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Sequence analysis of divergent canine coronavirus strains present in a UK dog population

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ABSTRACT

Forty faecal samples were tested by RT-PCR using coronavirus consensus primers to determine faecal shedding of canine coronavirus (CCoV) and canine respiratory coronavirus (CRCoV) in a dog population housed at a rescue centre.

Seven samples were positive for CCoV while all samples were negative for CRCoV. Sequence analysis of five CCoV strains showed a high similarity with transmissible gastroenteritis virus (TGEV) at the N-terminus of the spike protein. All strains contained an open reading frame for the nonstructural protein 7b, which is not present in TGEV, indicating that the strains were related to the previously described CCoV strain UCD-1. Two samples contained CCoV strains with 5′ spike sequences most similar to type II CCoV while one sample was found to contain type I CCoV.

Primers directed to the N gene allowed specific detection of all CCoV strains analysed in this study.

This investigation shows that CCoV strains containing spike proteins similar to TGEV are present in the UK dog population. PCR primers directed to conserved regions of the CCoV genome are recommended for detection of CCoV in clinical samples due to high genetic variability.

1. Introduction

The *Coronaviridae* family is organised in three groups according to antigenic and genetic similarities. In dogs, two distinct coronaviruses have been described; canine coronavirus (CCoV), which belongs to group 1 and canine respiratory coronavirus (CRCoV) belonging to group 2. Coronaviruses possess a single stranded, positive sense genome of 27–31 kb in length which contains a large open reading frame for the major nonstructural proteins at the 5′ end and the structural proteins as well as minor nonstructural proteins in the 3′ third of the genome. The structural proteins include the spike glycoprotein (S), the small envelope protein (E), the membrane protein (M) and the nucleocapsid protein (N) (Weiss and Navas-Martin, 2005).

CRCoV has been detected predominantly in respiratory samples but also occasionally in samples from the gastrointestinal tract (Decaro et al., 2007; Erles et al., 2003; Yachi and Mochizuki, 2006). A high prevalence of CRCoV has been found in kennelled dog populations in which infectious respiratory disease is common.

Canine coronavirus exhibits a predominantly enteric tropism and causes gastroenteritis particularly in young dogs (Pratelli, 2006). However a recently described strain, CCoV CB/05 has been shown to cause a fatal disease which was characterised by systemic spread of the virus (Decaro et al., 2008). CCoV is divided into two genotypes; type I is characterised by genetic similarity to feline coronavirus (FCoV) type I in the spike and membrane genes. One CCoV strain, UCD-1, contains a spike gene with a high sequence identity at the 5′ end to that of the porcine transmissible gastroenteritis virus (TGEV) (Wesley, 1999).

The aim of this study was to investigate faecal shedding of coronaviruses in kennelled dogs and to characterise detected coronavirus strains by sequence analysis.

2. Materials and methods

2.1. Clinical samples

Faecal samples were collected from dogs housed at a rehoming centre. None of the dogs showed clinical signs of gastroenteritis and the general health status of all dogs was good. In total faecal samples from 40 dogs were analysed.

2.2. RNA extraction and RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK). 25–50 mg of faeces was suspended in 350 μl Buffer RLT and centrifuged. The extraction was then performed on the supernatant as per the manufacturer's protocol for animal cells.

Reverse transcription was carried out using 7 μl of total RNA, 0.5 μg random hexameres (GE Healthcare) 1 μl of ImPromII reverse

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Table 1
Primer sequences.

Primer	Sequence 5′–3′	Position	GenBank accession number
CENP1	CTC-GTG-GYC-GGA-AGA-ATA-AT	7270-7289	D13096
CENP2	GCA-ACC-CAG-AMR-ACT-CCA-TC	7549-7530	D13096
CEPol-1	TCT-ACA-ATT-ATG-GCT-CTA-TCA-C	309-330	D13096
CESP-6	TTG-CAC-ATC-ATC-TCT-ATA-AGC	886-866	D13096
CESP-10	GAT-TTA-TCT-GAT-ARC-AGR-AAC-CA	941-919	AY307020
TGSP-2	TAA-TCA-CCT-AAM-ACC-ACA-TCT-G	20537-20516	NC_002306

The primer positions refer to the corresponding GenBank sequence.

transcriptase (Promega) and a $MgCl_2$ concentration of 3 mM in a total reaction volume of 20 μl.

For PCR, 2 μl of cDNA was added to a reaction mix of 1× PCR buffer, 2.5 mM $MgCl_2$, dNTP mix (final concentration of each deoxynucleotide 0.2 mM) and 1.25 U of Taq polymerase (Promega).

PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was carried out using the primers and conditions as described by Grone et al. (1996).

PCR for the specific detection of CRCoV was carried out using the nested PCR for the spike gene as described elsewhere (Erles et al., 2003).

