Canine respiratory viruses in New Zealand dogs

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Abstract

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Acute infectious tracheobronchitis (ITB) is an important health issue in dogs worldwide. It predominantly affects kenneled dogs but has also been described in pet dogs. The aetiology of the disease is complex and likely to involve various respiratory pathogens, as well as host- and environment-related factors. Traditionally, canine parainfluenza virus (CPiV), canine adenovirus type 2 (CAdV-2), and *Bordetella bronchiseptica* were regarded as most commonly involved in canine ITB, and are incorporated into available vaccines. Other bacterial species as well as canine herpesvirus type 1 (CHV-1), canine reoviruses, and canine distemper virus have also been detected in the samples from diseased dogs. Within the past two decades, several novel canine respiratory viruses that may contribute to canine ITB have been discovered. These include canine influenza viruses, canine respiratory coronavirus (CRCoV), canine pneumovirus (CnPnV), canine bocavirus, and canine hepacivirus.

It appears that the contribution of various respiratory pathogens to canine ITB differs between different geographical locations and hence, the availability of local data is important for the implementation of the most appropriate disease prevention strategies. To date, very little data are available on the respiratory pathogens circulating among dogs in New Zealand. As such, the objective of the present study was to identify what viruses circulate among local dog populations including determination of the presence/absence of the recently identified canine respiratory viruses and to determine which of these pathogens are likely to be aetiologically involved in canine ITB. The second objective was to characterise at the molecular level selected novel viruses identified.

In order to identify viruses associated with canine ITB, metagenomic shot-gun sequencing approach was used to determine what viral nucleic acids were present in the pooled samples prepared from oropharyngeal swabs collected from dogs with clinical signs of ITB (*n = 50*) and from healthy dogs (*n = 50*). Following shot-gun sequencing, assembly and mapping, sequences of CHV-1, CRCoV, CnPnV, canine picornavirus and influenza C virus were identified in the pooled sample from dogs with ITB, while none of these sequences

were identified in the pooled sample from healthy dogs. This is the first molecular identification of CnPnV, CRCoV, CanPV and influenza C virus in the New Zealand dog population.

Real-time PCR assays were then designed to assess the frequency of detection of five canine respiratory viruses (CPiV, CAdV-2, CHV-1, CRCoV and CnPnV) in individual oropharyngeal swab samples from dogs with signs of ITB and from healthy dogs. Infections with at least one canine respiratory virus were more commonly detected in dogs with signs of ITB (21/56 (37.50 %)) than in healthy dogs (15/60 (25.00 %)). Dogs with signs of ITB were most commonly positive for CnPnV (26.78 %) followed by CAdV-2 (8.92 %), CPiV (3.57 %), CHV-1 (3.57 %), and CRCoV (1.78 %). Only CnPnV (23.33 %) and CAdV-2 (5.00 %) were identified in samples from healthy dogs. The overall prevalence of CnPnV in the sampled population was 29/116 (25.00 %).

This research revealed the first molecular evidence for the presence of CRCoV and CnPnV in dogs in New Zealand. As such, these viral sequences were further analyzed. The attachment gene (G) of three CnPnVs (CnPnV NZ-007, CnPnV NZ-048 and CnPnV NZ-049) was sequenced to characterise CnPnV circulating among dogs in New Zealand. Sequence analysis of the CnPnV G gene revealed that both group A and group B subtypes of CnPnV circulate in New Zealand.

The genetic analysis of the 3’ genomic region of CRCoV from New Zealand (CRCoV NZ-046/16) revealed closer relation to the British CRCoV 4182, Italian CRCoV 240/05 and Chinese CRCoV BJ232 than to the Korean CRCoVs (K9, K37 and K39). A deletion of one nucleotide at the region between the genes encoding the spike protein and 12.8 kDa accessory protein in CRCoV NZ-046/16 resulted in deletion of a stop codon with subsequent translation of predicted 5.9 kDa and 2.7 kDa proteins instead of two accessory proteins (4.9 kDa and 2.7 kDa) encoded at that region by most other CRCoVs.

In order to gain some insight into the epidemiology of CRCoV among dogs in New Zealand, canine sera (*n = 100*) were randomly selected from diagnostic laboratory submissions on a monthly basis from March to December 2014, and analyzed for the presence of CRCoV antibodies using a commercial blocking ELISA with bovine

coronavirus antigen. Overall, 53 % of 1015 sera tested were positive for CRCoV antibody. The present study revealed an increase in the prevalence of CRCoV antibodies with age ($p = 0.014$).

The work presented in this thesis has contributed to our understanding of the viruses involved in canine respiratory disease in New Zealand. Although infections with CPiV, CAdV-2, and CHV-1 were detected, it appeared that recently discovered canine respiratory viruses, including CRCoV and CnPnV, may also play an important role in canine ITB in New Zealand. This may provide one explanation for the development of respiratory disease in some fully vaccinated dogs, as anecdotally reported by field veterinarians. If so, their potential role and local epidemiology should be investigated in future studies.

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Place: Palmerston North

Date:

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Abbreviations

µg	microgram
µL	microlitre
µM	micromolar
A-72	canine fibroblast cell lines
aa	amino acid
BCoV	bovine coronavirus
BLAST	Basic local alignment search tool
Bp	base pair
bRSV	bovine respiratory syncytial virus
CAdV-1	canine adenovirus type-1
CAdV-2	canine adenovirus type-2
CanPV	canine picornavirus
CBoV	canine bocavirus
cDNA	complementary DNA
CDV	canine distemper virus
CHV-1	canine herpesvirus type 1
CI	confidence interval
CIV	canine influenza virus
CnNPHV	canine hepacivirus
CnPnV	canine pneumovirus
CPE	cytopathic effect
CPiV	canine parainfluenza virus
CPV	canine parvovirus
Cq	quantification cycle
CRCoV	canine respiratory coronavirus
DMEM	Dulbecco's modified eagle medium
DMSO	diemethyl sulfoxide
DNA	deoxyribonucleic acid
E gene	envelope gene
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
G	attachment protein
GE	gene end
GS	gene start

HA	Hemagglutination
HCoV-OC43	human coronavirus OC43
HCV	hepatitis C virus
HE	hemagglutinin esterase
HECoV	human enteric coronavirus
HI	hemagglutination inhibition
HN	hemagglutinin-neuraminidase
hr	hour
hRSV	human respiratory syncytial virus
HRT-18	human rectal tumor-18
ICTV	International Committee on Taxonomy of Virus
IF	immunofluorescence
IFA	immunofluorescent antibody
IHC	Immunohistochemistry
ITB	infectious tracheobronchitis
kb	kilo base pair
kDa	kilo dalton
M gene	membrane gene
MDBK	Madin-Darby bovine kidney
MDCK	Madin-Darby canine kidney
MHV	mouse hepatitis virus
min	minute
mL	milliliter
mM	millimolar
MPV	murine pneumovirus
mRNA	messenger RNA
MRV	mammalian orthoreovirus
N gene	nucleocapsid gene
NA	neuraminidase
NCBI	National Center for Biotechnology Information
Neu5,9Ac_2	*N*-acetyl-9-*O*-acetylneuraminic acid
ng	nanogram
nm	nanometre
nt	nucleotide
NTR	non-translated regions
NZGL	New Zealand Genomics Limited
NZVP	New Zealand Veterinary Pathology

OD	optical density
OR	odds ratio
ORF	open reading frame
PBS	phosphate buffered saline pH 7.2
PCR	polymerase chain reaction
POI	percentage of inhibition
qPCR	real-time PCR/ quantitative PCR
RBC	red blood cells
RNA	ribonucleic acid
rpm	revolutions per minute
S gene	spike gene
sec	second
SV-5	Simian virus-5
TBE	tris-borate-EDTA
TRS	transcription regulatory sequence
U	unit
VNT	virus neutralization test
WGA	whole genome amplification
WSAVA	World Small Animal Veterinary Association

Chapter 1: GENERAL INTRODUCTION

Background

Infectious tracheobronchitis (ITB) is a common health problem among dogs, especially in the dogs from group housing such as in veterinary hospitals, breeding shelters and kennels. This disease is one of the major welfare and financial issues in intensive housing facilities, as ITB outbreaks can result in delays in re-homing and training programs. The most common clinical presentation of ITB infected dogs varies from subclinical to mild to moderate clinical respiratory signs lasting for several weeks. Some cases may develop into more severe form, such as bronchopneumonia, and in some cases it can be fatal (Pesavento *et al.*, 2008, Creevy, 2015).

Canine ITB can be caused by a large number of pathogens acting alone or synergistically. However, there is a lack of information on the prevalence of the different canine respiratory pathogens and their association with the signs of respiratory disease in New Zealand dogs. In addition, there has been almost no effort to investigate the presence of recently discovered, let alone unknown pathogens associated with respiratory disease in dogs in this country.

Knowledge of the causative agents responsible for canine ITB, among many veterinarians and dog owners, is limited to *Bordetella bronchiseptica*, canine parainfluenza virus (CPiV), canine adenovirus-2 (CAdV-2) and canine distemper (CDV). The current dog vaccines available in New Zealand include those pathogens, and are widely used either in intranasal or in parenteral formulations. Despite this, dog owners and veterinarians have seen a considerable number of ITB cases in vaccinated dogs in New Zealand, which has raised

concerns on the efficacy of currently available vaccines to prevent canine ITB cases in New Zealand.

In recent years, diagnostic investigations of canine ITB worldwide have shown involvement of other pathogens (canine respiratory coronavirus, canine pneumovirus, canine influenza virus, *Streptococcus zooepidemicus* and *Mycoplasma cynos*) (Erles *et al.*, 2003, Yoon *et al.*, 2005, Renshaw *et al.*, 2010). However, currently, there is very limited information available on the presence of these pathogens in dogs in New Zealand.

The present research was undertaken to identify the viruses involved in canine respiratory infections in dogs in New Zealand.

The research objectives of this thesis include:

1) To review the literature on infectious tracheobronchitis in dogs.
2) To detect viruses present in healthy dogs and dogs with ITB in New Zealand using shot-gun sequencing.
3) To determine the molecular prevalence of canine respiratory viruses (CRCoV, CPiV, CAdV-2, canine herpesvirus type-1 (CHV-1) and CnPnV) using real-time PCR assays.
4) To attempt isolation of CRCoV and CnPnV *in vitro*.
5) To characterize newly discovered viral sequences (CRCoV and CnPnV) obtained from dogs in New Zealand and to compare with their respective sequences obtained worldwide.
6) To determine the seroprevalence of CRCoV in New Zealand dogs.

Chapter 2: ACUTE INFECTIOUS TRACHEOBRONCHITIS: A REVIEW OF LITERATURE

Acute infectious tracheobronchitis, commonly referred to as 'kennel cough' or canine infectious respiratory disease, is described as any contagious, acute-onset of respiratory infection of dogs involving one or more of several viral or bacterial pathogens acting alone or synergistically, and mainly affecting the upper respiratory tract (Ford, 2012). This disease commonly affects dogs housed in groups such as breeding and boarding kennels, shelters or veterinary clinics.

Due to the overlapping clinical signs produced by different pathogens responsible for canine ITB, it is not possible to distinguish which pathogen is the cause of respiratory infection based on the clinical presentation. Most of the dogs naturally infected show mild to moderate respiratory signs, though rarely the infection can be severe, especially if complicated with secondary bacterial infection. The clinical presentation of canine ITB is as follows (Ford, 2012):

- Acute onset of dry, paroxysmal cough, often in association with gagging and retching behaviour
- High pitched "honking sound" in case of laryngitis
- Presence of tracheal sensitivity
- A serous to mucopurulent nasal and ocular discharge
- Occasional sneezing
- Fever, lethargy and inappetence is less common
- Severe cases of respiratory disease are often complicated by secondary bacterial infections leading to severe, potentially fatal bronchopneumonia

2.1. Overview of aetiology

Interest in the aetiology of canine ITB began after the first discovery of distemper in dogs in the 18th century (Jenner, 1809). While investigating the pathogenesis of distemper, *Bordetella bronchiseptica* was isolated in lung tissues, revealing bacterial involvement in the infectious respiratory disease of dogs (Ferry, 1910, Ferry, 1911). Experimental production of respiratory infection using *B. bronchiseptica* (Wright *et al.*, 1973, Thompson *et al.*, 1976, Bemis *et al.*, 1977) and isolation of this bacteria in natural infections thereafter, led to *B. bronchiseptica* being considered to be one of the primary pathogens of ITB in dogs (Thompson *et al.*, 1976, Goodnow *et al.*, 1983).

The complexity in the aetiology of canine ITB was noticed when other viral pathogens such as CAdV-2 (Ditchfield *et al.*, 1962), CPiV and CHV-1 (Binn *et al.*, 1967) were also recovered from the respiratory tissues of dogs with respiratory infections. The results from experimental studies indicated that CPiV, CAdV-2 and CHV-1 can have a role in development of canine ITB (Karpas *et al.*, 1968, Rosenberg *et al.*, 1971, Wright *et al.*, 1972). Since then, the association of canine ITB with infection by *B. bronchiseptica*, CPiV, CAdV-2 and CHV-1 either alone or synergistically has been reported globally; and these pathogens have been considered as the traditional pathogens responsible for ITB in dogs. The subsequent development and implementation of vaccines against *B. bronchiseptica*, CPiV and CAdV-2 has reduced the occurrence of canine ITB outbreaks.

With the beginning of 21st century, other pathogens have been reported to be involved in infectious respiratory disease of dogs. These findings have led to an expanded understanding of the aetiology of canine ITB, thereby improving surveillance and management of this disease. Canine respiratory coronavirus was identified in a large

rehoming shelter in the UK that was experiencing outbreaks of respiratory disease despite regular vaccination against *B. bronchiseptica*, CPiV and CAdV-2 (Erles *et al.*, 2003). In addition to this, isolation of canine influenza virus from racing greyhounds in Florida, with moderate to severe respiratory signs, suggested influenza A virus as a contributor to the canine ITB disease complex (Crawford *et al.*, 2005). Subsequently, CnPnV was detected in dogs with acute respiratory disease in the USA (Renshaw *et al.*, 2010). Next generation sequencing on respiratory samples obtained from affected dogs led to the identification of canine bocavirus (Kapoor *et al.*, 2012) and non-primate canine hepacivirus (Kapoor *et al.*, 2011). There is a continued interest in finding other microbial agents involved in the aetiology of canine ITB.

The aetiological agents in canine ITB are not limited to the involvement of viruses. Bacteria such as, *M. cynos* (Rosendal, 1971) and *Streptococcus equi* subsp. *zooepidemicus* (Garnett *et al.*, 1982), previously reported in respiratory infections, have also been identified in outbreaks of severe respiratory disease in dogs (Chalker *et al.*, 2004, Pesavento *et al.*, 2008, Byun *et al.*, 2009). Although these bacteria are present in healthy dogs, their involvement in the respiratory disease can increase its severity (Chalker, 2005, Acke *et al.*, 2015).

Other bacteria isolated from the lower respiratory tract of dogs include *Pasteurella* spp., *Pseudomonas* spp. and various coliforms (Angus *et al.*, 1997). Sprague *et al.* (2009) detected *Chlamydia psittaci* in a dog breeding facility in Northern Germany that was experiencing recurrent respiratory and reproductive problems. However, the role of these pathogens in canine ITB remains unclear.

The recent molecular diagnostic investigations of canine ITB have suggested that canine ITB has a far more complex aetiology than was speculated earlier. Nonetheless, respiratory disease outbreaks due to 'traditional' pathogens like CPiV, CAdV-2, CHV-1 and *B. bronchiseptica* are still reported (Schulz *et al.*, 2014b, Weese and Stull, 2013, Kumar *et al.*, 2015) and the use of vaccines against *B. bronchiseptica*, CPiV and CAdV-2 is still recommended. It is likely that many of the newly identified agents were always present and were causing respiratory infections in dogs much earlier; however, recent advances in pathogen detection methods, particularly the use of molecular methods may have resulted in their identification and confirmation of involvement in canine ITB. Routine use of vaccines and antimicrobials may also have exerted a selective pressure on pathogens affecting dogs which may have led to the discovery and re-emergence of canine respiratory pathogens with time. These recent findings inevitably have an important implication on the development of future kennel cough vaccine design.

The microbial agents that have been reported to be involved with signs of acute ITB are listed in Table 2.1.

Table 2.1. List of microbial agents identified in dogs with acute respiratory infection with the country of discovery.

Pathogen		Country	Reference
Viruses	Canine distemper	UK	(Rockborn, 1958)
	Canine reovirus- 1	USA	Lou *et al.* (1963)
	Canine adenovirus- 2	Canada	Ditchfield *et al.* (1962)
	Canine parainfluenza virus	USA	Binn *et al.* (1967)
	Canine herpesvirus-1	USA	Binn *et al.* (1967)
	Canine influenza virus H3N8	USA	Dubovi *et al.* (2004)
	Canine respiratory coronavirus	UK	Erles *et al.* (2003)
	Canine pneumovirus	USA	Renshaw *et al.* (2010)
	Non-primate canine hepacivirus	USA	Kapoor *et al.* (2011)
	Canine bocavirus	USA	Kapoor *et al.* (2012)
	Canine influenza virus H3N2	Korea	Song *et al.* (2008)
Bacteria	*Bordetella bronchiseptica*	USA	Ferry (1911)
	Streptococcus equi subsp. *zooepidemicus*	USA	Garnett *et al.* (1982)
	Mycoplasma cynos	Denmark	Rosendal (1971)

2.2. Incubation and shedding time reported for canine respiratory agents

Most of the viruses multiply rapidly at initial stages of infection before clinical signs are apparent. The absence or low magnitude of host cell mediated immune response during incubation period makes it easier to isolate the virus. Reported incubation periods and shedding times for canine respiratory agents are listed in the Table 2.2.

Table 2.2. Reported incubation period and shedding time of canine respiratory agents.

