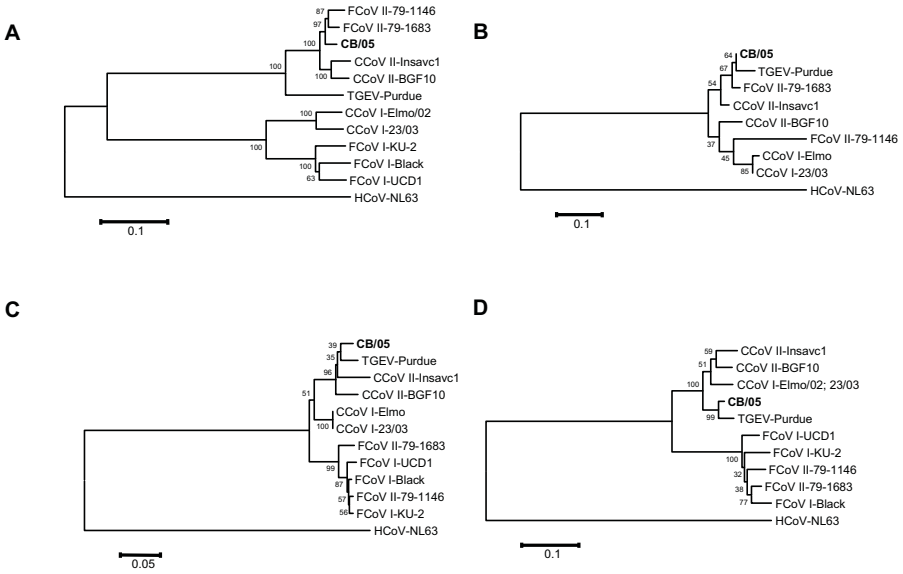
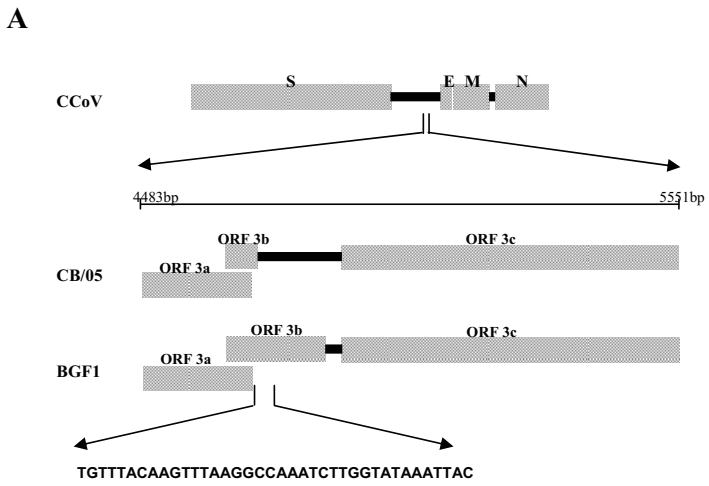


Fig. 1. Neighbor-joining trees based on the S (**A**), E, (**B**), M (**C**) and N (**D**) proteins of group I coronaviruses. Accession numbers of the strains used for phylogeny are reported in Table 2. The trees are rooted on group II coronavirus HCoV-NL63 (accession no. NC_005831) and drawn to scale. A statistical support was provided by bootstrapping over 100 replicates. The scale bars represent the numbers of amino acid substitutions.

DISCUSSION

The emergence of a pantropic variant of CCoV, reported in the present study, is paradigmatic of the evolution of coronaviruses. The ORF2 of PRCoV has a 200-aa deletion in the N-terminus with respect to TGEV, from which it presumably had arisen. Most likely, this deletion is responsible for the change in the viral pathobiology (42). Nevertheless, minor amino acid differences in the sequence of the spike protein have been shown to change the virulence of even very closely related TGEV isolates (37). The enteric biotype of FCoV, feline enteric coronavirus (FECV), causes persistent infections of the intestinal mucosa that may lead to point mutations in the S gene and/or deletions in the group-specific genes 3c, 7b (45) or 7a (25). Those mutations are considered responsible for changes in the tropism of the virus, which acquired the ability to infect monocytes/macrophages and to cause a systemic, fatal disease of cats (feline infectious peritonitis, FIP). An FIPV mutant lacking the group-specific genes 3abc or 7ab resulted in

live attenuated vaccines protective against FIP (19). Similar drastic shifts of tissue tropism have been observed with murine coronaviruses (22). Adaptation to humans of the recently recognized SARS-associated coronavirus (SARS-CoV) appears to be related to minor genome mutations, consisting of a 29-nt deletion in the genome of a wild-mammal coronavirus, that resulted in the translation of two different ORFs, 10 and 11, instead of the single ORF10 (17).



B

```
                    *         20         *         40
CB/05 : MLNLVSLLLKKSIVIQLFDITF------------------ : 22
BGF10 : .....A..............VYKFKAKSWYKLPFETRLR : 40

                    *         60         *
CB/05 : ------------------------------ :  -
BGF10 : IIEQTKPKALSVTKQAKTDYRKTAILNSMRK : 71
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Fig. 2. A. Schematic representation of the 3' end of the viral genome of CCoV strains CB/05 and BGF10. The 38-nt deletion in ORF3b of strain CB/05 is reported. **B.** Comparison between the amino acid sequences of nsp3b of strains CB/05 and BGF10.

The association of strain CB/05 to a severe, fatal disease of dogs, together with the isolation of the virus from organs with extensive lesions, strongly suggests that CCoV also may have changed its tropism and acquired the ability to spread from the enteric tract to the internal organs.

CCoV strain CB/05 displayed intact structural and nonstructural proteins, with a spike protein closely related to that of other type II CCoVs and FCoVs, mostly of FCoV type II

strain 79-1683 (95% of aa identity). In contrast to reference type II CCoVs and FCoVs, several point mutations were found in the N-terminus of the S protein of strain CB/05. However, most of these amino acid changes were not associated to increased pathogenicity or change in tropism since the same substitutions were observed in enteric CCoVs recovered from recent outbreaks of canine gastroenteritis. Only residues Pro-73, Asn-125 and Ala-407 were unique to strain CB/05, although a substitution at position 125 (Asp to His instead Asn) was found in the virulent strain BGF10.

A conspicuous change observed in the CB/05 genome was the truncated form of nsp3b, but whether the deletion in the ORF3b is involved in the change of virulence should be assessed by using reverse genetics systems similar to those recently used for FIPV (18). Further studies also are required to verify whether the truncated protein is translated. Recently, a 61-64-conspicuous nucleotide deletion in the intergenic S-3a region was tentatively associated to increased virulence of the enteric CCoV strain BGF10 (38). Similar mutations also have been shown to influence the level of nsp3b translation in TGEV (30). However, the same or more extended deletions have been found in strain CB/05 as well as in almost all nonvirulent strains circulating in Italy (C. Buonavoglia, unpublished data). Moreover, in strain BGF10 a long nsp3b protein (79-aa longer than the 171-aa protein of strain Insavc-1) is present, in contrast with the truncated protein predicted for the virulent strain CB/05.

Two cases of fatal coronavirus disease in pups without evidence of co-infection by CPV-2 have been reported by Evermann et al. (12). CCoV infection was demonstrated by immunohistochemistry on gut sections and electron microscopy of intestinal contents. Although CPV-2 was not identified in those cases, the gross lesions and histopathological changes were suggestive of CPV infection. It is possible, however, that the moderate depletion and necrosis of lymphoid tissues (thymus, spleen, lymph nodes and gut-associated lymphoid tissues) in both pups could be related solely to a pantropic CCoV infection. However, this hypothesis cannot be supported since investigations for CCoV were not carried out on the internal organs.

The growth characteristics of strain CB/05 were evaluated by using cell lines commonly used for isolation of canine viral pathogens. Viral growth occurred only on canine A-72 and feline fcwf cells, with higher titres found in the former cell line. Despite the high similarity to FCoV strain 79-1683 in the S protein, responsible for viral attachment on cell receptors (14, 26), strain CB/05 did not grow on feline CrFK cells. This

finding is in contrast with observations on other type II CCoV and FCoV strains which are easily adapted to CrFK cells (1, 31).

In this study, we have analyzed a pantropic variant of CCoV associated with a fatal disease of dogs characterized by leukopenia, gastroenteritis and severe lesions in the major organs. Our findings suggest that the novel pantropic CCoV variant can cause a fatal disease of dogs, whose clinical and diagnostic features should be recognized by veterinarians since the clinical course may resemble CPV-2 infection. In contrast, the gross lesions of this new CCoV disease are very similar to those observed in dogs with infectious canine hepatitis caused by CAdV-1.

Further investigation would provide new insights into the molecular mechanisms responsible for the change in viral pathobiology and into the pathogenic and immunological aspects of the pantropic CCoV infection.

ACKNOWLEDGEMENTS

This study was supported by grants of Ministry of Education, University and Research to N.D. (PRIN 2005, project "Il coronavirus del cane: aspetti molecolari e patogenetici") and of Ministry of Health to M.T. (Ricerca corrente 2003, project "Studio del coronavirus del cane come modello animale per le ricombinazioni genetiche dei coronavirus"). The authors are grateful to the undergraduate students Giuseppe Grandolfo and Angela Segreto for their excellent assistance with part of the experimental work.

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Experimental infection of dogs with a novel strain of canine coronavirus causing systemic disease and lymphopenia

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Veterinary Microbiology, 2008; 128, 253-260

ABSTRACT

A pantropic canine coronavirus (CCoV) strain (CB/05) has been recently associated to a fatal outbreak of systemic disease in young dogs. We report the clinical, virological and serological findings in dogs experimentally infected with strain CB/05. The dogs, three 2.5-month-old and two 6-month-old pups, were successfully infected, shedding viral RNA with their faeces for the entire observation period (21 days) and displaying systemic clinical signs resembling those observed during the course of natural infection. Leucopenia (acute lymphopenia) occurred in all infected dogs, with values dropping below 60% of the initial counts. Considering the severity of the CB/05-induced disease, two of the youngest pups were euthanized for ethical reasons at days 8 -9 postinfection, whereas the other pups underwent a slow but progressive improvement of their clinical status with complete recovery. At postmortem examination, remarkable lesions were observed in the internal organs of the euthanized pups, that tested positive for CCoV by real-time RT-PCR and virus isolation on cell cultures. All pups seroconverted for CCoV, as shown by the high optical density values and antibody titres detected by ELISA and virusneutralization tests, respectively. The present study confirms that strain CB/05 is highly pathogenic for dogs, being able to induce a severe disease (and in some cases the death) even in experimental conditions.

INTRODUCTION

Coronaviruses (CoVs) are enveloped, single-stranded RNA viruses which included in three different antigenic groups. CoVs infecting dogs comprise canine enteric coronavirus (CCoV) (10) and the newly recognized canine respiratory coronavirus (CRCoV) (7, 11), belonging to group 1 and group 2 CoVs, respectively. Two CCoV genotypes have been identified so far, namely CCoV type I and CCoV type II, which are responsible for the occurrence enteritis in dogs and are frequently associated in mixed infections (6, 16). Although its tropism is restricted to the gastroenteric tract, CCoV has been recently associated to systemic disease followed by fatal outcome in pups (1). Severe clinical signs were observed in the affected pups, whereas necropsy examination revealed remarkable gross lesions in lungs, liver, spleen, and kidneys. Virological and bacteriological investigations failed to detect common canine pathogens. Unexpectedly, CCoV type II RNA was detected at very high titres in the internal organs of the dead pups and the virus (strain CB/05) was isolated on canine cell cultures. The association of strain CB/05 to a severe, sometimes fatal disease of dogs, together with the isolation of the virus from organs with severe lesions, strongly suggests that CCoV has changed its tropism, acquiring the ability to spread from the enteric tract to the internal organs (8).

In this study, we report the results of the experimental infection with isolate CB/05 in pups with different age, showing that in contrast with classical CCoVs, this virus is able to cause systemic disease followed by fatal outcome in younger pups.

