

Serological and molecular evidence that canine respiratory coronavirus is circulating in Italy

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Abstract

Canine respiratory coronavirus (CRCoV) is a group II coronavirus that was firstly identified in lung samples of dogs with canine infectious respiratory disease (CIRD) in UK in 2003. We report for the first time the identification of CRCoV in Italy, together with serological evidence that the virus has been circulating in the Italian dog population as from 2005. Serological investigations on 216 dog sera, carried out by an ELISA test using the strictly related bovine coronavirus (BCoV) as antigen, revealed an overall CRCoV seroprevalence of 32.06% in the last 2 years. RT-PCR targeting the S-gene of CRCoV was carried out on 109 lung samples collected from carcasses of dogs submitted for diagnostic investigations. Positive results were obtained from the lungs of a dog of the Apulia region that was co-infected with canine parvovirus type 2. Sequence analysis of the S-gene fragment amplified by RT-PCR (595 bp) showed similarity to group II coronaviruses, with the highest nucleotide identity (98%) to the only CRCoV strain currently available in the GenBank database (strain T101). The results of the present study show that CRCoV is present also in continental Europe, although further studies are required to determine the real pathogenic potential of the virus.

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1. Introduction

Coronaviruses are enveloped, single-stranded RNA viruses, that are responsible for diseases of variable

severity in animals and humans and are currently classified into three antigenic groups (Enjuanes et al., 2000). To date, three different coronaviruses have been identified in dogs (Erles et al., 2003; Pratelli et al., 2003). Canine coronaviruses (CCoVs) type I and II are included in group I coronaviruses and their evolution is tightly intermingled with that of feline coronaviruses (Pratelli et al., 2003). Molecular

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methods able to distinguish between the two genotypes have detected at high frequency mixed infections caused by both genotypes (Pratelli et al., 2004; Decaro et al., 2005c). Usually, they cause infection of the enteric tract with occurrence of mild to severe diarrhoea in pups, although fatal infections with viral spread to internal organs have been reported (Buonavoglia et al., 2006).

Recently, a canine respiratory coronavirus (CRCoV) has been detected in dogs, which belongs to group II coronaviruses, sharing up to 96.0% amino acid (aa) conservation in the spike protein with bovine coronavirus (BCoV) and human coronavirus (HCoV) OC43 (Erles et al., 2003). Group II coronaviruses also include porcine haemagglutinating encephalomyelitis virus (HEV), mouse hepatitis virus (MHV), sialodacryoadenitis virus (SDAV), human enteric coronavirus (HECV) 4408 (Enjuanes et al., 2000), and the newly recognised HCoV-HKU1 (Woo et al., 2005). The human coronavirus associated with severe acute respiratory syndrome (SARS-CoV), initially defined as prototype of a new group IV, has been placed more recently within group II coronaviruses, in a subgroup IIb (Gorbalenya et al., 2004; Weiss and Navas-Martin, 2005).

Unlike the enteric coronaviruses CCoV type I and II, CRCoV is responsible for mild respiratory signs and is recognised as aetiological agent of canine infectious respiratory disease (CIRD) together with *Bordetella bronchiseptica*, canine adenovirus type 1 and 2, canine parainfluenzavirus, canine herpesvirus, reoviruses and influenzaviruses (Erles et al., 2004). CRCoV has been detected firstly in UK and more recently in Canada (Ellis et al., 2005) and Japan (Kaneshima et al., 2006). Serological evidence has been obtained that CRCoV has been circulating also in other countries, including USA, Ireland and Greece (Priestnall et al., 2006).

The present study aimed to establish evidence of the presence of CRCoV in Italy by means of serological and molecular investigations.

2. Materials and methods

2.1. Serum and tissue samples

Two-hundred-sixteen canine serum samples collected during years 1994–2006 were tested for

CRCoV antibodies. Most of the sera ($n = 203$) had been submitted from veterinarians for assessment of serology against canine parvovirus type 2 (CPV-2) and/or canine distemper virus (CDV), whereas additional 13 samples were recruited from previous studies (Pratelli et al., 2002; Elia et al., 2003). The sera had been collected from dogs housed in kennels ($n = 157$) or pet dogs ($n = 59$), whereas the age of the sampled dogs was <6 months ($n = 136$) or >6 months ($n = 80$). A total of 109 lung samples collected from the carcasses of dogs submitted to post-mortem examination and diagnostic investigations were tested for CRCoV RNA. Carcasses were collected by academic institutions and private veterinarians from different geographic regions of Italy during the last 3 years (2004–2006).