Canine respiratory agent	Incubation period (days)	Shedding (days)	Reference
Canine parainfluenza virus	8-14	2-7	Lazar *et al.* (1970)
Canine adenovirus type 2	1-2	5-10	Swango *et al.* (1970), Wright *et al.* (1972)
Canine herpesvirus - 1	6-10	3-18	Karpas *et al.* (1968)
Canine respiratory coronavirus	Not known	2-10	Mitchell *et al.* (2012)
Canine influenza virus - H3N8 strain	1-4	7-10	Deshpande *et al.* (2008)
Canine influenza virus - H3N2 strain	1-8	1-8	Song *et al.* (2009)
Canine pneumovirus	3 (in mice)	3-7	Percopo *et al.* (2011)
Bordetella bronchiseptica	6-10	1-21	Thompson *et al.* (1976)

2.3. Canine respiratory viruses

2.3.1. Traditionally known canine respiratory viruses

2.3.1.1. Canine parainfluenza virus

Taxonomic classification:

Order: *Mononegavirales*

Family: *Paramyxoviridae*

Sub family: *Paramyxovirinae*

Genus: *Rubulavirus*

Species: ***Canine parainfluenza virus 5***

Canine parainfluenza virus, also known as Simian virus-5 (SV-5), is primarily associated with ITB in dogs. It was first reported in laboratory dogs (Binn *et al.*, 1967) and sentry dogs with respiratory infections (Crandell *et al.*, 1968). Since then, the virus has been confirmed to be associated with respiratory infections in dogs either alone or with other viruses and bacteria (Crandell *et al.*, 1968, Appel and Percy, 1970, Binn *et al.*, 1979, Wagener *et al.*, 1984).

The CPiV genome is a single-stranded, non-segmented, negative sense RNA of approximately 15,246 nucleotide (nt) long (Karron and Collins, 2007).

CPiV replicates in the epithelial cells of the nasal mucosa, pharynx, trachea and bronchi. Virus entry into epithelial cells is initiated by the attachment of viral hemagglutinin-neuraminidase (HN) glycoprotein to *N*-acetylneuraminic (sialic) acid of the host cell membrane. Once bound HN interacts and causes necessary conformational change in the fusion glycoprotein resulting in fusion of the virus envelope and host cell membrane (Karron and Collins, 2007). The fusion between virus-host cell membranes allows viral nucleocapsid to enter the host cell cytoplasm, where viral replication occurs and results in the development of intracytoplasmic inclusion bodies (Baumgärtner *et al.*, 1991). Boulan and Sabatini (1978) have also shown budding at the apical (luminal) surface of CPiV infected polarized epithelial cells; however this has not been confirmed in dogs naturally infected with CPiV.

CPiV associated respiratory disease outbreaks have been characterised by mild to moderate signs of disease such as low-grade rise in rectal temperature, dry, harsh, hacking cough, watery nasal discharge, pharyngitis and tonsillitis (Crandell *et al.*, 1968, Appel and Percy,

1970). As natural respiratory infections are often complicated by other pathogens, it is hard to ascribe signs attributable to CPiV alone. Dogs experimentally infected with CPiV showed mild signs of respiratory infection (Rosenberg *et al.,* 1971). Following experimental CPiV infections in dogs, Lemen *et al.* (1990) noticed a transient airway hyper responsiveness to aerosolised histamine, while Wagener *et al.* (1983) reported denudation and peribronchiolar lymphocytic infiltration in larynx, trachea and bronchi.

Researchers have shown that the route of CPiV inoculation has a role in the development of respiratory infection. Dogs parenterally inoculated with CPiV showed no respiratory signs, however, aerosol and contact exposure produced abnormal respiratory signs and all dogs developed multifocal pulmonary petechiation 3 or 4 days post-infection (Appel and Percy, 1970).

Hemagglutinin inhibition (HI) and virus neutralization (VNT) antibodies against CPiV infection have been demonstrated in naturally infected dogs (Crandell *et al.*, 1968). Following intranasal inoculation with CPiV, both HI and VNT antibodies develop by day 7 post-infection. The CPiV titer peaks by day 21 post-infection and then declines (Lazar *et al.*, 1970). The duration of the CPiV specific antibody immune response has not been extensively studied, however, Appel and Percy (1970) reported a decline in the VNT titer by 3-4 months after experimental CPiV infection.

CPiV propagate *in vitro* in a variety of cell lines including Madin-Darby Canine Kidney (MDCK), canine fibroblast (A-72), African green monkey kidney (Vero), feline kidney and human embryonic kidney cell lines (Meguro *et al.*, 1979). CPiV produces a typical cytopathic effect (CPE) (i.e. small focal multinucleated syncytial giant cells and

progressive cellular degeneration) within 48 - 72 hr post infection. CPiV propagation in cell cultures can be confirmed by molecular methods or definitive staining with immunofluorescence or immunohistochemistry (Damian *et al.*, 2005) or a hemagglutination test, as CPiV can agglutinate human, chicken, guinea pig, rat, rabbit, dog, cat and sheep erythrocytes (Crandell *et al.*, 1968, Black and Lee, 1970). On inoculation in an embryonated chicken egg, CPiV propagated in the amniotic cavity without embryonic death, while the virus failed to replicate in the allantoic cavity (Crandell *et al.*, 1968).

2.3.1.2. Canine adenovirus

Taxonomic classification:

Order: *Unassigned*

Family: *Adenoviridae*

Genus: *Mastadenovirus*

Species: *Canine adenovirus 1*

Canine adenovirus 2

Two types of adenoviruses have been reported in dogs: canine adenovirus type-1 (CAdV-1) (Green *et al.*, 1930) and canine adenovirus type-2 (CAdV-2) (Ditchfield *et al.*, 1962). CAdV-1 causes infectious canine hepatitis and systemic disease, while CAdV-2 causes a localized respiratory infection and is one of the primary causes of ITB in dogs (Ditchfield *et al.*, 1962).

The canine adenovirus genome is non-enveloped, single linear molecule of double-stranded DNA (Valentine and Pereira, 1965). CAdV-1 is 30,536 nt long and CAdV-2 is 31,323 nt long. Sequence comparison of CAdV-1 and CAdV-2 reveals variation in the E3 region of

CAdV genome, which is responsible for the biological differences between the two canine adenoviruses (Linné, 1992).

Despite the fact that CAdV-1 primarily causes acute hepatitis in dogs, it has been recovered from throat swabs, tonsils and tracheal tissues of dogs with respiratory disease (Binn *et al.*, 1967, Wright *et al.*, 1972) suggesting that CAdV-1 also has an affinity for respiratory mucosa. Similarly, CAdV-2 primarily replicates in the respiratory epithelium (Swango *et al.*, 1970), but it has also been detected in brain tissue of dogs with neurological signs (Benetka *et al.*, 2006) and the alimentary tract of dogs with enteritis (Macartney *et al.*, 1988, Hamelin *et al.*, 1985). However, the pathogenesis of CAdV-2 in neurological disease and enteritis is not clearly understood.

The route of entry of CAdV-2 plays an important role for development of respiratory disease. Experimental intravenous/intramuscular administration of CAdV-2 virus in healthy dogs does not produce any clinical signs of respiratory disease (Fairchild *et al.*, 1969). Intranasal inoculation of CAdV-2 resulted in a harsh, dry hacking cough within 3 days post-infection; and other clinical signs including marked depression, anorexia, dyspnea, muscular trembling and serous nasal discharge were also observed (Swango *et al.*, 1970). Histopathological examination of respiratory tissues from dogs experimentally challenged with CAdV-2 via the aerosol route also showed moderate to severe proliferative, interstitial pneumonia (Swango *et al.*, 1970). These histopathological changes in the respiratory tissues can be observed from day 3 post-infection, and CAdV-2 can be recovered from the lungs until day 10 post-infection (Swango *et al.*, 1970).

Canine adenovirus type-2 replicates in non-ciliated epithelial cells, mucosal and submucosal epithelial cells of bronchi, mucosal and submucosal epithelial cells of the trachea, and type-2 alveolar epithelial cells (Castleman, 1985). The virus produces large metachromatic intranuclear inclusion bodies in bronchial epithelium and alveolar septal cells (Swango *et al.*, 1970).

Although CAdV-1 and CAdV-2 have different clinical presentations, they are antigenically (Marusyk, 1972) and genetically related (Morrison *et al.*, 1997, Davison *et al.*, 2003). CAdV-1 and CAdV-2 can be differentiated by their hemagglutination properties and the immunodiffusion test (Marusyk and Yamamoto, 1971, Marusyk, 1972); and molecularly by restriction endonuclease analysis (Assaf *et al.*, 1983, Hamelin *et al.*, 1984), DNA hybridization (Marusyk and Hammarskjöld, 1972), PCR (Hu *et al.*, 2001) and sequencing (Davison *et al.*, 2003).

The hexon protein of CAdV contains major neutralizing epitopes and is responsible for the antibody mediated immune response (Willcox and Mautner, 1976). Tribe and Wolff (1973) and Fairchild *et al.* (1969) have shown that dogs immunized with hexon antigens of either CAdV-1 or CAdV-2 are cross-protected against both CAdV-1 and CAdV-2 infections. Furthermore, Appel *et al.* (1975) showed that attenuated CAdV-2 vaccines are able to induce protective immunity to both CAdV-1 and CAdV-2 in puppies containing maternal antibodies to CAdV-2.

CAdV-2 was first isolated in primary dog kidney cells (Ditchfield *et al.*, 1962). Yamamoto (1966) reported that CAdV-2 can only propagate in canine primary cells and continuous canine cell lines; while propagation on human, rhesus monkey and bovine primary cells

and their cell lines, such as HeLa, FL, KB and Madin-Darby bovine kidney (MDBK), were unsuccessful. Cytopathic effect characterised by rounding and clumping of cells, was observed after 16 hr post-infection and was more pronounced after 48 hr post-infection (Yamamoto, 1966). Yamamoto (1966) reported the growth characteristics for CAdV-2 to be similar to other adenoviruses by having a long and latent release period, with peak CAdV-2 virus titers obtained at 40 hr and 96 hr in cells and supernatant fluid, respectively. The virus particles tend to aggregate at the periphery of the nucleus and release by protrusion of nuclear membrane, which is a peculiarity of CAdV-2 infection (Yamamoto, 1969).

2.3.1.3. Canine herpesvirus

Taxonomic classification:

Order: *Herpesvirales*

Family: *Herpesviridae*

Subfamily: *Alphaherpesvirinae*

Genus: *Varicellovirus*

Species: ***Canine herpesvirus 1***

Herpesviruses are linear double-stranded, enveloped DNA viruses of ~126-234 kb in size (Wildy *et al.*, 1960). Canine herpesvirus (CHV-1) is a member of *Alphaherpesvirinae* subfamily, genus *Varicellovirus* (Maclachlan and Dubovi, 2010). CHV-1 only infects the members of the *Canidae* family. The prevalence of CHV-1 is reported to range from 1% to 8% in Netherlands, and 45.75% in the Belgium dog population (Reading and Field, 1998, Rijsewijk *et al.*, 1999, Ronsse *et al.*, 2002).

CHV-1 was first reported in dogs as a cause of severe morbidity and mortality in fetuses and neonates (Carmichael *et al.*, 1965, Spertzel *et al.*, 1965, Stewart *et al.*, 1965). Since then, CHV-1 has been identified as an important pathogen associated with reproductive, respiratory and ocular diseases in dogs (Wright and Cornwell, 1968, Ledbetter *et al.*, 2006, Ledbetter *et al.*, 2009a, Kawakami *et al.*, 2010). It was recently reported that CHV-1 has more affinity to the genital mucosa compared to the respiratory mucosa (Li *et al.*, 2016).

The clinical signs of herpesvirus infection in dogs are age-dependent. CHV-1 infection in young dogs may cause slight nasal discharge, acute paroxysmal coughing (Karpas *et al.*, 1968) or fatal generalized necrotizing disease (Carmichael *et al.*, 1965). Fetal death and still birth (Hashimoto *et al.*, 1982), conjunctivitis and dendritic ulcerative keratitis are reported in older dogs (Ledbetter *et al.*, 2006, Ledbetter *et al.*, 2009a, Ledbetter *et al.*, 2009b, Ledbetter *et al.*, 2009c).

The primary role of CHV-1 in the development of canine ITB remains controversial. Experimental infection by the intranasal route has shown to cause mild clinical signs of rhinitis and pharyngitis in puppies (Appel *et al.*, 1969). Recently, Kumar *et al.* (2015) also reported a strong relationship between CHV-1 and acute respiratory disease in four dogs that had no history of any stressful events or use of immunosuppressive therapy or disease. However, due to the ability of CHV-1 to remain latent in the host, the causative role of CHV-1 cannot be clarified.

Latent infection is a common feature of herpesviruses and sites of latency vary for different herpesviruses. Dogs recovered from symptomatic or asymptomatic CHV-1 infections can remain latently infected. During latency, CHV-1 can be identified in regional sensory

ganglia (trigeminal, lumbo-sacral, and vestibular), tonsils, parotid salivary glands and lymphocytes of regional lymph nodes (retropharyngeal lymph node) (Okuda *et al.*, 1993, Burr *et al.*, 1996, Miyoshi *et al.*, 1999). Latently infected dogs can be of concern in high density areas such as kennels, where CHV-1 may get re-activated due to stress and can lead to horizontal transmission between dogs. CHV-1 can also be re-activated in dogs with a history of herpesvirus infection using immunosuppressive drugs such as prednisolone (Okuda *et al.*, 1993). A severe outbreak of canine ITB due to CHV-1 infection alone was reported in a referral animal medical center in Japan, which was attributed to the immunosuppressed status of several dogs owing to prednisolone therapy or the stress caused either by hospitalization, surgery, chemotherapy or radiation (Kawakami *et al.*, 2010).

CHV-1 has a relatively narrow host range *in vivo* and *in vitro* compared to other members of *Alphaherpesvirinae*. CHV-1 has been grown successfully on fetal mink lung cell lines and canine derived cell lines, like MDCK and canine fibroblast (A-72) cell lines (Peterson and Goyal, 1988, Kazuo Nakamichi, 2000); while other cell lines including ovine fetal lung, ferret kidney and turkey embryo kidney remained non-permissive to CHV-1 infection (Peterson and Goyal, 1988). Cytopathic effect is characterised by the production of small foci of rounded, refractile cells with large, distinct, intranuclear inclusion bodies (Strandberg and Carmichael, 1965).

2.3.1.4. Canine distemper virus

Taxonomic classification:

Order: *Mononegavirales*

Family: *Paramyxoviridae*

Sub family: *Paramyxovirinae*

Genus: *Morbillivirus*

Species: ***Canine distemper virus***

Canine distemper virus (CDV) is an enveloped virus containing single stranded, negative sense RNA genome enclosed in a helical nucleocapsid. CDV is a causative agent of a severe systemic disease in dogs; and infection with this virus is characterized by a variety of clinical signs affecting respiratory, gastrointestinal and neurological systems (Appel, 1969).

Implementation of canine distemper vaccination may have eradicated CDV in New Zealand dogs (Hill, 1999). However, periodic surveillance of CDV is required to investigate the circulation of CDV in New Zealand dogs and continued vaccination is still recommended.

2.3.2. Recently described canine respiratory viruses

2.3.2.1. Canine respiratory coronavirus

Taxonomic classification:

Order: *Nidovirales*

Family: *Coronaviridae*

Sub family: *Coronavirinae*

Genus: *Betacoronavirus*

Species: ***Betacoronavirus 1***

Subspecies: ***Canine respiratory coronavirus***

Coronaviruses belong to the *Coronaviridae* family of order *Nidovirales*. The family comprises of two sub-families: *Coronavirinae* and *Torovirinae*. Currently, the subfamily *Coronavirinae* is classified into four genera based on their genetic and serologic properties namely: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* (Woo *et al.*, 2012b). The viruses belonging to the *Alphacoronavirus* and *Betacoronavirus* genus infect a wide range of mammalian species ranging from bats to humans, while the viruses belonging to the *Gammacoronavirus* genus primarily infect birds. Recently described *Deltacoronavirus* genus includes avian and porcine coronaviruses (Woo *et al.*, 2012b).

CRCoV was first detected in shelter dogs with respiratory disease in the UK (Erles *et al.*, 2003). CRCoV is genetically and serologically distinct from the canine enteric coronavirus, which belongs to the genus *Alphacoronavirus* and causes enteric disease in dogs (Erles *et al.*, 2003, Decaro *et al.*, 2007).

CRCoV is genetically similar to bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV-OC43) (Erles *et al.*, 2007). According to the 9th report of the International Committee on Taxonomy of Viruses (ICTV), these viruses are placed under the same species, *Betacoronavirus 1*. From this point, the coronaviruses belonging to *Betacoronavirus 1* species will be discussed in detail.

Coronavirus genomes are non-segmented, positive sense, single-stranded RNA molecules ranging from 27 to 31.5 kb. The genome has a 5' cap (Lai and Stohlman, 1981), a 3' poly-adenylated tail (Yoshiaki *et al.*, 1977) and are polycistronic, which generate a nested set of subgenomic RNAs with common 5' and 3' sequences (Siddell, 1995).

The genomic organization of BCoV is shown in the Figure 2.1. Two-thirds of the 5' end of the BCoV genome consists of two large replicase open reading frames (ORF), designated as ORF1a and ORF1b (Lai, 1990). The ORF1b gene is placed at -1 frame with respect to ORF1a gene (Lai, 1990). The expression of ORF1b is mediated by ribosomal frame-shifting, where the ribosome slips one nucleotide backward prior to encountering the ORF1a stop codon and continues the translation of ORF1b thus producing a single ORF1ab polyprotein having RNA-dependent RNA polymerase and RNA helicase activity (Brierley *et al.*, 1987). The 3' genomic region of the BCoV genome includes five structural protein genes: hemagglutinin esterase (HE) gene, spike (S) gene, envelope (E) gene, membrane (M) gene and nucleocapsid (N) gene (Sturman and Holmes, 1983). Several ORFs that encode for accessory proteins are located between the replicase gene and the HE gene, and between the S and E genes (Sturman and Holmes, 1983).

Each of the coronaviral genes are preceded at the 5' end by transcription regulatory sequences (TRS) (Zúñiga *et al.*, 2004). The viral polymerase binds to the TRS and transcribes each gene in discontinuous sub-genomic sets of messenger RNA (mRNA) (Britton and Cavanagh, 2008). All mRNAs contain a common leader sequence at the 5' end which are encoded by the 5' end of the coronavirus genome.

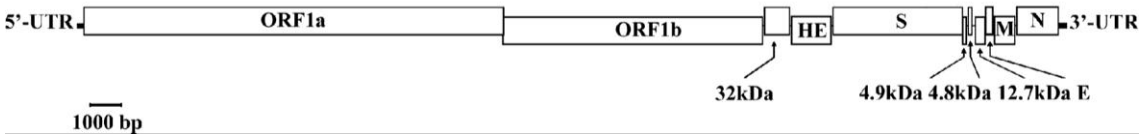


Figure 2.1. Genome organization of bovine coronavirus (BCoV) genome. The boxes indicate the open reading frames (ORF) of the genes for replicase, structural and accessory proteins. Letters in the boxes indicate gene name ORF1a and ORF1b are genes for polymerase; HE: hemagglutinin esterase; S: spike; E: envelope; M: membrane; N: nucleocapsid genes. The non-structural proteins in BCoV genome contains 32 kDa, 4.9 kDa, 4.8 kDa and 12.7 kDa (This figure is taken from Alekseev *et al.* (2008)).