MATERIALS AND METHODS

Cells and virus

Canine fibroma A-72 cells were grown in Dulbecco's minimum essential medium supplemented with 10% foetal calf serum. Strain CB/05 was isolated from the lungs of a dead pup (117/05-C) and adapted to growth on A-72 cells. At the 3rd passage, the virus was titrated on cell cultures and inocula containing $10^{6.25}$ $TCID_{50}$/ml of viral suspension were stored at -70°C. Contaminations by other canine pathogens, such as canine parvovirus type 2 (CPV-2), canine distemper virus (CDV) and canine adenoviruses (CAdVs), were ruled out by specific molecular assays (4, 9, 12).

Experimental study

The experimental study was performed according to the animal health and well-being regulations and was authorized by the Ministry of Health of Italy (authorization no. 53/2005-C). Six mixed-bred female dogs including four 2.5-month-old (*n* = 1-4) and two 6-month-old (*n* = 5, 6) pups were housed at the "Infectious Disease Unit" of the Animal Hospital, Faculty of Veterinary Medicine of Bari. The dogs had tested negative for CCoV RNA by a real-time RT-PCR assay (3) carried out on the faeces and for CCoV antibodies by an ELISA test (15) carried out on serum samples. All dogs were housed individually in separate boxes, fed twice daily with a commercial dry dog food and provided water *ad libitum*. After an acclimatization period of 5 days, 5 animals (*n* = 1, 2, 3, 5, 6) were administered oronasally 3 ml of a viral suspension of strain CB/05, with a titre of $10^{6.25}$ $TCID_{50}$ and 7.85 x 10^7 RNA copies per ml, whereas one dog (*n* = 4), 2.5-month old, was maintained uninfected by oronasal administration of 3 ml of sterile saline solution.

Clinical score, virus isolation and real-time RT-PCR

The clinical condition of each dog was monitored daily for 21 days. A scoring system was devised taking into account rectal temperatures, total white blood cell (WBC) counts, appearance of clinical signs (vomiting, diarrhea, depression, loss of appetite, dehydration), following the scheme adopted in a previous study (5) and derived by Nakamura et al. (14), with some modifications (Table 1).

Due to ethical reasons, dogs whose total clinical score reached a value ≥ 15 were euthanized by intravenous administration of 10 mg/kg of body weight of Zoletil 100 (Virbac S.r.l., Italy) followed by 0.5 ml/kg body weight of Tanax (Intervet Italia, Italy).

EDTA-treated blood samples were collected daily for total and differential WBC counting and for testing for CCoV RNAemia by real-time RT-PCR (3). The presence of the viral RNA in the blood was also evaluated at hours 3, 6, 9, 12 and 18 after inoculation. Plasma samples were prepared daily to evaluate free viral RNAemia and weekly to determine CCoV antibody titres by virus neutralization (VN) and ELISA tests (15).

To evaluate the viral shedding in the faeces, the rectal swabs collected daily from the control dog and from the dogs inoculated with strain CB/05 were subjected to RNA extraction using QIAamp® Viral RNA Mini Kit (Qiagen S.p.A.). In addition, tissue samples from parenchymatous organs were withdrawn from the two dead pups (Table 2).

Table 1. Scoring system for clinical signs of CB/05 infection[a]

Clinical signs	Daily score
Rectal temperature (°C)	
≤ 37.6 °C	3
37.7 - 38.7 °C	0
38.8 - 39.7 °C	1
39.8 - 40.0 °C	2
≥ 40.1 °C	3
WBC count[b]	
75-60	1
59-45	2
44-30	3
≤30	4
Diarrhea	
Mucoid	1
Fluid	2
Dysenteric	3
Haemorrhagic	4
Loss of appetite	
Dysorexia	1
Anorexia	3
Vomiting (episodes/day)	
1	1
>1	3
Depression	
Mild	1
Moderate	2
Severe	3
Dehydration	
Mild	1
Moderate	2
Severe	3

[a] Adapted from reference 5.

[b] Values are expressed as percentages of the WBC counts determined prior to inoculation.

RNA was extracted from the WBC pellets using QIAamp® RNA Blood Mini Kit (Qiagen S.p.A.), from the plasma samples using QIAamp® Viral RNA Mini Kit and from the tissue samples using QIAamp® RNeasy Mini Kit. Attempts to isolate the virus in A-72 cells were carried out on rectal swabs of all infected pups and on organs of the sacrificed animals as described previously (8).

Real-time RT-PCR targeting the M gene of CCoV type II (GenBank accession number D13096) was carried out on the RNA extracts as described elsewhere (6). The genotype-specific RT-PCR assays were undertaken in an i-Cycler iQ™ Real-Time Detection System (Bio-Rad Laboratories Srl, Milan, Italy) and the data were analyzed with the appropriate sequence detector software (version 3.0). After reverse transcription, triplicates of the CCoV type II standard dilutions and RNA templates were simultaneously subjected to real-time analysis. The 50 µl-reaction mixture contained 25 µl of IQ™ Supermix (Bio-Rad Laboratories Srl), 600 nM of each primers CCoVII-F (TAGTGCATTAGGAAGAAGCT) and CCoVII-R (AGCAATTTTGAACCCTTC), 200 nM of probe CCoVII-Pb (FAM -CCTCTTGAAGGTGTGCC-TAMRA) 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 48°C (type II-specific assay) for 30 s and extension at 60° C for 1 min.

Evaluation of antibody response to CCoV

Plasma samples from inoculated dogs were tested in parallel by virus neutralization (VN) and ELISA tests (15).

For VN test, duplicates of serial twofold dilutions of heat-inactivated plasmas (starting from dilution 1:2) were mixed with 100 $TCID_{50}$ of the isolated strain CB/05 in 96-well microtitre plates. After preincubation at room temperature for 90 min, 20,000 A-72 cells were added to each well. The plates were read after 4 days of incubation at 37°C. VN titres were calculated with the Karber method and expressed as the highest plasma dilution that was able to neutralise the virus.

For ELISA, microtitre plates were coated with CCoV antigen (enteric strain S-378) and, after treatment with blocking solution (0.2% gelatin in carbonate buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6]) and repeated washing, the 1:50 dilutions of the plasma samples were added to each well. Then the plates were incubated for 90 min at 37°C,

washed four times and incubated for 60 min at 37°C with anti-dog IgG-goat peroxidase conjugates (Sigma-Aldrich srl, Milan, Italy). After another washing cycle, 10 mg of freshly prepared substrate, 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]diammonium salt (ABTS, Sigma-Aldrich srl) in 50 ml of 0.05 M phosphate citrate buffer (pH 5.0) was placed in each well and the optical density at 405 nm (OD_{405}) was determined. Positive and negative controls were used as described previously (15).

RESULTS

Clinical signs and WBC counts

Neither clinical signs or viral shedding were observed in the control dog, whose leukocyte counts did not draw away from the baseline values. All the inoculated animals displayed severe clinical signs similar to those observed in dogs infected naturally, although the outcome of the disease was different on the basis of the age (Fig. 1). In fact, two out of the 3 youngest pups (dogs 1 and 3) had to be euthanized for ethical reasons, at day 8 and 9 postinfection (p.i.), respectively, while both the 6-month-old pups recovered from the disease, albeit very slowly. Irrespective of the final outcome, i.e, euthanasia or recovery, clinical signs were remarkably similar in all inoculated animals.

The 2.5-month-old dogs that underwent a fatal outcome (Fig. 1a) showed fever at days 1-2 p.i., with a peak of 39.9°C at day 2 p.i. (dog 1), and from day 1 to 6 p.i., with a peak of 40.0°C at day 3 p.i. (dog 3). Depression (day 3 to 8 p.i.), anorexia/dysorexia (day 3 to 9 p.i.), haemorrhagic diarrhea (day 2 to 7 p.i.), and vomiting (days 4-5 p.i.) also occurred. Leukopenia appeared at day 3 (dog 1) or 2 p.i. (dog 3), with total WBC counts remaining below 60% of the baseline values until euthanasia. Acute lymphopenia was observed in both dogs, with lymphocyte numbers dropping below 60% of the initial counts from day 3 p.i. until death (mean, 16.3%; 0.9 x 10^3 lymphocytes/µl at day 8 p.i.). Postmortem examination revealed severe changes in the intestines and major organs, which were very similar to those observed in dogs infected naturally (data not shown).

The 2.5-month-old dog that survived (Fig. 1b) displayed less severe symptoms, consisting of fever (up to 39.2°C) from day 1 to 5 p.i. and at days 11-12 p.i., and mucoid diarrhea (day 2 to 6 p.i.). Total WBC counts dropped below the 60% of the initial counts from day 3 to 6 p.i., whereas severe lymphopenia (below 60% of baseline values) was registered from

day 1 to 8 p.i, with a peak at day 5 (19%; 1.6 x 10^3 lymphocytes/µl). Subsequently, the total number of peripheral blood lymphocytes started to rise again and the clinical signs subsided. A mild loss of appetite occurred from day 1 to 6 p.i.

In the two 6-month-old pups (Fig. 1c), fever showed a biphasic course, with a first peak at day 2 p.i. of 39.8 and 40.1°C in dogs 5 and 6, respectively. A transient remission was observed from day 5 to 8 p.i. (dog 5) and at day 5 p.i. (dog 6), and a second episode of pyrexia occurred from day 9 to 14 p.i. with a peak of 39.5°C at day 10 p.i. (dog 5), and from day 6 to 8 p.i. with a peak of 39.4°C at day 7 p.i. (dog 6). Depression was observed between days 3 and 8 p.i., whereas vomiting appeared only sporadically in the same period (days 3, 4, 8 p.i. in dog 5; days 1, 3, 8 p.i. in dog 6), with 1-4 episodes per day. Both dogs displayed anorexia (days 3 to 7 p.i.), mucoid or fluid diarrhea (days 3 to 10), and leucopenia, with WBC values dropping below 60% of the baseline from day 3 to 6 (dog 5) or 3 to 8 p.i. (dog 6). Lymphocytes dropped below 60% of the initial cell counts from day 3 to 6 p.i. in dog 5 an from day 3 to 8 p.i. in dog 6 (mean, 32.2%; 1.6 x 10^3 lymphocytes/µl at day 3 p.i.). Starting from day 10 (dog 5) or 9 p.i. (dog 6), the clinical conditions of the dogs improved progressively, with a complete recovery at days 15-16 p.i.

The control (uninfected) pup (Fig. 1d) remained in a good clinical status during the entire observation period and no variations in total WBC and lymphocyte counts were observed.

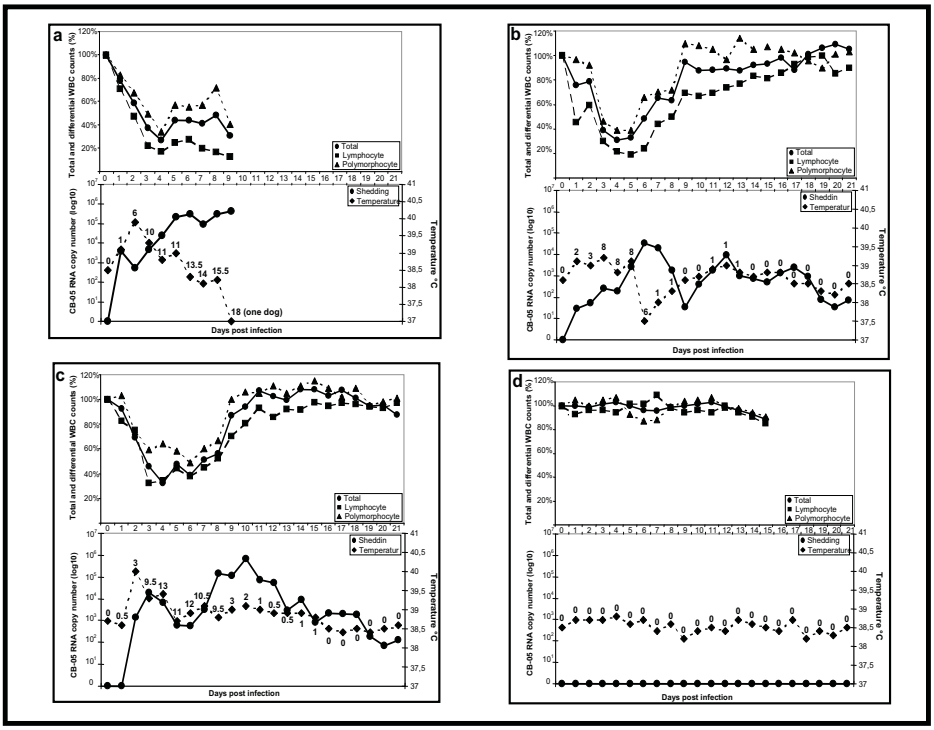

Fig. 1. Disease progression in dogs infected experimentally with strain CB/05. (**a**). 2.5-month-old dogs that were euthanized (dogs 1 and 3, means); (**b**). 2.5-month-old dog that survived (dog 2); (**c**). 6-month old dogs (dogs 5 and 6, means); (**d**). control dog (dog 2). Dogs inoculated oronasally with CB-05 strain were monitored for up to 21 days for total WBC, lymphocyte and polymorphocyte counts (top graphs). In addition, fever, viral RNA shed in faeces and clinical score where determined (bottom graphs). Total WBC, lymphocyte, and polymorphocyte counts are presented as percentages of the cell counts determined at day 0. Viral RNA titres as determined by real-time RT-PCR are expressed as log copy numbers (log_{10}) per µl of template. Clinical scores were calculated as shown in Table 1 and are reported for each day in correspondence of the temperature curves.

