

## Molecular characterization of a virulent canine coronavirus BGF strain<sup>☆</sup>

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### Abstract

Molecular characterisation of a canine coronavirus (CCoV) isolate (BGF), associated with an outbreak of diarrhoea in puppies, showed 92.7% identity with attenuated Insavc-1 strain. Canine coronavirus BGF revealed a full length non-structural protein 3b (nsp 3b), associated with virulence in other coronaviruses, and a highly divergent region at the amino terminal domain of the membrane protein that may be implicated in avoiding the host immune reaction. This new canine coronavirus strain could help to identify virulence factors in coronavirus. © 2004 Elsevier B.V. All rights reserved.

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Canine coronavirus (CCoV) is a member of the Coronaviridae family of the Nidovirales order (Enjuanes et al., 2000). Coronaviruses are single stranded positive sense RNA viruses between 28 and 31 kb in length. CCoV is classified as group I by antigenic and genetic relationships (Enjuanes et al., 2000; Sanchez et al., 1990). CCoV has been experimentally demonstrated to produce mild gastroenteritis in dogs (Tennant et al., 1991). Clinically, CCoV has usually been found in conjunction with other organisms like canine adenovirus type 1 (Pratelli et al., 2001) or canine parvovirus type 2 (Carmichael, 1999). Changes in the sequences of structural spike glycoprotein (Ballesteros et al., 1997; Hingley et al., 2002; Phillips et al., 2002; Sanchez et al., 1999) and the non-structural protein 3b (nsp 3b) (Kim et al., 2000; Wesley et al., 1990) have been associated with differences in coronavirus strain virulence.

In 2002, an epizootic outbreak of diarrhoea occurred in our Beagle breeding colony. Dogs affected were between 6 and 10 weeks of age. Clinical signs consisted of diarrhoea, with or without mucus, haematochezia, lethargy, dehydration, vomiting, anorexia, pale mucous membranes and an elevated or depressed rectal temperature. Samples from 106 affected animals were taken. *Campylobacter jejuni* sub. *jejuni* was found in 68%, *Isospora rivolta* in 9%, and *Giardia*

*canis* in 5% of them. CCoV RNA was analysed by use of RT-PCR (Pratelli et al., 1999) and found to be present in all 106 samples.

*Campylobacter jejuni* sub. *jejuni* was also isolated from some asymptomatic animals, as has been found by other studies (Fleming, 1983; Fox et al., 1983, 1984, 1988).

Sera from both normal dogs, and dogs in outbreaks of enteritis in kennels usually reveal a high rate of antibodies to CCoV. Seroprevalence studies in several countries indicate that prevalence rates vary from 0 to 80%; in one large study, 45% seroprevalence rates were reported in normal dogs, in contrast to 61% in diarrheic dogs, generally higher rates were observed in enzootically infected kennels (Pratelli, 2000; Tennant et al., 1993).

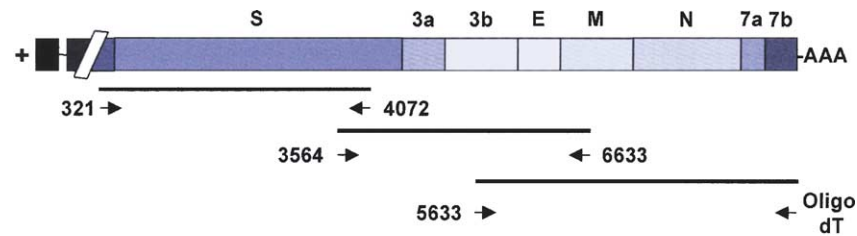
Recently, canine coronavirus strains that segregate separately from CCoV-Insavc 1 have been isolated from dogs with gastroenteritis (Naylor et al., 2002; Pratelli et al., 2003b). Here, we report the molecular characterization of 9.2 kb of the CCoV-BGF strain genome encoding the spike (S), envelope (E), membrane (M) and nucleoprotein (N) structural proteins. CCoV-BGF was found to have a 92.7% identity with CCoV Insavc-1 strain (Horsburgh et al., 1992) over the whole 9.2 kb analysed and had a full length non-structural protein 3b of 250 amino acids and a highly divergent region at the amino terminal end of the membrane protein.

