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Molecular characterization of a virulent canine coronavirus BGF strain

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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.

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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 (Carman, 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., 1995).

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.
RNA was extracted from faecal samples using RNaseasy (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, 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 Hot-Start Taq polymerase (QIAGEN, West Sussex, UK). PCRs were carried out as follows: 50 ng of cDNA template from reverse transcription (Fig 1A) was added to a PCR mix containing 2 mM dNTPs, 10 pmol of each primer, and 2.5 units of HotStart Taq polymerase (QIAGEN, West Sussex, UK) to a final concentration of 1.5 mM MgCl₂. 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). Sequences were edited and analysed using EditSeq and SeqMan of Lasergene programs (DNAStar Kostanz, 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
The full-length nsp3b protein could be partially responsible for the increased in virulence that appears to be associated with the attenuation of the Insavc-1 strain as a control (Festing et al., 2002; Sola et al., 2003a), although it is difficult to discard a recombination event with feline infectious peritonitis virus (FIPV) in that case.

The nsp3 protein was a polypeptide of 250 amino acids. When compared to that of the Insavc-1 strain, the primary 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 1–71 (93.5%).

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, 47–49, 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%).

When compared to that of the Insavc-1 strain, the nucleotide sequence contained 22 point mutations and the protein 9 amino acid changes. A transmembrane domain was found between residues 49–66, 73–95 and 99–121.

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The nucleoprotein was 382 amino acids long. 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 predicted between residues 199–219, 226–248 and 255–275. The nsp3 protein was a polypeptide of 250 amino acids.

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%).

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The membrane protein was 262 amino acids long. 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.

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The CCoV-BGF membrane protein accumulates most of the changes when compared to the Insacv-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 involve 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 variants 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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