Detection of CCoV RNA and CCoV antibodies

The uninfected dog tested constantly negative for CCoV RNA. All the CCoV-infected dogs tested negative for other common pathogens of dogs, including CCoV type I (6), CDV (9), CAdVs (12) and CPV-2 (4).

The faecal shedding of the infected dogs followed the similar pattern, although higher viral RNA titres were obtained from the two sacrificed animals in comparison to survivors (Fig. 1). The pups that succumbed shed virus starting between days 1 and 3 p.i. and lasting until the day of death, with a peak at day 6 (titre of 4.97 x 10^5 RNA copy numbers/µl of template) or 9 (titre of 8.72 x 10^5 RNA copy numbers/µl of template). After their death, CCoV type II RNA was detected in the organs at titres slightly lower than those observed in the dogs of the natural outbreak (Table 2).

Table 2. CCoV RNA titres in the organs of dogs succumbed to natural or experimental infection with strain CB/05

	117/05-A[a]		117/05-B[a]		117/05-C[a]		Dog 1[b]		Dog 3[b]	
	RRT-	VI	RRT-	VI	RRT-	VI	RRT-	VI	RRT-	VI
Tissue/sample	PCR		PCR		PCR		PCR		PCR	
Faeces	$6.12x10^5$	+	$9.24x10^4$	+	$3.17x10^5$	+	$5.14x10^4$	+	$1.09x10^5$	+
Lung	$1.08x10^6$	+	$2.74x10^4$	+	$2.32x10^6$	+	$6.79x10^3$	-	$2.99x10^4$	+
Spleen	$4.46x10^6$	+	$1.20x10^5$	+	$6.87x10^6$	+	$5.48x10^4$	+	$3.07x10^4$	+
Liver	$9.02x10^4$	+	$5.98x10^4$	+	$3.12x10^5$	+	$1.93x10^4$	+	$4.70x10^3$	-
Kidney	$7.54x10^5$	+	$1.40x10^4$	+	$2.53x10^5$	+	$5.11x10^3$	-	$2.94x10^3$	-
Mesenteric lymph node	$8.09x10^4$	+	$2.01x10^4$	+	$5.17x10^4$	+	$2.19x10^4$	-	$3.61x10^4$	+
Brain	$5.23x10^3$	-	$9.52x10^2$	-	$1.25x10^4$	-	$3.32x10^2$	-	$6.00x10^2$	-

RRT-PCR, real-time RT-PCR. Results are expressed as CCoV type II RNA copy number/µl of template.

VI, virus isolation. Results are expressed as positive (+) or negative (-).

[a]Dogs succumbed to natural infection (8).

[b]Dogs euthanized after experimental infection (this study).

The 2.5-month-old pup that recovered shed CCoV RNA starting from day 1 p.i. (titre of 2.97 x 10^1 RNA copy numbers/µl of template) and lasting for the entire observation period (21 days), with a peak at day 6 p.i. (3.24 x 10^4 RNA copy numbers/µl of template). Shedding of CCoV in the faeces of the two 6-month-old dogs was observed from day 2 p.i. (mean titre, 1.40 x 10^3 RNA copy numbers/µl of template) to day 21 p.i. (last day of

observation) reaching the maximal mean value of 6.79 x 10^5 RNA copy numbers/µl of template at day 10 p.i.

Surprisingly, CCoV RNA was never detected in the blood of the 6-month-old pups, as well as in the euthanized animals, in whose organs remarkable viral RNA titres were found. Traces of CCoV RNA were detected only in the blood of the survived 2.5-month-old pup between days 7 and 10 p.i., with plasma viral titres ranging from 5.54 x 10^0 to 9.30 x 10^1 RNA copies/ml of template.

The virus was successfully isolated on cell cultures from the rectal swabs of all inoculated dogs (data not shown) and from some organs of the euthanized pups (Table 2).

All the infected animals seroconverted for CCoV, whereas antibodies were not detected in the control dog (Fig. 2d). In the dogs that were euthanized the antibody titres were determined only at day 7 p.i., with VN titres of 1:8 and OD values of 0.089 (geometric means, Fig. 2a). In survivors, the maximal antibody titres were reached at days 14 and 21 p.i. by VN and ELISA test, respectively (Fig. 2b and 2c).

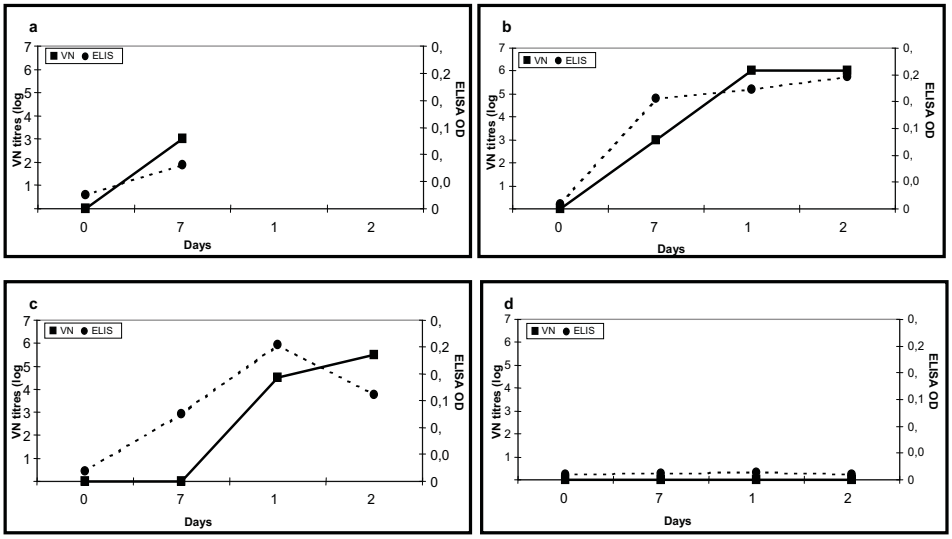

Fig. 2. Antibody responses in dogs infected experimentally with strain CB/05. A. 2.5-month-old dogs that were euthanized (dogs 1 and 3, means); B. 2.5-month-old dog that survived (dog 2); C. 6-month-old dogs (dogs 5 and 6, means); D. control dog (dog 4). Antibody responses are presented as geometric means of virus neutralising (VN) titres and ELISA optical density (OD) values.

DISCUSSION

In a previous study, a pantropic variant of CCoV was associated to a fatal disease of dogs, characterized by leucopenia, gastroenteritis and severe changes in the internal organs (1). The disease induced by strain CB/05 in pups of the natural outbreak was reproduced in dogs infected experimentally. All inoculated dogs were successfully infected, as shown by the occurrence of faecal shedding, seroconversion and severe clinical signs. The viral excretion was similar to those observed during enteric CCoV infection (3, 6), but the course of disease was more severe, as clinical signal characteristic of systemic infection were observed in the infected dogs. The pantropism of the virus was confirmed by the presence of gross lesions in the internal organs of the dead dogs, as well as by the detection of viral RNA in those tissues. Interestingly, CCoV RNA was detected also in the brain of the dead dogs. In contrast, enteric CCoV has been never associated to systemic infection, although the virus has been isolated previously from some tissues (tonsils, lungs and liver) of experimentally infected pups (17).

Two of the three inoculated 2.5-month-old pups had to be euthanized after few days postinfection, whereas the other dogs (the remaining dog of the same age and the two dogs six-month of age) recovered after a severe disease. Considering that dogs infected naturally were all between 45 and 56 days of age, it could be hypothesized that the age of the infected animals plays a role in determining the fate of CB/05 infection, with a very severe clinical course in the youngest pups. Moreover, in the experimental infection, the organs of the dead dogs contained lower CCoV RNA titres with respect to dogs infected naturally, so that virus isolation was not obtained from all PCR-positive tissues. Despite the drop of the WBC counts registered in all infected dogs and the detection of the viral RNA in the internal organs of the sacrificed dogs, free or cell-associated CCoV RNAemia was not found at any time either in euthanized or survived dogs, with the exception of the recovered 2.5-month-old pup which showed very low RNA viral titres in the plasma between days 7 and 10 p.i. Thus, at this moment, the mechanisms of lymphopenia and viral spread to internal organs remained unknown. Albeit strange, our findings are compatible with those obtained from cats experimentally infected with feline infectious peritonitis virus (FIPV). Cats succumbed to FIPV display very high viral titres in the haemolymphatic tissues (13), in contrast with the low loads detected in the blood (2). In our study, CB/05-infected dogs were found to contain lower viral loads in the lymphoid tissues in

comparison to FIPV-infected cats, thus likely accounting for the undetected viral RNAemia in euthanized dogs.

In conclusion, we have confirmed the pantropism of strain CB/05, reproducing the natural disease even in experimental conditions. Further studies will contribute to better understand the epidemiological distribution and the pathogenetic mechanisms of the virus, including the possible involvement of the different lymphocyte classes.

ACKNOWLEDGEMENTS

This work was supported by grants from Ministero dell'Istruzione, dell'Università e della Ricerca: PRIN 2005 (N.D.), project "Il coronavirus del cane: aspetti molecolari e patogenetici"; PRIN 2006 (C.B.), project "Infezione del cane da coronavirus pantropico: aspetti epidemiologici, patogenetici e molecolari".

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Recombinant canine coronaviruses related to transmissible gastroenteritis virus of swine are circulating in dogs

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Journal of Virology, 2009; 83, 1532-1537.

ABSTRACT

Four canine coronavirus type II (CCoV-II) strains were identified in the gut and internal organs of pups which had died of acute gastroenteritis. The CCoV-II strains were strictly related to porcine transmissible gastroenteritis virus (TGEV) in the N-terminal domain of the spike protein, whereas in the other parts of the genome, a higher genetic relatedness to recent CCoV-II isolates was observed. Experimental infection of dogs with a TGEV-like isolate induced mild gastroenteritis without any systemic involvement. By virus neutralization tests, antigenic differences between reference and TGEV-like CCoVs were found. Our data support the potential recombinant origin of the TGEV-like CCoVs.

Coronaviruses (CoVs) are able to cause a variety of different clinical forms of disease in a wide range of species (11). Currently, they are classified into three groups based on antigenic and genetic relatedness. CoVs infecting dogs have been reported that belong to groups 1 and 2, namely canine coronavirus (CCoV) and canine respiratory coronavirus (CRCoV), respectively (3). CCoV is an enteropathogen which has been known since the early 1970s (1) and includes two different genotypes, CCoV-I and CCoV-II, with different genetic and biological properties (19, 22). CCoV-II strains with uncommon virulence have been described (12, 13, 23), including a pantropic variant causing systemic disease in pups (2, 4, 8). In addition, recombinant viruses have been reported between CCoV-II and CCoV-I (12) or porcine transmissible gastroenteritis virus (TGEV) (27). In this paper, we report the genomic, biological and antigenic characterization of four type II CCoVs with the N-terminal domain of the S protein highly divergent from classical CCoV strains but strictly related to TGEV.