The genomic organization of CRCoV is similar to the BCoV genome (Lim *et al.*, 2013). However, there is disparity in the number and size of accessory proteins encoded at the region between the S and E genes of CRCoV (Erles *et al.*, 2007, Lorusso *et al.*, 2009). At this region, BCoV encodes for three accessory proteins of 4.9 kDa, 4.8 kDa and 12.7 kDa (Fig. 2.1). While, most of the CRCoV sequences from the UK (CRCoV-G9142), Italy (CRCoV-240/05), Korea (CRCoV-K9, -K37 and -K39) and China (CRCoV-BJ232), have three accessory proteins of 4.9 kDa, 2.7 kDa and 12.7 kDa (Lorusso *et al.*, 2009, An *et al.*, 2010a, Lu *et al.*, 2017) (Fig.2.2). Mutations in the region between the S and E genes has been observed in some CRCoV sequences (CRCoV-4182, -T0715, -T1030 and -T1207), resulting in two accessory proteins of 8.8 kDa and 12.8 kDa (Erles *et al.*, 2007) (Fig. 2.2). The role of accessory proteins of CRCoV is not well understood, but may have a role in natural infections.

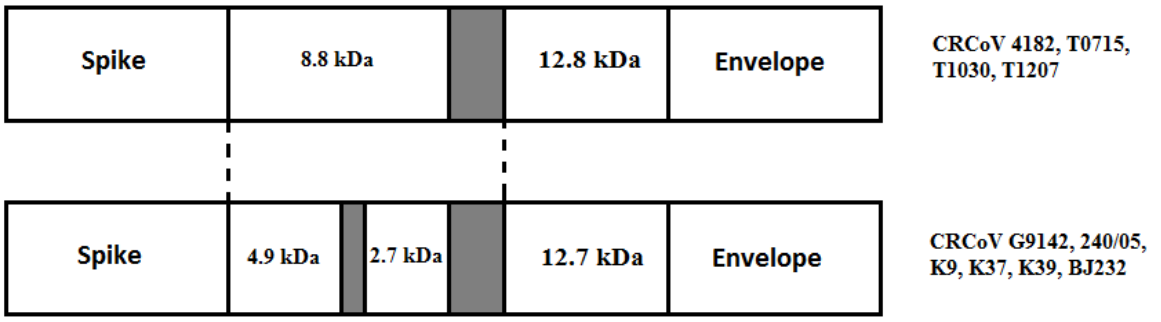


Figure 2.2. Comparison of the region between spike and envelope gene of CRCoV-4182, T0715, T1030, T1207, CRCoV-G9142, 240/05, K9, K37, K39 and BJ232. The region between the spike and envelope includes accessory genes of different molecular size. The region shaded in grey is the non-coding regions (This figure is modified from Lorusso *et al.* (2009).

2.3.2.1.1. Pathogenesis

CRCoV has an affinity for respiratory tissue and is frequently detected in trachea, nasal tonsils and lung lavage samples in experimentally infected dogs (Mitchell *et al.*, 2012). Trachea, nasal tonsils and the nasal cavity are the main sites for CRCoV infection (Mitchell *et al.*, 2012).

The mechanism of entry of CRCoV is not well understood but it is thought to be similar to BCoV. CRCoV is hypothesized to recognize *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2) receptors on the specific host cells for its attachment (Erles *et al.*, 2007). Prior to viral and host cell receptor attachment, the S protein undergoes cleavage into two functionally distinct subunits, S1 and S2 (Abraham *et al.*, 1990a). The S1 subunit binds to specific host cell receptor and causes dissociation of S1 from remaining S2 subunit (Zelus *et al.*, 2003, Taguchi and Matsuyama, 2002, Matsuyama and Taguchi, 2002). This in turn exposes the fusion peptide (FP), which interacts with the host cell membrane and brings two heptad repeats (HR-N and HR-C) together to form a stable α-helical six stranded structure (Fig. 2.3). This ultimately causes destabilization of cell lipid bilayers surrounding

the virus and cell leading to the fusion and release of viral nucleocapsid into the host cell cytoplasm (Tripet *et al.*, 2004).

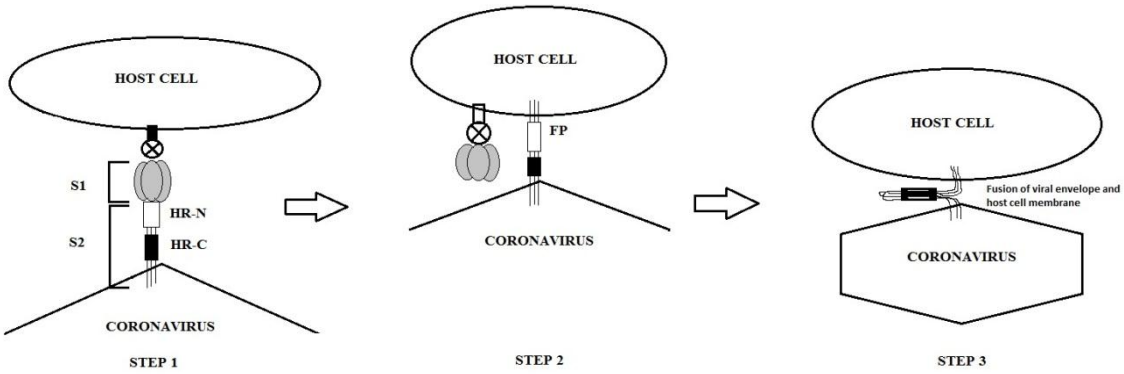


Figure 2.3. Diagrammatic representation of different stages of coronavirus S glycoprotein during the virus entry. S protein is denoted S1 and S2 for its N- and C-terminal domains; *Step 1* - the S1 domain binds the cell receptor, *Step 2* - the N-terminal (S1) domain is dissociated to expose the fusion peptide (FP) region, and *Step 3* - the collapsed S2 domain draws the viral and cellular membranes together causing fusion and release of the viral nucleocapsid into the host cell. HR-N and HR-C denote coiled coils at the N terminus and C terminus of the S2 domain. *X* denotes the host cell surface receptor. (This figure is adapted from the original figure in Tripet *et al.* (2004)).

Histopathological changes can be observed as early as three days after CRCoV infection (Mitchell *et al.*, 2012). Histopathological examination of respiratory tissues from experimentally challenged dogs with CRCoV showed inflammation in the nares and trachea, and shortening and clumping or loss of tracheal cilia (Mitchell *et al.*, 2012). Perivascular and peribronchiolar lymphoid aggregates were also observed in the lungs (Mitchell *et al.*, 2012).

2.3.2.1.2. Disease and clinical signs

CRCoV causes respiratory disease in dogs following experimental infections. In one study, clinical signs of mild respiratory disease (nasal discharge, sneezing and coughing) were observed in all dogs (*n=30*) following experimental intranasal inoculation of CRCoV

(Mitchell *et al.*, 2012). Viral shedding from the oropharynx was detected in the experimentally infected dogs from day two to day 10 post-infection (Mitchell *et al.*, 2012).

CRCoV infections in dogs can alter host gene expression. Suppression of mRNA level of the three pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-6 and interleukin-8) was observed from 24 - 72 hr in CRCoV inoculated dogs (Priestnall *et al.*, 2009). The levels were raised for each cytokine at 96 hr post-infection (Priestnall *et al.*, 2009). Down regulation of pro-inflammatory cytokines by CRCoV may predispose the host to secondary infections.

2.3.2.1.3. *In vitro* growth

Coronaviruses are difficult to isolate *in vitro* (Storz *et al.*, 1981, Frana *et al.*, 1985, Wicht *et al.*, 2014). Factors such as specific cell receptors, proteases and an acidic pH can modulate the entry of coronavirus into the host cells (Toth, 1982, Frana *et al.*, 1985). The trypsin-like proteases mediate viral attachment to the cell receptors by cleaving of the S protein into its subunits (Storz *et al.*, 1981).

The human rectal tumor (HRT-18) cell line is commonly used for isolation of BCoV and HCoV-OC43 (Guy *et al.*, 2000, St-Jean *et al.*, 2004). Madin-Darby Bovine Kidney (MDBK), Vero and porcine kidney 15 continuous cell lines have also been successfully used for BCoV isolation (Dea *et al.*, 1980).

For CRCoV, cell lines including A-72, Vero cells, baby hamster kidney cells (BHK-21), Chinese hamster ovary cells, MDCK and MDBK have failed to propagate CRCoV, suggesting a lack of appropriate receptors for the virus or absence of other factors required for CRCoV growth (Erles *et al.*, 2007). So far, successful isolation of CRCoV has only

been possible in the HRT-18 cell line and its clone HRT-18G, with supplementation of trypsin in the growth media (Erles *et al.*, 2007, An *et al.*, 2010a). CRCoV growth in cell culture is typically without syncytia or production of CPE (Erles *et al.*, 2007), hence CRCoV *in vitro* growth has to be confirmed using molecular or serological techniques.

2.3.2.1.4. Epidemiology

CRCoV was first reported in a UK rehoming facility in 2003 (Erles *et al.*, 2003). However, the detection of CRCoV in archival respiratory tissues of dogs necropsied in the late 1990s suggests that the virus circulated in the dog populations earlier than its first identification (Ellis *et al.*, 2005).

Serological evidence of CRCoV exposure in dogs has since been reported in the UK, USA, Canada, Republic of Ireland, Italy, Japan, Korea, Greece, and New Zealand (Kaneshima *et al.*, 2006, Priestnall *et al.*, 2006, Decaro *et al.*, 2007, Knesl *et al.*, 2009, An *et al.*, 2010b) (Fig. 2.4). The highest CRCoV seroprevalence was detected in Canada (59.1%) (Priestnall *et al.*, 2006), while the lowest seroprevalence was detected in Korea (12.8%) (An *et al.*, 2010b).

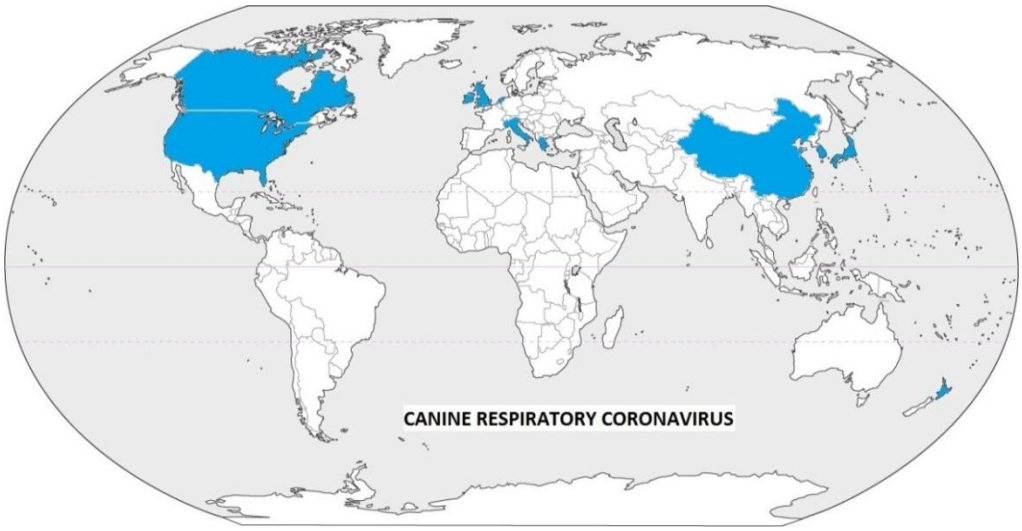


Figure 2.4. Geographical distribution of canine respiratory coronavirus (CRCoV). Countries shaded in blue indicate reported presence based on virological, molecular or serological evidence of CRCoV.

2.3.2.2. Canine pneumovirus

Taxonomic classification:

Order: *Mononegavirales*

Family: *Paramyxoviridae*

Subfamily: *Pneumovirinae*

Genus: *Pneumovirus*

Species: ***Canine pneumovirus***

The *Pneumovirinae* subfamily consists of two genera, *Pneumovirus* and *Metapneumovirus*. Human respiratory syncytial virus (hRSV) is the type species in the *Pneumovirus* genus and the genus includes other important veterinary pathogens including bovine respiratory syncytial virus (bRSV), caprine respiratory syncytial virus, ovine respiratory syncytial virus, and murine pneumovirus (MPV).

Canine pneumovirus (CnPnV) is a novel virus isolated from dogs with acute respiratory disease and sequence analysis of CnPnV has placed this virus within the *Pneumovirus*

genus of the subfamily *Pneumovirinae* under the family *Paramyxoviridae* (Decaro *et al.*, 2014a). It is closely related to MPV (Renshaw *et al.*, 2010).

Pneumovirus virions are typically spherical and are approximately 150-200 nm in diameter (Joncas *et al.*, 1969, Compans *et al.*, 1967). Virions are enveloped and contain a herringbone shaped helically symmetrical nucleocapsid.

The CnPnV genome is a linear, negative-sense, single stranded RNA molecule and is 14,884 nt long (Decaro *et al.*, 2014a). The genome organization of CnPnV is depicted in Figure 2.5. The genome consists of 10 genes that encode for 12 putative proteins (Decaro *et al.*, 2014a). The coding regions are flanked by leader and trailer regions at the 3' and 5' ends (genome sense), respectively (Decaro *et al.*, 2014a). Non-translated regions (NTR) are located between the protein coding regions. Within the NTR are the gene start (GS) and gene end (GE) sequences that define the transcriptional boundaries on the negative strand template (Fig. 2.5).

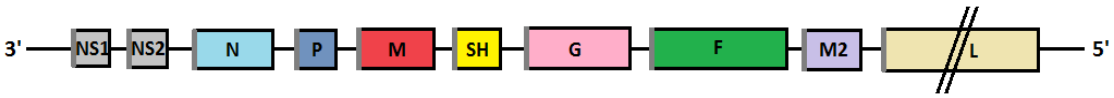


Figure 2.5. Genome organization of canine pneumovirus. The non-strucutral gene 1 (NS1), non-structural gene 2 (NS2), nucleocapsid gene (N), phosphoprotein (P), matrix gene (M), small hydrophobic gene (SH), attachment gene (G), fusion gene (F), M2 gene (M2) and large polymerase gene (L) are shown as rectangles with shaded bars at each end representing gene start (GS) (grey band) and gene end (GE) (black band) transcriptional signals. (This figure is adapted and modified from (Decaro *et al.*, 2014a).

2.3.2.2.1. Pathogenesis

The mechanism of entry and pathogenesis of CnPnV is not well understood. The primary targets of all pneumoviruses are respiratory epithelial cells (Cook *et al.*, 1998), and it is likely that the target entry of CnPnV may be similar. The attachment protein (G) of hRSV

interacts with the host cell receptor to initiate viral infection (Krusat and Streckert, 1997). It is hypothesized that the G protein of CnPnV interacts with a host cell receptor.

Experimental models of CnPnV infection in wild-type BALB/c mice involving intranasal inoculation of virions, resulted in replication of CnPnV in lung tissues (Percopo *et al.*, 2011). CnPnV infection was able to elicit local production of pro-inflammatory cytokines, including macrophage inflammatory protein 1 alpha (MIP-1α), monocyte chemoattractant protein-1 (MCP-1) and interferon gamma (IFNγ) at day 6 post-infection (Percopo *et al.*, 2011). A similar pro-inflammatory cytokine response has been observed in other pneumoviral infections (Rosenberg and Domachowske, 2008). On histopathology, there was a focal perivascular neutrophilic infiltrate (Percopo *et al.*, 2011), similar to other pneumoviral infections (Rosenberg and Domachowske, 2008). However, although CnPnV has proven its ability to initiate respiratory infections in rodent species (Percopo *et al.*, 2011), further experimental studies are required to understand the pathogenesis of this virus in dogs.

2.3.2.2.2. *In vitro* growth

To date, CnPnV has only been isolated in canine A-72 cell lines (Renshaw *et al.*, 2010). Cytopathic effect was characterised by scattered foci of rounded cells with syncytia and vacuolization, and CPE was visible after three to four passages (Renshaw *et al.*, 2010). The virus initially replicated poorly in the cell culture, however with continuous passaging CPE was confirmed (Renshaw *et al.*, 2011).

2.3.2.2.3. Epidemiology

CnPnV was first detected in 2010 during a retrospective study of respiratory disease in dogs from two animal shelters in the USA (Renshaw *et al.*, 2010). CnPnV was later detected in dogs with acute respiratory infections in the USA (Renshaw *et al.*, 2011), the UK (Mitchell *et al.*, 2013) and Italy (Decaro *et al.*, 2014b) (Fig. 2.6). The serological prevalence of CnPnV antibodies in the UK and Republic of Ireland dogs was reported to be 50.2% (314/625 dogs) (Mitchell *et al.*, 2013). Extensive serological surveys and surveillance studies are required to determine the epidemiological presence of CnPnV worldwide.

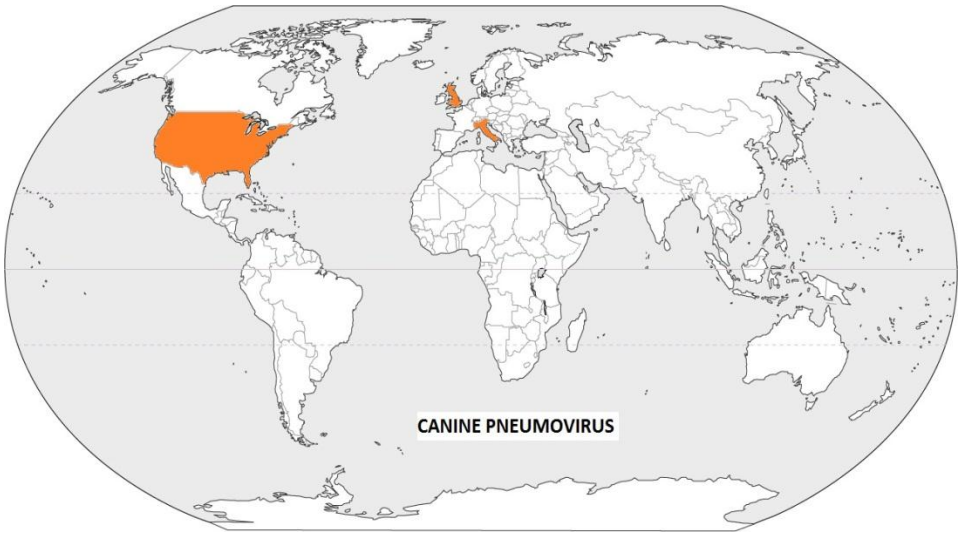


Figure 2.6. Geographic distribution of canine pneumovirus (CnPnV). Countries shaded in orange indicate reported virological and molecular presence of CnPnV.