2.2. Screening of serum samples for CRCoV antibodies

Dog sera were tested for CRCoV antibodies by means of an ELISA test using bovine coronavirus strain 9WBL77 as antigen.

For antigen preparation, BCoV was cultivated on human rectal tumour (HRT-18) cells grown in Dulbecco's minimal essential medium (D-MEM) added with 10% foetal calf serum. When the monolayer was confluent, the medium was removed and the cells were washed two times with FCS-free medium plus trypsin ($5 \mu\text{g/ml}$) and inoculated with BCoV strain 9WBL77. After an incubation of 60 min at 37°C , the inoculum was replaced with fresh serum-free medium plus trypsin ($5 \mu\text{g/ml}$). The infected cells were harvested 96 h post-infection (in the presence of cytopathic effect and strong haemagglutinating activity for mouse erythrocytes) and clarified by centrifugation at $3000 \times g$ for 20 min to remove the cell debris.

Ninety-six-well microtitre plates were coated with $100 \mu\text{l}$ per well of a hyperimmune guinea pig serum specific for BCoV diluted 1:500 in carbonate buffer ($15 \text{ mM Na}_2\text{CO}_3$, 35 mM NaHCO_3 [pH 9.6]) and then incubated overnight at $>4^\circ\text{C}$ with slow shaking. After four washes with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), $100 \mu\text{l}$ of BCoV antigen diluted 1:5 in PBS-T plus 1% yeast extract was added per well and the plates were incubated for 1 h at 37°C . The washing cycle was

repeated and 1:50 dilutions in PBS-T of each serum sample were added in duplicate. The plates were then incubated for 90 min at 37 °C and, after additional four washes, 100 µl/well of goat anti-dog IgG-horseradish peroxidase conjugates (Sigma–Aldrich srl, Milan, Italy) was added and the plates were incubated for 1 h at 37 °C. After repeated washes, 100 µl of freshly prepared substrate 2,2'-azino-di-(3-ethylbenzothiazoline sulphonate) (ABTS, Sigma-Aldrich) plus 25 µg/100 ml H₂O₂ was placed in each well and a final incubation of 25 min at room temperature was performed. After adding the stop buffer solution (SDS 1%), the optical density at 405 nm (OD₄₀₅) was determined using an automatic ELISA reader (Bio-Rad Laboratories srl, Milan, Italy). The cutoff value (OD < 0.058) was defined as the mean OD plus three standard deviations calculated with the negative serum samples collected from 10 dogs that had tested negative for CRCoV RNA by RT-PCR and for CRCoV antibodies by virus neutralisation using BCoV and HRT-18 cells (Kaneshima et al., 2006) and haemagglutination inhibition (HI) (data not shown).

2.3. Evaluation of cross-reactivity between CCoV and CRCoV antibodies

In order to rule out any cross-reactivity between CRCoV and CCoV antibodies, ELISA was carried out on serum samples collected at days post-infection 0 and 21 from five dogs infected experimentally with CCoV type II strain CB/05 (Buonavoglia et al., 2006 and unpublished data).

2.4. Haemagglutination inhibition (HI) assay

For HI, two-fold dilutions (from 1:10 to 1:640) of each serum sample in PBS were made in 96-well V-plates, adding 25 µl of BCoV antigen (corresponding to eight HA units) to each serum dilution. After an incubation period of 45 min at room temperature, 50 µl of a 0.7% suspension of mouse erythrocytes was added to each well. HI test results were read after 2 h of incubation at room temperature. The HI antibody titres were expressed as the reciprocals of the highest dilutions of the test sera that inhibited completely the haemagglutinating activity of the virus.

2.5. RNA extraction

Viral RNA was extracted from tissue samples using the QIAamp[®] RNeasy Mini Kit (Qiagen S.p.A., Italy), following the manufacturer's instructions. Template RNAs were eluted in 50 µl of RNase-free water and stored at –70 °C until their use.

2.6. Screening of tissue samples for CRCoV nucleic acid

Detection of CRCoV RNA was carried out using SuperScript[™] One-Step RT-PCR for Long Templates (Life Technologies, Invitrogen, Milan, Italy) and primers specific for the spike protein gene (Erles et al., 2003). The following thermal protocol was used: reverse transcription at 50 °C for 30 min, inactivation of Superscript II RT at 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s, with a final extension at 68 °C for 10 min. The PCR products were detected by electrophoresis through a 1.5% agarose gel and visualisation under UV light after bromide ethidium staining.