Identification of TGEV-like CCoV strains. Between December 2005 and March 2008, four dogs which had died of gastroenteritis were submitted to our lab for routine diagnostic investigations. The dogs were a 14-week-old great dane pup (341/05), a 10-week-old chihuahua pup (174/06), an 11-week-old mixed-breed pup (430/07), and a 13-week-old maltese pup (119/08). Dogs 174/06 and 119/08 had been imported from Hungary a few days before the onset of clinical signs. Intestinal contents and tissue samples collected from the dead dogs were tested by conventional or real-time polymerase chain reaction (PCR) assays for the detection of the main viral pathogens of dogs as previously described (8). CCoV was detected in the fecal samples or intestinal contents of all the pups examined, with viral RNA titers ranging from 1.37×10^5 to 2.38×10^7 μl^{-1} of template. Further genotyping by type-specific TaqMan assays (10) showed the presence of both CCoV types I and II in the gut of dogs 430/07 and 119/08, whereas the specimens from the other two dogs were found to contain only genotype II. Surprisingly, CCoV-II RNA was also detected in the internal organs of all of the dogs, albeit with variable tissue distribution (data not shown). It is noteworthy that all pups were positive for canine parvovirus (CPV) by real-time PCR (7). Subsequent characterization by means of type-specific minor groove binder (MGB) probe assays (5, 6, 9) showed that dogs 341/05, 430/07 and 119/08 were co-infected by CPV-2a, whereas a classical CPV-2 (vaccinal) strain was detected in the gut of pup 174/06.

The 3' end of the genome of the four CCoV-II strains detected in the lung samples was amplified and analyzed as previously described (8), and the nt sequences were deposited in GenBank under accession numbers EU856361 to EU856362 and EU924790 to EU924791. As expected, all of the predicted genes but open reading frame 3b, ORF3c, and ORF7b were preceded by the repeated intergenic sequence CTAAAC. The spike (S) protein was 1,457 amino acid long in all the strains that were analyzed, in contrast to classical type II CCoVs and FCoVs that displayed a shorter protein (1451-1454 aa). In the S protein, the aa identities among the CCoV strains sequenced in this study ranged between 95.1 and 98.9%, and the identity of these strains to other type II CCoVs was only 79.9 to 82.8%. Surprisingly, a higher genetic relatedness to TGEV was found, whereas other group 1a CoVs were proven to be less related. An exceptionally high identity to TGEV was evident in the N terminus (Table 1).

Table 1. Amino acid identities (%) of TGEV-like CCoVs to group 1a CoV reference strains in main nonstructural and structural proteins

CoV strain	Accession number	Amino acid identity (%) to TGEV-like CCoVs							
		Pp1ab[a]	Pp1a[a]	S	S N terminus[b]	S C terminus[c]	E	M	N
TGEV-Purdue	NC_002306	98.0-98.2	97.4	91.5-92.1	80.2-81.9	94.3-94.9	89.0-96.3	91.9-95.0	95.0-95.8
TGEV-TS	DQ201447	ND	NA	91.2-91.9	80.5-82.3	93.9-94.7	89.0-97.5	91.9-93.8	94.7-95.5
TGEV-96-1933	AF104420	NA	NA	89.0-89.3	76.3-77.7	92.0-92.5	87.8-95.1	90.8-91.6	93.1-93.7
PRCoV-RM4	Z24675	NA	NA	77.0-77.5	12.0-12.7	92.8-93.4	89.0-97.5	91.9-93.1	93.1-93.9
PRCoV-86-137004	X60089 (S); X55980 (E, M, N)	NA	NA	77.2-77.7	12.7-13.4	92.8-93.4	89.0-97.5	92.3-93.5	93.4-94.2
CCoV-II-UCD1	AF116248	NA	NA	NA	81.6-82.3	NA	NA	NA	NA
CCoV-II-Insavc-1	D13096	NA	NA	79.9-81.1	22.6-24.3	93.6-95.1	86.5-95.1	90.0-90.4	91.8-92.1
CCoV-II-CB/05	NA (pp1ab, pp1a); DQ112226 (S, E, M, N)	98.7	97.9-98.3	81.5-82.8	21.9-24.0	95.7-98.0	91.4-100	94.6-99.2	97.6-100
CCoV-II-BGF10	AY342160	NA	NA	80.3-80.9	24.0-36.4	93.9-94.7	79.2-87.8	89.7-91.2	92.6-92.9
CCoV-I-Elmo/02	AY307020 (S); AY426983 (E); NA (M, N)	NA	NA	41.3-41.9	17.9-19.2	48.9-49.1	79.2-85.3	87.9-89.0	88.4-89.0
CCoV-I-23/03	AY307021 (S); AY426984 (E); AY548235 (M, N)	NA	NA	41.6-42.1	15.5-17.4	49.1-49.3	80.4-86.5	87.9-89.0	88.4-89.0
FCoV-II-79-1146	NC_007025	94.5-94.8	87.8-88.1	80.5-81.5	21.3-24.0	95.3-95.7	71.9-78.0	83.6-84.4	76.7-77.2
FCoV-II-79-1683	X80799 (S); FCY13921 (E, M, N)	NA	NA	81.1-81.8	21.9-24.3	95.8-96.1	89.0-97.5	85.6-87.9	78.0-78.5
FCoV-I-KU-2	D32044 (S); AAB47501 (M); AB086881 (N)	NA	NA	41.3-41.8	17.4-18.4	48.0-48.4	NA	82.1-82.8	75.3-75.6
FCoV-I-Black	EU186072	92.9-93.2	88.2-88.5	40.8-41.3	17.7-18.7	47.5-47.8	71.9-78.0	82.5-84.0	76.1-76.4
FCoV-I-UCD1	AB088222 (S); AB086902 (M, N)	NA	NA	41.3-41.7	17.8-19.1	48.1-48.5	NA	81.3-81.7	76.1-77.2

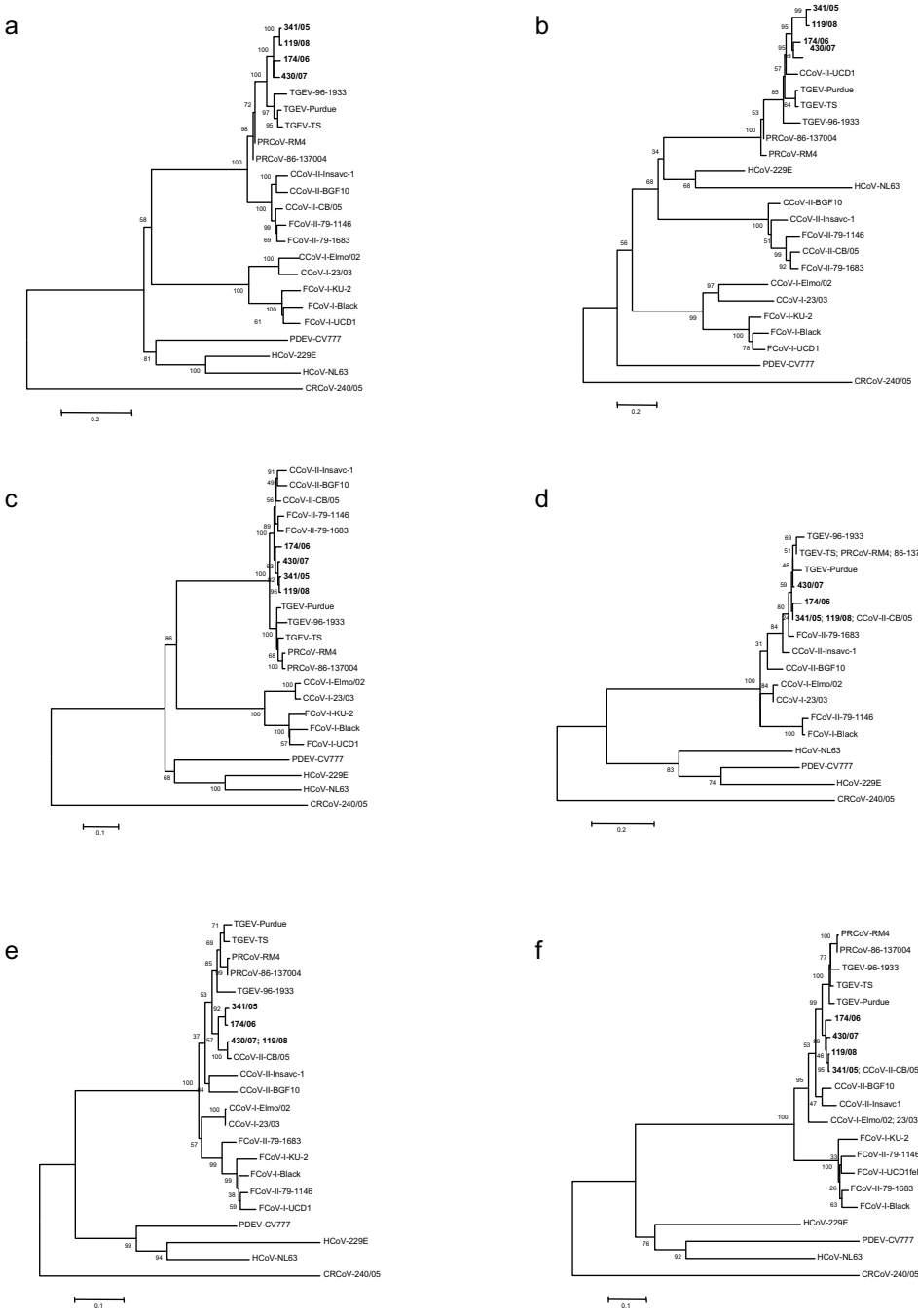
[a] Only partial C-terminal sequences of strains 341/05 and 174/06 were analyzed. [b] Residues 1-282 of the S protein of TGEV-like CCoVs.

[c] Residues 283-1457 of the S protein of TGEV-like CCoVs.

ND, not done; NA, not available.

Analysis of the other structural proteins, including the small envelope (E), membrane (M), and nucleocapsid (N) proteins, did not show atypical findings, with the exception of the E protein of strain 430/07, which was 75 instead of 82 aa long, due to a 21-nt deletion in the 5' end of the encoding gene. Analogously, the nonstructural proteins were conserved with respect to CCoV-II except for a 154-nt deletion in ORF7b of strain 341/05 leading to a shorter accessory protein 7b.

By phylogenetic analysis of the full-length sequence of the S protein conducted using Mega3 (17), group 1a CoVs clustered into two main clades. The first clade included CCoV/FCoV-I, whereas swine CoVs and CCoV/FCoV-II segregated into two separate clusters within the same clade (Fig. 1a). Interestingly, the four TGEV-like CCoVs formed a monophyletic group within the same cluster of TGEV and porcine respiratory coronavirus (PRCoV), which was separated from other type II CCoVs. This pattern of segregation was more evident in the N terminus, where the atypical CCoVs segregated together with the old CCoV strain UCD1 within the cluster including swine CoVs (Fig. 1b). In contrast, in the C terminus, the TGEV-like CCoVs clustered together with extant CCoV-II isolates and separately from TGEV/PRCoV (Fig. 1c). Analysis of the E, M, and N proteins showed a constant segregation of the TGEV-like CCoVs with typical CCoV-II strains (Fig. 1d to f).