2.3.2.3. Canine influenza virus

Taxonomic classification:

Order: *Unassigned*

Family: *Orthomyxoviridae*

Genus: *Influenzavirus A*

Species: ***Canine influenza virus***

Canine influenza virus (CIV) belongs to the genus *Influenzavirus A* under the family *Orthomyxoviridae.* The first canine respiratory outbreak due to CIV was reported in racing greyhounds in Florida, USA in 2004 (Crawford *et al.*, 2005). The CIV-H3N8 strain was reported to be responsible for the USA respiratory outbreaks (Crawford *et al.*, 2005). Subsequently, CIV-H3N2 strain was isolated in dogs with severe respiratory disease in Korea (Song *et al.*, 2008). Other strains including H5N1 (Lin *et al.*, 2012), H5N2 (Zhan *et al.*, 2012), H3N1 (Songserm *et al.*, 2006, Chen *et al.*, 2010) and H1N1 (Lin *et al.*, 2012) have also been sparsely reported in dogs. At present, CIV-H3N8 and CIV-H3N2 are considered to be important influenza A viruses in dogs.

Influenza A viruses are enveloped virions of 80 to 120 nm in diameter and have a single-stranded, negative sense RNA genome. The entire influenza A virus genome is 13,588 nt long and is contained on eight RNA segments (Palese and Shaw, 2007). The genome encodes 12 proteins: two surface glycoproteins, two matrix proteins, one nucleoprotein, three polymerase complex proteins and four non-structural proteins (Palese and Shaw, 2007).

The nucleoprotein and three polymerase complex proteins: polymerase basic 1, polymerase basic 2 and polymerase acidic, form a ribonucleoprotein complex (Heggeness *et al.*, 1982,

Compans and Duesberg, 1972). The two surface glycoproteins, hemagglutinin (H) and neuraminidase (N), and two matrix proteins, matrix 1 (M1) and matrix 2 (M2), are integral membrane proteins on the lipid bilayer (Compans *et al.,* 1970).

2.3.2.3.1. Pathogenesis

The primary targets of CIV are respiratory epithelial cells (Crawford *et al.*, 2005). CIV attaches to sialic acid α-2,3 receptors present in the epithelial cells of upper respiratory tract (Daly *et al.*, 2008). The hemagglutinin protein is involved in virus attachment to the host cell receptor leading to the fusion of virus-host cell membrane and penetration of the viral genome into the host cell cytoplasm (Palese and Shaw, 2007). The N protein also has ability to attach the virus to host cell receptors.

In experimental infection, CIV can efficiently replicate in the tracheal epithelium and result in upper respiratory tract lesions, causing damage to the ciliary epithelium (Gonzalez *et al.*, 2014). The clinical signs of CIV are similar to other respiratory viruses including mild anorexia, coughing, depression, nasal and ocular discharge (Deshpande *et al.*, 2008, Song *et al.*, 2009). Some dogs with CIV infection can develop necrotizing and hyperplastic tracheitis and bronchitis with involvement of submucosal glands, further leading to pneumonia (Castleman *et al.*, 2010). In the initial stages, pneumonia is characterised by infiltration of neutrophils in alveolar spaces and loss of alveolar type-1 epithelial cells (Jung *et al.*, 2010). Alveolar thickening and infiltration of alveolar macrophages is also observed in the later stages of CIV infection (Song *et al.*, 2009, Jung *et al.*, 2010).

2.3.2.3.2. *In vitro* growth

The presence of sialic acid α-2,3 receptors and tryptic proteases are the prerequisites for influenza A virus propagation. Both these requirements are present in abundance in embryonated chicken eggs (Govorkova *et al.,* 1999) and hence, embryonated chicken eggs have been extensively used for propagation of influenza A viruses (Burnet and Bull, 1943, Payungporn *et al.,* 2008). Likewise, MDCK cell lines also contain sialic acid α-2,3 receptors on their surface (Gaush and Smith, 1968, Govorkova *et al.,* 1999). The propagation of influenza A virus in MDCK cell lines also requires additional supplementation with trypsin protease in the growth medium (Tobita *et al.,* 1975).

2.3.2.3.3. Epidemiology

Respiratory outbreaks due to CIV-H3N8 have been endemic among dogs in USA (Hayward *et al.,* 2010) and the overall seroprevalence of CIV-H3N8 was reported to be 49% in the high risk dog populations in some States of the USA (Anderson *et al.,* 2013). However, the seroprevalence of CIV-H3N8 was reported to be 0% in the tested dog population from Europe (Schulz *et al.,* 2014a), New Zealand (Knesl *et al.,* 2009); and 0.4% in Canada (Kruth *et al.,* 2008), indicating a low risk of CIV-H3N8 infections in these countries at that time.

Canine respiratory disease outbreaks due to CIV-H3N2 strain have been reported in the USA (Creevy, 2015), Korea (Song *et al.,* 2008), China (Wang *et al.,* 2013) and Thailand (Bunpapong *et al.,* 2014). The prevalence of CIV-H3N2 antibodies was reported to be higher (6.71% by ELISA in South China, and 20.2-33% by VNT in Northeast China) in China (Qi *et al.,* 2011, Zhang *et al.,* 2012a) compared to the prevalence reported in Korea (0.49%) (Lee *et al.,* 2009) and Japan (2.12%) (Said *et al.,* 2011).

2.3.3. Other viruses

2.3.3.1. Canine Bocavirus

Taxonomic classification:

Order: *Unassigned*

Family: *Parvoviridae*

Sub family: *Parvovirinae*

Genus: *Bocaparvovirus*

Species: ***Canine bocavirus***

Bocaviruses are small, non-enveloped, icosahedral virions containing linear, single stranded DNA molecules. The virus commonly infects the respiratory and gastrointestinal systems of animals and humans. According to the 9th report of ICTV, canine bocavirus (CBoV) belongs to the *Bocaparvovirus* genus of the subfamily *Parvovirinae* under the family *Parvoviridae*. This genus includes bovine parvovirus, canine minute virus, porcine bocavirus, gorilla bocavirus and human bocavirus.

CBoV was first identified in 2012 during a metagenomic study to characterise the respiratory microbiome of diseased dogs (Kapoor *et al.*, 2012). Genetic analysis of CBoV sequences identified three different genetic variants CBoV-A, CBoV-B, CBoV-C in dogs (Kapoor *et al.*, 2012). The CBoV-A variants were prevalent among healthy dogs, while CBoV-B and CBoV-C were highly prevalent in dogs with respiratory disease (Kapoor *et al.*, 2012). However, a clear relationship between CBoV and respiratory disease in dogs has not yet been established.

2.3.3.2. Canine Hepacivirus

Taxonomic classification:

Order: *Unassigned*

Family: *Flaviviridae*

Genus: *Hepacivirus*

Species: ***Canine hepacivirus***

Kapoor *et al.* (2011) first discovered a canine homolog of human hepatitis C virus in dogs from respiratory outbreaks in the USA. This virus was named canine hepacivirus (CnNPHV) and is currently grouped as a non-primate hepacivirus. Genetic analysis has confirmed that CnNPHV is closely related to hepatitis C virus (HCV) (Kapoor *et al.*, 2011).

Canine hepacivirus has been detected in the hepatic and respiratory systems of dogs (El-Attar *et al.*, 2015), suggesting a wider tissue tropism. Considering liver to be the primary site for HCV, the identification of CnNPHV in respiratory tissues of dogs is intriguing. More research is required to determine the pathogenesis of CnNPHV infection in dogs.

To date, CnNPHV has only been reported in dogs with respiratory infection from the USA (Kapoor *et al.*, 2011) and the UK (El-Attar *et al.*, 2015).

2.3.3.3. Canine Reovirus

Taxonomic classification:

Order: Unassigned

Family: *Reoviridae*

Subfamily: *Spinareovirinae*

Genus: *Orthoreovirus*

Species: Mammalian orthoreovirus –

Canine reovirus

Mammalian reoviruses (MRV) are non-enveloped, double stranded RNA viruses included in the genus *Orthoreovirus* within the family *Reoviridae*. MRV has a broad host range and is responsible for either symptomatic or asymptomatic infection in mammals (Maclachlan and Dubovi, 2010).

Three serotypes for MRV (MRV-1, MRV-2 and MRV-3) have been recognized based on neutralization and hemagglutination test (Lerner *et al.*, 1962). All three serotypes have been detected in dogs (Holzinger and Griesemer, 1966, Binn *et al.*, 1977, Kokubu *et al.*, 1993, Decaro *et al.*, 2012). The MRV-1 was isolated from dogs with pneumonia in association with CDV or CPiV (Lou *et al.*, 1963). MRV-2 was recovered from dogs with respiratory infections (Binn *et al.*, 1977), while MRV-3 has been recovered from dogs with diarrhea (Kokubu *et al.*, 1993, Decaro *et al.*, 2005). This suggests that MRV has tropism for both the enteric and respiratory system of dogs.

Although MRV was isolated in dogs with respiratory disease, attempts to experimentally reproduce MRV infection in disease-free dogs were unsuccessful (Holzinger and Griesemer, 1966). This indicates that MRV may not induce direct pathogenic activity and may act as a synergistic pathogen aggravating disease.

2.4. Detection of canine respiratory viruses

Detection of virus critically depends on several factors including the time of sample collection, method of collection, collection site and storage during transportation of specimens to the laboratory. The time of sample collection is an important factor, as viral

shedding is more pronounced before clinical signs are observed. The current existing methods used in diagnostic virology are described in brief.

2.4.1. Isolation of virus in cell culture

Cell culture is often considered to be the gold standard for the diagnosis of viral infections. This technique was first used to grow vaccinia virus in 1913 (Steinhardt *et al.,* 1913). Even now this technique is widely used in most virology laboratories.

Most of the canine respiratory viruses grow on MDCK cell lines with specific growth requirements for certain viruses (Cornwell *et al.,* 1970, Moloney *et al.*, 1985, Gaush and Smith, 1968, Danskin, 1973). *In vitro* growth requirements of individual canine respiratory viruses were discussed earlier in this literature review. Viral growth can be detected by observing CPE under light microscopy, by hemadsorption or by hemagglutination test (HA). The presence of viruses does not always produce a CPE and requires confirmation by other diagnostic techniques such as fluorescent antibody staining or molecular methods.

The advantage of cell culture is that it provides a viable isolate, which can be used for further serological assays, vaccine production and experimental studies. However, this technique cannot be used for rapid diagnosis of an infection due to various reasons. Firstly, cell culture requires several days for confirmation of the results which may lead to delayed diagnosis and treatment. Secondly, different viruses require different growth requirements in order to propagate virus *in vitro*, which can sometimes be challenging and may give false negative results (Maclachlan and Dubovi, 2010). Finally, as one cell line cannot support the growth of all medically relevant viruses, maintaining several types of cell lines can become onerous, expensive and time consuming. Considering the disadvantages of cell culture,

culture-independent techniques for pathogen detection have been developed for rapid diagnosis of viral infections in diagnostic laboratories.

2.4.2. Serological assays

In most of the viral infections, serological testing in an animal is more reliable to determine recent infection than mere detection of the virus. Serological tests are designed to detect host antibody responses developed against infection. The commonly used serological assays to detect host antibody response are enzyme-linked immunosorbent assay (ELISA), VNT, HA, HI, and the indirect fluorescent antibody test (IFA) (Gardner, 1977).

A serological response to infection is usually not detected during the acute clinical phase and there is a need to rely on convalescent samples. However, collecting sera at the onset of infection (acute phase) and later after two weeks (convalescent phase) can help to infer the causative agent involved. During infection, the host antibody response can be detected by the presence of IgM and IgG antibodies in the host serum. The IgM antibody response is seen during the early stages of infection, while the IgG antibody response is observed during later stages of infection.

Serological assays help to understand the nature of the host immune response to viral infections. These assays can be employed to determine if the animal was recently exposed to a pathogen or had a subclinical infection, or to ascertain if an animal had responded to vaccination, or to determine if a specific virus could be linked to a clinical event (Maclachlan and Dubovi, 2010). These serological assays are routinely used for epidemiological surveys, disease control and eradication programs.

The commonly used serological tests for detection of viral infections in canine ITB are described in Table 2.3.

Table 2.3. Serological tests used for detection of antibodies for various canine respiratory viruses.

Virus	Serological tests	References
Canine parainfluenza	HI, VNT, ELISA	(Erles *et al.*, 2004, Cornwell *et al.*, 1976)
Canine adenovirus-2	HI	(Swango *et al.*, 1969)
Canine influenza virus	HI, VNT, ELISA	(Anderson *et al.*, 2012, Lee *et al.*, 2012)
Canine respiratory coronavirus	ELISA	(Priestnall *et al.*, 2006, Priestnall *et al.*, 2007)
Canine pneumovirus	ELISA	(Mitchell *et al.*, 2013)
Canine herpesvirus	VNT, ELISA	(Reading and Field, 1999, Rijsewijk *et al.*, 1999)

2.4.3. Detection of viral antigens

Viral antigens can be detected by immunofluorescence (IF) staining, immunohistochemistry (IHC) and enzyme immunoassay. Although these techniques do not require a viable virus, the timing of sample collection may affect the results.

Immunofluorescence staining

Immunofluorescence (IF) staining can be used for detection of viral antigens in frozen tissue sections, cultured cells, cytology smears taken directly from an animal and tissue biopsies (Maclachlan and Dubovi, 2010). This technique is particularly useful for viruses that do not produce CPE, but cannot be used on formalin fixed tissues.

Mucosal biopsies of upper respiratory tract, genital tract, eye or skin can be used for detection of viral antigen (Maclachlan and Dubovi, 2010). Shih *et al.* (1999) demonstrated

antigens of influenza A virus, parainfluenza virus, respiratory syncytial virus and adenovirus from throat and nasopharyngeal swabs inoculated in cultures containing MDCK cells using IF staining. This technique has also been used to identify CnPnV on A-72 cell cultures (Renshaw *et al.*, 2010).

Immunohistochemistry

Immunohistochemistry (IHC) is a method for localizing specific antigens in tissues or cells based on antigen-antibody recognition. In immunofluorescence technique to detect corresponding antigens in frozen tissue sections was first reported by Coons *et al.* (1941). IHC can be used to detect corresponding antigens in formalin fixed tissue sections (Heyderman, 1979).

Immunohistochemistry is an important application in pathology which facilitates comparison of viral antigen distribution with lesions in the tissue sections. The antigens of CDV, CAdV-2, CPiV and CRCoV have been detected using IHC in formalin fixed lung tissues of dogs with respiratory disease (Damian *et al.*, 2005, Ellis *et al.*, 2005). This technique can be used for definitive identification of viral antigens within cells of the respiratory tract.

2.4.4. Nucleic acid detection

Over the past three decades, molecular diagnostic techniques have become increasingly common in research and routine diagnostic laboratories. Use of these techniques has made the diagnosis of viral infections much easier due to their sensitivity, specificity, speed, and ability to identify pathogens that are difficult to culture (Ou *et al.*, 1988, Mackay *et al.*, 2002). One of the techniques that have been routinely used for detection of pathogens in

many research and diagnostic laboratories is polymerase chain reaction (PCR). PCR was first developed by Mullis (1987), and is based on the principle of enzymatic replication of nucleic acid sequences.

The subsequent development of real-time PCR has had a significant impact on detection of pathogens (bacteria, viruses, fungus and parasites) in diagnostic microbiology (Mackay, 2004). The major application of real-time PCR is in virology, where these assays can detect viral load in a clinical sample. Furthermore, ablity to multiplex has allowed simultaneous measurement of different viral nucleic acid targets in a single sample. This technique helps to better identify co-infections and is particularly useful in multi-etiological diseases such as canine ITB (Mochizuki *et al.*, 2008, Schulz *et al.*, 2014b). Currently, various commercial laboratories have established multiplex PCR platforms for detecting known respiratory pathogens in clinical samples.

The main limitation of PCR and other nucleic acid amplification tests is that they are agent specific and require prior knowledge about the target sequence for primer design. Hence, no signals will be detected if the primer sequence does not match any organism contained in the sample. Thus, PCR cannot detect the presence of unknown pathogens in a clinical sample.

2.4.5. Diagnostic metagenomics

All the techniques described above are generally target-specific and lack the ability to detect unknown pathogens. Furthermore, each assay requires onerous optimization and standardization. These limitations have recently been overcome by culture-independent sequence based techniques known as “metagenomics” (Handelsman *et al.*, 1998).

Metagenomics uses a shot-gun sequencing approach to understand the microbiome of a given sample. Metagenomics allows sequencing of all the pathogens present in a clinical sample and can be useful to identify unknown pathogens present in a particular infection.

Various steps are involved in viral metagenomic studies, which include sample preparation, sequence-independent amplification, high throughput sequencing, and bioinformatics analysis to investigate viral flora in a sample. Viral nucleic acids only constitute a small proportion of the total nucleic acids present in the sample. Hence, one of the most important and crucial tasks is to prepare the samples for viral metagenomic study. To prepare samples for viral metagenomics, filtration using a 0.22 μm filter is a common procedure for removing bacteria (Thurber *et al.*, 2009). Since viral nucleic acid is surrounded by the viral capsid, treating samples with nucleases prior to nucleic acid extraction will degrade host nucleic acid, without degrading the viral nucleic acid present in a sample (Allander *et al.*, 2001).

Following nucleic acid extraction, amplification of the nucleic acids is performed in a sequence-independent manner in order to simultaneously multiply several viral genomes, including highly divergent and completely novel viruses (Ambrose and Clewley, 2006). Sequencing is often carried out to identify the viral nucleic acids. This requires constructing shot-gun libraries and sequencing them, usually using a high throughput sequencer (Breitbart and Rohwer, 2005). There are a number of high throughput sequencers commercially available, which include 454 GS-FLX by Roche, HiSeq and MiSeq by Illumina, SOLiD and Ion Proton by Life Technologies and PacBio RS by Pacific Biosciences. The choice of sequencer is based on availability and the type of study to be conducted.