2.7. Virus isolation

The RT-PCR positive samples were homogenised (10%, w/v) in D-MEM plus antibiotics (penicillin 5000 IU/ml, streptomycin 2500 µg/ml, amphotericin B 10 µg/ml) and trypsin (5 µg/ml) and inoculated onto confluent HRT-18 cells following the same protocol as for BCoV. After 3 days of incubation at 37 °C, the inoculated cells were tested for CRCoV antigen by an immunofluorescence (IF) assay using a dog serum positive for CRCoV antibodies and by RT-PCR (Erles et al., 2003). Each sample was passaged three times prior to be considered as negative for CRCoV.

2.8. Sequence analysis and phylogeny

The PCR-amplified product of the S-gene of strain 240/05 was sequenced in both directions by Genome Express (Meylan, France) and the obtained sequences were assembled and analysed using the BioEdit software package (Hall, 1999) and the NCBI's (<http://www.ncbi.nlm.nih.gov>) and EMBL's (<http://www.ebi.ac.uk>) analysis tools. GenBank accession number DQ66240 was assigned to the sequenced

fragment. Phylogenetic and molecular evolutionary analyses were conducted using Mega3 (Kumar et al., 2004). Phylogenetic trees, based on the S-gene fragment of CRCoV strain 240/05, were elaborated using both parsimony and neighbor-joining methods, supplying a statistical support with bootstrapping over 100 replicates.

3. Results

3.1. Detection of CRCoV antibodies

By using the BCoV-based ELISA, antibodies against CRCoV were detected in the Italian dog population only in the last 2 years, with seroprevalence rates comprised between 38.33% (year 2005) and 26.76% (year 2006), whereas no evidence for CRCoV circulation was found in previous years (1994–2004). The OD values detected by ELISA ranged from 0.063 to 0.229.

The influence of age on CRCoV seropositivity was evaluated by distributing the samples of the last 2 years by the age of the tested dogs, taking into account an age <6 months ($n = 100$) or >6 months ($n = 31$). The CRCoV prevalence was higher in dogs >6 months of age (51.61%) than in younger dogs (26.00%).

All the 13 sera that had been tested previously by CCoV ELISA (Pratelli et al., 2002) resulted negative for CRCoV antibodies.

3.2. Evaluation of cross-reactivity between CCoV and CRCoV antibodies

All the sera collected from the dogs infected experimentally with CCoV tested negative by the BCoV-based ELISA before the challenge and remained negative after 21 days post-infection. In contrast, CCoV antibody titres, calculated using CCoV-specific ELISA (Pratelli et al., 2002), underwent a considerable increase (data not shown). Thus, the cross-reactivity between CCoV and CRCoV using the BCoV-based ELISA was definitively ruled out.

3.3. Comparison between ELISA and HI

Out of 216 sera examined, 36 and 42 samples tested positive by HI and ELISA, respectively. Only 10 sera

resulted positive by both assays, whereas concordant negative results were obtained for 148 samples. Taking ELISA as the ‘gold standard’, HI relative sensitivity and specificity were 23.81 and 85.06%, respectively. Most HI positive results were obtained from samples having ELISA OD values >0.100.

3.4. Detection of CRCoV RNA

By RT-PCR analysis of the tissue samples, CRCoV RNA was detected in the lungs of a 60-day-old German shepherd of the Apulia region. The dog (240/05) had died with signs of severe haemorrhagic gastroenteritis but without any respiratory distress, although at necropsy small areas of bronchopneumonia were observed. A TaqMan-based real-time PCR assay (Decaro et al., 2005b), carried out on the intestinal content of this dog, identified the nucleic acid of canine parvovirus type 2 (CPV-2), which was characterised as CPV-2c by means of an MGB probe assay (Decaro et al., 2005a, 2006). Moreover, CCoV type II RNA was detected in the intestinal content by means of genotype-specific real-time RT-PCR (Decaro et al., 2005c).

By testing other tissue samples from the dead dog, CRCoV RNA was found also in the spleen, mesenteric lymph nodes and intestine, but not in the liver, kidney and bone marrow.

All attempts failed to isolate on cell cultures the CRCoV strain from the RT-PCR positive samples, as demonstrated by the IF and RT-PCR results obtained from the third passages on HRT-18 cells of all tissue homogenates.

3.5. Sequence analysis and phylogeny

The specificity of the amplification was confirmed by sequence analysis of the RT-PCR product generated by primer pair Sp1/Sp2. The 595-bp fragment analysed showed the highest nucleotide (nt) identity (98%) to the only CRCoV strain currently available in the GenBank database (strain T101) and to the strictly related viruses BCoV, HCoV-OC43 and HECV-4408 (97% of nt identity), whereas a high divergence was observed from the group I coronavirus CCoV type II (4% of nt identity) (Table 1).