a
341/05
119/08
174/06
430/07
TGEV-96-1933
TGEV-Purdue
TGEV-TS
PRCoV-RM4
PRCoV-86-137004
CCoV-II-Insavc-1
CCoV-II-BGF10
CCoV-II-CB/05
FCoV-II-79-1146
FCoV-II-79-1683
CCoV-I-Elmo/02
CCoV-I-23/03
FCoV-I-KU-2
FCoV-I-Black
FCoV-I-UCD1
PDEV-CV777
HCoV-229E
HCoV-NL63
CRCoV-240/05
0.2
b
341/05
119/08
174/06
430/07
CCoV-II-UCD1
TGEV-Purdue
TGEV-TS
TGEV-96-1933
PRCoV-86-137004
PRCoV-RM4
HCoV-229E
HCoV-NL63
CCoV-II-BGF10
CCoV-II-Insavc-1
FCoV-II-79-1146
CCoV-II-CB/05
FCoV-II-79-1683
CCoV-I-Elmo/02
CCoV-I-23/03
FCoV-I-KU-2
FCoV-I-Black
FCoV-I-UCD1
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CCoV-II-Insavc-1
CCoV-II-BGF10
CCoV-II-CB/05
FCoV-II-79-1146
FCoV-II-79-1683
174/06
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TGEV-Purdue
TGEV-96-1933
TGEV-TS
PRCoV-RM4
PRCoV-86-137004
CCoV-I-Elmo/02
CCoV-I-23/03
FCoV-I-KU-2
FCoV-I-Black
FCoV-I-UCD1
PDEV-CV777
HCoV-229E
HCoV-NL63
CRCoV-240/05
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TGEV-96-1933
TGEV-TS; PRCoV-RM4; 86-137004
TGEV-Purdue
430/07
174/06
341/05; 119/08; CCoV-II-CB/05
FCoV-II-79-1683
CCoV-II-Insavc-1
CCoV-II-BGF10
CCoV-I-Elmo/02
CCoV-I-23/03
FCoV-II-79-1146
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FCoV-II-79-1683
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FCoV-I-Black
FCoV-II-79-1146
FCoV-I-UCD1
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PRCoV-86-137004
TGEV-96-1933
TGEV-TS
TGEV-Purdue
174/06
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CCoV-II-BGF10
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Fig. 1. Phylogenetic analysis of group 1 CoVs. Neighbor-joining trees based on the spike protein full-length (**a**), N-terminual (**b**) and C-terminal (**c**) sequences, and the envelope (**d**), membrane (**e**), and nucleocapsid (**f**) proteins of group 1 CoVs. For phylogenetic tree construction, the group 1a CoVs listed in Table 1 and the following CoV strains were used (GenBank accession numbers are in parentheses): human coronavirus (HCoV) 229E (NC_002645), porcine epidemic diarrhea virus (PEDV) CV777 (NC_003436), and HCoV-NL63 (NC_005831). The tree is rooted on the group 2 CoV canine respiratory coronavirus (CRCoV) 240/05 (EU999954). Statistical support was provided by bootstrapping more than 1,000 replicates. The scale bars indicate the estimated numbers of amino acid substitutions per site.

The partial nucleotide sequence of ORF1a and the complete nucleotide sequence of ORF1b of strains 341/05 and 174/06 (accession numbers EU856361 to EU856362) were determined using primers amplifying overlapping fragments. In total, 21,347 and 21,395 nucleotides (nt) were sequenced from strains 341/05 and 174/06, respectively, representing about three-fourths of the entire genome of group 1a CoVs. In the partial C-terminal domain of pp1ab (4,239 aa), strains 341/05 and 174/06 were 98.8% identical and displayed approximately the same identity to CCoV-II-CB/05. In contrast, less similarity to TGEV, FCoV-II, and FCoV-I was found (Table 1). Analysis of the nearly full-length genome (more than 21,000 nt) confirmed the higher genetic relatedness to CCoV-II-CB/05 (95.8% nt sequence identity). By a SimPlot analysis (18), the potential recombinant origin of the TGEV-like CCoVs was evident. In fact, their genetic distance from TGEV was approximately the same as that from CCoV in the entire sequence, except at the 5' end of the S gene (Fig. 2a). By visual inspection of the S-gene alignments, the putative acceptor and donor sites involved in the double recombination between CCoV-II and TGEV were identified (Fig. 2b).

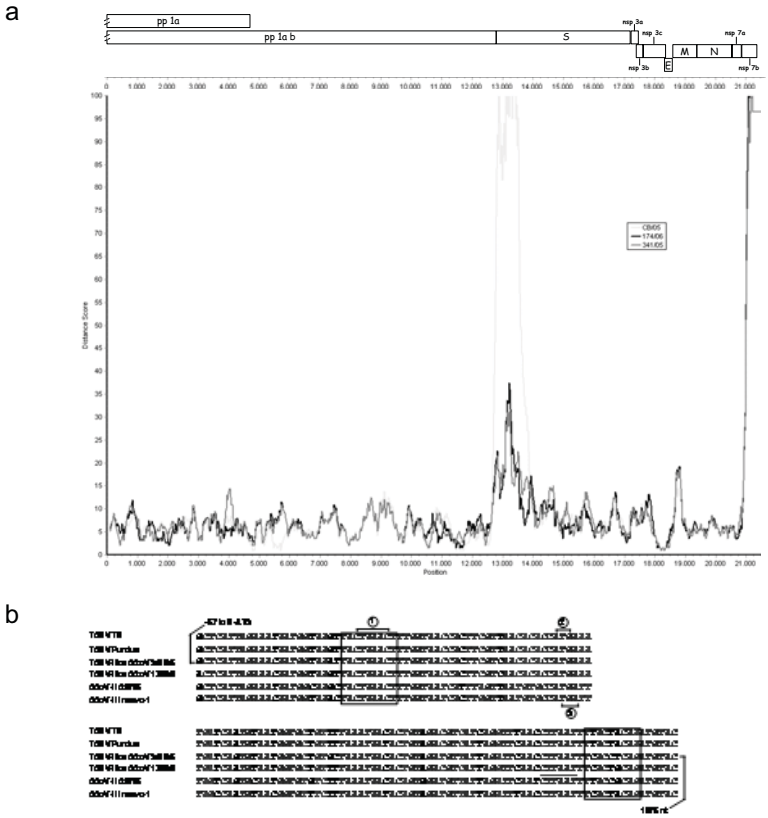

Fig. 2. Recombinant origin of the TGEV-like CCoVs. **(a)** SimPlot analysis of the nucleotide sequences of the nearly full-length genome of the TGEV-like CCoVs. Each point plotted is the percent genetic similarity within a 200-nt-wide sliding window centered on the position plotted with a step size of 20 nt, and Hamming correction on each curve represents a comparison of the sequence data of TGEV-like CCoVs 341/05 and 174/06 and CCoV-II-CB/05 to the reference sequence data of TGEV-Purdue. (**b**) Nucleotide sequence alignment of TGEV, TGEV-like CCoV, and CCoV-II genomes at the 5' end of the S-encoding gene. Indicated are the S-gene transcription regulating sequence TRS (circled number 1), the S-gene initiation translation codon (circled number 2), and the ORF1b termination codon (circled number 3). The potential acceptor and donor sequences involved in the recombination event are boxed or underlined. Blue, red, and green letters indicate nucleotides conserved among all viruses, nucleotides conserved among TGEV and TGEV-like CCoV genomes, and typical nucleotides of CCoV origin, respectively. A putative location of the acceptor site is the sequence 22-TTACTAAAC-30, which has high identity (seven out of nine nucleotides) with that present in the proposed donor site (1061-TTACTACAA-1069). Alternatively, small sequence domains present in the TGEV-like CCoVs 341/05 (1021-CTAAAT-1026) and 174/06 (1054-CTTAAT-1059) are observed in the potential donor sites that could act as a slow-down signal and promote recombination between these sequences and others with high identity that are present at the acceptor sites, such as the sequence 25-CTAAAC-30.

Biological and antigenic characterization of the TGEV-like CCoV strains. Virus isolation attempts from lung samples using canine fibroma A-72 cells (8) were successful with all TGEV-like CCoVs, except for strain 119/08, which possessed lower viral RNA titers in the internal organs and intestinal content of the dead dog. All virus isolates induced a cytopathic effect in the inoculated monolayers and were recognized by a monoclonal antibody (MAb) that bound the N protein of CCoV, FCoV and TGEV. In contrast, no viral growth was observed when using swine testicular cells, that were permissive for the reference TGEV strain, Purdue. In order to evaluate the pathogenic potential of TGEV-like CCoVs in dogs, the plaque-purified isolate from sample 341/05 was used to infect experimentally three antibody-defined beagle pups, whereas an additional pup served as control. All inoculated pups shed virus with their feces during the observation period (Fig. 3), whereas no trace of viral RNA was detected in the blood or nasal samples. Clinical signs observed in the infected dogs were indicative of a classical enteric coronavirosis, consisting of mild diarrhea lasting 2 to 3 days. Neither leukopenia nor other signs of systemic involvement were observed in any infected pup.

The sera obtained from dogs experimentally infected with strain 341/05 or with the classical CCoV-II enteric strain S378 (10) were tested against homologous and heterologous viruses using a virus neutralization (VN) test (21). Poor cross-protection between homologous and heterologous antibody titers was evident. In fact, in dogs infected with strain 341/05, the homologous VN titer (reciprocal of the geometric mean) was 20.16 and the heterologous titer was 6.35, whereas dogs infected with strain S378 displayed homologous and heterologous antibody titers of 40.32 and 10.08, respectively. In addition, the three TGEV-like CCoV plaque-purified isolates were characterized using a panel of TGEV MAbs previously developed (14, 16). The viruses reacted against three MAbs, two of which neutralize both TGEV and CCoV, whereas the third MAb had been shown to bind TGEV but not CCoV (24).

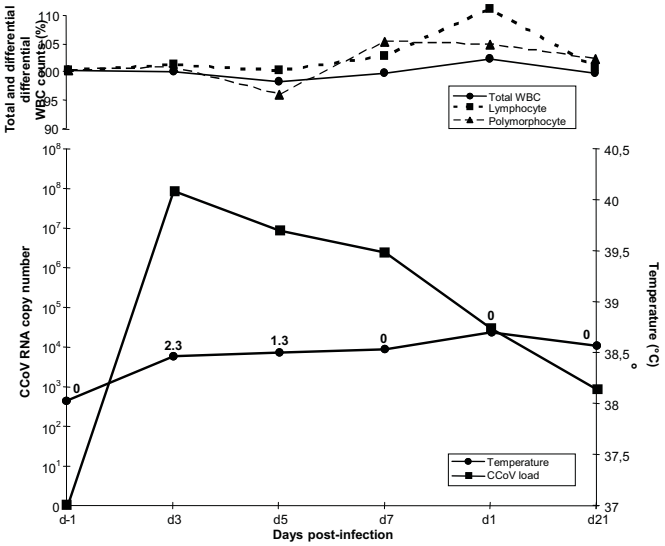

Fig. 3. Results of the experimental infection of dogs with a TGEV-like CCoV. Three 12-week-old beagle pups were inoculated oronasally with 2 ml of strain 341/05 (titer of $10^{6.25}$ 50% tissue culture infective doses ml^{-1}) and monitored for up to 21 days for total white blood cell (WBC), lymphocyte, and polymorphocyte counts (top graph). In addition, fever, viral RNA shed in feces and clinical scores were determined (bottom graphs). Total WBC, lymphocyte, and polymorphocyte counts are presented as percentages of the cell counts determined at day 0. Viral RNA titers as determined by real-time RT-PCR are expressed as copy numbers per microliter of template. Clinical scores were calculated as previously described (4) and are reported for each day with referebce to the temperature curves.

We have identified CCoVs with a potential double-recombinant origin through partial S-gene exchange with TGEV. Recombination involving the S gene has been described for FCoV (15) and CCoV (12, 27). A TGEV-like CCoV, strain UCD1, has been previously reported, but only partial S-gene sequences were determined, thus preventing a complete genomic characterization (27). In contrast, we have determined and analyzed the 3' end of four TGEV-like CCoVs and the nearly full-length genome of two of those strains. In addition, strain UCD1 was detected in the feces of a dog with diarrhea, whereas we detected the recombinant viruses in the feces/intestinal contents and in the internal organs as well.

A major mechanism driving CoV genetic evolution is represented by the high-frequency of homologous RNA recombination, which is frequently mediated by a copy-choice mechanism based on sequence homology flanking the recombination sites (20, 28).

In addition, the necessary discontinuous RNA synthesis required during RNA transcription to generate the subgenomic mRNAs in CoVs, is associated to highly conserved sequences preceding each gene and representing a slow-down or stop signal for RNA synthesis (25). This sequence, in the case of group 1a CoVs, is CTAAAC (26, 28). Therefore, in the sequence domains where recombination has taken place, it is likely to be small segments of sequence identity between the donor and the acceptor sequences. In fact, observation of the sequence domains involved in the recombination event revealed the presence of segments of sequence homology (Fig. 2b), although single, well-defined cross-over points could not be definitively determined.

