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Genomic analysis of canine pneumoviruses and canine respiratory coronavirus from New Zealand

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ABSTRACT

Aims: To isolate canine respiratory coronavirus (CRCoV) and canine pneumovirus (CnPnV) in cell culture and to compare partial genomic sequences of CRCoV and CnPnV from New Zealand with those from other countries.

Methods: Oropharyngeal swab samples from dogs affected by canine infectious respiratory disease syndrome that were positive for CnPnV (n = 15) or CRCoV (n = 1) by virus-specific reverse transcriptase quantitative PCR (RT-qPCR) in a previous study comprised the starting material. Virus isolation was performed in HRT-18 cells for CRCoV and RAW 264.7 and Vero cells for CnPnV. The entire sequence of CnPnV G protein (1,266 nucleotides) and most (8,063/9,707 nucleotides) of the 3′ region of CRCoV that codes for 10 structural and accessory proteins were amplified and sequenced. The sequences were analysed and compared with other sequences available in GenBank using standard molecular tools including phylogenetic analysis.

Results: Virus isolation was unsuccessful for both CRCoV and CnPnV. Pneumovirus G protein was amplified from 3/15 (20%) samples that were positive for CnPnV RNA by RT-qPCR. Two of these (NZ-048 and NZ-049) were 100% identical to each other, and 90.9% identical to the third one (NZ-007). Based on phylogenetic analysis of the G protein gene, CnPnV NZ-048 and NZ-049 clustered with sequences from the USA, Thailand and Italy in group A, and CnPnV NZ-007 clustered with sequences from the USA in group B. The characteristics of the predicted genes (length, position) and their putative protein products (size, predicted structure, presence of N- and O-glycosylation sites) of the New Zealand CRCoV sequence were consistent with those reported previously, except for the region located between open reading frame (ORF)3 (coding for S protein) and ORF6 (coding for E protein). The New Zealand virus was predicted to encode 5.9 kDa, 27 kDa and 12.7 kDa proteins, which differed from the putative coding capacity of this region reported for CRCoV from other countries.

Conclusions: This report represents the first characterisation of partial genomic sequences of CRCoV and CnPnV from New Zealand. Our results suggest that the population of CnPnV circulating in New Zealand is not homogeneous, and that the viruses from two clades described overseas are also present here. Limited conclusions can be made based on only one CRCoV sequence, but the putative differences in the coding capacity of New Zealand CRCoV support the previously reported variability of this region. The reasons for such variability and its biological implications need to be further elucidated.

Abbreviations: BCoV: Bovine coronavirus; CIRDS: Canine infectious respiratory disease syndrome; CnPnV: Canine pneumovirus; CRCoV: Canine respiratory coronavirus; FBS: Fetal bovine serum; HA: Hemagglutination; MPV: Murine pneumonia virus; ORF: Open reading frame; RT-qPCR: Reverse transcriptase quantitative PCR; TRS: Transcription regulatory sequence

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Canine respiratory coronavirus; canine pneumovirus; New Zealand canine respiratory disease; sequence analysis; phylogeny

Introduction

Canine pneumovirus (CnPnV) and canine respiratory coronavirus (CRCoV) are two of several pathogens that have been implicated in the development of canine infectious respiratory disease syndrome (CIRDS), also known as "kennel cough." The disease most commonly affects dogs housed in groups (Schulz *et al.* 2014; Mitchell *et al.* 2017). Affected dogs present with acute onset of a hacking cough, accompanied in some cases by fever, sneezing, and nasal or ocular discharge. There is no surveillance for CIRDS in New Zealand and hence there are no reliable data on the incidence of CIRDS in this country. Anecdotally, however, outbreaks of kennel cough occur here, and there has been a perception among veterinarians that the numbers of CIRDS cases have increased within the past few years (Anonymous 2022).

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Traditionally, CIRDS has been linked to infections with *Bordetella bronchiseptica*, canine parainfluenza virus, and canine adenovirus type 2 (McCandlish *et al.* 1978), but several other infectious agents have been detected from affected dogs in the past 20 years. These include CRCoV (Erles *et al.* 2003), CnPnV (Renshaw *et al.* 2010), canine influenza virus (Pecoraro *et al.* 2014), canine bocavirus (Kapoor *et al.* 2012) and canine hepacivirus (Kapoor *et al.* 2011), as well as bacteria (*Streptococcus equi* subsp. *zooepidemicus* (Chalker *et al.* 2003) and various *Mycoplasma* species (Chalker *et al.* 2004)). The exact role of these agents in the development of CIRDS needs to be elucidated as most have been detected both from clinically ill and healthy dogs, underscoring the impact of host-, environment- and pathogen-related factors on the expression of disease (Mitchell *et al.* 2017; Maboni *et al.* 2019). Of the CIRDS-associated pathogens, canine influenza virus, canine bocavirus, and canine hepacivirus have never been detected in New Zealand (Sowman *et al.* 2018; More *et al.* 2021).