These characteristics of a strain found to be strongly associated with a significant disease outbreak should help to identify factors associated with virulence in CCoV.

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(A)

Name	Sequence (5' to 3')	Position <sup>a</sup>	Orientation
321	GCTCTATCACATAACTCAGTCC	-185(-163)	Forward
3564	ATGCGTTAGGTCTCAATCCC	3563-3583	Forward
4072	ATCTCTGGGATTGAGACCTAACG	3566-3589	Reverse
5633	ATCTATGACGTTCCCTCGGG	5633-5653	Forward
CCoV2	TCTGTTGAGTAATCACCAGCT	6612-6633	Reverse
Oligo dT	TTTTTTTTTTTTTTTTT		Reverse

(B)

Fig. 1. (A) Schematic representation of the cloning process for the last third of CCoV-BGF genome. (B) Oligonucleotides used for the cloning. (<sup>a</sup>) Nucleotide position starting from the first codon of the spike gene.

RNA was extracted from faecal samples using RNeasy (QIAGEN, West Sussex, UK) according to the manufacturer's instructions.

cDNA of 9 kb length containing the structural proteins S, E, M and N was cloned from a faecal positive sample using pCR 2.1 (Invitrogen, Paisley, UK), according to the manufacturer's instructions, in three large partially overlapping fragments (Fig. 1A and B). RT-PCR consisted of an initial reverse transcription using Superscript III (Invitrogen, Paisley, UK), according to the manufacturer's instructions, followed by a PCR reaction using the Expand Long Template PCR system (Roche, East Sussex, UK) according to the manufacturer's instructions. Once the consensus sequence was obtained, overlapping RT-PCRs of approximately 600 nucleotides were designed and run using HotStart Taq polymerase (QIAGEN, West Sussex, UK) in PCR buffer 1x (QIAGEN, West Sussex, UK) to a final concentration of 1.5 mM MgCl<sub>2</sub>. PCR conditions consisted of an initial activation step of 95 °C for 15 min followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 55–60 °C, depending on the primer pair, for 45 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products for cloning and sequencing were cleaned using QIAquick (QIAGEN, West Sussex, UK). The most representative viral population was obtained by sequencing three independent RT-PCR reactions from three randomly chosen clinical samples. Direct sequencing from clinical samples was done to avoid in vitro selection of attenuated strains (Sanchez et al., 1999).

Sequencing was done using the Big Dye Version 1.0 Sequence Reaction Kit (Applied Biosystems, Foster City, CA,

USA). Samples were sequenced by use of an ABI Prism 3700 Capillary Sequencer (Applied Biosystems, Foster City, CA, USA).

Sequences were edited and analysed using EditSeq and SeqMan of Lasergene programs (DNASStar Konstanz, Germany). PSORT II (Nakai and Horton, 1999), SignalP-HMM (Nielsen et al., 1997) and TMHMM (Sonnhammer et al., 1998) were used to predict subcellular localization sites, signal peptides and transmembrane helices, respectively. A search against the PROSITE database was done to identify the different protein domains (Falquet et al., 2002). A sequence similarity search was performed for the different structural proteins using the Fasta3 program (Pearson and Lipman, 1988) against the SWall-SPTR protein sequence database (EMBL). Phylogenetic analyses were done using Phylip 3.6 through the Phylogeny Interface Environment (PIE) at the Rosalind Franklin Centre for Genomics Research (Fig. 2).