Although the TGEV-like CCoVs were detected in the internal organs of naturally infected dogs, experimental infection failed to cause systemic involvement or virus dissemination through the blood. Thus, the CPV coinfection observed in the natural outbreaks could have played a certain role in TGEV-like CCoV spreading to internal organs. In addition, the antigenic differences observed between reference and recombinant CCoVs may have some implications for prophylaxis programs, as dogs administered classical CCoV vaccines may be susceptible to infection (disease?) caused by TGEV-like CCoVs. The detection of these viruses in different time periods (at least 4 years) and geographic areas (at least Italy and Hungary) have let us to presume that TGEV-like CCoVs are effectively circulating in the dog population. Taking into account the genetic and antigenic differences between classical and recombinant viruses, we propose the further division of CCoV-II into two different subtypes, CCoV-IIa and CCoV-IIb, including reference and TGEV-like CCoV-II isolates, respectively.

ACKNOWLEDGEMENTS

The authors are grateful to undergraduate student Mauro Di Pilato for his excellent assistance with part of the experimental work and to P.J. Collins (CIT, Department of Biology, Cork, Ireland) for the English revision of the manuscript. The MAb used for the IF assay and CCoV strain S378 were kindly donated by G. Chappuis (Merial, Lyon, France) and L.E. Carmichael (James Baker Institute for Animal Health, Cornell University, Ithaca, NY), respectively. This work was supported by grants from the Italian Ministry of Health, Ricerca finalizzata 2007, project "Mammalian coronaviruses: molecular epidemiology, vaccine development and implications for animal and human health" (Nicola Decaro) and from the Italian Ministry of Education, University and Research, PRIN 2006, project “Infezione del cane da coronavirus pantropico: aspetti epidemiologici, patogenetici e molecolari” (Canio Buonavoglia).

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Epilogue

In 2003, a new infectious human disease emerged in southern China, called severe acute respiratory syndrome (SARS), whose causative agent was rapidly identified as a novel coronavirus, namely SARS-CoV (4). Serological and genetic evidence supported a zoonotic origin for SARS-CoV (7, 16). At first, the reservoirs of SARS-CoV were suspected to be some wild carnivore species, including palm civet and other carnivores from live animal markets. However, subsequent investigations showed that palm civets and other wild carnivores are involved in the direct transmission of SARS-CoV to humans, acting as intermediate hosts, whereas the true reservoir is represented in fact by some bats, mainly horseshoe bats (9, 16).

The 2003 SARS-CoV outbreak dramatically illustrates the pathogenic potential of CoVs. While the unusual pathogenicity of SARS-CoV may be explained at least in part from the fact that it is a zoonotic infection (a case of an ill-adapted virus being introduced in a new naïve host population), there are CoVs that acquire high pathogenicity and the capacity to cause lethal infection in their own natural host. Perhaps, the best known example is feline infectious peritonitis virus (FIPV), a hypervirulent variant of the apathogenic biotype feline enteric coronavirus (eFCoV) (for a review, see reference 5). In immunocompetent cats *e*FCoVs cause asymptomatic persistent infections; viral replication is limited and the virus is restricted to the intestinal mucosa (6). In contrast, in cats immunocompromised as a consequence of other viral infections (feline immunodeficiency virus, feline leukaemia virus) or stressing conditions, FCoV replication is unleashed and virulent mutants (FIPVs) spontaneously arise that establish a rampant systemic infection. This change in pathogenicity appears to correlate with a shift in host cell tropism. Accordingly, unlike their parental virus *e*FCoV, FIPV strains efficiently infect and replicate in monocytes/macrophages (13, 14). Efficient infection of macrophages *in vitro* has been linked to changes in the carboxyterminal domain of the S protein (12). However, at the moment, the genetic changes directly responsible for the shift in virulence are not known (5). Deletions or inactivation of some (sub)group accessory genes, including ORF7a and ORF3c, were detected in FIPV strains, probably as a consequence of the shift from *e*FCoV to FIPV (8, 15). These findings, however, may merely indicate that some genes that are necessary during replication and persistence of *e*FCoV in the intestinal mucosa, are dispensable during systemic infection by FIPV (5).

One of the main findings presented in this thesis is that transisition from low-to-high virulence correlating with a shift from enteric to systemic infection is not unique to

FCoV. Like *e*FCoVs, CCoVs generally cause mild enteric infections (Chapters 2-3). In Chapter 4-5, however, we describe a hypervirulent CCoV variant CB/05. that was isolated in 2005 during an outbreak of a new fatal systemic disease in pups (1). Clinical signs were fever (39.5-40°C), lethargy, loss of appetite, vomiting, haemorrhagic diarrhea, severe leukopenia and neurological signs (ataxia, seizures) followed by death within 2 days after the onset of the symptoms. Necropsy examination revealed severe gross lesions in lungs, liver, spleen, and kidneys. Virological and bacteriological investigations on the parenchymatous organs failed to detect common canine pathogens, whereas CCoV type I and type II were identified in the intestinal content of all pups by genotype-specific real-time RT-PCR assays. Unexpectedly, CCoV type II RNA was also detected at high titres in lungs, spleen, liver, kidney and brain. A CCoV type II strain (CB/05) could be recovered in A-72 tissue culture cells from all tissues examined, with the exception of the brain. Immunohistochemistry using a CCoV-specific monoclonal antibody detected CCoV antigen in all tissues. Sequence analysis of the 3' genome end of the pantropic CCoV strain, including ORFs 2 (S gene), 3a, 3b, 3c, 4 (E gene), 5 (M gene), 6 (N gene), 7a and 7b, showed that strain CB/05 is strictly related to CCoV type II, although a higher identity to FCoV type II strain 79-1683 was found in the S protein. Interestingly, a 38-nt deletion was detected in ORF3b of strain CB/05, which could lead to the translation of a truncated protein (2). Experimental infection of seronegative pups with strain CB/05 reproduced the disease with occurrence of severe clinical signs, including pyrexia, anorexia, depression, vomiting, diarrhea and leukopenia (3). However, the outcome of infection, apparently depended on the age of the infected animal. Dogs, 6 months of age, slowly recovered from the disease, whereas two-out-of three 2.5-month-old pups had to be euthanized because of the severity of the CB/05-induced infection. The systemic involvement was confirmed by the presence of gross lesions in the internal organs of the dead dogs, as well as by the detection of viral RNA in those tissues, including brains, albeit at lower titres as compared to those detected in dogs succumbed to natural infection (Chapter 6). Traces of viral RNA were detected in the blood of a single dog, although further unpublished studies have demonstrated that detectable RNemia (viral RNA in white blood cells) can occur easily during CB/05 experimental infection (Decaro et al., unpublished).

As in the case of FIPV, the genetic changes responsible for the exceptional pathogenicity of strain CB/05 have not been yet determined. So far, only the 3’ end of the viral genome has been sequenced, revealing some features in which strain CB/05 differes

from all other CCoV strains characterized thus far (for instance, the ORF3b deletion and particular mutations in the spike protein), but, at the end of the day, the potential involvement of these or other changes in the increased pathogenicity can only be established through reverse genetics in combination with experimental infection..

The emergence of new CCoV genotypes and pathotypes in dogs raises intriguing questions about the need for the development of specific vaccines prepared with the new virulent strains. Previous studies demonstrated that inactivated vaccines currently used against enteric CCoV are poorly effective, whereas an experimental modified-live virus (MLV) vaccine administered oronasally was able to induce complete protection from disease as well as from infection (10). Preliminary data indicated that there is poor cross-reaction between the S genes of CCoV types I and II (11) and that even the CCoV type II-based MLV vaccine does not prevent infection of dogs after challenge with a CCoV type I strain (Buonavoglia et al., unpublished). However, the lack of tissue culture systems that support *in vitro* propagation of CCoV type I hampers the development of conventional homologous CCoV-I vaccines, and hence innovative and more expensive approaches may be required, such as those used for production of recombinant vaccines. Moreover, considering that strong immunity induced by natural infection with enteric CCoV was not able to protect pups from challenge with pantropic CCoV (Decaro et al., manuscript in preparation), the efficacy of currently used vaccines based upon enteric CCoV strains will likely be limited against pantropic CB/05-like viruses.

Future investigations should provide new insights into the molecular mechanisms responsible for the change in pathobiology of CCoV and into the pathogenic and immunological aspects of CCoV-induced systemic disease. At the same time, constant epidemiological surveillance will help a timely identification of additional CoV strains with novel genetic and biological properties. The lessons still to be learned will be valuable to predict and counter changes in coronavirus pathogenicity in general and hence, they will be of both human clinical and veterinary importance.

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Nederlandese samenvatting

Coronavirussen (CoV's; orde *Nidovirales*, familie *Coronaviridae*) zijn in hoge mate onderhevig aan genetische evolutie door de voortdurende accumulatie van mutaties en als gevolg van recombinatie met verwante familieleden. Op grond van genetische en antigene eigenschappen worden de CoV's verdeeld in drie groepen. Hiervan zijn de groep 1 virussen nog weer verder onderverdeeld in subgroepen 1a en 1b, met in subgroep 1a de nauw verwante kattencoronavirussen (feliene CoV's; FCoV's), hondencoronavirussen (caniene CoV's; CCoV's) en varkenscoronavirussen (het zgn. transmissible gastroenteritis virus [TGEV] en het porciene respiratoir CoV [PRCV]). Het doel van dit proefschrift was om de epidemiologische, evolutionaire en pathobiologische eigenschappen van CCoV's te bestuderen als modellen voor de evolutie en complexiteit van coronavirussen in het algemeen. Momenteel zijn er van de CCoV's twee verschillende genotypen bekend, n.l. CCoV type I (CCoV-I) en type II (CCoV-II). Deze zijn tot ca. 96% genetisch identiek, waarbij hun divergentie hoofdzakelijk is terug te voeren op verschillen in het gen dat codeert voor het zgn. virale spike (S) eiwit. Daarnaast beschikken type I CCoV's over een onlangs ontdekt open leesraam ('open reading frame' of ORF), ORF3, dat verondersteld wordt te coderen voor een door geïnfecteerde cellen uitgescheiden geglycosyleerd eiwit.

Voor de detectie en kwantificering van CCoV RNA werd een *real-time* reverse transcriptase-polymerase ketting reactie (RT-PCR) test opgezet, gericht op het gen voor het virale membraan (M) eiwit. Deze op zgn. TaqMan chemie gebaseerde test bleek niet alleen specifiek en reproduceerbaar, maar bovendien ongeveer 1 log gevoeliger dan conventionele RT-PRCR tests. Ook voor de specifieke onderscheiding en karakterisering van de twee CCoV genotypen werden moleculaire methoden ontwikkeld gebaseerd op het vóórkomen van puntmutaties in de M en S genen. Het betrof hier vier conventionele en twee *real-time* RT-PCR tests. Uitgebreide analyse van fecesmonsters van grote aantallen honden met diarree liet zien dat de beide genotypen veelvuldig voorkomen en regelmatig ook tegelijkertijd in dergelijke zieke honden kunnen worden aangetroffen.

