

# Isolation and sequence analysis of canine respiratory coronavirus

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## Abstract

Canine respiratory coronavirus (CRCoV) has frequently been detected in respiratory samples from dogs by RT-PCR. In this report the first successful isolation of CRCoV from a dog with respiratory disease is described. The isolate CRCoV-4182 was cultured in HRT-18 cells but failed to replicate in a number of other cell lines. The nucleotide sequence of the 3'-terminal portion of the CRCoV genome was determined including all open reading frames from the NS2 gene to the N gene. Comparison with other coronavirus sequences showed a high similarity to bovine coronavirus (BCoV). The region between the spike and the E gene was found to be the most variable and was used for phylogenetic analysis of several CRCoV strains. CRCoV-4182 showed a mutation within the non-structural protein region downstream of the S gene leading to the translation of an 8.8 kDa putative protein comprising a fusion of the equivalent of the BCoV 4.9 kDa protein to a truncated version of the BCoV 4.8 kDa protein. The culture of CRCoV will enable analysis of the expression and function of this and other CRCoV proteins as well as allowing the study of the role of CRCoV in the aetiology of canine infectious respiratory disease.

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## 1. Introduction

Canine respiratory coronavirus (CRCoV) was originally detected by RT-PCR in respiratory samples from a population of dogs at a rehoming centre with a high incidence of infectious respiratory disease (Erles et al., 2003). Subsequent studies found that serum antibodies to the virus were common in the canine population of the United Kingdom, Republic of Ireland, USA and Japan (Kaneshima et al., 2006; Priestnall et al., 2006). The role of CRCoV in the canine infectious respiratory disease complex is still under investigation. Phylogenetic analyses and the presence of a haemagglutinin esterase gene placed CRCoV in group 2 of the *Coronaviridae* family. Coronaviruses are enveloped viruses with a large (27–32 kb) single-stranded, positive-sense RNA genome. The structural proteins located within the viral envelope are the spike protein (S), membrane protein (M), envelope protein (E) and the haemagglutinin esterase (HE) protein, which is only present in members of group 2 of the coronaviruses. The nucleocapsid protein (N) associates with the viral RNA to form the helical nucleocapsid (Spaan et

al., 1988). Two large open reading frames (ORFs) at the 5' end of the genome encode the coronavirus replicase proteins. Additional non-structural proteins of group 2 coronaviruses such as bovine coronavirus (BCoV) and mouse hepatitis virus (MHV) are located between the polymerase gene and the HE gene and between the S and E gene. Coronaviruses produce 3' coterminal sets of mRNA of which in general only the first gene at the 5' end is translated. All mRNAs contain a common leader sequence at the 5' end which is encoded by the 5' end of the coronavirus genome. Coronavirus genes are preceded at the 5' end by transcription regulatory sequences (TRS) that commonly include the consensus sequence CYAAAC (Hofmann et al., 1993; Zuniga et al., 2004).

Canine respiratory coronavirus was initially characterised by sequence analysis of RT-PCR products generated from RNA isolated from tissue samples. The CRCoV spike gene showed a 97.3% nucleotide identity with the spike gene of bovine coronavirus (BCoV) and 96.9% identity with human coronavirus OC43 (HCoV-OC43) (Erles et al., 2003).

Despite frequent detection of CRCoV in tissue samples by RT-PCR, isolation of the virus on canine cell lines as well as on the human adenocarcinoma cell line HRT-18 was at first unsuccessful (Erles et al., 2003; Kaneshima et al., 2006). HRT-18 cells are commonly used for the isolation of BCoV from bovine respi-

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ratory and faecal samples and support amongst others the growth of HCoV-OC43, porcine haemagglutinating encephalomyelitis virus (HEV) as well as equine coronavirus (ECoV) (Guy et al., 2000; Sasseville et al., 2002; St.-Jean et al., 2004; Tsunemitsu and Saif, 1995).

We report here the first isolation of CRCoV from a canine respiratory sample as well as sequence analysis of the CRCoV genome from the NS2 gene to the N gene.

## 2. Material and methods

### 2.1. Cell lines

A72, BHK-21, CHO, DH82, MDBK and MDCK cells were maintained in MEM, Vero cells were maintained in DMEM and HRT-18 cells in RPMI (Sigma). All media contained 5% FCS (PAA Laboratories), 100 U ml<sup>-1</sup> penicillin and 0.1 mg ml<sup>-1</sup> streptomycin (Sigma).

### 2.2. Clinical samples

Respiratory samples from dogs were collected as part of an ongoing study into the causes of canine infectious respiratory disease. A post mortem examination was carried out in 2003 on a 3-month-old dog that had suffered from canine infectious respiratory disease and had been euthanased after developing severe respiratory distress. A lung wash was carried out using Hank's balanced salt solution (Sigma). In addition a tracheal sample was taken (T4182). Tracheal samples, obtained in 2004 from three other dogs housed at the same kennel (T0715, T1030, and T1207) and identified as positive for CRCoV RNA, were used for comparative sequence analysis. Tracheal sample T101 had been obtained in 2000 at the same kennel as the tracheal samples listed above and the complete CRCoV spike gene sequence of this sample is available at GenBank (AY150272). A CRCoV positive pharyngeal swab (G9142) from a dog housed at a training centre had been obtained in 2001 (Erles and Brownlie, 2005).

### 2.3. Virus isolation

A 25 cm<sup>2</sup> culture flask containing HRT-18 cells was washed with 2 ml of serum-free RPMI containing 1 µg ml<sup>-1</sup> trypsin (Sigma) and inoculated with 2 ml of lung wash. After 1 h at 37 °C the inoculum was removed and replaced with 5 ml of serum-free RPMI containing 1 µg ml<sup>-1</sup> trypsin.