Canine pneumovirus is an enveloped, negative sense, single-stranded RNA virus classified within the family *Pneumoviridae*, genus *Orthopneumovirus* (Rima *et al.* 2017). Pneumoviruses encode 9–11 proteins, including a heavily glycosylated attachment protein (G protein), which has a high degree of sequence variation within and between different species of pneumoviruses (Kumagai *et al.* 2021; Yu *et al.* 2021). CnPnV was first identified during a diagnostic investigation of acute infectious respiratory disease in dog breeding colonies in the USA (Renshaw *et al.* 2010, 2011). Since this initial report, the virus has been detected from dogs with respiratory disease in Thailand (Piewbang and Techangamsuwan 2019), China (Song *et al.* 2021), the UK, the Republic of Ireland (Mitchell *et al.* 2013a), and Italy (Decaro *et al.* 2014a). Based on serological evidence, infection with CnPnV is also common in many other European countries (Day *et al.* 2020). In a recently reported New Zealand-based study, CnPnV sequences were detected in 14/56 (25%) nasopharyngeal swabs collected from CIRDS-affected dogs and from 17/60 (28%) clinically normal dogs by reverse transcriptase quantitative PCR (RT-qPCR) (More *et al.* 2021).

Canine respiratory coronavirus is an enveloped positive sense, single-stranded RNA virus classified within the order *Nidovirales*, genus *Betacoronavirus* and species *Betacoronavirus 1* (Erles *et al.* 2003). Typical for all nidoviruses, coronavirus genes are expressed from the 3′ co-terminal sets of mRNA generated through a process of discontinuous transcription (Figure 1) (Pasternak *et al.* 2006). Transcription regulatory sequences (TRS) are crucial for that process. The TRS are located at the 5′ end of the coronavirus genome, as well as upstream of structural and accessory genes located in the 3′ end of the genome, and commonly include a consensus sequence CYAAAC (Hofmann *et al.* 1993). The virus was first detected in samples collected from dogs with severe respiratory disease in a large re-homing centre in the UK in 2003 (Erles *et al.* 2003). Since then, CRCoV has been identified in other geographical areas including Italy (Lorusso *et al.* 2009), Korea (An *et al.* 2010), Japan (Soma *et al.* 2008), and China (Hong *et al.* 2015). Based on serological evidence, CRCoV infection is common among dogs in New Zealand, with more than half of the dogs sampled in one study testing positive for CRCoV antibodies (More *et al.* 2020). Despite this relatively high level of infection, the virus has never been isolated in New Zealand and only 1/116 dogs tested in a recent molecular survey was positive for CRCoV RNA by RT-qPCR (More *et al.* 2021).

The aim of the current study was to culture CRCoV and CnPnV from samples collected from CIRDS-affected dogs that were positive for viral RNA in the previous study (More *et al.* 2021) and to compare

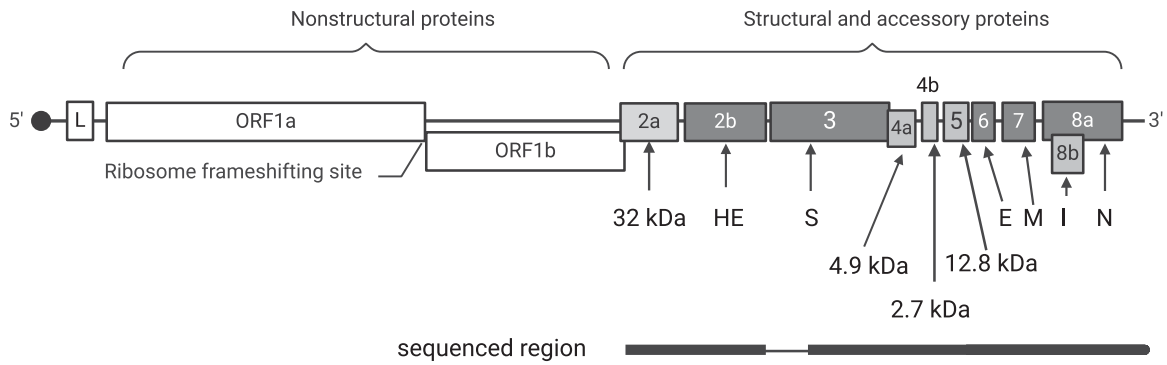

Figure 1. Schematic representation of the organisation of the genome of canine respiratory coronavirus (CRCoV; based on isolate BJ232; GenBank accession KX432213). The open reading frames (ORF) are numbered within or above the rectangles, and the protein products of the structural and accessory genes are specified below. The positive-sense, single-stranded, 30.8 kb RNA genome is capped at the 5′ end and expressed through a process of discontinuous transcription. A common leader sequence (L) encoded at the 5′ of the genome is present at the 5′ end of each mRNA. The region sequenced in the current study is shown below the genome, with a thin line indicating the missing sequence.

partial genomic sequences of CRCoV and CnPnV form New Zealand with those from other countries.