Beide CCoV genotypen zijn geassocieerd met milde, tijdelijke enteritis in hondenpups. In 2005 echter werd er in Italië een hoogvirulente variant van CCoV type II gerapporteerd, die in pups een systemische infectie veroorzaakte met fatale afloop. De klinische symptomen bestonden uit koorts (39.5-40°C), lethargie, ontbreken van eetlust, braken, bloederige diarree, ernstige leukopenie en neurologische afwijkingen (ataxie, toevallen), leidend tot de dood binnen 2 dagen na het begin van de verschijnselen. Bij *post-mortem*

analyse bleken de dieren zeer ernstige laesies te hebben in longen, lever, milt en nieren. Virologisch en bacteriologisch onderzoek van de parenchymatische weefsels leverde geen aanwijzingen op voor de aanwezigheid van bekende hondenpathogenen anders dan CCoV type I en II, welke d.m.v. genotype-specifieke *real-time* RT-PCR konden worden aangetoond in de darminhoud van alle pups. Verrassend genoeg werd CCoV type II bovendien gedetecteerd in de longen, lever, nieren en hersenen. Uit alle onderzochte weefsels met uitzondering van hersenen kon een CCoV type II virus (stam CB/05) op A-72 cellen worden geïsoleerd. In alle weefsels kon CCoV m.b.v. een CCoV-specifiek monoclonaal antilichaam immunohistochemisch worden aangetoond. Sequentieanalyse van het 3'eind van het genoom van de pantrope CCoV stam - ORFs 2 (S gen), 3a, 3b, 3c, 4 (E gen), 5 (M gen), 6 (N gen), 7a en 7b - toonde aan dat de stam CB/05 in dit gebied een hoge mate van sequentie-identiteit heeft met andere type II CCoV's. Experimentele infectie van seronegatieve pups met het geïsoleerde CB/05 virus bleek de ernstige ziekte te reproduceren, met klinische symptomen als hoge koorts, anorexie, depressie, braken, diarree en leukopenie. De ernst van de infectie bleek leeftijdsafhankelijk. Terwijl de wat oudere (6 maanden) hondjes - zij het langzaam - herstelden, moesten twee van de drie 2½ maand oude puppies wegens ernstige ziekte worden geëuthanaseerd. Het pantropisme van het virus werd bevestigd door de aanwezigheid van ernstige laesies in de interne organen van de overleden honden alsook door de detectie van viraal RNA in de weefsels, met inbegrip van de hersenen, zij het in lagere titers dan in honden overleden aan de natuurlijke infectie. Sporen van viraal RNA werden waargenomen in het bloed van één van de honden, hoewel later, nog ongepubliceerd vervolgonderzoek aantoonde dat RNemie (viraal RNA in bloed) gemakkelijk detecteerbaar is tijdens CB/05 virusgeïnduceerde experimentele infectie.

De evolutie van CCoV's en TGEV is sterk gecorreleerd. Er is gepostuleerd dat TGEV is voortgekomen uit CCoV-II d.m.v. cross-species transmissie, een stelling die wordt ondersteund door de hoge genetische verwantschap van deze virussen en door de aanwezigheid van ORF3 sequentierestanten in de genomen van CCoV-II en TGEV. Nieuwe CCoV-II stammen werden geïsoleerd uit vier pups die tevens een coïnfectie met hondenparvovirus doormaakten. Sequentie- en fylogenetische analyses van het 3'-terminale deel (8,8 kb) van hun virale genoom liet zien dat de geïdentificeerde CCoV-II stammen in het N-terminale domein van het S eiwit nauw verwant waren aan TGEV terwijl ze in het C-terminale domein en in genen coderend voor andere structurele eiwitten

en voor accessoire eiwitten een hogere verwantschap met klassieke CCoV-II isolaten hadden. Uit sequentieanalyse van vrijwel het gehele genoom van twee TGEV-achtige stammen bleek dat het replicasecomplex van die virussen eveneens van caniene herkomst is. Drie TGEV-achtige CCoV's werden geïsoleerd op hondencelcultures, terwijl deze stammen niet groeiden op varkenscellen. Experimentele infectie van honden met een TGEV-achtig isolaat induceerde een klassieke gastroenterale aandoening zonder enige systemische verspreiding, daarmee de TGEV-achtige virussen differentiërend van het recent gerapporteerde pantropische CCoV. Antigene verschillen tussen klassieke en TGEV-achtige CCoV's konden worden aangetoond d.m.v. neutralisatietests met TGEV-specifieke mnoclonale antilichamen alsook met sera van experimenteel geïnfecteerde honden. Deze waarnemingen ondersteunen de mogelijk recombinante oorsprong van de TGEV-achtige stammen, welke ontstaan kunnen zijn door dubbelrecombinatie tussen CCoV-II en TGEV waarbij uitwisseling van het 5'-terminale deel van het S gen plaatsvond. Als gevolg daarvan is het CCoV-II genotype verder verdeeld in de twee subtypen CCoV-IIa en CCoV-IIb, het laatste subtype de TGEV-achtige virussen betreffend.

Toekomstig onderzoek dient gericht te zijn op het verkrijgen van nieuwe inzichten in de moleculaire mechanismen verantwoordelijk voor de veranderingen in pathobiologie van CCoV en in de pathogenetische en immunologische aspecten van CCoV-geïnduceerde systemische ziekte. Tegelijkertijd zal continue epidemiologische surveillance de tijdige identificatie mogelijk maken van additionele CoV stammen met nieuwe genetische en biologische eigenschappen. De lessen welke we nog te leren hebben zullen belangrijk zijn voor het voorspellen van en reageren op veranderingen in pathogeniciteit van coronavirussen in het algemeen en zullen derhalve zowel van veterinair als van humaan klinisch belang zijn.

Abstract in italiano

I coronavirus (ordine *Nidovirales*, famiglia *Coronaviridae*) possiedono una spinta evolutiva eccezionale dovuta all'accumulo di mutazioni ed ai processi di ricombinazione omologa tra virus correlati. I coronavirus sono organizzati in tre gruppi antigenici dei quali il gruppo 1 è suddiviso nei sottogruppi 1a e 1b. Il primo include virus altamente correlati, in particolare i coronavirus felini (FCoV), i coronavirus del cane (CCoV, il virus della gastroenterite trasmissibile del suino (TGEV) e la sua variante coronavirus respiratorio del suino (PRCoV). Lo scopo della presente tesi è stato quello di studiare gli aspetti epidemiologici, evoluzionistici e patobiologici di CCoV, i quali sono paradigmatici della evoluzione e complessità di tutti i coronavirus. Ad oggi si conoscono due distinti genotipi di CCoV, il tipo I (CCoV-I) ed il tipo II (CCoV-II), i quali possiedono una identità genetica superiore al 96%e divergono solo nel gene della proteina degli spikes (S). Inoltre, CCoV-I presenta una nuova *open reading frame* (ORF), ORF3, che codifica per una proteina glicosilata probabilmente secreta dalle cellule infette.

È stato messo a punto un test real-time RT-PCR sul gene della proteina di membrana per la ricerca e la quantificazione dell'RNA di CCoV. Il test, che è basato sulla chimica TaqMan, si è dimostrato specifico, riproducibile e più sensibile di un logaritmo rispetto ad un test RT-PCR convenzionale. Sono stati anche messi a punto metodi molecolari per la ricerca e caratterizzazione dei genotipi CCoV, i quali sono basati su mutazioni puntiformi riscontrate nei geni delle glicoproteine di membrana e degli spikes. Queste metodiche comprendono quattro test RT-PCR convenzionali e due test real-time RT-PCR. L'analisi di campioni fecali prelevati da cani con diarrea ha dimostrato che i due genotipi CCoV sono frequentemente responsabili negli stessi animali di infezioni simultanee (causate da entrambi i genotipi).

Entrambi i genotipi sono stati associati ad enterite di lieve entità seguita dalla guarigione spontanea dei cani infetti. Nel 2005, è stata segnalata in Italia una variante altamente virulenta di CCoV-II (stipite CB/05), la quale ha causato una malattia sistemica seguita dalla morte dei cuccioli infetti. I segni clinici comprendevano febbre (39.5-40°C), letargia, perdita di appetito, vomito, diarrea emorragica, grave leucopenia e sintomi neurologici (atassia, convulsioni), i quali si sono risolti con la morte a distanza di due giorni dall'esordio della sintomatologia. All'esame anatomo-patologico sono state osservate gravi lesioni nei polmoni, nel fegato, nella milza e nei reni. Gli esami virologici e batteriologici eseguiti sugli organi interni non hanno dimostrato la presenza dei più comuni patogeni del cane, mentre l'RNA di CCoV-I e CCoV-II è stato messo evidenza nel

contenuto intestinale mediante l'impiego di test real-time RT-PCR genotipo-specifici. Inaspettatamente l'acido nucleico di CCoV-II è stato rilevato a titoli elevati in polmoni, milza, fegato, reni e cervello. Lo stipite CCoV-II (CB/05) è stato isolato su cellule di cane A-72 a partire da tutti i tessuti esaminati ad eccezione del cervello e gli antigeni virali sono stati evidenziati in tutti gli organi mediante immunoistochimica. L'analisi di sequenza della estremità 3' del genoma virale, incluse le ORF 2 (gene S), 3a, 3b, 3c, 4 (gene E), 5 (gene M), 6 (gene N), 7a e 7b, ha dimostrato che lo stipite CB/05 possiede un elevato grado di identità genetica rispetto a classici stipiti CCoV-II. La malattia osservata in condizioni naturali è stata riprodotta sperimentalmente in cuccioli sieronegativi per CCoV, i quali hanno manifestato sintomi gravi tra cui febbre, anoressia, depressione, vomito, diarrea e leucopenia. L'esito dell'infezione è comunque dipeso dall'età, in quanto i cuccioli di sei mesi sono andati incontro ad una guarigione lenta ma progressiva, mentre i cuccioli di 2,5 mesi sono stati sottoposti ad eutanasia a causa delle gravi condizioni cliniche in cui versavano. Il carattere pantropico del virus è stato confermato dalla lesioni anatomo-patologiche a carico degli organi interni e dalla presenza dell'RNA virale negli stessi tessuti, incluso il cervello,m sebbene a titoli più bassi rispetto a quelli osservati in corso di infezione naturale. Tracce di RNA virale sono state evidenziate nel sangue di un solo cucciolo, anche se studi successivi (non pubblicati) hanno dimostrato che la presenza transitoria del virus nel sangue degli animali infettati sperimentalmente è di comune riscontro.

L'evoluzione di CCoV e TGEV è strettamente interconnessa. È stato infatti ipotizzato che TGEV possa derivare da CCoV-II a seguito di trasmissione cross-specie e tale ipotesi è supportata dalla elevata identità genetica esistente tra i due virus e dalla presenza di residui di ORF3 sia in CCoV-II che in TGEV. Stipiti CCoV-II atipici sono stati isolati da quattro cuccioli con infezione concomitante sostenuta dal parvovirus del cane. Mediante l'analisi di sequenza della estremità 3' del genoma virale, tali stipiti sono risultati strettamente correlati a TGEV nella regione amino-terminale della proteina degli spikes, mentre il dominio carbossi-terminale della stessa proteina e le altre regioni del genoma virale hanno dimostrato una identità più elevata nei confronti dei classici stipiti CCoV-II. L'analisi dell'intero genoma di due stipiti TGEV-like ha dimostrato che anche il complesso della replicasi virale è tipico di CCoV-II. Tre dei quattro stipiti sono stati adattati alla crescita su cellule di cane, mentre nessuno stipite è cresciuto su cellule di derivazione suina. L'infezione sperimentale di cuccioli con un isolato virale ha determinato

l'insorgenza di malattia enterica di lieve entità che si riscontra in corso di classica coronavirosi del cane, per cui gli stipiti TGEV-like sono risultati possedere caratteristiche patobiologiche diverse rispetto alla variante pantropica. Mediante virus-neutralizzazione con anticorpi monoclonali e sieri di cani con infezione sperimentale, sono state osservate notevoli differenze sul piano antigenico tra stipiti CCoV classici e TGEV-like. I risultati ottenuti supportano la probabile origine ricombinante degli stipiti TGEV-like, i quali potrebbero essersi originati mediante un processo di doppia ricombinazione tra CCoV-II e TGEV a livello della estremità 5' del gene S. Pertanto CCoV-II è stato ulteriormente suddiviso in due sottotipi, CCoV-IIa e CCoV-IIb, i quali comprendono rispettivamente stipiti classici e ricombinanti.

Ulteriori indagini si rendono indispensabili per comprendere la basi molecolari dell'evoluzione patobiologica di CCoV e gli aspetti patogenetici ed immunologici della malattia indotta da questo virus. Allo stesso tempo, la continua sorveglianza epidemiologica potrà favorire la identificazione di ulteriori stipiti CCoV con nuove caratteristiche genetiche e biologiche. I dati ottenuti potranno essere utili per prevedere e fronteggiare ulteriori cambiamenti patobiologici dei coronavirus in generale, per cui saranno importanti dal punto di vista della medicina sia umana che veterinaria.

Acknowledgements

This thesis owes its existence to the help, support, and inspiration of many people. In the first place, I would like to express my sincere appreciation and gratitude to Prof. Canio Buonavoglia for his continuous encouragement and guidance during these years. His uncompromising quest for excellence significantly shapes everyone at my institute. My young associate professorship is mainly due to his support and expertise. I owe special gratitude to all personnel (colleagues, PhD students, post-docs and technicians) of the Infectious Diseases Unit of the Department of Veterinary Public Health (University of Bari). The discussions and cooperations with all of them have contributed substantially to this work.