After 5 days the cells were analysed for the presence of CRCoV by using a nested RT-PCR for the CRCoV spike gene (primers Sp1–2 and Sp3–4) as described previously (Erles et al., 2003) as well as immunofluorescence (see below). The culture supernatant was inoculated onto fresh HRT-18 cells for a total of 13 passages.

### 2.4. Inoculation of cell lines with CRCoV

Cell lines grown in 6 well plates were washed with 1 ml of serum-free medium containing penicillin, streptomycin and 1 µg ml<sup>-1</sup> trypsin (sf-medium) and then inoculated with 100 µl

of CRCoV in a total of 1 ml sf-medium for 1 h at 37 °C and 5% CO<sub>2</sub>. The inoculum was removed, the cells were washed with sf-medium and fresh sf-medium was added. The cells were analysed at several time points for CRCoV replication by immunofluorescence and RT-PCR.

The canine cell lines tested for susceptibility to CRCoV infection were the fibroblast cell line A72, the canine kidney epithelial cell line MDCK and the macrophage cell line DH-82. In addition Vero cells (African green monkey kidney), BHK-21 cells (hamster kidney), CHO cells (hamster ovary) and MDBK cells (bovine kidney) were used.

### 2.5. Immunofluorescence staining

Cells were seeded onto coverslips and inoculated as described above. Immunofluorescence analysis was performed 24, 48 and 120 h post infection. The cells were fixed in methanol/acetone (2:1) for 10 min at –20 °C and air-dried. After rehydration in PBS for 5 min the coverslips were placed on 30 µl of chicken anti-BCoV antibody (Churchill Applied, Huntingdon, UK) 1:100 in PBS or a canine serum positive for CRCoV antibodies (previously determined by ELISA) at a dilution of 1:30 in PBS. After 1 h at 37 °C the coverslips were washed in PBS (3 × 5 min) and incubated with FITC-labelled anti-chicken IgG or anti-dog IgG, respectively, for 1 h at 37 °C. The coverslips were washed in PBS as above and mounted onto a slide using 50% glycerol in PBS.

### 2.6. RNA extraction and RT-PCR

RNA was extracted from cells using the RNeasy kit (Qiagen) following the manufacturer's protocol for cultured cells. The RNA was eluted into 30 µl of RNase-free water. Reverse transcription was performed using 7 µl of RNA, 0.5 µg random hexamers (GE Healthcare), 1 µl of ImPromII reverse transcriptase (Promega) and a MgCl<sub>2</sub> concentration of 3 mM in a total reaction volume of 20 µl.

For sequence analysis overlapping PCR products were generated using Pfu polymerase as described previously (Erles et al., 2003). Primers were designed using BCoV consensus sequences as well as available CRCoV sequences (Table 1). For amplification of the NS2 gene the forward primer NS32-1 located in the polymerase gene and reverse primer NS32-2 located in the HE gene were used. The HE gene was amplified using forward primer HE-3 located in the NS2 gene and reverse primer HE-4 located in the spike gene. For the amplification of the CRCoV spike gene, primers Sp-5, Sp-6, Sp-7 and Sp-8 were used as previously described (Erles et al., 2003) in addition to primers Sp-9 (forward, located in the HE gene) and Sp-10 (reverse, located in the non-structural gene region between the spike and the E gene). For amplification of the non-structural region between the spike and the E gene primers Sp-7 (forward, located in the spike gene) and BSM (reverse, located in the E gene) were used. The regions coding for the E protein as well as the membrane protein were amplified using the forward primer M-1 located in the NS12.8 gene and reverse primer M-2 located in the nucleocapsid protein gene. For analysis of the nucleocapsid protein gene forward

Table 1  
Primers used for sequence analysis of CRCoV

Primer	Sequence	Position in CRCoV-4182
NS32-1	5'-CAT-TTT-GGA-CGG-TTT-TCT-GC	1–20
NS32-2	5'-ACA-TTG-GTA-GGA-GGG-TTA-TCA	1272–1252
HE-3	5'-TGC-GTA-AAG-AGC-TAG-AAG-AAG-GTG-A	991–1015
HE-4	5'-AAA-AGC-CAT-TGG-TAA-GGA-A	2523–2505
Sp-9	5'-CGG-TCA-TAA-TTA-TTG-TAG-TTT-TGT	2400–2423
Sp-10	5'-GGA-GCC-AAT-AAA-TCA-AAG-ACG-AAC	6600–6577
BSM	5'-CAC-ATA-CCG-CAA-AGT-TGA-ATA-CAC	7407–7384
NSR-1	5'-GGG-ACT-AGT-TGT-TTT-AAG-AAA-TG	6487–6509
NSR-2	5'-CAA-GCA-TGA-CTA-GGA-ACT-CTA-C	7082–7061
M-1	5'-AGA-GTT-CCT-AGT-CAT-GCT-TGG-TG	7063–7085
M-2	5'-AGG-ACG-CTC-TAC-TAC-TGG-ATT-GCT-TAC	8275–8249
NP-1	5'-GTA-ATT-ACC-GAC-TGC-CAT-CAA-CC	8153–8175
NP-2	5'-TGG-TAA-CTT-AAC-ATG-CTG-GCT-CTT	9815–9792
NP-6	5'-GTT-ACT-AGC-GAC-CCA-GAA-GAC-TCC	8685–8662
BCV-LS	5'-GAG-CGA-TTT-GCG-TGC-GTG-CAT-CCC-GC	Hofmann et al. (1993)

primer NP-1, located in the membrane protein gene, and reverse primer NP-2, located in the 3' untranslated region, were used.

Primers NSR-1 (forward, located in the spike gene) and NSR-2 (reverse, located in the ORF for the 12.8 kDa protein) were used for the amplification of an approximately 600 bp PCR fragment for comparative analysis of the non-structural protein regions of CRCoV and BCoV strains.