One of the most challenging task is analysis of vast amount of sequencing data obtained from high throughput sequencers. For viral metagenomic data analysis, host and bacterial sequences are omitted and remaining sequences are assembled. After assembly, the programs such as Blastn® and Blastx® are used to perform homology searches for known viruses. Although these methods are used to detect known and highly divergent viruses, they often fail to detect completely novel viruses as they do not have any similarity to the sequences deposited in database. Various programs, such as MEGAN (Huson *et al.*, 2007), have also been developed that can help with both data analysis as well as visualizing results.

In the field of virology, metagenomics was first used to understand the marine viral communities (Breitbart *et al.,* 2002). Subsequently, numerous researchers had used this platform to detect viruses in various samples (Nakamura *et al.*, 2009, Dunowska *et al.*, 2012, Lysholm *et al.*, 2012, van den Brand *et al.*, 2012, Bodewes *et al.*, 2014, Ng *et al.*, 2015). A number of pathogens that were previously unknown including canine bocavirus (Kapoor *et al.*, 2012), nidovirus (Dunowska *et al.*, 2012), arenavirus (Briese *et al.*, 2009), canine kobuvirus and sapovirus (Li *et al.*, 2011) have been detected using this approach. Moreover, this approach has also been used for the detection of known viral pathogens (e.g. ebola virus, influenza and norovirus) in clinical samples (Nakamura *et al.*, 2009, Towner *et al.*, 2008).

Metagenomics contributes to the detection of known and unknown microbial agents; however, mere presence of an agent does not necessarily indicate its role in a clinical disease. Hence, metagenomics should be used as a starting point for microbial agent detection, which in combination with traditional and molecular techniques will help to

ascertain possible pathogen-disease association. The combination of next generation sequencing and PCR has already been used in various studies to determine an association between microbial agent and disease (Li *et al.*, 2011, Xu *et al.*, 2011, Dunowska *et al.*, 2012, Zhang *et al.*, 2012b, Ng *et al.*, 2015).

2.5. Timeline for canine respiratory agent discovery

The timeline summary of the discovery of canine respiratory agents reveals a substantial number of new and previously undiscovered microbial agents that have been identified during the last few decades (Fig. 2.7). The development of molecular diagnostic techniques has helped immensely to define the aetiology of canine ITB. Of the canine respiratory viruses recently identified, it seems likely that CRCoV and CnPnV were present earlier to their discovery. However, lack of diagnostic measures and knowledge may have hampered their detection in the past. Applying metagenomics may lead to discovery of even more microbial agents in future.

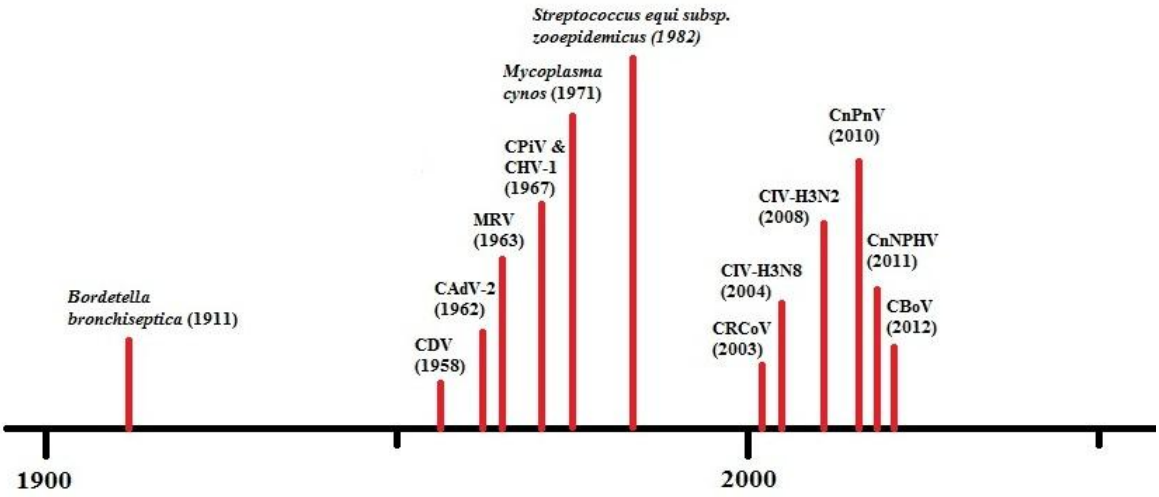


Figure 2.7. Timeline for the identification of canine respiratory agents. The X-axis represents the years.

2.6. Current literature on canine respiratory pathogens in New Zealand

New Zealand, being geographically isolated from other landmasses, has an advantage in being free from some of the most important pathogens currently present around the world, including rabies virus. This is primarily due to the strict biosecurity, and stringent policies on the importation of animals coming into New Zealand. Secondly, rigorous vaccination programs and the absence of native or endemic reservoirs may have led to the eradication of some pathogens in New Zealand, for example CDV (Hill, 1999). Furthermore, some pathogens present in New Zealand are genetically different from the pathogens identified in other countries. One illustration of this is the presence of only one canine parvovirus (CPV) subtype (CPV-2a) in New Zealand dogs (Ohneiser *et al.*, 2015) compared to other CPV subtypes present worldwide.

The current literature on the presence of canine respiratory pathogens in New Zealand is limited. Veterinarians in New Zealand have reported a rise in ITB cases in the dogs vaccinated against *B. bronchiseptica*, CPiV and CAdV-2 (Potter *et al.*, 2009, Meyer *et al.*, 2010). It is not clear if the canine respiratory outbreaks in New Zealand are caused due to the traditional respiratory pathogens, such as *B. bronchiseptica*, CPiV, CAdV-2 and CHV-1, or by recently described canine ITB pathogens. A lack of thorough diagnostic investigations in these cases has prevented confirmation of the aetiology of respiratory disease in New Zealand dogs.

Recently, a longitudinal pilot study conducted in New Zealand reported that 27.6% (13/47) of dogs with signs of acute respiratory disease were positive for at least one of CAdV-2, CPiV, *M. cynos*, *B. bronchiseptica* or CHV-1 using a commercial multiplex PCR panel (Sowman *et al.*, 2013), while the dogs were negative for the presence of CRCoV. The study

did not look for the presence of CnPnV and CIV in the same study population. Serological testing on the paired sera revealed seroconversion to CRCoV in 68.0% (32/47) dogs with respiratory signs suggesting an involvement of CRCoV in the development of signs of ITB in these dogs (Sowman *et al.*, 2013). These findings highlight the possibility of new viruses such as CnPnV, CRCoV, CBoV, and CnNPHV being involved in canine ITB in New Zealand.

So far, diagnostic investigations conducted in New Zealand did not include detection of CRCoV, CnPnV, CBoV, CnNPHV and canine reovirus in dogs with signs of ITB. Hence, it is not known if these pathogens are associated with canine ITB in New Zealand. A broad pathogen survey with metagenomics approach would be ideal to investigate the causative agents involved, and to understand the prevalence of ITB pathogens in dogs in New Zealand. Only once the relative importance of different pathogens and their epidemiology is understood, can interventions to reduce disease incidence be improved.

In order to understand the pathogens involved in canine respiratory outbreaks in New Zealand, the following survey was undertaken to establish which respiratory viruses are associated with the development of respiratory signs.

Chapter 3: SAMPLE COLLECTION FOR MOLECULAR SURVEY

3.1. Introduction

Canine ITB is commonly reported in shelter dogs, or in dogs with a history of kenneling or during dog racing (Pesavento and Murphy, 2014). The pathogens capable of causing ITB in dogs include viruses, bacteria and fungi (Mochizuki *et al.*, 2008). Unfortunately, the majority of canine ITB cases are never thoroughly investigated to determine the aetiology. If they are investigated, it is usually when the owner decides that the disease has become severe and the animal requires medical attention. When the respiratory disease becomes severe, it is often difficult to prove a viral aetiology. This is because viruses are often difficult to isolate after the onset of clinical signs due to their limited shedding time, and viral infections often get complicated by secondary bacterial infection (McCandlish *et al.*, 1978).

The list of possible canine respiratory viruses, as described above in Chapter 2, includes canine parainfluenza virus, canine adenovirus type-2, canine herpesvirus type-1, canine influenza virus, canine respiratory coronavirus, canine pneumovirus, canine bocavirus, canine reovirus, and canine hepacivirus (Dubovi, 2010, Erles *et al.*, 2003, Hong *et al.*, 2015, Renshaw *et al.*, 2010, Mitchell *et al.*, 2013, Decaro *et al.*, 2014b). So far, there has not been a comprehensive study undertaken to investigate all canine respiratory viruses present among dogs in New Zealand.

In order to undertake a molecular survey for canine respiratory viruses present among dogs in New Zealand, oropharyngeal swab samples were collected from dogs with signs of ITB and from healthy dogs. The sample collection described in this section was used for metagenomic shot-gun sequencing (Chapter 4) and real-time PCR (Chapter 5).

Subsequently, the positive samples were also used for virus isolation (Chapter 6) and molecular characterization of CnPnV (Chapter 7) and CRCoV (Chapter 8).

3.1.1. Study protocol

The study was performed as a prospective investigation and the study protocol was approved by the Animal Ethics Committee, Massey University (Approval number: MUAEC Protocol 12/35).

Private veterinary clinics throughout New Zealand were contacted for their participation. Sample packs consisting of a swab with viral transport medium (Copan Diagnostics, Murrieta, USA), a letter (Appendix A) explaining the purpose of the study, a questionnaire form for owners and veterinarians (Appendix B), and instructions for sampling and transportation of samples (Appendix C) along with an addressed prepaid courier envelope were distributed among participating clinics. The samples were collected from March 2014 to February 2016.

3.1.2. Study population

The study population consisted of two groups of dogs; 1) dogs from private households and, 2) kenneled dogs. Oropharyngeal swabs were collected from dogs with signs of ITB and from healthy dogs from both private households and kenneled dogs. Inclusion criteria for sick dogs were the presence of respiratory signs; including nasal discharge, coughing, sneezing, altered respiration, pyrexia or lymphadenopathy for no more than 14 days; and not having been recently vaccinated against CPiV, *B. bronchiseptica* and CAdV-2. Dogs with other medical conditions of the respiratory tract including pleural effusion, neoplasia or cardiac disease were excluded from the study. Clinically healthy dogs included in the

study had no history of respiratory signs within the last 6 months and had not been vaccinated within 4 weeks before sampling.

3.1.3. Study population statistics

The list of healthy dogs and dogs with signs of ITB is given in Appendix D. Oropharyngeal swabs were collected from 116 dogs, out of which 50.8% (*n=59*) of samples were collected from dogs from private households and 49.2% (*n=57*) from kenneled dogs. The number of sick and healthy dogs from private households and kenneled dogs are given in Table 3.1.

Table 3.1. Number of dogs with signs of infectious tracheobronchitis (ITB) and healthy dogs sampled from private households and kenneled dogs.

Dogs from private households n = 59 (50.8%)		**Kenneled dogs n = 57 (49.2%)**	
Dogs with signs of ITB	**Healthy dogs**	**Dogs with signs of ITB**	**Healthy dogs**
35	24	21	36

In the dogs with ITB, 42.9% were female and 57.1% were male; while in the healthy group, 58.3% were female and 41.7% were male. The median age of dogs with signs of ITB was 3.5 years (ranging from one year to 15 years) and the median age of healthy dogs was 2 years (ranging from 5 months to 14.5 years). Age was not documented for six dogs with signs of ITB and two healthy dogs.

The clinical signs documented in dogs with signs of ITB included cough (87.5%), nasal discharge (12.5%), sneezing (19.6%), altered respiration (3.5%), pyrexia (1.78%) and submandibular lymphadenopathy (28%).

3.1.4. Sampling procedure

Samples were collected from the oropharyngeal region of healthy dogs and dogs with signs of ITB. For oropharyngeal sampling, the samples were collected by gently rolling the swab on the tonsiller region of the oropharynx. The swabs were placed into 1 mL of universal transport medium (Copan Diagnostics, Murrieta, USA) and veterinarians were asked to store samples at 4 °C until delivery. The collected samples were couriered to the laboratory on ice as soon as possible. Upon arrival, the samples were processed immediately.

3.1.5. Sample processing

Each oropharyngeal swab sample was present in 1 mL of transport media, and the sample was thoroughly mixed by vortexing for 15 sec followed by brief centrifuge. Further, all samples were aliquoted into three volumes 200 µL, 200 µL and ~600 µL. In order to avoid frequent freeze-thaw cycles, the aliquots were placed in separate cryo-vial tubes, labeled accordingly, and were stored at -80 °C. The samples were labeled based on the health status of dogs; 56 samples collected from dogs with signs of ITB were labeled from ITB-001 to ITB-056, while 60 samples collected from healthy dogs were labeled from HEAL-001 to HEAL-060.

One of the 200 µL aliquots from each sample was used for metagenomic shot-gun sequencing (Chapter 4). Another 200 µL aliquot from each sample was used for real-time PCR analysis (Chapter 5). The remaining aliquot (~600 µL) from each sample was stored at -80 °C for future virus isolation (Chapter 6).

Chapter 4: DETECTION OF VIRUSES FROM THE OROPHARYNGEAL REGION USING A METAGENOMIC SHOT-GUN SEQUENCING APPROACH

4.1. Introduction

Infectious tracheobronchitis is a disease of multifactorial aetiology in dogs. Traditionally, it has been most commonly linked to infection with pathogens including *B. bronchiseptica*, CPiV, CAdV-2 and CDV (Binn *et al.*, 1967, Crandell *et al.*, 1968, Fairchild *et al.*, 1969, Wright *et al.*, 1973). Within the last 20-30 years, several new pathogens have been identified in clinical samples from dogs with respiratory disease. These include CRCoV (Erles *et al.*, 2003), CnPnV (Renshaw *et al.*, 2010), CIV (Yoon *et al.*, 2005), CBoV (Kapoor *et al.*, 2012) and canine hepacivirus (Kapoor *et al.*, 2011). While the exact role of many of these pathogens needs to be elucidated, this recent expansion on the number of canine respiratory pathogens suggests the possibility that microbial agents that contribute to canine ITB may yet need to be discovered.

The diagnostic techniques commonly employed for virus detection, including cell culture, PCR or IHC, require prior knowledge of what viruses may be present in a clinical sample. These techniques fail to identify new pathogens or even variants that are potentially involved in a disease; and hence, a broad diagnostic approach, such as metagenomics, is required to identify new pathogens. Viral metagenomics has been described in detail in Chapter 2 (Section 2.4.5). Several new pathogens including canine bocovirus (Kapoor *et al.*, 2012), nidovirus (Dunowska *et al.*, 2012), arenavirus (Briese *et al.*, 2009), canine kobuvirus and sapovirus (Li *et al.*, 2011) have been identified using this approach.

The aim of this study was to determine which respiratory viruses circulate among selected dog populations in New Zealand using a culture-independent metagenomic approach. It was hoped that this approach would allow for detection of not only viruses that have been previously identified in New Zealand (Sowman *et al.*, 2013), but also any potentially new ones.

4.2. Materials and methods

4.2.1. Samples

The oropharyngeal swab samples were collected from dogs with signs of ITB and from healthy dogs as described in section 3.1.3. Swab samples from dogs with signs of ITB (ITB 001-050, *n=50*) and from healthy dogs (HEAL 001-050, *n=50*) were processed for metagenomic shot-gun sequencing.

4.2.2. Pre-enrichment

Aliquots (200 µL) of each individual oropharyngeal swab sample, prepared and stored as described in section 3.1.5, were used in this study. An uncontaminated sample of the viral transport media used for sampling was processed alongside clinical samples as a negative control. The 200 µL aliquots were centrifuged at 10,000 × g for 1 min at room temperature and the supernatant was filtered using a 0.2 µM syringe filter (Membrane solutions®, Kent, USA). A 100 µL aliquot of the filtrate from each sample was then enriched for viral sequences by treatment with 2 units of Turbo DNase1 (Ambion Life technologies, Foster City, USA) in 1 x Turbo DNase buffer in a final volume of 150 µL for 60 min at 37 °C. An additional 2 units of DNase1 were added after the first 30 min of incubation. The nuclease treatment was stopped by addition of equal amount (150 µL) of lysis buffer from the nucleic acid extraction kit.

Viral nucleic acids were extracted from the above 150 µL DNase-treated aliquots using ZR-Duet™ DNA/RNA MiniPrep (Zymo Research, Irvine, USA) according to the manufacturer's instructions. The kit allows parallel extraction of high quality genomic DNA and total RNA from samples.

4.2.3. Nucleic acid quantification

Quantity and quality of nucleic acids at various stages of preparation throughout the protocols described in this chapter were assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). Samples were regarded to be of acceptable quality if the 260/280 ratio was approximately 1.8 and 2.0 for DNA and RNA, respectively. When indicated, DNA- and RNA- specific kits were also used to assess the quantity of nucleic acids using a Qubit 2.0 flurometer (Invitrogen, Carlsbad, USA).

4.2.4. Sample pooling

Due to financial constraints, it was elected to pool the samples and carry out sequencing on the pooled sample instead of individual samples. The DNA and RNA extracted from dogs with signs of ITB (*n=50*) and from healthy dogs (*n=50*) were pooled into four groups: DNA from healthy dogs (NGS1), DNA from clinically affected dogs (NGS2), RNA from healthy dogs (NGS3) and RNA from clinically affected dogs (NGS4). The control DNA and RNA samples were named as NGS5 and NGS6, respectively. The controls used in this study were sterile viral transport media and were processed parallel with other samples, in order to detect contamination during sample processing. The following steps were carried out for DNA and RNA samples:

4.2.4.1. DNA sample processing

To create NGS1 and NGS2 pools, about 20 µL of extracted DNA (section 4.2.2) from individual samples were pooled. The pooled samples were concentrated by ethanol precipitation. Briefly, one tenth volume of sodium acetate pH 5.2 (final concentration of 0.3 M) was added to each of the pooled DNA samples and the content of the tubes were mixed well. Glycogen (1 µg/µL) was also added as a carrier to each tube followed by two volumes of absolute ethanol. The contents were mixed and the tubes were placed at -80 °C for 30 min. The DNA was then pelleted by centrifugation at 16,000 × g for 15 min at 4 °C. The supernatant was carefully decanted and 1 mL of 70% ethanol was added to each tube. The DNA pellet was washed gently by inverting the tube several times, and pelleted again by centrifugation at maximum speed for 5 min. The supernatant was carefully decanted and the DNA pellet was air dried. The pellet was re-suspended in 10 µL of Tris-HCL and stored at -20 °C for further steps.