I am very grateful to Prof. Peter Rottier and Dr. Raoul de Groot (Virology Division of the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University), for giving me the opportunity to work and live in The Netherlands for some months as a visiting scientist. They provided for an optimum working environment at the institute and allowed me to have an international PhD in Utrecht. All people working in their lab are also acknowledged for their precious assistance during my stay in Utrecht.

Lastly, I would like to thank my family for the continuous support. I wish to express my love and gratitude to my parents for always giving me the opportunity to make my personal choices without any external interference. Above all, I express my full gratitude to the most important persons in my life: my beloved Lori for accepting and loving me despite my harsh personality and my jewel Giovanni for his lovable smiles opening my heart every day. I dedicate this thesis to both of them.

Ringraziamenti

La presente tesi è il risultato del lavoro, del supporto e del contributo intellettuale di molte persone. Tra queste vorrei esprimere la mia stima sincera e tutta la mia gratitudine al Prof. Canio Buonavoglia per il supporto e la guida generosamente offertimi in tutti questi anni. La sua continua ricerca dell'eccellenza coinvolge e, per certi versi, travolge continuamente tutti noi. Se sono diventato professore associato ad una giovane età, lo si deve soprattutto al forte sostegno ed alla competenza scientifica con i quali mi ha costantemente supportato. I miei ringraziamenti vanno anche a tutti i colleghi ed amici (docenti, dottorandi, assegnasti e tecnici) della Sezione di Malattie Infettive del Dipartimento di Sanità Pubblica e Zootecnia dell'Università di Bari per aver contribuito in maniera rilevante alla mia attività di ricerca.

Sono profondamente grato al prof. Peter Rottier e al Dr. Raoul de Groot (Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University) per avermi dato l'opportunità di lavorare e vivere in Olanda per alcuni mesi. Li ringrazio per aver creato un ambiente di lavoro accogliente e per avermi proposto di conseguire il PhD ad Utrecht. Sono grato anche a tutto il personale del loro laboratorio per la preziosa collaborazione durante la mia permanenza ad Utrecht.

Infine, vorrei ringraziare la mia famiglia per avermi sempre supportato. In particolare, ringrazio i miei genitori per avermi dato la possibilità di prendere le mie decisioni senza alcuna interferenza esterna. Soprattutto, esprimo la mia gratitudine alle persone più importanti della mia vita: la mia amata Lori, che sopporta il mio non facile carattere, ed il mio gioiello Giovanni, i cui splendidi sorrisi ogni giorno spalancano le porte del mio cuore.

Curriculum vitae

Nicola Decaro was born in Bari on November 11, 1973. In 2000, he graduated in Veterinary Medicine at the Faculty of Bari, Italy, with a dissertation on canine coronavirus epidemiology, and awarded the "Sigillum Universitatis Barensis", as the best student graduated at the Faculty of Veterinary Medicine of Bari in the academic year 1999-2000. Between 2000 and 2004, he received a doctorate fellowship in “Infectious diseases of carnivores” under the supervision of Professor Canio Buonavoglia. He was in charge of the same Faculty as assistant professor (2002-2004) and associate professor (current position) of Infectious Diseases of Animals. From 2008 he has been Director of the Specialization School of Infectious Diseases of Animals. He is or has been coordinator of several research projects on the infectious diseases of animals. His research interest includes the infectious diseases of small and large animals, with particular emphasis on the development of diagnostic assays, surveillance and molecular analyses on the viral pathogens of dogs (canine coronavirus and canine parvovirus). He is author and/or co-author of 156 scientific manuscripts (103 on peer-reviewed international journals) and member of the editorial boards of *Journal of Veterinary Diagnostic Investigation*, *Journal of Microbiological Methods* and *Veterinaria*.

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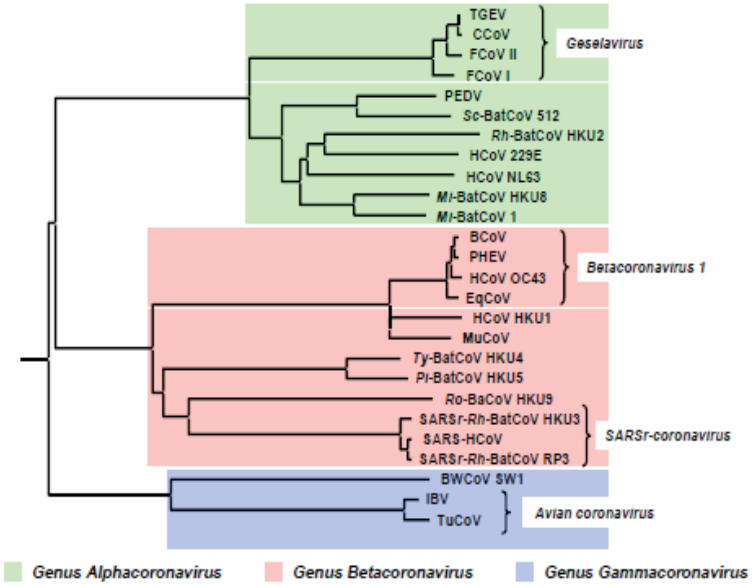
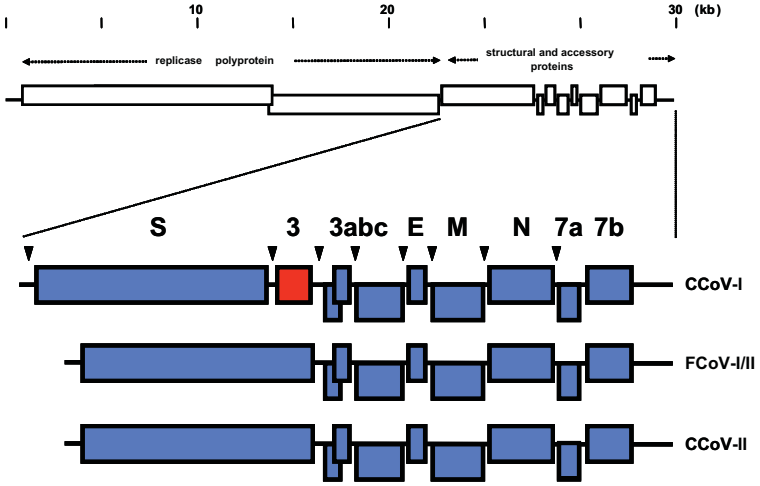
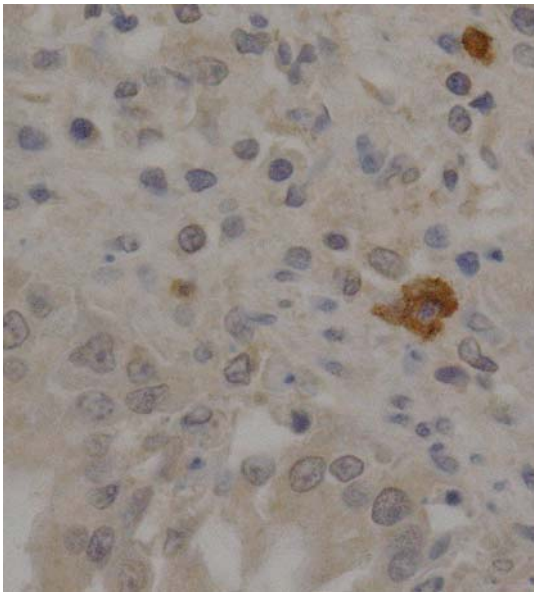
Colour figures



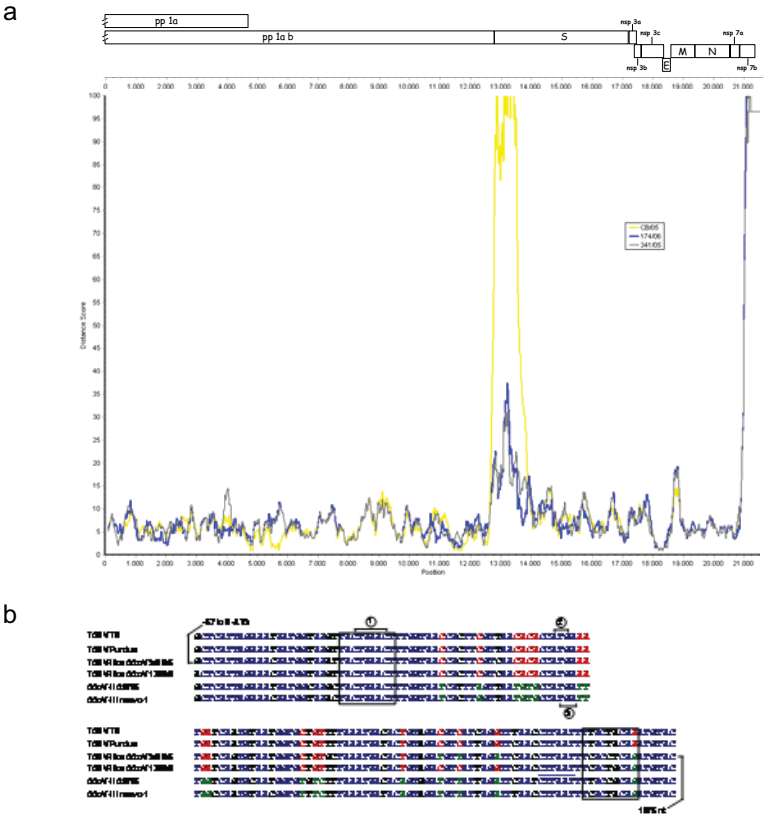
Chapter 1, Fig. 1. Rooted neighbour-joining tree inferred from multiple amino acid alignments of the RdRp, illustrating the relationships between the proposed coronavirus genera and between the viruses lumped in the new species *Geselavirus*, *Betacoronavirus 1*, *SARS-related coronavirus* and *Avian Coronavirus* (from reference 9).



Chapter 1, Fig. 2. Schematic representation of the genomes of CCoVs and FCoVs depicting the genetic differences among the CCoV genotypes. Genes encoding for structural and non-structural proteins are shown in grey and white, respectively. ORF sizes are not drawn to scale. The arrows indicate the transcription regulating sequences preceding each CoV gene (courtesy Raoul J. de Groot).



Chapter 4, Fig. 1. Immunohistochemical detection of canine coronavirus antigen (brown staining) in canine lung tissue by a specific monoclonal antibody (magnification ×400).



Chapter 7, Fig. 2. Recombinant origin of the TGEV-like CCoVs. **(a)** SimPlot analysis of the nucleotide sequences of the nearly full-length genome of the TGEV-like CCoVs. Each point plotted is the percent genetic similarity within a 200-nt-wide sliding window centered on the position plotted with a step size of 20 nt, and Hamming correction on each curve represents a comparison of the sequence data of TGEV-like CCoVs 341/05 and 174/06 and CCoV-II-CB/05 to the reference sequence data of TGEV-Purdue. (**b**) Nucleotide sequence alignment of TGEV, TGEV-like CCoV, and CCoV-II genomes at the 5' end of the S-encoding gene. Indicated are the S-gene transcription regulating sequence TRS (circled number 1), the S-gene initiation translation codon (circled number 2), and the ORF1b termination codon (circled number 3). The potential acceptor and donor sequences involved in the recombination event are boxed or underlined. Blue, red, and green letters indicate nucleotides conserved among all viruses, nucleotides conserved among TGEV and TGEV-like CCoV genomes, and typical nucleotides of CCoV origin, respectively. A putative location of the acceptor site is the sequence 22-TTACTAAAC-30, which has high identity (seven out of nine nucleotides) with that present in the proposed donor site (1061-TTACTACAA-1069). Alternatively, small sequence domains present in the TGEV-like CCoVs 341/05 (1021-CTAAAT-1026) and 174/06 (1054-CTTAAT-1059) are observed in the potential donor sites that could act as a slow-down signal and promote recombination between these sequences and others with high identity that are present at the acceptor sites, such as the sequence 25-CTAAAC-30.