### 2.7. Analysis of mRNA transcription of the CRCoV nucleocapsid protein gene

Forward primer (BCV-LS), based on the BCoV leader sequence (Hofmann et al., 1993), was used in combination with a reverse primer (NP-6) towards the nucleoprotein gene sequence. PCR was carried out using Taq polymerase (Promega) and a final concentration of 1.5 mM MgCl<sub>2</sub>. The conditions for the PCR were as follows: initial denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 1 min, 55 °C for 40 s and 72 °C for 40 s and a final extension at 72 °C for 10 min. The expected size of the PCR product was 524 bp.

PCR products were analysed on 1% agarose gels and visualised by ethidium bromide.

For sequence analysis PCR products were cloned into the pT7blue2 vector. Plasmid DNA was submitted to The Sequencing Service (University of Dundee, UK).

### 2.8. Haemagglutination test

Chicken blood was obtained diluted 1:2 in Alsever's solution (Harlan Sera-Lab). Red blood cells were washed in PBS and diluted to 1% in PBS. A two-fold dilution series of culture supernatants of CRCoV infected HRT-18 cells in PBS was prepared. Fifty microlitres of red blood cell suspension and 50 µl of each virus dilution were mixed in a 96 well plate and incubated at 4 °C overnight.

### 2.9. Virus titration

HRT-18 cells were seeded onto a 96 well plate at  $4 \times 10^4$  cells per well. After inoculation of the cells with ten-fold dilutions of

CRCoV in sf-RPMI the plates were incubated at 37 °C, 5% CO<sub>2</sub> for 5 days. The cells were washed with PBS, fixed with 80% acetone in dH<sub>2</sub>O for 10 min at –20 °C and air-dried. For detection of CRCoV the plates were incubated with a canine serum positive for CRCoV antibodies over night at 4 °C, washed (3 × 5 min with PBS) and incubated with a peroxidase-labelled anti-dog IgG antibody (Sigma, 1:500 in PBS) for 1 h at 37 °C. DAB substrate (Sigma) was added; colour development was stopped after 30 min at RT by washing with dH<sub>2</sub>O.

### 2.10. Accession numbers

The sequence of CRCoV isolate 4182 has been assigned GenBank accession number DQ682406. For phylogenetic analysis the following nucleotide sequences were used: U00735 (BCoV Mebus), AF058942 (BCoV-LY138), AF239310 (BCoV BCO 43277), AY078417 (HEV-67N), AY903460 (HCoV-OC43), AY316300 (ECoV-NC99) and NC006852 (MHV-JHM).

### 2.11. Sequence analysis

Analysis of sequence data was carried out using VectorNTI Advance 10 (Invitrogen). Nucleic acid and amino acid similarity searches were performed using FASTA (Pearson, 1990).

### 2.12. 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 tree was drawn using the Treeview program (Page, 1996).

### 2.13. Protein analysis

Prediction of N-glycosylation sites and phosphorylation sites of CRCoV proteins was carried out using the PROSITE program (Bairoch et al., 1997) located at the

PredictProtein server (<http://www.predictprotein.org/>). For O-glycosylation sites the NetOGlyc3.1 program was used (<http://www.cbs.dtu.dk/services>) (Julenius et al., 2005).

Transmembrane helices were predicted using the PHDhtm program (Rost et al., 1996) at the PredictProtein server as well as the TMpred program at EMBnet (<http://www.ch.embnet.org/>) (Hofmann and Stoffel, 1993). Signal peptide predictions were carried out using the SignalP 3.0 program (<http://www.cbs.dtu.dk/>) (Dyrlov Bendtsen et al., 2004).

### 3. Results

#### 3.1. Susceptibility of cell lines to CRCoV infection

CRCoV strain 4182 was isolated from a dog, euthanased in 2003 following severe respiratory disease, by inoculating a lung wash sample obtained from the carcass onto HRT-18 cells. CRCoV replication was detected after the second passage on HRT-18 cells by using RT-PCR (Fig. 2) and immunofluorescence with an antibody against BCoV as well as a canine CRCoV-antibody positive serum (Fig. 1).

Despite the addition of trypsin no syncytia or other visible signs of a cytopathic effect of CRCoV infection were observed on HRT-18 cells. The trypsin concentration of  $1 \mu\text{g ml}^{-1}$  was the highest concentration that could be used without detachment of HRT-18 cells from the culture surface. Despite the lack of a cytopathic effect HRT-18 cells were found to be a suitable culture system reproducibly producing titres of up to  $10^6$  TCID<sub>50</sub>/ml. Due to the absence of a cytopathic effect an immunoperoxidase assay to determine CRCoV titres was developed.

CRCoV infected cell culture supernatants were incubated with chicken erythrocytes and found to have haemagglutinating activity. The haemagglutination assay may be useful as a

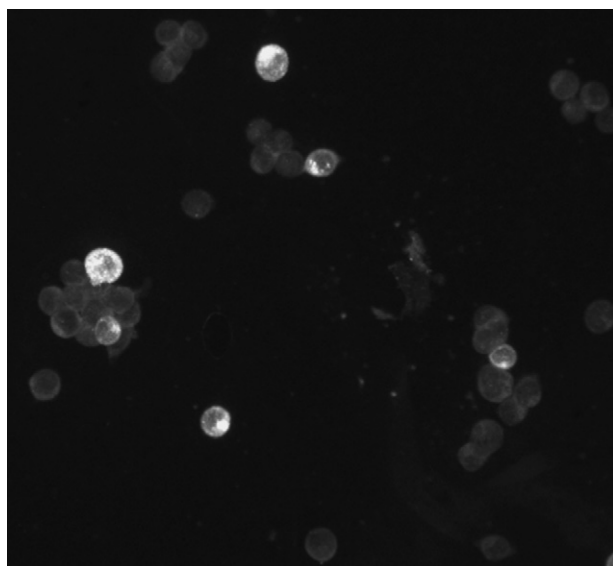


Fig. 1. Detection of CRCoV in HRT-18 cells by immunofluorescence assay using a polyclonal antiserum against bovine coronavirus.