4.2.4.1.1. Whole genome amplification (WGA)

NGS1 and NGS2 DNA was further amplified by WGA using an Illustra GenomiPhi V2 DNA amplification kit (GE Healthcare, Bangkok, Thailand), as per manufacturer's instructions. Briefly, 1 µL (5-10 ng of DNA) of each sample was added to 9 µL of sample buffer and incubated at 95 °C for 3 min. The tubes were immediately placed on ice and 9 µL of reaction buffer and 1 µL of enzyme mix was added, and incubated at 30 °C for 1.5 hr, followed by inactivation at 65 °C for 10 min. The amplified genomic DNA was obtained in 20 µL of reaction tube. For DNA clean-up, 180 µL of elution buffer (10 mM Tris-HCL, pH 7.4) was added to 20 µL of WGA amplified genomic DNA, and processed using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Basel, Switzerland), as per

manufacturer's instructions. The DNA was finally eluted in 50 µL of the elution buffer (10 mM Tris-HCL, pH 7.4).

4.2.4.1.2. DNA quality and quantity check

The quality and quantity of NGS1 and NGS2 DNA were assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). The quantity of DNA was also assessed using the Qubit® dsDNA BR Assay Kit (Life technologies, Carlsbad, USA). To further assess the quality, 150 ng of each genomic DNA preparation was subjected to electrophoresis through a 1% ethidium bromide stained agarose gel along with a high DNA mass ladder (Invitrogen, Carlsbad, USA). The NGS1 and NGS2 samples (23 ng/µL in a total volume of 20 µL each) were submitted to the Massey Genome Service (Massey University, Palmerston North) for shot-gun sequencing (Illumina MiSeq™).

4.2.4.2. RNA sample processing

RNA samples were processed in an RNase free environment. NGS3 and NGS4 pools were created by pooling 25 µL of DNase treated RNA (section 4.2.2) from each individual swab sample. The pooled RNA samples were cleaned up and concentrated using NucleoSpin® RNA clean-up XS (Macherey-Nagel GmbH & Co. KG, Dueren, Germany), as per the manufacturer's instructions. The quality of RNA was assessed using NanoDrop 2000 spectrophotometer. The quantity of RNA was assessed using Qubit® RNA BR Assay Kit.

The total quantity of RNA in NGS3 and NGS4 was lower than 500 ng, which was needed to perform double-stranded cDNA synthesis. Hence, each individual swab sample (section 4.2.2) that was processed by centrifugation and filtration was used for re-extraction of RNA

by Trizol LS® reagent (Life Technologies, Carlsbad, USA). The DNase treatment, RNA pooling and processing were performed as described above.

4.2.4.2.1. Double-stranded cDNA synthesis

4.2.4.2.1.1. Oligonucleotides

The 96 non-ribosomal hexanucleotides (Endoh *et al.*, 2005) were commercially synthesized in a 96 well plate (Integrated DNA Technologies, Coralville, USA). The oligonucleotide plate was briefly spun at 500 × g for 5 min. The first oligonucleotide was diluted with 720 µL of water (100 µM). The content of the well was mixed by pipetting up and down and transferred into the next well. The process was repeated until all the oligonucleotides were diluted. The final preparation contained 96 non-ribosomal oligonucleotides at 100 µM concentration.

4.2.4.2.1.2. Double-stranded cDNA synthesis

The cDNA synthesis for pooled RNA samples (NGS3 and NGS4) was carried out using Maxima H Minus Double-Stranded cDNA synthesis kit (Thermo Scientific, Waltham, USA), according to the manufacturer's protocol. For the first-strand synthesis, 450-500 ng RNA was mixed with 200 pmol of 96 non-ribosomal hexanucleotides and incubated at 65 °C for 5 min. Further, 5 µL of 4 X first-strand reaction mix and 1 µL of enzyme mix were added to each tube and incubated at 25 °C for 10 min, then at 50 °C for 30 min. The reaction was terminated by incubation tube at 85 °C for 5 min. The second-strand synthesis was performed by adding 10 µL of 5 X second-strand reaction mix and 2.5 µL of second-strand enzyme mix to the first-strand cDNA synthesis reaction mixture; and incubating at 16 °C for 60 min. The second-strand synthesis reaction was terminated by adding 6 µL of

0.5 M EDTA, pH 8.0 to each reaction tube. All the incubation steps were performed in the PCR machine (Veriti Thermal cycler, Applied Biosystems).

The blunt-end double-stranded cDNA was purified using phenol chloroform extraction followed by ethanol precipitation using standard methods (Sambrook *et al.*, 2001). The cDNA pellet was dissolved in 10 µL of 10 mM Tris-HCL, pH 7.4 (Roche Diagnostics GmbH, Basel, Switzerland) and quantified using NanoDrop 2000 spectrophotometer.

4.2.4.2.2. Whole genome amplification

The NGS3 and NGS4 cDNA were further amplified in WGA reaction using Illustra GenomiPhi V2 DNA amplification kit (GE Healthcare, Bangkok, Thailand), as per the manufacturer's instructions. The amplified cDNA was cleaned up using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Basel, Switzerland) and the cDNA was eluted in 50 µL of elution buffer (10 mM Tris-HCL, pH 7.4). The procedure for whole genome amplification and amplified cDNA clean-up was the same as described in section 4.2.4.1.1.

4.2.4.2.3. DNA quality and quantity check

The quality and quantity of NGS3 and NGS4 cDNA were assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). The quantity of cDNA was also assessed using the Qubit® dsDNA BR Assay Kit (Life technologies, Carlsbad, USA). To further assess the quality, 150 ng of cDNA from NGS3 and NGS4 were subjected to electrophoresis through a 1% ethidium bromide stained agarose gel along with a high DNA mass ladder (Invitrogen, Carlsbad, USA). Aliquots (20 µL each) of NGS3 (52 ng/µL) and

NGS4 (42.7 ng/µL) were submitted to the Massey Genome Service (Massey University, Palmerston North) for shot-gun sequencing (Illumina MiSeq™).

4.2.5. Shot-gun sequencing

The library preparation and shot-gun sequencing procedures were performed by the Massey Genome Services (Massey University, Palmerston North). All four sample libraries (NGS1, NGS2, NGS3 and NGS4) and two controls (NGS5 and NGS6) were prepared using the Illumina TrusSeq™ DNA Nano library preparation_V1 (Illumina, San Diego, USA) method. DNA from each sample were sheared and size fractionated. Fragments of 400-500 bp in length were then ligated to Illumina adapters with a unique barcode per sample. Each library was enriched for products that were fully ligated using streptavidin beads. The libraries were then pooled by equal molarity before loading onto the Illumina MiSeq™ sequencer. A pool containing all six libraries was run in Illumina MiSeq 2 × 250 base PE runs, version 2 chemistry. A total of three runs were conducted to generate 36-45 million reads.

4.2.6. Bioinformatics analysis

The analyses described in section 4.2.6.1 to section 4.2.6.3 were primarily performed by a bioinformatician (Dr Patrick Biggs, NZGL and IVABS, Massey University, Palmerston North). The analysis described in section 4.2.6.3.1 and 4.2.6.3.2 was performed by me under the bioinformatician's guidance.

4.2.6.1. Data pre-processing

Initial read analysis

The reads obtained from the Illumina MiSeq runs were analyzed with an in-house quality control tool that performed read quality analysis and visualization (SolexaQA++[1] (Cox *et al.*, 2010) and FastQC[2]), PhiX removal with Bowtie2[3] (Langmead and Salzberg, 2012), the SamToFastq.jar program from the Picard suite[4] and adapter removal through the "fastq-mcf" program from the ea-utils suite of tools[5]. In addition, the reads were analyzed with FastQScreen[6] as a further check for any potential Illumina adapters and cloning vector contamination. Given the observed numbers of reads, the analyses were only performed on the four experimental libraries (NGS1, NGS2, NGS3 and NGS4).

Read merging

Merging of all reads was performed on the "processed" reads, which were devoid of adaptor and vector sequences. Based on the sample file names, the four experimental libraries (NGS1, NGS2, NGS3 and NGS4) from each of the three runs were merged together as two sequential operations, so in other words, initially the sequences from run 1 were merged with run 2 to make a set of files in a folder called "run12". These sequences were then merged in the same way with the data from run 3 to make a folder called "mergedAll". All further analyses were performed on "mergedAll" data for each of the four libraries.

[1] http://solexaqa.sourceforge.net/
[2] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[3] http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
[4] http://picard.sourceforge.net/
[5] http://code.google.com/p/ea-utils/; version 1.1.2-621
[6] http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/

4.2.6.2. Mapping based approaches using Bowtie2

This approach was attempted to understand the sequence data in detail. Three datasets were used for mapping: "dog", "viruses", and "bacteria". The aim was to see how many reads were mapped to the canine genome as well as to the selected bacterial and viral genomes by performing end-to-end and local alignment using Bowtie2 (Langmead and Salzberg, 2012). The end-to-end alignment aligns the entire query sequence with target sequence, while local alignment aligns a substring of query sequence to a substring of the target sequence.

The three subsets for mapping were as follows:

Dog:

The source for the Bowtie2 indexing was the CanFam3.1[7] (GCA_000002285.2) assembly from the Ensembl genome browser, as listed on the information webpage[8].

Bacteria:

The three bacterial genome sequences used for initial mapping are given in the Table 4.1.

Viruses:

Viral sequences of interest were obtained from the GenBank. The list of 32 viral sequences used for mapping is given in the Table 4.2.

Table 4.1. List of bacterial sequence of interest used for initial mapping.

GI	RefSeq	Description
225867617	NC_012470.1	*Streptococcus equi* subsp. *zooepidemicus* H70
412337338	NC_019382.1	*Bordetella bronchiseptica* 253, complete genome
433624204	NC_019949.1	*Mycoplasma cynos* C142 complete genome

[7] http://www.ensembl.org/Canis_familiaris/Info/Index
[8] http://www.ensembl.org/Canis_familiaris/Info/Annotation

Table 4.2. List of viral sequences used for initial mapping.

Accession number	Description
AC_000003	Canine adenovirus 1, complete genome
AC_000020	Canine adenovirus type 2, complete genome
AY781188	Mammalian orthoreovirus 3 strain T3D/04 lambda 3 (L1) gene, partial cds
AY785910	Mammalian orthoreovirus 3 strain T3D/04 sigma 1 (S1) gene, partial cds
EU127500	Influenza A virus (A/canine/Korea/GCVP01/2007(H3N2)) segment 4 hemagglutinin (HA)
EU127501	Influenza A virus (A/canine/Korea/GCVP01/2007(H3N2)) segment 6 neuraminidase (NA) gene, complete cds
JF744991	Canine hepacivirus AAK-2011 polyprotein gene, complete cds
JF744996	Canine hepacivirus AAK-2011 isolate CHV-K136 helicase NS3 gene, partial cds
JF744997	Canine hepacivirus AAK-2011 3' UTR
JQ181557	Canine hepacivirus core protein gene, partial cds
JQ692591	Canine bocavirus strain HK831F, complete genome
JX195341	Influenza A virus (A/canine/Guangdong/1/2011(H3N2)) segment 5 nucleocapsid protein (NP) gene, complete cds
JX860640	Canine respiratory coronavirus strain K37, complete genome
KF155142	Influenza A virus (A/canine/Korea/MV1/2012(H3N2)) segment 1 polymerase PB2 (PB2) gene, complete cds
KF155144	Influenza A virus (A/canine/Korea/MV1/2012(H3N2)) segment 3 polymerase PA (PA) and PA-X protein (PA-X) genes, complete cds
KF155148	Influenza A virus (A/canine/Korea/MV1/2012(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds
KF155149	Influenza A virus (A/canine/Korea/MV1/2012(H3N2)) segment 8 nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds
KM359815	Influenza A virus (A/canine/NY/1623.1/2010(H3N8)) segment 4 hemagglutinin (HA) gene, complete cds
KM359830	Influenza A virus (A/canine/NY/3699/2010(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds
KM359848	Influenza A virus (A/canine/NY/3699/2010(H3N8)) segment 5 nucleocapsid protein (NP) gene, complete cds
KM359852	Influenza A virus (A/canine/PA/111788.2/2009(H3N8)) segment 8 nuclear export protein (NEP) and nonstructural protein 1 (NS1) genes, complete cds
KM359856	Influenza A virus (A/canine/PA/111788.2/2009(H3N8)) segment 3 polymerase PA (PA) and PA-X protein (PA-X) genes, complete cds
KM359860	Influenza A virus (A/canine/PA/111788.2/2009(H3N8)) segment 2 polymerase PB1 (PB1) and PB1-F2 protein (PB1-F2) genes, complete cds
KM359864	Influenza A virus (A/canine/PA/111788.2/2009(H3N8)) segment 1 polymerase PB2
KP281713	Canine bocavirus isolate 13D003, complete genome
KP281720	Canine bocavirus isolate 14Q216, complete genome
KP893891	Parainfluenza virus 5 isolate CC-14, complete genome
KP981644	Canine coronavirus strain CB/05, complete genome
NC_001734	Canine adenovirus, complete genome
NC_001921	Canine distemper virus, complete genome
NC_025344	Canine pneumovirus strain dog/Bari/100-12/ITA/2012, complete genome
NC_030117	Canid herpesvirus 1 strain 0194, complete genome

4.2.6.3. Taxonomic classification

Two different metagenomic classifying algorithms were used to analyze the reads from each library. These classifiers were: DIAMOND (Buchfink *et al.*, 2015) and Kraken[9] (Wood and Salzberg, 2014). These classifications were primarily performed to obtain viral sequences present in each sample library for further analysis.

4.2.6.3.1. Assembly of viral reads from DIAMOND analysis

The four samples were run through DIAMOND process to generate necessary files for further analysis and visualization in Megan6 (Tamura *et al.*, 2013). Megan6 graphically shows the number of sequences aligned to bacteria, viruses and dog sequences. The viral reads were extracted from each library. The extracted viral reads were assembled in Geneious version 10.2.3. The assembled contigs were aligned to the viral proteome database using BLASTx. The viral sequences were mapped to the reference viral sequences to determine the number of reads to each sequence (Geneious version 10.2.3.).

4.2.6.3.2. Kraken data analysis

An excel file containing microbial reads found in all the four samples upto the genus level was provided by the bioinformatician. The microbial reads obtained in all the four samples were analyzed using a cutoff of 1. The number of viral reads of interest present in each sample was determined for comparative analysis.

4.3. Results

4.3.1. RNA quantity before cDNA synthesis

The total quantities of RNA in the pooled NGS3 and NGS4 samples before cDNA synthesis were 151 ng and 100.7 ng, respectively during the first attempt. These values

[9] http://ccb.jhu.edu/software/kraken/

were lower than the required RNA quantity (500 ng) required for cDNA synthesis using Maxima H Minus Double-Stranded cDNA synthesis kit. Hence, the total RNA was re-extracted from each individual swab sample using Trizol LS® reagent. The total quantities of RNA obtained for NGS3 and NGS4 pooled samples after Trizol LS® extraction were 456 ng and 523 ng, respectively. Thus, the RNA quantities obtained from NGS3 and NGS4 in the second attempt were acceptable for performing cDNA synthesis.

4.3.2. DNA quality and quantity check for all pooled samples sent for sequencing

All the pooled samples (NGS1, NGS2, NGS3 and NGS4) and two controls (NGS5 and NGS6) submitted for shot-gun sequencing passed the quality check (Table 4.3) and were used for library preparation.

Table 4.3. Quality check (QC) for all the samples submitted for shot-gun sequencing.

Sample Name	DNA Concentration (ug/mL)	RNA Concentration (ug/mL)	Protein Concentration (ug/mL)	% RNA contamination	% Protein contamination	Pass Qubit QC check Y=Yes N=No
NGS1	27.80	<0.02	<1.0	-	-	Y
NGS2	19.70	<0.02	<1.0	-	-	Y
NGS3	19.70	<0.02	<1.0	-	-	Y
NGS4	42.80	<0.02	<1.0	-	-	Y
NGS5 (control)	0.10	<0.02	<1.0	-	-	Y
NGS6 (control)	<0.1	<0.02	<1.0	-	-	Y
DNA_Standard (10µg/mL)	10.00	n/a	n/a	n/a	n/a	n/a
RNA_Standard (10µg/mL)	n/a[#]	10.00	n/a	n/a	n/a	n/a
Protein_Standard (200µg/mL)	n/a	n/a	201.00	n/a	n/a	n/a

[#] n/a is not applicable

4.3.3. Sequencing data

4.3.3.1. Data pre-processing

The total reads obtained in NGS1 and NGS2 sample libraries (containing pooled DNA samples) were comparatively lower than those obtained in NGS3 and NGS4 sample libraries (containing pooled cDNA samples) (Table 4.4). The control samples (NGS5 and NGS6) showed very few sequences justifying their exclusion from further bioinformatics analysis. The percentage of total read counts obtained in each sample libraries from three runs are shown in Table 4.4. The sequencing run report and quality check report are given in Appendix E and Appendix F, respectively.

Table 4.4. The total number of reads and their percentage obtained in samples libraries NGS1, NGS2, NGS3, NGS4, NGS5 and NGS6 from three runs.

Sample library	Run 1		Run 2		Run 3		Total	
	Read counts	**Percentage of total reads (%)**	**Read counts**	**Percentage of total reads (%)**	**Read counts**	**Percentage of total reads (%)**	**Read counts**	**Percentage of total reads (%)**
NGS1	3,496,298	16.78	2,664,564	16.71	2,404,030	16.87	8,564,892	16.78
NGS2	2,360,829	11.33	2,449,606	15.36	2,277,036	15.98	7,087,471	13.89
NGS3	8,238,758	39.55	5,951,387	37.32	5,255,610	36.88	19,445,755	38.11
NGS4	6,735,307	32.33	4,879,628	30.60	4,309,683	30.24	15,924,618	31.21
NGS5	n/a[#]	n/a	1,315	0.01	2,031	0.01	3,346	0.01
NGS6	n/a	n/a	775	0.00	1,705	0.01	2,480	0.00
Total	**20,831,192**	**1.000**	**15,947,275**	**1.000**	**14,250,095**	**1.000**	**51,028,562**	**1.000**

[#]n/a is not applicable

4.3.3.2. Mapping-based approach using Bowtie2

Mapping against dog genome

The number of reads mapped against the dog genome was greater for cDNA sample libraries (NGS3 and NGS4) compared to DNA libraries (NGS1 and NGS2) (Table 4.5). There was no difference in the number of reads mapped against dog genome in the sample libraries of healthy dogs (NGS1 and NGS3) and clinically affected dogs (NGS2 and NGS4).