The spike protein was a polypeptide of 1453 amino acids, two amino acids longer than that of the attenuated Insavc-1 strain. When compared to Insavc-1, the nucleotide sequence contained 325 point mutations and the protein 90 amino acid changes. A putative signal peptide—probably cleaved between Cys-18 and Thr-19 by the signal peptidase—was represented by the first 18 amino acids. A transmembrane domain was found between residues 1393 and 1415. The protein contained 36 potential N-glycosylation sites, 1 potential leucine zipper domain (1346–1367) that is highly conserved among coronaviruses (Luo et al., 1999), and 1 cysteine rich region (1408–1436). The best identity was obtained against CCoV Insavc-1 (93.8%). Minor amino acid differences in the sequence of the spike protein have been shown to change the virulence of even very close isolates (Sanchez et al., 1999). In the phylogenetic analysis, three different canine coronavirus clusters were identified for the

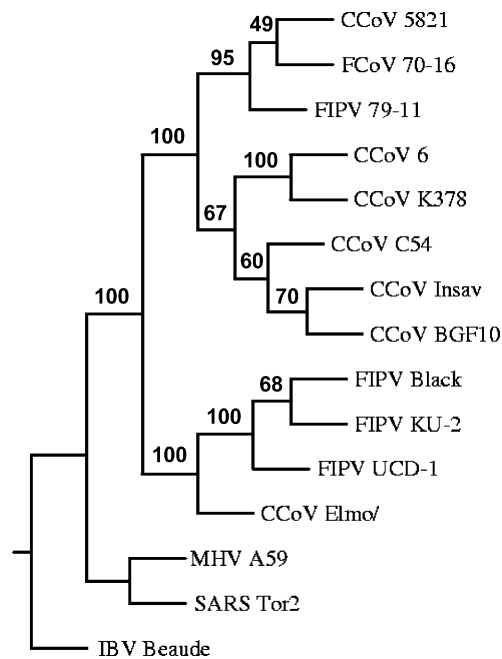


Fig. 2. Phylogenetic tree of spike protein of canine and feline coronaviruses. Phylogenetic tree was constructed by the protein sequence parsimony method (protpars) using Phylip 3.6 through the Phylogeny Interface Environment (PIE) at the Rosalind Franklin Centre for Genomics Research. Bootstrap values were calculated on 1000 replicates randomising the input order 41 times. The tree was rooted with MHV A59, SARS Tor2 and IBV Beaudette. FIPV, feline infectious peritonitis virus; FCoV, feline coronavirus; CCoV, canine coronavirus; MHV, mouse hepatitis virus; SARS, severe acute respiratory syndrome; IBV, infectious bronchitis virus.

spike protein. CCoV-BGF spike protein fitted into the cluster of canine coronaviruses containing no feline sequences (Fig. 2).

The nsp3 protein was a polypeptide of 250 amino acids. When compared to that of the Insavc-1 strain, the nucleotide sequence contained 18 point mutations and the protein 9 amino acid changes. Three transmembrane domains were found between residues 49–66, 73–95 and 99–121.

The envelope protein was a polypeptide of 82 amino acids, 2 amino acids longer than that of the attenuated Insavc-1 strain. When compared to Insavc-1, the nucleotide sequence contained 22 point mutations and the protein 9 amino acid changes. A transmembrane domain was found between residues 20–42. The best identity was obtained against CCoV Insavc-1 (89%).

The membrane protein was 262 amino acids long. When compared to that of the Insavc-1 strain, the nucleotide sequence contained 91 point mutations and the protein 37 amino acid changes. Four transmembrane domains were predicted between residues 5–27, 47–69, 76–98 and 113–135 (Fig. 3A). The protein contained two potential glycosylation sites and three potential phosphorylation sites. The best identity was obtained against CCoV 259/01 (88.2%).