Materials and methods

Source of samples

Oropharyngeal swab samples from dogs affected by CIRDS that were positive for CnPnV (n= 15) or CRCoV (n = 1) by virus-specific RT-qPCR in the previous study (More *et al.* 2021) constituted the starting material. The samples were stored at –80°C.

Virus isolation

Virus isolation was attempted in HRT-18 cells for CRCoV and in RAW 264.7 and Vero cells for CnPnV. The HRT-18 and Vero cell lines were commercially obtained from American Tissue Culture Collection (Manassas, VA, USA), while RAW 264.7 was donated by Dr. Patrick Reading (Melbourne, Australia). All three cell lines were grown in Advanced Dulbecco's modified Eagle medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 2% v/v fetal bovine serum (FBS; Gibco Life Technologies, New York, NY, USA), 1% v/v antibiotic solution (5,000 U/mL penicillin and 5,000 µg/mL streptomycin; Thermo Fisher, Waltham, MA, USA), and 1% v/v Glutamax (Gibco Life Technologies) using standard laboratory procedures (Freshney 2005). Maintenance medium, where used, was the same as the growth medium, but with 1% v/v FBS. For isolation of CRCoV, the inoculum was supplemented with trypsin to 1 µg/mL, and the cells were maintained in maintenance medium containing 1 µg/mL trypsin. All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5–6 days and observed daily for the presence of viral cytopathic effect. All cultures were passaged at least twice before considered negative.

The cell lysate from the third passage was used as a template for virus-specific RT-qPCR. Lysate from the fourth passage of CRCoV culture was subjected to hemagglutination (HA) test using 1% washed chicken red blood cells at 4°C until HA was observed in positive control wells containing bovine coronavirus (BCoV). Negative controls consisted of cell lysate from non-inoculated HRT-18 cells. Cultures were considered positive for CRCoV if both RT-qPCR and HA tests were positive, and positive for CnPnV when cell lysates from the third passage were positive for CnPnV RNA by RT-qPCR (performed as reported previously; More *et al.* 2021).

Nucleic acid extraction and cDNA synthesis

Total nucleic acids were extracted from the samples using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Basel, Switzerland) as described previously (More *et al.* 2021). In order to obtain long cDNA fragments, RNA was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics GmbH). The reaction consisted of 1 µg of RNA, 60 µM random hexamer primers, 4 µL of 1 X reaction buffer, 20 U of RNase inhibitor, 1 mM of deoxyribonucleotide mix and 10 U reverse transcriptase in a total reaction volume of 20 µL. The tubes were incubated at 25°C for 10 minutes, 55°C for 30 minutes and 85°C for 5 minutes.

Amplification and analysis of CnPnV G protein gene

The CnPnV G protein gene was amplified using overlapping primers previously described by Glineur *et al.* (2013) (Supplementary Table 1). Each 25 µL PCR reaction consisted of 2.5 mM $MgCl_2$, 0.3 mM of each dNTP, 0.5 units of KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA) and 0.3 mM of each forward and reverse primer. The cycling conditions consisted of the initial denaturation at 95°C for 3 minutes followed by 30 cycles of denaturation at 98°C for 20 seconds, annealing at 67°C or 64°C for 15 seconds and extension at 72°C for 1 minute, with the final extension step at 72°C for 1 minute. PCR products of the expected size were excised from the gel following electrophoresis. DNA was recovered using a freeze-squeeze method and sent for sequencing to a commercial provider (Massey Genome Service, Massey University, Palmerston North, NZ). Sequencing was performed using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 310 genetic analyser (Applied Biosystems). Obtained sequences were assembled and analysed using Geneious version 10.2.3 (Kearse *et al.* 2012). Nucleotide and amino acid sequence alignments were performed using either Clustal or Muscle algorithms within Geneious. A phylogenetic tree was constructed using the maximum likelihood method in MEGA 11 (Tamura *et al.* 2021) and visualised using Interactive Tree of Life (Letunic and Bork 2021).

Amplification and analysis of the 3' end of the CRCoV genome

The 9,815 nucleotides from the 3' genomic region of CRCoV comprising 10 open reading frames (ORF) coding for structural and accessory proteins (Figure 1) was targeted for amplification using previously published overlapping primers (Supplementary Table 2). Each PCR reaction included 1 X Platinum SuperFi Green PCR master mix (Invitrogen, Carlsbad, CA, USA), 0.5 µM of each primer and 2 µL of cDNA in a total reaction volume of 10 µL. The cycling conditions comprised the initial denaturation at 98°C for 30

seconds, followed by 30 cycles of denaturation (98°C for 10 seconds), annealing (various temperatures for 10 seconds) and extension (72°C for 2 minutes), followed by final extension (72°C for 5 minutes).