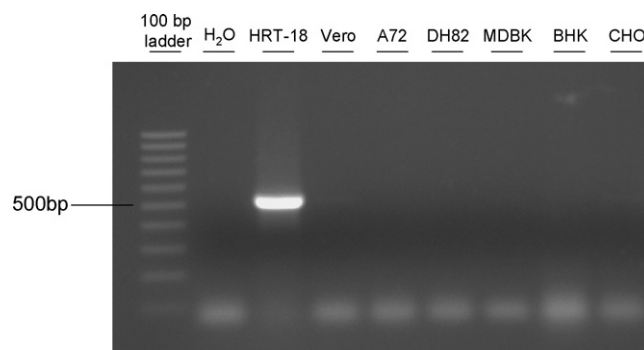


Fig. 2. Susceptibility of cell lines for infection with CRCoV. The cell lines were infected with CRCoV and total RNA was isolated 48 h post infection. RT-PCR for the detection of CRCoV nucleoprotein gene mRNA was performed using the primers BCV-LS and NP-6.

tool for the identification of CRCoV positive cultures if other isolates also prove to be non-cytopathic.

Cell lines other than HRT-18 were tested for their susceptibility to CRCoV infection. Three canine cell lines, derived from fibroblasts, kidney epithelial cells and macrophages, were tested. In addition Vero cells, BHK-21 cells, CHO cells and MDBK cells were used. All cell lines were analysed by immunofluorescence assay and RT-PCR for the nucleocapsid protein mRNA at 24, 48 and 120 h after infection. In addition for A72 cells and Vero cells the supernatants were passaged three times before analysis of the final passage. However none of the cell lines tested was susceptible to CRCoV infection (Fig. 2).

#### 3.2. Amino acid changes in the CRCoV spike protein after adaptation to HRT-18 cells

After 13 passages on HRT-18 cells total RNA was extracted from CRCoV infected cells and the complete sequence of the spike gene was determined. From the post mortem case that was used for isolation of CRCoV-4182 a tracheal sample was also obtained for RNA extraction and PCR to determine the spike gene sequence (T4182). These sequences were compared to the spike gene sequence from CRCoV-RNA positive tracheal sample T101 (GenBank AY150272). In total eight nucleotide and amino acid positions were found to be changed in the spike gene after 13 passages when compared to the RNA sequences obtained from both tracheal samples (Table 2). Comparing the complete spike gene sequences from tracheal sample T4182 to the sequence obtained from tracheal sample T101, collected from the same population 3 years previously, 24 nucleotide changes were found leading to amino acid changes in 16 positions (Table 2).

#### 3.3. Sequence analysis of CRCoV from the NS2 gene to the nucleocapsid gene

The predicted amino acid sequences of CRCoV proteins were compared to the corresponding proteins of two BCoV strains (LY-138 and Mebus), HCoV-OC43 (VR-759), HEV (VW572), ECoV (NS99), MHV (JHM), rat sialodacryoadenitis virus (SDAV-681) and human coronavirus HKU-1. The amino



Table 2  
Amino acid changes in the spike protein of CRCoV

Amino acid	T101	T4182	Isolate 4182 p13
36	I	V	V
74*	T	T	I
166*	D	D	N
171*	M	M	I
175	N	S	S
192	P	S	S
210	S	F	F
267*	F	F	S
501	F	S	S
692	G	A	A
757	W	C	C
758	G	V	V
769	T	S	S
786	P	S	S
792	H	Q	Q
827	P	S	S
974	A	V	A
977	F	S	S
983*	P	P	R
985*	S	S	P
1018*	S	S	A
1063	K	R	R
1300*	Y	Y	F
1360	S	L	L

The amino acid sequence of the complete spike ORF of CRCoV isolate 4182 was compared to the spike amino acid sequence obtained from CRCoV-RNA extracted from tracheal samples T101 and T4182. Amino acid positions that acquired changes during culture of CRCoV isolate 4182 in HRT-18 cells are indicated by an asterisk.

acid identities are given in Table 3. Due to the variability of the proteins expressed in the region between the S gene and the E gene nucleotide rather than amino acid identities were calculated. In addition to CRCoV-4182 at passage 13, CRCoV cDNA from samples T4182, T0715, T1030, T1207 and G9142 was analysed for this region.

Fig. 3 shows the genomic organisation of the region between the S and the E gene for BCoV strain Mebus compared to CRCoV strain 4182. The region was divided into three parts for sequence analysis. Region A (297–305 bp) corresponded to the ORFs for the 4.9 and 4.8 kDa proteins of BCoV, region C corresponded to the partial ORF (137 bp) of the BCoV 12.7 kDa protein and region B was the non-coding sequence located in

between these ORFs (79–83 bp). Region A was found to be the most variable amongst the CRCoV strains with nucleotide identities of 96–99%. When comparing CRCoV to BCoV strains LY-138, Mebus and BCO.43277 nucleotide identities of 89–93% were calculated. Regions B and C were identical for all CRCoV strains analysed but showed nucleotide identities of 86–92% and 95–97%, respectively, when compared to the above BCoV strains.

Comparison of the cDNA sequences of T4182 with CRCoV-4182 after 13 passages in HRT-18 revealed no nucleotide changes in the non-structural region.