The nucleoprotein was 382 amino acids long. When compared to that of the Insavc-1 strain, the nucleotide sequence contained 86 point mutations and the protein

23 amino acid changes. The protein contained 3 potential glycosylation sites, 13 potential phosphorylation sites, and a bipartite nuclear targeting sequence (199-KKLGVDTEKQQQRARSK-255). The nucleoprotein of transmissible gastroenteritis virus (TGEV), mouse hepatitis virus (MHV) and infectious bronchitis virus (IBV) have been shown experimentally to localise in the nucleolus (Chen et al., 2002; Wurm et al., 2001). The best identity was obtained against CCoV 1–71 (93.5%).

The CCoV BGF strain was found to be closely related to the attenuated Insavc-1 strain (overall amino acid identity of 92.7%) (Fig. 2). We compared both strains because data for all the structural proteins are only available for those two strains and also because CCoV-Insavc-1 is attenuated and a very well characterized strain (Horsburgh et al., 1992) that has been used as a vaccine. Previously, a strain of CCoV have been classified as “novel third CCoV subgroup” (Naylor et al., 2002) based on sequence data from a small part of the highly variable 5' terminal spike region. Based on the whole spike sequence phylogeny, we found two different subgroups. The first contains only the CCoV recovered from diarrheic dogs in Italy (Pratelli et al., 2003a), although it is difficult to discard a recombination event with feline infectious peritonitis virus (FIPV) in that case.

The second group contains the remaining CCoVs sequenced to date, including CCoV-BGF (Fig. 2). We found that for the other three structural proteins, the CCoV-BGF is not grouped with the known CCoV sequences. A lack of data from the last third of the genome containing the coronavirus structural proteins of the different CCoV isolates prevented a powerful phylogenetic analysis. Nevertheless, the high homology between Insavc-1 and BGF strains could help to identify CCoV genomic regions involved in virulence.

A deletion of 61 bp (isolates 5 and 10) to 64 bp (isolate 27) was found in the intergenic S-3a region. Deletions in this region have already been described in virulent TGEV variants (McGoldrick et al., 1999) and may be associated with increased nsp 3b translation levels. The length of the transcription regulatory sequences has been shown to influence the level of transcription and translation in TGEV (Alonso et al., 2002). Interestingly, a nsp 3b protein of 250 amino acids is present, instead of the truncated form of 171 amino acids described for the attenuated Insavc-1 strain (Horsburgh et al., 1992). The truncated nsp 3b has been associated in survey studies with attenuated strains (Horsburgh et al., 1992; Kim et al., 2000; Wesley et al., 1990). The only reported experimental study of virulence of coronavirus group 1 involving the nsp 3b protein is difficult to interpret because of lack of experimental details such as animal health status, and the absence of administration of a known attenuated strain as a control (Festing et al., 2002; Sola et al., 2003). Therefore, we think that an increased amount of the full-length nsp 3b protein could be partially responsible for the increased in virulence that appears to be associated with CCoV-BGF.