The PCR products were gel-purified and sequenced as described above for the CnPnV G gene. Obtained sequences were assembled and analysed using Geneious version 10.2.3 (Kearse *et al.* 2012). Putative ORF were identified based on the ORF predictor tool within Geneious combined with the position of the corresponding ORF in CRCoV-4182 from the UK (DQ682406); CRCoV-BJ232 from China (KX432213); CRCoV 240/05 from Italy (EU999954); and CRCoV K37 (EU983106), CRCoV K39 (EU983107), and CRCoV K9 (EU983105) from Korea, which was assessed based on multiple sequence alignments. The TRS for each gene of CRCoV was predicted based on locations previously described (Erles *et al.* 2007; Lorusso *et al.* 2009; An *et al.* 2010). The phylogenetic analysis was conducted using the nucleotide sequence of the partial S gene and the entire 3′ genomic sequence of the New Zealand CRCoV and overseas sequences available in GenBank. Nucleotide sequences were aligned using Clustal algorithms within Geneious. Maximum likelihood phylogenetic trees were generated using MEGA 11 (Tamura *et al.* 2021) and visualised using the Interactive Tree of Life program (Letunic and Bork 2021).

Putative N-glycosylation sites were predicted by using the NetNGlyc 1.0 server and O-glycosylation sites by using NetOGlyc 4.0 server (Julenius *et al.* 2005).

The partial sequence of CRCoV NZ-046/16 has been deposited in GenBank under accession number MH249786. The New Zealand sequence of CnPnV protein G was deposited in GenBank under accession numbers MK121747, MK121748 and MK121749.

Results

Virus isolation

All virus isolation attempts were unsuccessful. Neither CRCoV nor CnPnV were isolated from RT-qPCR-positive samples.

Sequence analysis of the CnPnV G gene

Out of the 15 samples positive for CnPnV by RT-qPCR, the G protein gene was amplified from only three samples: ITB-007, ITB-048 and ITB-049. These three samples had the highest calculated copy number of the CnPnV M gene per µL of template: 117 (ITB-048), 503 (ITB-049) and 933 (ITB-007), with the remaining M gene-positive samples containing low levels of target RNA (<10 copies per µL of template) based on previously reported data (More *et al.* 2021). The signalment and clinical history for these three dogs are presented in Table 1.

Of the three CnPnV G gene sequences, NZ-048 and NZ-049 were identical to each other, and each was 90.9% identical to NZ-007. The G gene of CnPnV NZ-007 was 1,266 nucleotides long and encoded a predicted protein of 421 amino acids; while the G gene of CnPnV NZ-048 and 049 was 1,245 nucleotides long each and encoded a predicted protein of 414 amino acids, similar to other CnPnV sequences deposited in GenBank, except for CnPnV 126724-10-4DC (GenBank accession KC495967), which was 419 amino acids long. Differences in the predicted lengths in G proteins were due to the position of the stop codon, with the longer G proteins containing an additional 5–7 amino acids at the C-terminal.

Based on phylogenetic analysis of the G protein, two New Zealand sequences (CnPnV NZ-048 and CnPnV NZ-049) clustered with sequences from the USA, Thailand and Italy in group A and one New Zealand sequence (CnPnV NZ-007) clustered with sequences from the USA in group B (Figure 2).

Sequence analysis of the partial CRCoV sequence

The target sequence was amplified with 9/10 primer pairs designed to amplify the entire 3′ end of the viral genome. The PCR with Sp-9 and Sp-10 primers, designed to generate a 4,200 bp region comprising the entire S gene, was unsuccessful. Hence, this region was attempted to be amplified using four separate PCR reactions to generate shorter (~ 1 kb) overlapping fragments of the S gene (S1F/R, S2F/R, S3F/R and S4F/R; Supplementary Table 2). Of those, only primers S3F/R and S4F/R produced expected PCR products using the initial amplification conditions. Further optimisation of the PCR reactions with the remaining two primer pairs was not possible as there was no CRCoV-positive sample left. Subsequently, only a partial sequence of the region coding for the S protein was obtained (2,457 out of the predicted 4,092 nucleotides).

Table 1. Signalment of dogs that were the source of samples used for canine pneumovirus glycoprotein G sequencing.

	Signalment					
Dog ID	Breed	Age (years)	Sex	Clinical signs	Duration of disease (days)[a]	History
ITB-007	Shih Tzu	4	Male	Occasional coughing, serous nasal discharge	3	Recently kennelled
ITB-048	Bichon Frisé,	1.5	Male	Frequent coughing	4	Recently kennelled
ITB-049	Bichon Frisé	6	Male	Frequent moist, productive cough, sneezing	2	Recently kennelled

[a]At the time of sample collection.