### 3.4. Analysis of the NS2 gene

The ORF for the NS2 gene was 837 nt long and encoded a 32.3 kDa protein. A transcription regulatory sequence (TRS) was present seven nucleotides prior to the AUG start codon (CUAAAC). Analysis of the CRCoV NS2 protein sequence using the PROSITE motif search revealed five potential phosphorylation sites at amino acid positions 18, 123, 141, 200 and 273 as well as an N-glycosylation site at amino acid 241.

### 3.5. Analysis of the HE gene

The haemagglutinin-esterase gene of CRCoV had a length of 1275 nt resulting in a 47.7 kDa protein. The start codon was preceded by a TRS (CUAAAC) which was separated from the AUG codon by nine nucleotides. The protein contained eight potential N-glycosylation sites and a potential signal peptide was identified at amino acids 1–18 with a potential cleavage site between amino acids 18 and 19. The N-terminal 389 amino acids were predicted to be outside the viral envelope with a transmembrane region at amino acids 390–410 and an internal segment from amino acid 411–424 using PHDhtm. Using TMpred the transmembrane helix was predicted for amino acids 394–415. The putative active site for esterase activity, FGDS (Kienzle et al., 1990), was present at amino acids 37–40.

### 3.6. Analysis of the S gene

Translation of the 4092 nt ORF of the spike gene resulted in a 151.1 kDa protein. The AUG codon was directly preceded by the TRS CUAAAC. The spike protein of CRCoV is likely to be gly-

Table 3  
Amino acid identities of CRCoV proteins compared with the corresponding proteins of other group 2 coronaviruses

Strain	Percent amino acid identity with CRCoV							
	NS2	HE	S	12.8	E	M	N	I
BCoV-LY138	98.2	97.2	96.8	97.2	98.8	98.3	98.9	96.6
BCoV Mebus	96.8	97.4	95.7	97.2	97.6	97.8	98.7	94.7
HCoV-OC43 VR-759	95.3	92.9	90.1	91.7	96.4	91.7	97.5	92.2
HEV-VW572	93.8	88.7	81.7	86.2	97.6	93.9	94.7	89.4
ECoV-NC99	n/a <sup>a</sup>	72.9	80.5	82.6	90.5	90.4	91.1	76.0
MHV-JHM	52.8	60.2	61.4	53.3	65.9	83.3	71.2	49.0
SDAV-681	53.0	60.0	64.6	55.4	67.5	83.8	71.7	43.1
HKU-1	–	57.4	63.9	46.8	54.4	81.4	64.7	42.9

<sup>a</sup> Sequence data not available.

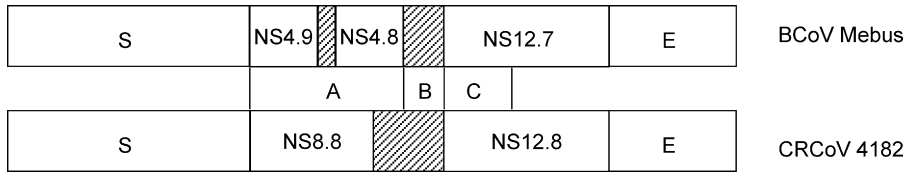


Fig. 3. Comparison of the genomic organisation of the region located between the S and the E genes of BCoV strain Mebus and CRCoV strain 4182. Hatched boxes represent non-coding sequences. A–C indicate the three regions used for comparative nucleotide sequence analysis between CRCoV and BCoV strains.

cosylated similar to other coronaviruses. Prosite motif search revealed 19 potential N-glycosylation sites. The amino acid changes that accumulated in the spike gene during passaging in HRT-18 cells did not lead to a change of any of the predicted N-glycosylation sites. The protein furthermore contained a potential cleavage recognition sequence (RRSR) at residues 765–768 which is present in BCoV and HCoV-OC43, whose spike proteins are cleaved into an S1 and S2 subunit (Abraham et al., 1990a; Mounir and Talbot, 1993b). A potential N-terminal signal peptide was identified at amino acids 1–14 by SignalP-NN or at amino acids 1–17 by SignalP-HMM. Analysis of the protein structure using PHDhtm identified residues 1310–1329 to be the most likely out of three potential transmembrane regions. The most likely transmembrane region as predicted by TMpred was located at amino acids 1311–1329.

3.7. Analysis of the non-structural protein region between the spike gene and the 12.8 kDa protein gene

The coding sequence for potential non-structural proteins, within cDNA derived from four strains obtained at the same kennel between 2003 and 2004, was analysed. A deletion of two nucleotides prior to the stop codon that terminates the 4.9 kDa protein of BCoV was detected. This resulted in the absence of this stop codon and the presence of 12 additional amino acids not found in BCoV leading to the translation of one joint protein of

8.8 kDa (Figs. 3 and 4). This mutation was not found in CRCoV strain G9142.

However all canine strains analysed shared a nucleotide change within the sequence coding for the equivalent of the 4.8 kDa protein of BCoV leading to a truncation of this protein. The CRCoV strains T4182, T0715, T1030 and T1207 therefore coded only for one putative non-structural protein of 8.8 kDa whereas strain G9142 had one protein of 4.9 kDa and one of 2.7 kDa.

The 4.9 kDa protein of BCoV has been shown to lack a start codon in its mRNA (Hofmann et al., 1993). The closest transcription regulatory sequence in CRCoV (CCAAAC) was located 317 nucleotides upstream of the start codon. BCoV uses a TRS (CUAAGT) three nucleotides downstream of the start codon which is also present in CRCoV.

Structural analysis of the 8.8 kDa protein predicted a transmembrane region located at amino acids 29–50 with the C-terminus of the protein being outside the viral envelope. The AUG codon for the putative 2.7 kDa protein of strain G9142 was preceded by the sequence CCAAAC.