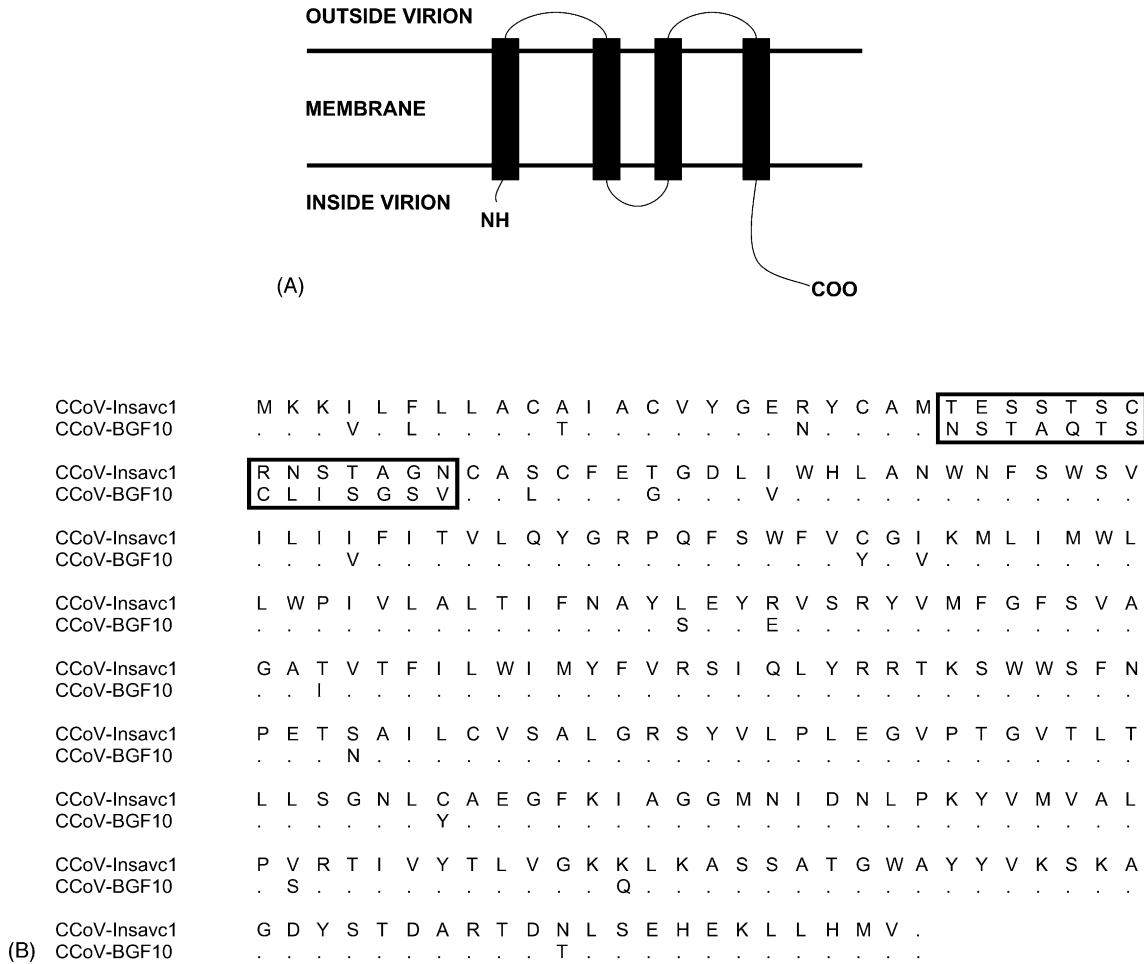


Fig. 3. (A) Topology of the CCoV-BGF membrane protein as predicted by TMHMM method. (B) Comparison of the membrane protein sequence from CCoV Insavc-1 and CCoV-BGF10 with the highly variable sequence highlighted.

The CCoV-BGF membrane protein accumulates most of the changes when compared to the Insavc-1 strain at the 5' end of the gene. A region of high divergence was found between residues 24 and 37 of the membrane protein (Fig. 3B). This region has previously been shown to be highly variable in other isolates and it has been postulated that it may be involved in escaping the host immune response in CCoV (Pratelli et al., 2002). The importance of the membrane protein in eliciting the humoral host immune response is not clear because monoclonal antibodies against TGEV membrane protein do not completely neutralise virus infection (Sanchez et al., 1990; Risco et al., 1995). We assume that the presence of an additional membrane domain in CCoV-BGF, predicted by TMHMM (Fig. 3A), could be helping the virus to avoid degradation by host proteases (Armstrong et al., 1984) and therefore to evade the T-cell response.

Canine coronavirus is highly seroprevalent in the UK (Tennant et al., 1993). Field samples will identify more accurately virulence factors such as the spike and nsp 3b proteins. In vitro and in vivo studies using the newly generated infectious cDNAs (Almazan et al., 2000; Yount et al., 2000) will help to study not only virulence but also protein vari-

ants like the highly variable amino terminal domain of the M protein that could be involved in escaping the host immune response. Generation of a CCoV-BGF infectious cDNA is currently under development in our laboratory.

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