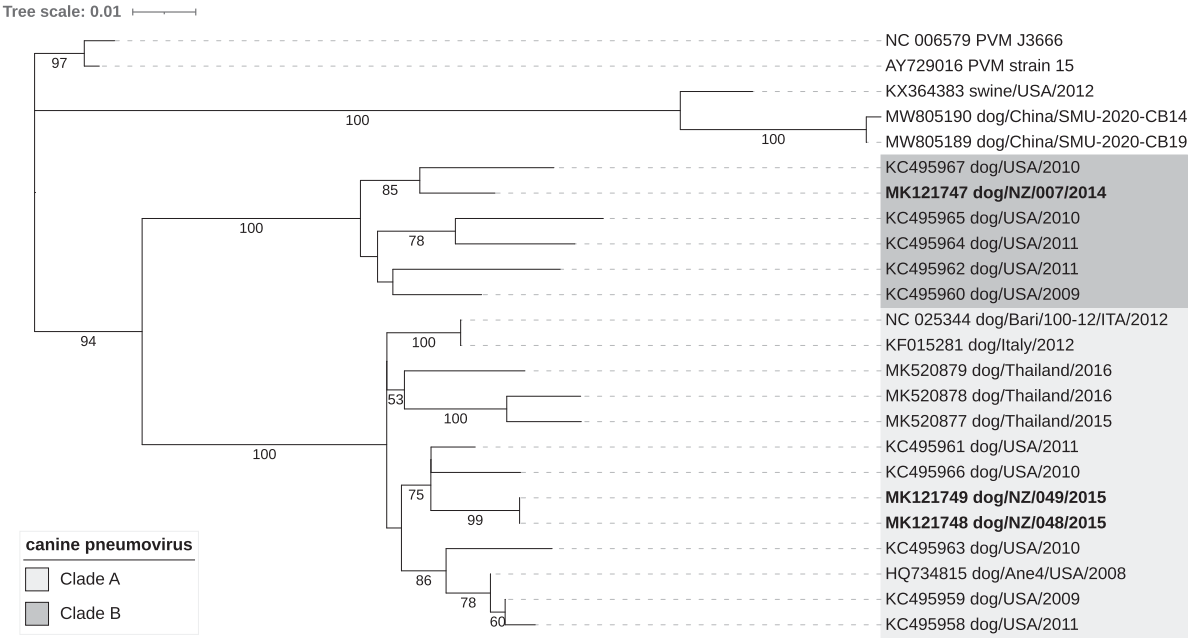

Figure 2. Evolutionary relationships of canine pneumoviruses based on the analysis of glycoprotein G amino acid sequences (n = 24). The evolutionary history was inferred by using the maximum likelihood method and the JTT matrix-based model (Jones *et al.* 1992). The tree with the highest log likelihood (−2,648.85) is shown. The percentage of trees in which the associated taxa clustered together in a bootstrap test (100 replicates) is shown below the branches (only values ≥ 50% are shown). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.6156)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with 90% site coverage were eliminated (i.e. <10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option)). There were a total of 414 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.* 2021). Tree was visualised using the Interactive Tree of Life online tool (Letunic and Bork 2021).

The characteristics of the predicted genes (length, position) and their putative protein products (size, predicted structure, presence of N- and O-glycosylation sites) of CRCoV NZ.046/16 (Table 2) were consistent with those reported for CRCoV previously (Erles *et al.* 2007; An *et al.* 2010), with the exception of the region located between the ORF3 (coding for S protein) and ORF6 (coding for E protein). Sequence variation within this region has been reported previously, with predicted expression of two or three differently sized proteins

Table 2. Coding capacity and putative protein products expressed from the 3′ end of CRCoV 046/16.

				Protein product			
ORF	Coding region[a]	TRS sequence	TRS position	Name	Length (aa)	N-glycosylation (aa position)[b]	O-glycosylation (aa position)[c]
2a	301–1137	CUAAAC	288–293	32 kDa	278	161, 241	–
2b	1,149–2,423	CUAAAC	1,134–1,139	Haemagluttinin esterase (HE)	424	54, 89, 153, 236, 301, 316	373
3	<4,073–6,529	NA		Partial spike (S)	>818	104, 131, 151, 169,194, 649,679, 689[d]	221, 224, 227[d]
4a	6,519–6,677	CCAAAC	6,150–6,155	5.9 kDa	52	–	–
4b	6,688–6,765	Unknown	Unknown	2.7 kDa	25	–	–
5	6,896–7,225	CUAAAC	6,818–6,823	12.7 kDa	109	18	–
6	7,212–7,466	CCAAAC	7,083–7,088	Envelope (E)	84	–	–
7	7,481–8,173	CCAAAC	7,472–7,477	Membrane (M)	230	26	–
8a	8,183–9,529	CUAAAC	8,170–8,175	Nucleocapsid (N)	448	17, 336, 408	2, 4, 9, 10, 11, 14, 15, 33, 38, 48, 50, 51, 55, 95, 167, 168, 177, 180, 194, 198, 200, 201, 202, 205, 206, 209, 210, 213, 215, 219, 223, 225, 226, 249, 255, 258, 275, 390, 410, 416, 423, 427, 432
8b	8,244–8,867	CUAAAC	8,170–8,175	Internal N (I)	207	24	3, 5, 9, 11, 116, 117, 121, 125

[a]Within partial CrCoV genome sequence, GenBank accession MH249786.
[b]Predicted using NetNGlyc – 1.0 with a default cut-off of 0.5.
[c]Predicted using NetOGlyc - 4.0 with a default cut-off of 0.5.
[d]Positions within the partial sequence comprising the 3′ end of the spike protein coding region.
aa = amino acid; NA = not applicable (as the 5′ sequence of the S protein gene was not determined); ORF = open reading frame; TRS = transcription regulatory sequence.