3.8. Analysis of the ORF for the 12.8 kDa protein

The complete sequence of the gene corresponding to the BCoV 12.7 kDa non-structural protein was determined. A TRS (CUAAAC) was located 72 nucleotides upstream of the start

		*	20	*	40		
CRCoV-T4182	:	-----	MTTKFVSDLLAPDDILHPSNHVKLI	IRPIEVEHI	IKA	: 38	
CRCoV-G9142	:	-----	MTTKFVSDLLAPDDILHPSNHVKLI	IRPIEVEHI	IKA	: 38	
BCoV-Mebus	:	-----	MTTKFVFDLLAPDDILHPSNHVK	LIIRPIEVEHI	IIA	: 37	
BCoV-LY138	:	-----	MTTKFVFDLLAPDDILHPSNHVKLI	IRPIEVEHI	IIA	: 38	
BCoV-BCO.43277	:	-----	MTTKFVFDLLAPDDILHPSNHVN	LIIRPI	-----	: 29	
MHV-JHM	:		MALIGPKTTIAAVF	IGPFLVACMLG	IGLVYLLQLQVQIFHVKDTIRV	: 47	
		*	60	*	80	*	
CRCoV-T4182	:		TTIPALVGTVSYIITSKPVM	MATTIGGTDYTNIMPSTVFTTVY	----	: 81	
CRCoV-G9142	:		TTIPAF	-----	MPMATTIGGTDYTNIMPSTVFTTVY	----	: 69
BCoV-Mebus	:		TTMPAV	-----	MPMATTIDGTDYTNIMPSTVSTTVYLGCS	: 72	
BCoV-LY138	:		TTMPAV	-----	MPMATTIDGTDYTNIMPSTVSTTVYLGGS	: 73	
BCoV-BCO.43277	:		-----	MPMATTIEGADYTNIMPITVFTTVYLGVS	----	: 58	
MHV-JHM	:		TGKPA	---	TVSYTTSTPVTPVATTL	DGGTYYTLIRPTSSYTRVYLGSS	: 91
		100	*	120	*	140	
CRCoV-T4182	:	-----	-----	-----	-----	: -	
CRCoV-G9142	:	-----	-----	-----	-----	: -	
BCoV-Mebus	:		IGIDTSTTGFTCF	FSRY	-----	: 88	
BCoV-LY138	:		IGIDTSTTGFTCF	SWY	-----	: 89	
BCoV-BCO.43277	:		IGIDTSTTGFTCF	FSRY	-----	: 74	
MHV-JHM	:		RGFDTSTFGPK	TLDTYITSSKPHLNSGR	PYTLRHLPKYMT	PPATWRFG	: 138

Fig. 4. Alignment of the predicted amino acid sequence of the putative CRCoV-4182 8.8 kDa protein and the putative CRCoV-G9142 4.9 kDa and 2.7 kDa proteins with the corresponding proteins of MHV-JHM and BCoV strains Mebus, LY-138 and BCO 43277.

codon however in BCoV the unusual TRS, GUAGAC, 21 nucleotides further downstream, is used (Hofmann et al., 1993). This TRS was also present in all CRCoV strains analysed. Structural analysis did not predict any transmembrane regions.

### 3.9. Analysis of the E gene

The small membrane protein E of CRCoV was encoded by a 255 nt ORF and had a molecular weight of 9.6 kDa. A TRS (CCAAAC) was located 123 nucleotides upstream of the AUG codon. Sequence analysis of a PCR product created using the primer for the leader sequence and the reverse primer BSM located in the E gene confirmed that this TRS is present in the mRNA for the E protein in CRCoV. SignalP-HMM calculated a signal anchor at positions 1–34. The PHDhtm program predicted two transmembrane regions located between residues 14–38 (outside to inside) and 43–60 (inside to outside) whilst TMpred calculated only one transmembrane region at amino

acids 18–36 (outside to inside). Both programs predicted the N-terminus of the protein to be outside the viral envelope. No glycosylation sites were found using PROSITE.

### 3.10. Analysis of the M gene

The length of the ORF for the matrix protein of CRCoV was 693 nucleotides encoding a protein of 26.4 kDa. Three nucleotides prior to the start codon a TRS (CCAAAC) was found. SignalP HMM predicted a signal anchor at amino acids 1–46. PHDhtm and TMpred predicted similar models for the location of the membrane protein within the viral envelope. The model predicted by PHDhtm was as follows; the residues calculated by TMpred are given in brackets: the N-terminus is located outside, residues 28–45 (25–46) are an outside to inside transmembrane region, amino acids 51–75 (57–74) are an inside to outside transmembrane region, 80–103 (81–102) are an outside to inside transmembrane region and the C-terminus is located on