Phylogeny with the neighbor-joining method showed that strain 240/05 was tightly intermingled

Table 1
Nucleotide identity (%) of reference CoVs to strain 240/05 in the partial S-gene sequences

Antigenic group	Coronavirus strain	GenBank accession number	Nucleotide identity (%)
II	CRCoV-T101	AY150272	98
II	BCoV-Mebus	U00735	97
II	BCoV-ENT	AF391541	96
II	HCoV-OC43	Z32768	97
II	HECV-4408	L07748	97
II	HEV-VW572	NC_007732	74
II	HCoV-HKU1	NC_006577	68
II	SDAV-681	AF207551	67
II	MHV-A59	AY700211	64
I	CCoV II-CB/05	DQ112226	4

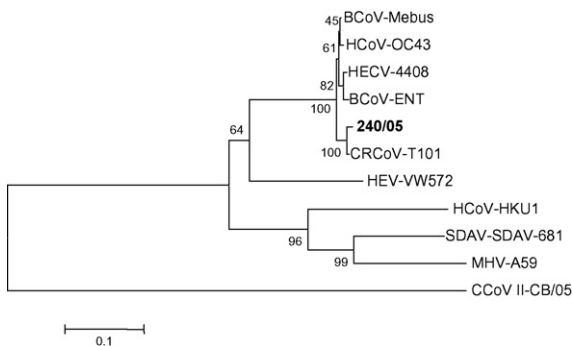


Fig. 1. Neighbor-joining tree based on partial S nucleotide sequences of coronaviruses. Accession numbers of the strains used for phylogeny are reported in Table 1. CCoV type II strain CB/05 (Buonavoglia et al., 2006) was used as outgroup. Bootstrap values were calculated and are indicated at each node. The scale bar represents 10 substitutions per 100 sequence positions.

with CRCoV strain T101, forming together a separate cluster within group II coronaviruses (Fig. 1). A similar tree topology was obtained using the maximum parsimony (data not shown).

4. Discussion

In this study, the presence of CRCoV in Italy was clearly demonstrated by using serology and RT-PCR. By means of serological testing, the prevalence of CRCoV in Italy was found to be similar to those assessed in UK (36%) and Republic of Ireland (30%) (Priestnall et al., 2006) rather than those observed in Japan (17.8%) (Kaneshima et al., 2006) and North

America (54.7%) (Priestnall et al., 2006). The BCoV-based ELISA test suggested that a group II coronavirus has been circulating in the Italian dog population not before 2005, whereas the first report of CRCoV identification in UK dates to 3 years ago (Erles et al., 2003). However, due to the absence of representativity of the samples examined, it should be taken into account that these data are only indicative of the real circulation of the virus. The seropositivity increased with the age of the dogs, since CRCoV antibodies were detected at higher frequency in dogs over 6 months of age with respect to younger dogs.

A HI test for CRCoV antibodies was also established taking advantage on the presence of the haemagglutinin/esterase protein in all group II coronaviruses that is responsible for haemagglutination of mouse erythrocytes. However, assuming the ELISA test as the 'gold standard', the HI test was proven to be poorly specific and sensitive, therefore not recommendable as an extensive diagnostic test. The poor specificity may be related to aspecific inhibitions of the haemagglutinating activity by substances present in the sera, whereas the lack in sensitivity may be a consequence of the high antibody titres probably required to prevent haemagglutination.

A definitive evidence of the presence of CRCoV in Italy was provided by the molecular identification of a S-gene fragment in the lungs of a CPV-positive dog, showing high identity to the analogous nucleotide sequences of the CRCoV strain detected by Erles et al. (2003). Despite the wide distribution of the virus in the organism, as determined by the RT-PCR positive results obtained from several tissues, attempts to isolate the virus on HRT-18 cells were unsuccessful. This finding could be explained with low amounts of virus present in the tissues or, alternatively, with virus inactivation during transportation or storage. A poor sensitivity of HRT-18 cells could be ruled out as this cell line has proved to be highly permissive for CRCoV growth (Erles et al., 2006). Although the dog was not reported to have respiratory signs, at necropsy some lesions were observed in the lungs. However, the involvement of CRCoV in the death of the infected dog could not be demonstrated, since a co-infection by CPV-2 was detected.

To date, outbreaks of CRCoV infection have been reported in UK (Erles et al., 2003), Japan (Kaneshima et al., 2006) and Canada (Ellis et al., 2005). The

present study reports the first detection of CRCoV in a country of the continental Europe. Enduring epidemiological surveillance will help a timely identification of future outbreaks and a more in-depth comprehension of the pathogenic potential of CRCoV in dogs.

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