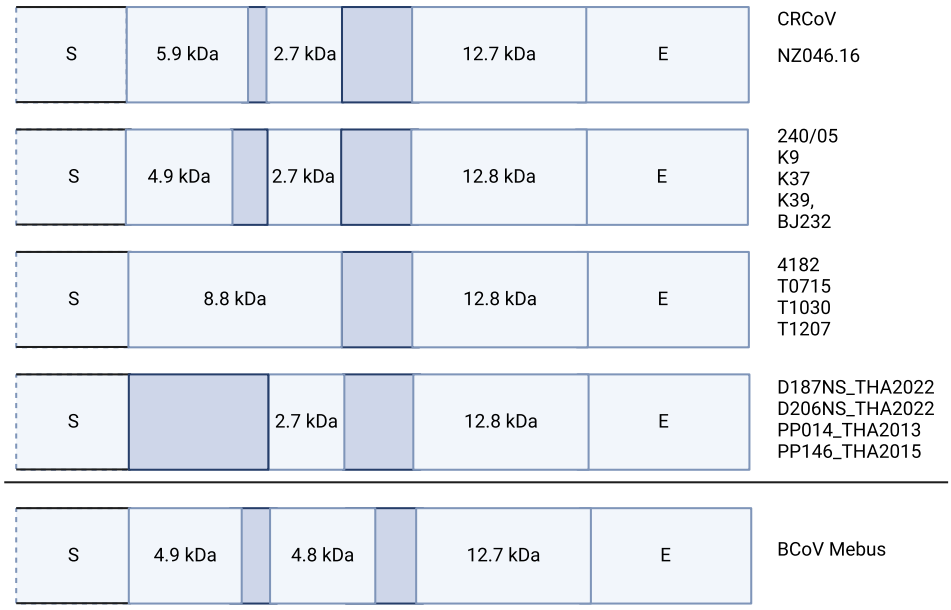

Figure 3. Schematic representation of the genomic organisation of the region between the spike (S) protein gene and the envelope (E) protein gene of canine respiratory coronaviruses (CRCoV) and bovine coronavirus (BCoV). Representative viruses are listed on the right. The GenBank accession numbers for these viruses are: 240/05 (EU999954), K9 (GQ918141), K37 (JX860640), K39 (GQ918143), BJ232 (KX432213), 4182 (DQ682406), D187NS_THA2022 (OQ621724), D206NS_THA2022 (OQ621723), PP014_THA2013 (OQ621712), PP146_THA2015 (OQ621713), BCoV Mebus (BCU00735). Isolates T0715, T1030 and T1207 are included based on description in Erles *et al.* (2007). Dark shading indicates non-expressed regions of the genome.

(Figure 3). A single base deletion (four instead of five uracil bases at nucleotide 6,647–6,650) in CRCoV NZ046/16 compared to CRCoV such as 240/05, BJ232, K9, K37 or K39 resulted in the absence of a stop codon and addition of eight amino acids to the accessory protein putatively encoded by ORF4a, with the predicted size of 5.9 kDa instead of 4.9 kDa in most other CRCoV. Several CRCoV sequences from the UK encode a putative 8.8 kDa protein in the same region, while CRCoV sequences from Thailand appear to lack the corresponding gene (Figure 3).

The putative 2.7 kDa accessory protein gene (ORF4b) was 94.9% to 98.7% identical to the corresponding genes of other CRCoVs where it was present, and the gene for 12.5 kDa accessory protein was conserved in the genomes of all CRCoV examined, with 97.3% to 99.4% identity between NZ CRCoV and CRCoV from other countries.

Based on phylogenetic analysis of the partial sequence of the S protein, CRCoV NZ-046/16 clustered with CRCoV sequences from the USA (Figure 4). When the phylogenetic analysis was repeated using the entire available 3′ sequence, the NZ-046/16 sequence clustered with sequences from the USA (USA 1 and USA 5) and Europe (240/05 from Italy and 4182 from the UK) (data not shown).

Discussion

This report represents the first characterisation of partial genomic sequences of CRCoV and CnPnV from New Zealand. The small number of sequences analysed makes it impossible to make any inferences on the likely sources of these viruses in New Zealand or the timing of their introduction. The duration of quarantine for dogs imported into New Zealand from countries other than Australia ranges from 10 to 60 days (Anonymous 2023). Shedding of CRCoV from the oropharynx was detected up to 11 days following experimental infection (Mitchell *et al.* 2013b). There are no data on the duration of shedding of CnPnV, but the risk of transmission of respiratory syncytial virus (a human pneumovirus) between infected and uninfected household members was highest within the first week of infection (Otomaru *et al.* 2021). However, respiratory syncytial virus may establish persistent infections in immunocompromised people, with long-term shedding of up to 334 days reported in one study (Lehners *et al.* 2016). Whether or not persistent infections with CnPnV in immunocompromised dogs can also be established needs to be elucidated. Altogether, the quarantine requirements are likely to have substantially reduced, but not fully removed, the risk of introduction of CRCoV and CnPnV to the country through infected dogs. Another possibility is the introduction of those viruses on fomites. Both CRCoV and CnPnV are enveloped viruses and therefore unlikely to survive well outside of their hosts. However, the presence of viable virus on inanimate surfaces for as short as a couple of days would, in theory, allow for the inadvertent introduction of such a virus into New Zealand on fomites such as contaminated footwear (Geng and Wang 2023).