For the detection of coronaviruses in faecal samples the following PCR protocols were used. For primers CENP 1 and CENP 2 directed to the CCoV nucleocapsid protein gene (Table 1) the following cycling conditions were used: 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 53 °C for 40 s and 72 °C for 30 s followed by a final extension at 72 °C for 10 min. The primers were designed to amplify a 280 bp product corresponding to 68–347 nt of the nucleocapsid gene of CCoV. The 5′ end of the spike gene was amplified using a consensus forward primer located in ORF1b (CEPol-1) and reverse primers specific for either TGEV (TGSp-2), CCoV type I (CESp-10) or CCoV type II (CESp-6). The primer sequences are given in Table 1. Primer pair CEPol-1-TGSp-2 amplified a 370 bp product corresponding to 20,168–20,537 nt of the TGEV Purdue genome, which consisted of 198 nt of the 3′ end of ORF1b and the first 172 nt of the spike gene. Primer pair CEPol-1-CESp-10 was designed to amplify a 1108 bp product consisting of 167 nt of the 3′ end of ORF1b and the first 941 nt of the spike gene. Primer pair CEPol-1-CESp-6 amplified a 578 bp product consisting of 197 nt of the 3′ end of ORF1b and the first 381 nt of the spike gene. The cycling conditions were 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min followed by a final extension for 10 min at 72 °C. In addition primers EL1F and EL1R directed to the spike gene of type I CCoV (Pratelli et al., 2004) were used, which amplify a 346 bp product corresponding to 3538–3883 nt of the spike gene of CCoV strain Elmo. A nested PCR primer set to the CCoV spike gene (Naylor et al., 2002) amplified a 1086 bp product using the outer primers, corresponding to 81–1166 nt of the spike gene of CCoV strain Insavc. The spike gene consensus primers 55 and 56 for all coronaviruses (Wise et al., 2006) amplified 3549–4188 nt of the spike gene of CCoV Insavc (640 bp product). Primers M5 and M6 amplified the complete sequence of the CCoV membrane protein gene (Pratelli et al., 2003). The consensus primers Conscoro 5 and Conscoro 6 were based on the polymerase gene (corresponding to 13,602–13,852 nt of the genome of TGEV strain Purdue). These primers detect both, CCoV and CRCoV (Erles et al., 2003). The region containing ORFs 7a and 7b was amplified using primers N3SN and R3AS as previously described (Wesley, 1999).

2.3. Analytic restriction digest of Conscoro PCR product

Using the Qiaquick PCR purification kit (Qiagen) 30 μl of the PCR product was purified. Restriction digest was performed using 15 μl of purified product and AluI (Promega) in the appropriate buffer for 1 h at 37 °C. The 251 bp product amplified by primers conscoro 5/6 contained a restriction site for AluI at position 124 for CRCoV generating two fragments of 124 and 127 bp length. In PCR products generated from CCoV the restriction site was located at position 43 and digest generated two products of 43 and 208 bp. The products were analysed by gel electrophoresis on 1.5% agarose gels and visualised using ethidium bromide.

2.4. Cloning and sequencing of PCR products

PCR products were cloned into the pGEM-T-easy vector (Promega). Plasmid DNA (600 ng) was sent to The Sequencing Service (University of Dundee, UK) for sequencing.

2.5. Phylogenetic analysis

DNA sequences were aligned using ClustalX (Thompson et al., 1997). Phylip (Phylogeny Inference Package, version 3.6) was used to perform bootstrap and maximum parsimony analyses (Felsenstein, 2004). A consensus tree was calculated using Consense (Phylip 3.6). The resulting trees were drawn using the Treeview program (Page, 1996).

2.6. GenBank accession numbers

The following accession numbers were assigned: partial spike protein gene 04-0709 (FJ009112), 03-3684 (FJ009113), complete M protein gene 04-0709 (FJ009114), 04-0377 (FJ009115), partial N protein gene 04-0377 (FJ009116), 04-0709 (FJ009117), 03-3684 (FJ009118).

3. Results

3.1. PCR analysis for CRCoV and CCoV

Forty faecal samples were collected from dogs housed at a rehoming kennel. The samples were analysed to determine the presence of canine coronaviruses in faecal samples. The results of all PCR analyses are summarised in Table 2.

All faecal samples were negative for CRCoV by nested PCR based on the CRCoV spike gene.

Two out of 40 samples were positive for CCoV by PCR using a nested primer set based on the spike gene of CCoV (Naylor et al., 2002).

PCR analysis using consensus coronavirus primers based on the polymerase gene detected seven positive samples. Subsequent restriction digests and sequence analyses determined that these samples contained CCoV. As the CCoV specific nested PCR directed to the spike gene had failed to detect five out of seven samples further sets of primers were tested to find a method for detecting UK strains of CCoV.

Primers M5 and M6 had previously been described to be able to detect type I and type II CCoV (Pratelli et al., 2003). Using these primers six out of seven samples were positive. PCR using a newly