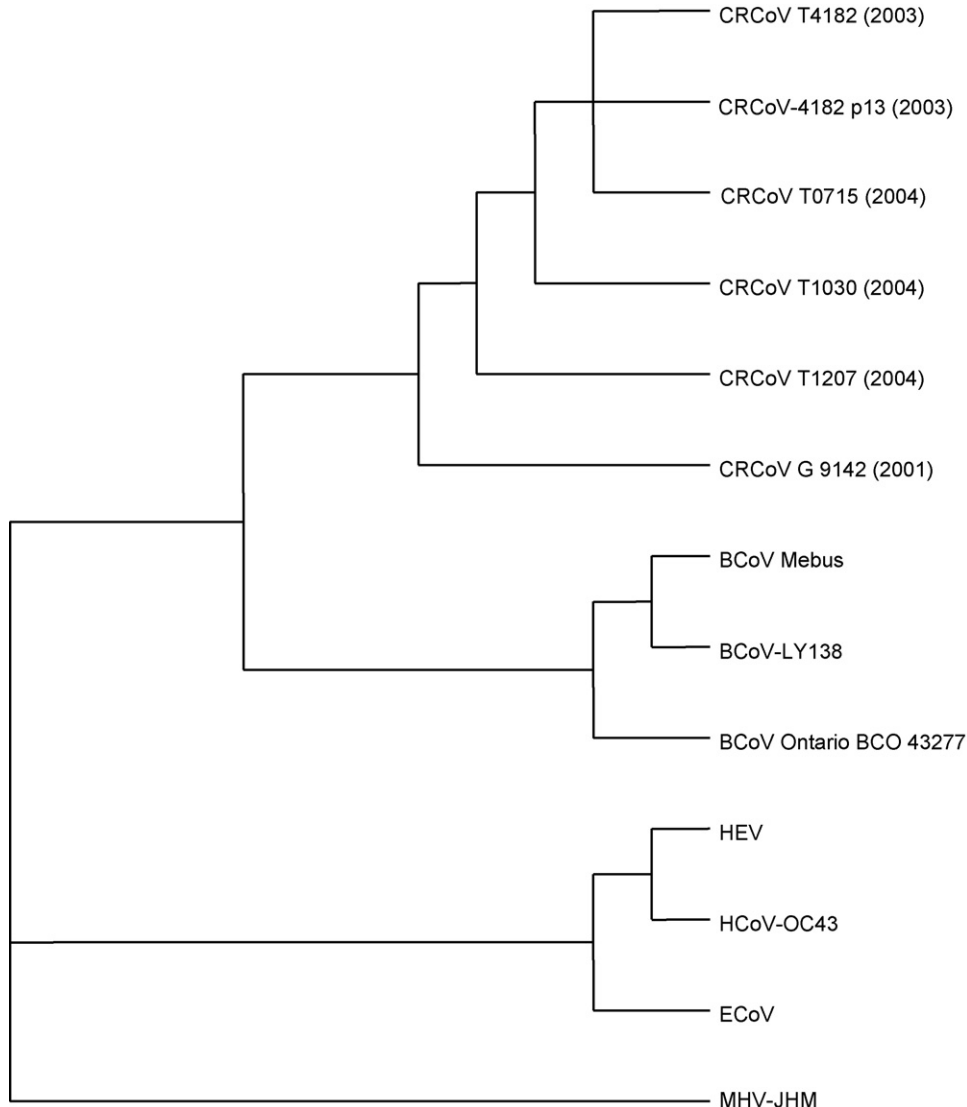


Fig. 5. Maximum parsimony phylogenetic tree of the non-structural region between the S and the E gene of CRCoV and other group 2 coronaviruses.

the inside of the envelope. Four potential O-glycosylation sites were identified at positions 2–6 as well as an N-glycosylation site at residue 26.

### 3.11. Analysis of the N gene

In addition to the genomic RNA of the nucleocapsid protein gene the PCR product obtained for the N mRNA was analysed. The leader–mRNA junction sequence was found to be identical to that of BCoV. The TRS CUA AAC was located seven nucleotides prior to the start codon. The 1347 nt ORF coded for a 49.4 kDa protein. No transmembrane regions were predicted.

### 3.12. Analysis of the internal ORF within the N gene

Translation of the internal 621 nt ORF within the N gene resulted in a 23.2 kDa protein. A potential transmembrane region at residues 142 to 162 was predicted by PHDhtm; however the TMPred program found no possible structural model that could incorporate a transmembrane helix.

### 3.13. Phylogenetic analysis

A maximum parsimony analysis was carried out for the nucleotide sequence of the non-structural region between the S and E genes. The analysed sequences started at the initiation codon for the 8.8 kDa protein (or its equivalent, respectively) and ended 137 nt downstream of the start codon of the 12.8 kDa protein ORF. The resulting phylogenetic tree is given in Fig. 5.

## 4. Discussion

This paper reports the first successful isolation of canine respiratory coronavirus. CRCoV has proven to be difficult to isolate; the virus used in this study grew readily on HRT-18 cells, a cell line also commonly used for the culture of bovine coronavirus and human coronavirus OC43 (St.-Jean et al., 2004; Tsunemitsu and Saif, 1995), but not on the three canine cell lines tested or any of the other cell lines derived from a variety of species. To increase sensitivity, a PCR assay for the detection of nucleocapsid protein gene mRNA was used in addition to detection of CRCoV proteins by immunofluorescence. The failure of CRCoV to replicate in these cell lines may indicate a lack of the appropriate receptor for CRCoV or that other necessary factors for CRCoV replication are not present. MDCK, MDBK and Vero cells have been successfully used for the culture of BCoV. We chose to use CRCoV isolate 4182 at passage 13 as sufficient virus to carry out the experiments was available at that passage. The CRCoV-4182 isolate might have become adapted to HRT-18 cells which may explain its inability to grow in the canine cell lines tested.

The spike gene was shown to have acquired eight nucleotide and amino acid substitutions after 13 passages in HRT-18 cells. These mutations may be random however it was notable that no synonymous nucleotide changes were found which may indicate positive selection for growth in HRT-18 cells.

The amino acid changes accumulated by the spike protein during growth in HRT-18 cells did not affect recognition of the virus in an immunofluorescence assay using polyclonal antisera raised against bovine coronavirus or sera from dogs with naturally acquired CRCoV-infections. The spike protein is required for the binding of coronavirus particles to specific receptors on the host cell surface. CRCoV was found to be able to agglutinate chicken red blood cells indicating interaction of a viral surface protein with sialic acid residues on the erythrocyte surface. Due to the similarity of the CRCoV spike protein to those of BCoV and HCoV-OC43, CRCoV is likely to bind to the same receptors on the cell surface, namely 9-O acetylated sialic acid (Schultze and Herrler, 1992).