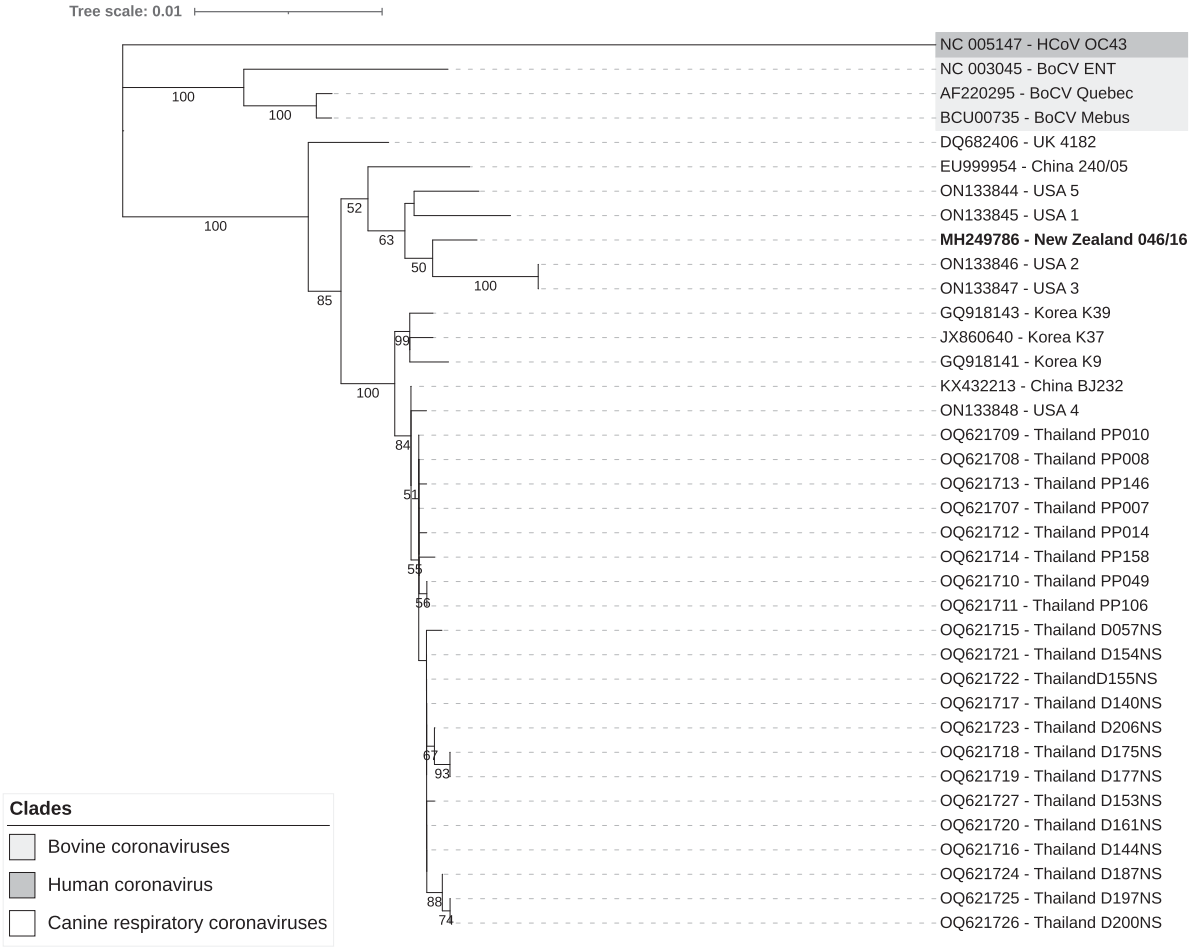

Figure 4. Evolutionary relationships of canine respiratory coronaviruses from various countries based on partial nucleotide sequences of the spike gene (n = 37). The New Zealand sequence is in bold. The evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (–5,109.77) is shown. The percentage of trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown below the branches for values ≥ 50%. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.1139)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with <90% site coverage were eliminated (i.e. <10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option)). There were a total of 2,457 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.* 2021). Tree was visualised using Interactive Tree of Life online tool (Letunic and Bork 2021).