Mapping against viral genome

The mapping of reads against selected 32 viral sequences did not work well. The total reads mapped to selected 32 viral sequences in all the sample libraries are shown in Table 4.6.

Mapping against three bacterial genome

The number of reads mapped against three selected bacterial sequences were low in all the sample libraries (Table 4.7). However, the reads from NGS3 sample library showed higher mapping against *B. bronchiseptica* reference sequence compared to other sample libraries (Table 4.7).

Table 4.5. The number of reads mapped against dog genome using Bowtie2 end-to-end and local mapping alignments in NGS1, NGS2, NGS3 and NGS4 sample libraries.

		Number of reads				Percentage of reads (%)			
	Mapping alignment	**NGS1**	**NGS2**	**NGS3**	**NGS4**	**NGS1**	**NGS2**	**NGS3**	**NGS4**
mapped	end-to-end	9,074	40,328	6,203,883	5,369,771	0.11	0.57	31.90	33.72
unmapped	end-to end	8,550,405	7,032,643	10,641,860	7,802,441	99.83	99.23	54.73	49.0
total		8,559,479	7,072,971	16,845,743	13,172,212	99.94	99.80	86.63	82.72
mapped	local	30,732	143,386	17,373,397	14,708,341	0.36	2.02	89.34	92.36
unmapped	local	8,516,498	6,923,349	1,927,218	1,093,415	99.43	97.68	9.91	6.87
total		8,547,230	7,066,735	19,300,615	15,801,756	99.79	99.71	99.25	99.23
Total number of reads		8,564,892	7,087,471	19,445,755	15,924,618	100.0	100.0	100.0	100.0

Table 4.6. The number of reads mapped against 32 selected viral sequences using Bowtie2 end-to-end and local mapping alignments in NGS1, NGS2, NGS3 and NGS4 sample libraries.

		Number of reads				Percentage of reads (%)			
	Mapping alignment	**NGS1**	**NGS2**	**NGS3**	**NGS4**	**NGS1**	**NGS2**	**NGS3**	**NGS4**
Mapped	end-to-end	-	238	11	16	0.00	0.003	5.6e-5	1.0e-4
Mapped	local	13	302	3,444	3,805	1.5e-4	0.004	0.017	0.023
Total number of reads		8,564,892	7,087,471	19,445,755	15,924,618	100.0	100.0	100.0	100.0

Table 4.7. The number of reads mapped against *Bordetella bronchiseptica, Streptococcus zooepidemicus* and *Mycoplasma cynos* genomes using Bowtie2 end-to-end and local alignments in NGS1, NGS2, NGS3 and NGS4 sample libraries.

			Number of reads				Percentage of reads (%)			
Bacterium	**Accession**	**Mapping alignment**	**NGS1**	**NGS2**	**NGS3**	**NGS4**	**NGS1**	**NGS2**	**NGS3**	**NGS4**
Bordetella bronchiseptica	NC_019382	end-to-end	4,073	607	45,026	16,757	0.048	0.009	0.232	0.105
Streptococcus zooepidemicus	NC_012470	end-to-end	146	173	4,133	2,675	0.002	0.002	0.021	0.017
Mycoplasma cynos	NC_019949	end-to-end	33	2,084	4,057	26,913	0.000	0.029	0.021	0.169
		Total	**4,252**	**2,864**	**53,216**	**46,345**	**0.050**	**0.040**	**0.274**	**0.291**
Bordetella bronchiseptica	NC_019382	local	41,206	8,695	1,141,208	521,599	0.481	0.123	5.869	3.275
Streptococcus zooepidemicus	NC_012470	local	3,957	1,683	90,341	86,871	0.046	0.024	0.465	0.546
Mycoplasma cynos	NC_019949	local	1,726	7,117	158,440	144,749	0.020	0.100	0.815	0.909
		Total	**46,889**	**17,495**	**1,389,989**	**753,219**	**0.547**	**0.247**	**7.148**	**4.730**
		Total number of reads	8,564,892	7,087,471	19,445,755	15,924,618	100.0	100.0	100.0	100.0

4.3.3.3. Taxonomic classification

The viral sequences obtained from DIAMOND and Kraken analysis are described below.

DIAMOND

Based on the DIAMOND process, four files were generated for each sample. These files, on visualization in Megan6, showed the taxonomic overview of the sequencing data in all the four libraries (NGS1, NGS2, NGS3 and NGS4).

The bacteriophage sequences were present in all the sample libraries. Apart from bacteriophages, viral sequences of CnPnV, CRCoV, influenza C virus, and canine picornavirus (CanPV) were identified in NGS4 sample library. No viral sequences of interest were identified in the NGS2 sample library, and in the pooled sample of healthy dogs (NGS1 and NGS3 sample libraries).

Kraken

The sequences that mapped to arachea, bacteria and viruses at a higher taxanomic level are shown in Table 4.8. When the viral data were analyzed, CnPnV, CRCoV, influenza C virus and CanPV were identified in NGS4 sample library, similar to DIAMOND analysis. Additionally, CHV-1 sequences were identified in the pooled DNA sample of diseased dogs (NGS2 sample library). None of these viral sequences were identified in the pooled sample from healthy dogs (NGS1 and NGS3 sample libraries).

Table 4.8. An overview of number of reads assigned (d_assigned) and unassigned (d_unassigned) in each sample library using Kraken database, where d_all = total number of reads. The number of reads from NGS1, NGS2, NGS3 and NGS4 sample libraries that were mapped to archaea, bacteria and virus sequences are also shown. The percentage of reads is shown using cut off at 1.

Taxonomy	**Taxa Level**	**NGS1**	**NGS2**	**NGS3**	**NGS4**
d_all	L0	8,564,892	7,087,471	19,445,755	15,924,618
d_assigned	L0	1,488,422	1,219,753	1,232,938	661,615
d_unassigned	L0	7,076,470	5,867,718	18,212,817	15,263,003
% mapped		17.38%	17.21%	6.34%	4.15%
Number of reads (Percentage of assigned reads)					
Archaea	**L1**	15 (0.00)	22 (0.00)	18,110 (1.48)	13,789 (2.12)
Bacteria	**L1**	1,481,358 (99.56)	1,211,938 (99.39)	1,196,071 (97.70)	628,762 (96.49)
Viruses	**L1**	6,466 (0.43)	7,464 (0.61)	10,079 (0.82)	9,054 (1.39)
Total of all mapped reads		1,487,839	1,219,424	1,224,260	651,605

A combined number of reads to selected viral sequences identified in dog with signs of ITB in either NGS2 or NGS4 sample library using DIAMOND and Kraken are shown in table 4.9.

Table 4.9. Viruses and their accession number identified in dogs with signs of ITB (NGS2 and NGS4 sample libraries) based on DIAMOND and Kraken analysis.

Virus	Family	GenBank accession no.	Number of reads	
			DIAMOND	Kraken
Canine Pneumovirus	*Paramyxoviridae*	NC_025344	170	85
Canine respiratory coronavirus	*Coronaviridae*	JX860640	10	2
Canine picornavirus	*Picornaviridae*	NC_016964	2	7
Influenza C virus	*Orthomyxoviridae*	LC125010	2	12
Canine herpesvirus1	*Herpesviridae*	NC_030117	-	23

4.4. Discussion

The viral sequences identified in dogs with signs of ITB were CHV-1, CRCoV, CnPnV, influenza C virus and CanPV. This represents the first identification of CnPnV, influenza C virus and CanPV in dogs in New Zealand.

The number of CnPnV sequences was higher than any other viral sequences present in the pooled sample of diseased dogs (NGS4 sample library) (Table 4.9). This may suggest that CnPnV was involved in the development of respiratory disease in the sampled dogs. However, the use of a pooled sample precludes such conclusion, as it is impossible to determine whether CnPnV reads (or any other viral reads present in the pooled sample) originated from one or more of the individual samples that contributed to the pool. Hence, testing of individual oropharyngeal swab samples would be required to determine the frequency of infection with specific viruses among sampled dogs. The results of testing individual swabs for CRCoV, CHV-1, CAdV-2, CPiV, and CnPnV are presented in Chapter 5. Hence, the possible involvement of those viruses in canine ITB in New Zealand, including the role of CnPnV, is discussed in more detail in Chapter 5.

A few influenza C viral sequences were found only in the pooled sample of disease dogs (NGS4 sample library). The role of this virus in canine respiratory infections is unknown. Influenza C virus is responsible for upper and lower respiratory infections in young children (Katagiri *et al.*, 1983, Katagiri *et al.*, 1987, Moriuchi *et al.*, 1991, Matsuzaki *et al.*, 2006). This virus has been previously reported in pigs (Yuanji *et al.*, 1983) and dogs (Manuguerra and Hannoun, 1992). Sero-epidemiological surveys strongly suggest that dogs can be naturally infected with influenza C virus (Manuguerra and Hannoun, 1992, Manuguerra *et al.*, 1993). Dogs experimentally infected with influenza C developed signs of nasal discharge, swelling of eyelids and epiphora (Ohwada *et al.*, 1986). Although influenza C virus has the ability to infect dogs, it is not yet clear if the same human agent or closely related agent causes infections in dogs. The detection of influenza C viral sequences in the pooled sample of diseased dogs in the present study indicates that some dogs may possibly have acquired this virus from humans.

In the present study, CanPV sequences were also identified in the pooled samples of diseased dogs (NGS4 sample library). CanPV has previously been reported in the fecal, nasopharyngeal and urine samples of clinically normal dogs (Woo *et al.*, 2012a). However, there are no reports on the presence of CanPV in dogs with respiratory disease. Considering the fact that human picornaviruses are associated with respiratory disease (Schieble *et al.*, 1967, Jacques *et al.*, 2006), it is possible that CanPV may have a similar role in dogs. Further studies are required to understand the pathogenesis of CanPV in respiratory infections of dogs.

Sample processing for viral metagenomic analysis can affect the number and type of viral sequences retrieved; and should be taken into consideration during the downstream analysis. The sample preparation for viral metagenomic studies require pre-enrichment

process in order to minimize bacterial and host genome load and increase the frequency of obtaining viral sequences from the sample. The bias related to sample preparation for metagenomic analysis has already been described (Thomas *et al.*, 2012). The potential bias related to pre-enrichment and nucleic acid extraction method observed in this study are discussed below.

A high proportion of canine sequences were obtained in the pooled cDNA-based samples (NGS3 and NGS4 sample libraries) (Table 4.5). This indicates that the use of 96 non-ribosomal hexanucleotides and employment of pre-enrichment steps incorporating centrifugation, filtration and DNase treatment to enrich for viral sequences was not fully successful in eliminating host origin sequences. The pre-enrichment steps used in our study did not involve RNase treatment of samples, thus host mRNA expressed genes present in the samples were not removed. This must have led to cDNA synthesis and thereby sequencing of host mRNA in the cDNA-based samples.

In this study, the quantity of RNA extracted using column-based nucleic acid extraction was low. Hence, RNA was re-extracted using Trizol LS® reagent, which yielded more RNA compared to the column-based extraction kit. Failure of column-based extraction kits to yield more RNA can be explained due to the following reasons. The successful extraction of RNA requires the use of carrier RNA. The column-based extraction kit used in this study did not use carrier RNA to bind the sample RNA that may have possibly resulted in the loss of RNA during the washing step. On the other hand, glycogen was used as a carrier during the extraction of RNA by Trizol LS® method which extracted enough RNA required for reverse transcription. Secondly, the RNA extracted using the column may have been degraded during processing or handling of samples. Thus, application of shot-gun sequencing for viral metagenomic studies

require highly efficient viral extraction methods and maintaining RNase-free environment.

Computational analyses of sequences are one of the hardest challenges in metagenomic studies, primarily because of handling a lot of sequence data (Fancello *et al.*, 2012). A variety of bioinformatics tools are available for each step in metagenomic analysis. Currently, there is no standard protocol established for the analysis of viral metagenomic data. In this study, the original intention was to use Bowtie2 to align the reads against canine reference genome and selected bacterial and viral reference sequences. However, mapping against a subset of viral sequences using Bowtie2 did not work well (Table 4.6). This was due to a small subset of viral reference sequences used for analysis of a large sequencing data. Hence, the results obtained from this attempt indicated that this was not an appropriate approach to map reads directly against the reference sequences of potential organisms of interest. Attempts should have been made to first remove all canine and bacterial sequences from the sequencing data and then align reads to the reference viral sequences. Hence, it is important to conduct a proper pipeline of procedures based on our requirements for bioinformatic analysis.

The taxonomic classifiers (DIAMOND and Kraken) used in this study displayed a better representation of host sequences and overall microbial taxa present in the samples. The percentage of viral sequences obtained by the two classifiers was comparatively lower than the bacterial sequences. The results obtained in Kraken analysis are shown in Table 4.8. There are several reasons for the low viral sequences present in the sample libraries. Firstly, pre-enrichment steps (centrifugation and filtration) or library preparation may have an effect on the relative abundance of viral sequences in the metagenomic samples. The viral genomes are smaller in size and are comparatively lower in number compared to the bacterial genomes; thus the chances of

losing viral sequences during sample processing and handling is relatively high. Alternatively, it is possible that the oropharyngeal swabs used in this study may not contain a large number of viral particles, and probably much fewer than other more invasive techniques such as tracheal lavage (Kaczorek *et al.*, 2017). Also, compared to the bacterial genomic reference database, the current viral genomic reference database is underrepresented (Klingenberg *et al.*, 2013). Hence, it is often difficult to get an accurate and realistic estimation of the percentage of viral sequences in metagenomic studies as most of the viral sequences are not aligned to the viral reference genome database. Viruses, especially RNA viruses, have a high tendency to undergo mutation, and hence the viral sequences may be highly divergent and not have homologs in the public database (Fancello *et al.*, 2012).

The DIAMOND taxonomic classifier used in this study identified CnPnV, CRCoV, CanPV and influenza C viral sequences (Table 4.9). In addition to these viruses, Kraken identified CHV-1 in NGS2 sample library. There was disparity in the results shown by the two taxonomic classifiers. This may be due to the different algorithms used by different taxonomic classifiers. DIAMOND classifier uses the whole National Center for Biotechnology Information (NCBI) *-nr* database for classification of sequences, whereas Kraken uses microbial subset of the NCBI BLAST non-redundant protein database *nr* for the classification of sequences. Thus results obtained from different classifiers should be analyzed with caution as there are chances of obtaining false positive results.

The current metagenomic study was conducted on the pooled sample rather than individual samples. Sample pooling may have resulted in loss of viral sequences during sample processing leading to no or very low detection of viral sequences. This can explain why canine viral sequences were not detected in the pooled sample of healthy

dogs. Due to financial constraints, it was not possible to use individual samples for metagenomic analysis. Testing of individual samples for the presence of viruses identified in this study would provide a valuable information on frequency of viral detection in both sick and healthy dog groups. The next chapter (Chapter 5) describes the individual testing of samples for the presence of five canine respiratory viruses (CPiV, CAV-2, CHV-1, CRCoV and CnPnV) in both healthy dogs and dogs with signs of ITB.

In conclusion, the data presented in this chapter provide evidence that CHV-1, CRCoV, CnPnV, influenza C virus and CanPV circulate among dogs in New Zealand. These viruses were identified only in the pooled DNA and cDNA samples of dogs with signs of ITB.

The work described in this chapter was presented at an international conference:

More G.; Dunowska M.; Biggs P.J.; Acke E. and Cave N. J. The upper respiratory tract microbiome and its potential role in canine respiratory infections. *Oral presentation at 97th Annual Conference of Research Workers in Animal Diseases*; (**2016**) Dec 4-6; Chicago, Illinois, USA.

Chapter 5: PREVALENCE OF CANINE RESPIRATORY VIRUSES USING REAL-TIME PCR

5.1. Introduction

While the list of aetiological agents shown to be responsible for canine ITB continues to expand, the reported prevalence of canine respiratory pathogens in different geographical areas varies. Schulz *et al.* (2014b) reported *B. bronchiseptica* (78.7%) and CPiV (37.7%) to be the most common pathogens associated with ITB in dogs in Southern Germany. A similar study conducted by Joffe *et al.* (2016) showed a high prevalence of *M. cynos* (81%) and CPiV (42%) in samples from the pharyngeal and nasal region of dogs with ITB compared with healthy dogs in Canada. Kaczorek *et al.* (2017) reported a high prevalence of CHV-1 (80%) followed by CPiV (67.5%), *B. bronchiseptica* (32.5%) and CAdV-2 (10%) in the tracheal lavage samples of dogs with ITB from the North-east region of Poland; no healthy dogs were tested in this study. In New Zealand, the pathogens responsible for canine ITB has not yet been fully described. Hence, a thorough survey is required to understand the prevalence of canine respiratory pathogens circulating among dogs in New Zealand.

In a longitudinal pilot study conducted in dogs in New Zealand, Sowman *et al.* (2013) reported 27.6% (13/47) of dogs with acute respiratory disease to be positive for at least one of CAdV-2, CPiV, *M. cynos*, *B. bronchiseptica* or CHV-1 using a commercial multiplex PCR panel. However, the researchers did not screen for other important respiratory pathogens such as CnPnV and CIV in the dogs with respiratory disease.

In chapter 4, a metagenomic shot-gun sequencing approach was used to describe the viral microbiome of oropharyngeal swabs from dogs with signs of ITB and from healthy dogs. A number of known viral sequences such as CnPnV, CRCoV, CanPV and influenza C virus were identified in the pooled oropharyngeal sample of dogs from the

ITB group. However, the actual prevalence of each respiratory virus in that population could not be determined by shot-gun sequencing data, as pooled samples were used for the analysis.

As such, the aim of the work presented in this chapter was to investigate the prevalence of CnPnV, CRCoV, CPiV, CHV-1 and CAdV-2 in dogs with acute respiratory disease and compare the data to the corresponding findings for healthy dogs.

5.2. Materials and methods

The work presented in this chapter included the oropharyngeal swab samples collected from 116 dogs described in section 3.1.3. The number of dogs with signs of ITB and healthy dogs from private households and kenneled dogs included in this study is described in the Table 3.1.

5.2.1. Viral RNA and DNA extraction

The viral nucleic acids were extracted from a 200 µL aliquot of each of the oropharyngeal swab samples (Section 3.1.5) using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Basel, Switzerland) according to the manufacturer's instructions. The kit allows purification of both high quality genomic DNA and total RNA from samples.