Haemagglutination in BCoV and HCoV-OC43 is mediated by the spike protein as well as the haemagglutinin esterase gene. CRCoV strain 4182 was found to encode a complete HE protein that contained the predicted esterase site. Due to its similarity with other coronavirus haemagglutinin esterases this protein is likely to contribute to CRCoV receptor binding and to act as a receptor destroying enzyme (Smits et al., 2005).

The CRCoV E protein appears to be translated from a separate mRNA similar to BCoV (Abraham et al., 1990b; Hofmann et al., 1993) which is in contrast to MHV where one mRNA contains ORF 5a, coding for a non-structural protein, and ORF 5b coding for the E protein (Budzilowicz and Weiss, 1987; Jendrach et al., 1999).

The membrane protein shows a high degree of conservation amongst group 2 coronaviruses owing to its important role in the assembly of viral particles. It has been shown that M and E proteins of MHV, when expressed in cells, were sufficient for the formation of virus-like particles (Vennema et al., 1996). The CRCoV membrane protein sequence was found to have potential O-glycosylation sites at the N-terminus and to span the viral envelope three times, similar to the membrane proteins of BCoV and HCoV-OC43 (Deregt et al., 1987; Mounir and Talbot, 1992).

The CRCoV N protein was found to be highly similar to that of other group 2 coronaviruses. The nucleocapsid protein gene contained an internal ORF coding for a protein with high similarity to the BCoV I protein (Senanayake et al., 1992). An I protein homologue is also present in MHV (Fischer et al., 1997), CoV HKU-1 (Woo et al., 2005), HEV and HCoV-OC43 but not in other coronavirus groups. In HCoV-OC43 the ORF for the I protein is disrupted leading to two potential proteins Ia and Ib (Vijgen et al., 2005). The protein has been detected in cells infected with BCoV where it was present mostly in the cytoplasm and to a small degree on the surface of the cells. Its function is unknown but it has been shown to be non-essential for MHV replication (Fischer et al., 1997).

An ORF for a 32 kDa NS2 protein was present in CRCoV. The NS2 protein of BCoV has been shown to be phosphorylated and to be located in the cytoplasm of infected cells (Cox et al., 1991). Recent research has placed this protein into the 2H phosphoesterase enzyme superfamily and has suggested that it is involved in RNA transcription (Mazumder et al., 2002). A deletion mutant of MHV that does not express the NS2 protein however was able to replicate in continuous cell lines indicating



that the NS2 is either not required for viral replication in vitro or that its function can be provided by certain host cell lines (Schwarz et al., 1990). Deletion of the NS2 and the HE gene led to attenuation of MHV in vivo whereas growth in cell culture was unaffected (de Haan et al., 2002).

The region between the spike gene and the gene for the 12.8 kDa non-structural protein was the most variable region when comparing CRCoV to BCoV. A deletion of two nucleotides within the gene for the homologue of the BCoV 4.9 kDa protein led to the loss of a stop codon and the generation of an 8.8 kDa protein that included the full length of the 4.9 kDa homologue and a truncated version of the 4.8 kDa protein homologue. It has been hypothesised previously that the 4.9 and 4.8 kDa proteins of BCoV may have arisen by mutation from an 11 kDa protein which is similar to the 15.2 kDa protein of MHV JHM (Abraham et al., 1990b). The mutation leading to the translation of an 8.8 kDa protein has so far only been detected in CRCoV strains circulating within a canine population at one kennel whereas a strain from a different canine population coded for a 4.9 kDa protein comparable to BCoV. These putative non-structural proteins are not present in HCoV-OC43 (Mounir and Talbot, 1993a) and the function of the equivalent 4.9 kDa and 4.8 kDa proteins in BCoV is unknown. Moreover the 4.9 kDa protein of BCoV may not be expressed due to the lack of a start codon in its mRNA (Hofmann et al., 1993). The same may apply to CRCoV as the closest transcription regulatory sequence was located 317 nucleotides prior to the start codon. The same TRS which is used by BCoV three nucleotides downstream of the start codon is also present in CRCoV. Analysis of the CRCoV mRNA for this region will be required to determine if the protein is likely to be expressed. It remains to be assessed if the presence of an 8.8 kDa protein changes the growth characteristics of the virus in vitro or if it alters the virulence of CRCoV in dogs. ORF 4, which codes for the corresponding non-structural proteins in MHV, has been deleted experimentally without a detrimental effect to the growth of the virus in cell culture (Ontiveros et al., 2001). Similarly, replication of MHV strain S in cell culture is comparable to other MHV strains despite the lack of expression of the ORF 4 product (Yokomori and Lai, 1991).

The 12.8 kDa putative non-structural protein showed much less variation amongst CRCoV strains and when comparing CRCoV to BCoV. This conservation may indicate that the 12.8 kDa protein and its equivalents play a more important role for the replication of these coronaviruses compared to the smaller non-structural proteins.

The role of the non-structural proteins of CRCoV as well as related coronaviruses remains to be elucidated. Proteins which have been found to be dispensable for growth in cell culture in some cases influence virulence of the virus in vivo (de Haan et al., 2002). More CRCoV isolates will be required to be able to compare their characteristics in vitro and in vivo.

The availability of a CRCoV isolate has enabled more detailed sequence analysis. All CRCoV genes analysed were found to be most closely related to BCoV-LY138 an isolate obtained in the USA in 1965. Previous sequence analysis of a partial region of the polymerase gene also showed the closest nucleotide similarity to be with BCoV. CRCoV is therefore

unlikely to have resulted from a recombination event. Phylogenetic analyses for the non-structural region between the S and E genes reported here placed CRCoV most closely to BCoV confirming analyses based on the partial polymerase gene (Erles et al., 2003) as well as for the complete spike and nucleoprotein genes (data not shown). It is possible that CRCoV originated from a transmission of BCoV to dogs. Sequence data from CRCoV strains from countries other than the UK however will be necessary to determine the origins of CRCoV.

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