Canine pneumovirus sequence analysis

Glycoprotein G was amplified from only 3/15 (20%) samples that previously tested positive for CnPnV on qRT-PCR (More *et al.* 2021). The qRT-PCR used in the study targeted the M protein gene, which is relatively conserved compared to the G protein gene, which is more variable (Slovic *et al.* 2017; Kumagai *et al.* 2021; Yu *et al.* 2021). This may be one reason for the discrepancies between results of the two tests. The three protein G-positive samples had the highest calculated copy number of the CnPnV M gene per µL of template, and hence low levels of target RNA may be another reason for unsuccessful amplification of a G protein sequence from the remaining 12/15 samples used in the current study (More *et al.* 2021).

Pneumovirus G protein is responsible for viral attachment to the host cell receptor and is a major target for neutralising antibodies (Levine *et al.* 1987; Ling and Pringle 1989). Two distinct groups (group A and B) of CnPnV have been described based on sequence variation in the G protein gene of several CnPnV sequences from the USA (Glineur *et al.* 2013). Viruses from both groups were detected in the current study, suggesting that the variability of CnPnV circulating in New Zealand is similar to that described in the USA.

It has been suggested that the close similarity of CnPnV with the rodent viruses (MPV-15 and MPV-J3666 in Figure 2) may indicate cross-species transfer of a rodent pneumovirus to dogs (Decaro *et al.* 2014b). Interestingly, pneumoviruses recently

detected from dogs in China were more similar to porcine pneumovirus than to murine pneumonia virus (MPV), raising a possibility that cross-species transmission may have also occurred from swine to dogs (Song *et al.* 2021). Alternatively, CnPnV and pneumoviruses of rodents, pigs and dogs may have co-evolved independently from a common ancestor. The limited number of CnPnV sequences available in GenBank precludes distinguishing between those possibilities.

Canine respiratory coronavirus

The sequence and genomic organisation of CRCoV is similar to that of BCoV (Erles *et al.* 2003, 2007). The most variable region is located between the spike (S) and envelope (E) genes. This region includes two or three small accessory proteins named based on their predicted molecular sizes. The UK CRCoV were predicted to encode two (8.8 or 4.9 kDa and 12.9 kDa) proteins in this region, while viruses from Italy and Korea were predicted to encode three proteins (4.9, 2.7 and 12.8 kDa) (Erles *et al.* 2007; Lorusso *et al.* 2009; An *et al.* 2010). Canine respiratory coronavirus sequences from Thailand were predicted to encode only 2.7 and 12.8 kDa accessory proteins (accessions OQ621707 to OQ621727, unpublished). The New Zealand CRCoV was predicted to encode three proteins of 5.9, 2.7 and 12.7 kDa in the same region, which is a combination that has not been previously described. This further highlights the variable nature of this genomic fragment. The expression and sizes of accessory proteins of different CRCoV need to be confirmed experimentally, as so far these are simply predictions based on the analysis of genomic sequences. The role of these putative proteins in the CRCoV life cycle and pathogenesis also remains to be elucidated.

Based on the phylogenetic analysis, CRCoV NZ-046/16 was more similar to CRCoV sequences from the USA than to sequences from Asia. However, it is difficult to draw any conclusions about evolutionary relationships between New Zealand CRCoV and viruses from other countries based on only one New Zealand sequence and a limited number of sequences from other countries. Such relationships will become clearer as more sequence data become available.

Virus isolation

Our attempts to isolate CRCoV and CnPnV from the oropharyngeal swab samples that were positive for these viruses by RT-qPCR were unsuccessful. This may reflect the absence of infectious viruses in the samples, with inactivation occurring during transportation or storage, or suboptimal culture conditions, particularly for CnPnV. Limited literature is available on *in vitro* growth requirements of CnPnV and so far, the virus has been successfully isolated only in canine fibroblast A-72 cells by some (Renshaw *et al.* 2010), but not other (Decaro *et al.* 2014b) researchers. Canine pneumovirus shares sequence similarity with MPV (Renshaw *et al.* 2011) and has been shown to replicate efficiently in the lung tissue of mice following experimental infection (Percopo *et al.* 2011; Glineur *et al.* 2013). Both Vero and RAW 264.7 cell lines were successfully used for the culture of MPV (Dyer *et al.* 2007), and hence we elected to use these cell lines to attempt CnPnV isolation. However, there are no data on susceptibility of these cell lines to CnPnV infection and hence, lack of successful isolation of the virus in the current study may reflect the choice of the cell lines used. The A-72 cells would have been preferred but were not available at the time of the study.

The HRT-18 cell line and its clone HRT-18G were shown to be supportive for the growth of CRCoV in previous studies, while several other cell lines including A-72, BHK-21, CHO, DH82, MDBK and MDCK were not (Erles *et al.* 2007; An *et al.* 2010). As CRCoV does not produce visible cytopathic effect in cell culture (Erles *et al.* 2007), RT-qPCR and HA were used to detect growth of the virus in the current study. Similar to our results, other researchers also failed to propagate CRCoV on HRT-18 cells from the samples that were positive for the virus using molecular methods (Yachi and Mochizuki 2006; Decaro *et al.* 2007; Lorusso *et al.* 2009; Lu *et al.* 2017), highlighting the difficulties in growing CRCoV *in vitro*.

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No potential conflict of interest was reported by the author (s).

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