5.2.2. Reverse transcription

In order to detect DNA viruses using real-time PCR, the nucleic acids extracted were used as template. However, to detect RNA viruses, RNA was first transcribed into cDNA using qSCRIPT™ (Quanta Bioscience, Gaithersburg, USA) according to the manufacturer's instructions. Briefly, the reaction mix consisted of 4 µL of qSCRIPT mix, 6 µL of RNA and remaining nuclease free water in a total volume of 20 µL, and was incubated at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min.

5.2.3. Endogenous control PCR

To monitor successful RNA extraction, a real-time PCR for eukaryotic 18S rRNA was performed. For 18S rRNA, the forward primer: 5′- TGTGCCGCTAGAGGTGAAATT-3′ and reverse primer: 5′- TGGCAAATGCTTTCGCTTT -3′ were used (Suzuki *et al.*, 2000). The real-time PCR was performed in a total volume of 10 µL containing 1 X concentration of PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, USA) with a final concentration of 0.4 µM of each primer and 1 µL of cDNA template. The cycling conditions were as follows: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec followed by melting curve analysis from 95 °C over 15 sec and 60 °C over 15 sec.

5.2.4. Primers designed for real-time PCR

Viral sequences for canine respiratory viruses were first searched in NCBI (Geer *et al.*, 2009). Using Interproscan (Zdobnov and Apweiler, 2001), protein sequences of each virus were scanned to identify conserved motifs and were targeted for primer design. Primers were designed using Geneious version 10.2.3. (Kearse *et al.*, 2012). The forward and reverse primers of 20-26 bp each producing an amplicon of 80-190 bp were selected for the real-time PCR assay. Later, uMelt^{SM} (Dwight *et al.*, 2011) was used to predict the melt curves for the predicted products for each primer pair, and to identify the possibility of formation of primer dimers, which reduces reaction efficiency. The target region was analyzed further for secondary structure formation at primer binding region using Mfold (Zuker, 2003). The NCBI primer BLAST (Altschul *et al.*, 1990) was performed as an additional quality control check to ensure that the primers were not complementary to non-target sequences.

Primers were commercially synthesized (Integrated DNA technologies, Coralville, USA) and supplied lyophilized. The primers were re-suspended with nuclease free water to make a stock primer solution of 100 µM and stored at -20 °C. A working primer solution of 20 µM was made for use in quantitative PCR reactions. The primers used for real-time PCR are listed in Table 5.1.

Table 5.1. Primer sequences, target region and positions with accession number used for real-time PCR for each virus.

Virus	Primer sequence	Target region	Position (Accession number)	Reference
Canine parainfluenza virus				
CPiV3 For	5'-AGGGTGCAGTTGACATGAGG-3'	Nucleocapsid	**1269 – 1404**	Designed for this study
CPiV3 Rev	5'-GAGAACGGGTTGACTCCTCC-3'		**(KP893891)**	
Canine adenovirus-2				
CAdV2 For	5' -TCATGCCGGGCTTCTACA CTAACG-3'	Penton base	**13,228 - 13,370**	Designed for this study
CAdV2 Rev	5'-ATCACAAATCCCTCCTGGTATGGT-3'		**(AC_000020)**	
Canine herpesvirus-1				
CHV For	5'-ACAGAGTTGATTGATAGAAGAGGTATG-3'	Glycoprotein B	**439 – 574**	(Decaro *et al.*, 2010)
CHV Rev	5'-CTGGTGTATTAAACTTTGAAGGCTTTA-3'		**(AF361073)**	
Canine respiratory coronavirus				
CrCoV_15264.F	5'-CGACTTGCGAATGAATGCGCACA- 3'	ORF1ab	**15264 – 15386**	Designed for this study
CrCoV_15360.R	5' –TTGCATCACCACTACTAGTGCCACCA- 3'		**(JX860640)**	
Canine pneumovirus				
CnPnV_M2geneF	5'- GTGTGCTGAAAAGTTACCTGGAAA- 3'	Matrix 2	**7825 – 7948**	Designed for this study
CnPnV_M2geneR	5'- GCGTTGCTATCTCTTGCTTGTATT- 3'		**(NC_025344)**	

5.2.5. Generation of standard curves

The standards for CAdV-2 were constructed using a vaccine CAdV-2; while for CHV-1 and CPiV, the standards were constructed from the pure culture available in the laboratory from previous studies. For CRCoV, 424 bp G-block was commercially obtained (Integrated DNA technologies, Coralville, USA) and used for constructing standards. For CnPnV, the standards were constructed by amplifying the PCR product from the positive samples. Each PCR product was amplified, and the sequence

confirmed by sequencing and cloning using the TOPO TA cloning kit (Thermo Fisher Scientific, Waltham, USA).

5.2.5.1. Transformation of cells

The cloning reaction was set up according to the TOPO TA cloning kit with pCR™-4 TOPO™ vector (Thermo Fisher Scientific, Waltham, USA) with slight modifications. The TOPO cloning reaction was set by adding reagents in the following order and mixed gently (Table 5.2).

Table 5.2. TOPO cloning reaction.

Reagents	Volume
PCR product	2 µL
Salt solution	1 µL
Water	2 µL
TOPO vector	1 µL
Total	**6 µL**

The TOPO cloning reaction was incubated at room temperature for 15 min and placed on ice. For transformation, 2 µL of TOPO cloning reaction was added to One Shot® chemically competent *E.coli*, mixed gently without pipetting and incubated on ice for 30 min. The cells were heat shocked at 42 °C for 30 sec and placed immediately on ice. About 250 µL of S.O.C medium (at room temperature) was added to the cells, and tubes were incubated at 37 °C while shaking (200 rpm) for 1 hr. An aliquot (10-50 µL) of the transformation reaction was plated on pre-warmed Luria agar plates containing 100 µg/mL of ampicillin. The plates were incubated overnight at 37 °C.

5.2.5.2. Colony screening

The plates were observed for colonies the next day. Individual colonies were picked up using sterile tooth picks and placed into microfuge tubes containing 10 µL of Luria broth (LB) with 50 µg/mL of ampicillin. The colonies were screened for the presence of

the desired insert by PCR using universal M13 primers. The colonies showing the right insert size (240 bp (M13 product) and PCR product size) were selected for further steps.

5.2.5.3. Plasmid extraction

An aliquot (5 µL) of each of the selected colonies were inoculated into 5 mL of LB containing 50 µg/mL of ampicillin. Bacteria were grown overnight at 37 °C with shaking (200 rpm). Plasmids were extracted from the cells using Roche high pure plasmid isolation kit (Roche Diagnostics GmbH, Basel, Switzerland) as per manufacturer's instructions. The extracted plasmid DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

5.2.5.4. Generation of standard curves

Plasmids were linearized with *PstI* enzyme in a total volume of 100 µL. Each reaction contained 10^9 copies/µL of plasmid, 1 unit of *PstI* enzyme and 10 µL of 10 X buffer. The reaction was incubated for 1hr at 37 °C. The amount of the plasmid stock solution to be used in each restriction enzyme digestion reaction was calculated using Finnzyme copy number calculator (Thermo Scientific, Waltham, USA). The reaction was inactivated by adding 20 mM final concentration of EDTA. The digested plasmids had 10^9 copies per µL of desired product and were sent for sequencing to confirm the identity of inserts. A 10-fold dilution series of each digested plasmid (1×10^7 to 1×10^1 copies/µL) was prepared and used to generate a standard curve for the target specific real-time PCR.

5.2.6. Real-time PCR

The real-time PCR (qPCR) for viral nucleic acids of CnPnV, CRCoV, CPiV, CHV-1 and CAdV-2 were performed on the MIC qPCR cycler (Bio Molecular Systems, Queensland, Australia) in a 10 µL final reaction volume. Each reaction contained 2 µL

of template, primer and 1 X master mix. The final concentrations of primers for each assay, master mix used and cycling conditions are listed in Table 5.3. All samples were screened in duplicate. For each real-time PCR run, relevant standards and a negative control (nuclease free water and dog DNA for screening of DNA virus and dog cDNA for screening of RNA virus) were included to check the performance of the assay.

The analysis of copy number, linear regression and melting curve analysis was performed for each experiment using MIC Software version 1.2. Runs with PCR efficiency < 90% and > 110% were repeated.

Table 5.3. Details on PCR master mix, primer concentration and cycling conditions used for each virus.

Virus	SYBr master mix	Primer concentration (µM)	Cycling conditions
Canine parainfluenza virus	PowerUp™ SYBR™ Green Master Mix[#]	0.4	50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 1s, 60 °C for 30s
Canine adenovirus-2	Luminaris HiGreen qPCR Master Mix[#]	0.5	50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 1s, 62 °C for 30s
Canine herpesvirus-1	PowerUp™ SYBR™ Green Master Mix[#]	0.4	50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 1s, 62 °C for 30s
Canine pneumovirus	PowerUp™ SYBR™ Green Master Mix[#]	0.4	50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 15s, 55 °C for 15s, 72 °C for 30s
Canine respiratory coronavirus	PowerUp™ SYBR™ Green Master Mix[#]	0.4	50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 15s, 60 °C for 30s

[#] manufactured by Thermo Fisher Scientific, Waltham, USA

5.2.7. Statistical analysis

Data were analyzed using the software package R version 3.1.0 (R Development Core team, 2014). The results obtained from real-time PCR between the two groups (dogs with signs of ITB and healthy dogs) were compared using Fisher's exact tests and χ^2 tests. To detect CnPnV shedding levels in the two groups (dogs with signs of ITB and healthy dogs), the copy number of CnPnV present in each positive sample was log transformed and normalized. Welch's two sample *t*-test was performed to ascertain the statistical significance between CnPnV shedding levels in dogs with ITB and healthy dogs. The level of significance was set at $p < 0.05$. Prevalence of the pathogens was calculated with a confidence interval (CI) of 95%.

5.3. Results

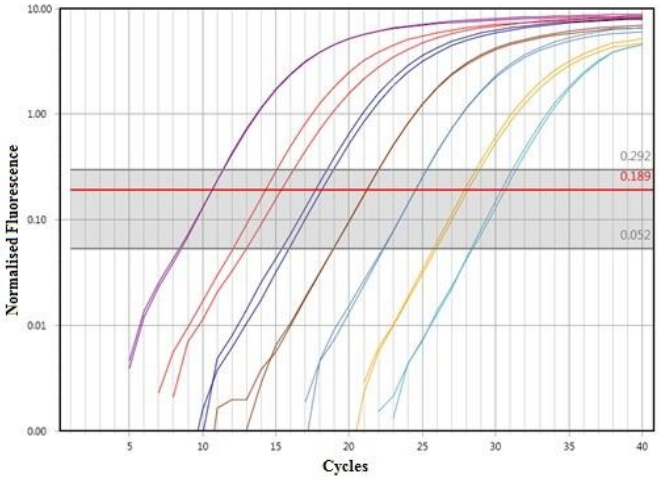
5.3.1. Real-time PCR

The primer efficiencies and amplicon sizes tested for each virus by quantitative PCR are given in the Table 5.4. The amplification curves, derivative melt curves and standard curves are presented below for CPiV qPCR (Fig. 5.1), CAdV-2 (Fig. 5.2), CHV-1 qPCR (Fig. 5.3), CnPnV qPCR (Fig. 5.4) and CRCoV qPCR (Fig. 5.5).

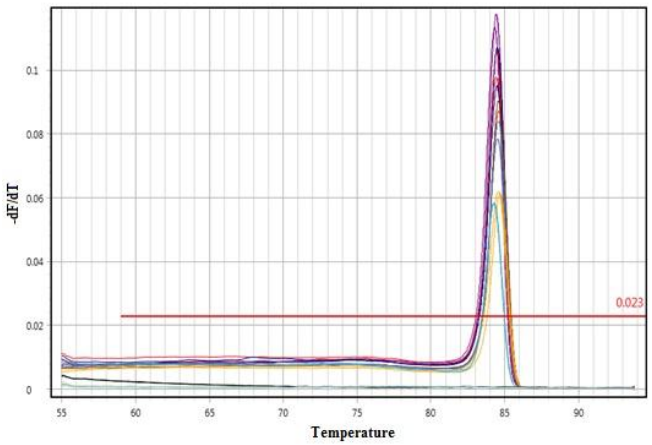
Table 5.4. Primer pairs, amplicon sizes, primer efficiency and R^2 tested by real-time PCR for each virus.

Virus	Primer pairs	Amplicon size (bp)	Efficiency (%)	R^2
Canine parainfluenza virus	**CPiV3 For** **CPiV3 Rev**	135	101	0.98
Canine adenovirus type 2	**CAdV2 For** **CAdV2 Rev**	142	95	0.99
Canine herpesvirus 1	**CHV For** **CHV Rev**	136	96	0.99
Canine respiratory coronavirus	**CrCoV_15264.F** **CrCoV_15360.R**	122	92	0.99
Canine pneumovirus	**CnPnV_M2geneF** **CnPnV_M2geneR**	123	94	0.99

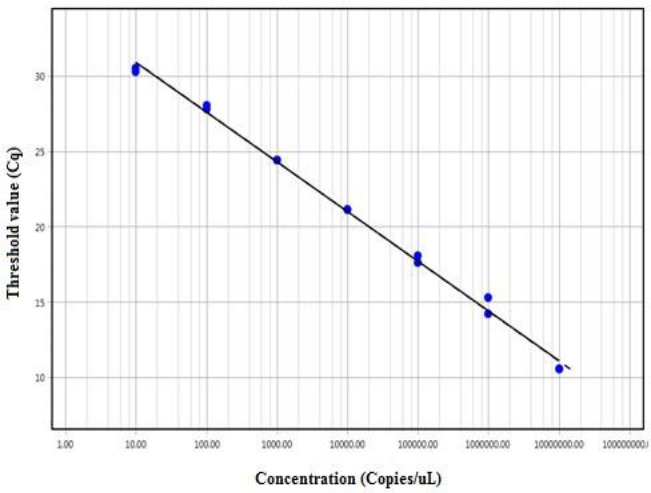
The samples positive for respiratory viruses using virus specific real-time PCR in dogs with ITB and healthy dogs are shown in the Table 5.5.



(A)

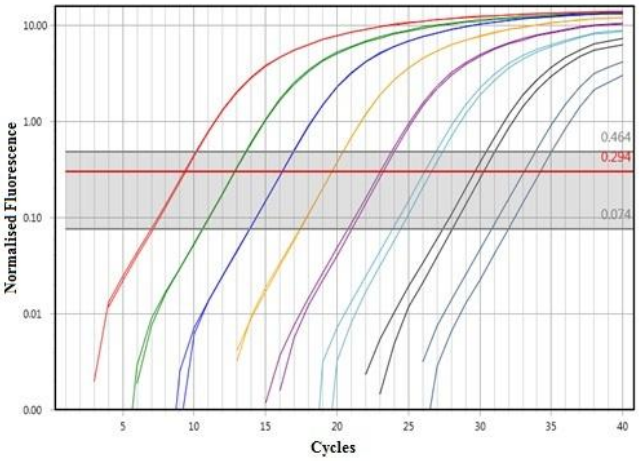


(B)

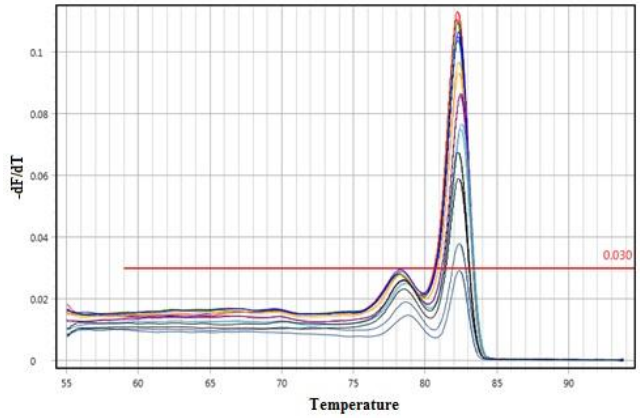


(C)

Figure 5.1. Real-time PCR amplification curve (A), derivative melt curve (B) and standard curve (C) for canine parainfluenza virus (CPiV) specific real-time PCR. Standard curve was prepared using seven 10-fold dilutions (10^7 to 10^1 copies/µL) of a linearized plasmid containing the target sequence, in duplicate. In graph A and B the dilutions (copies/µL) are denoted as violet- 10^7, red- 10^6, dark blue- 10^5, brown- 10^4, blue-10^3, orange- 10^2 and light blue- 10^1 and the red horizontal line is the threshold. Nuclease free water was used as a non-template control. The CPiV real-time PCR had efficiency 1.01 and R^2 0.99.



(A)



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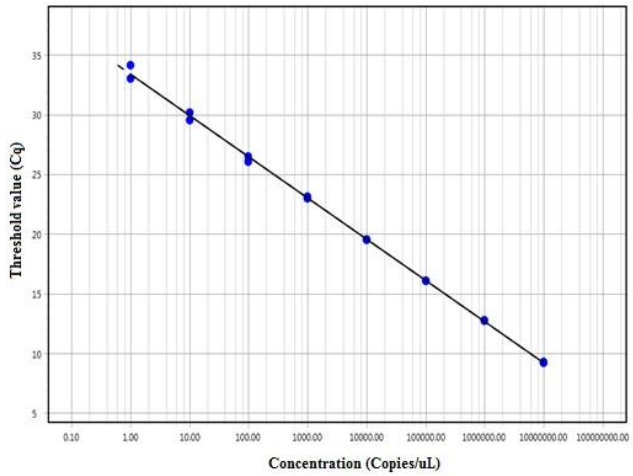
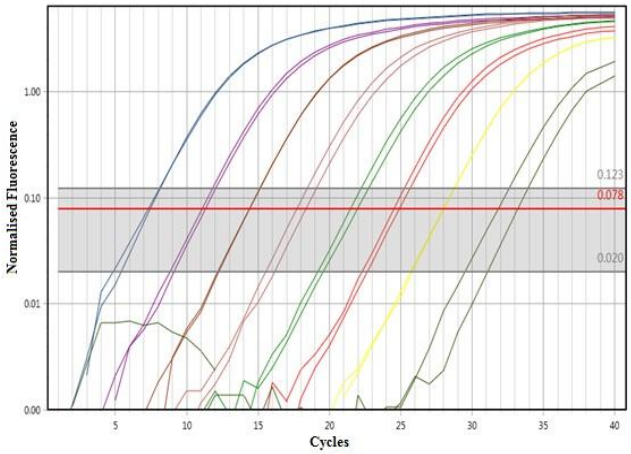
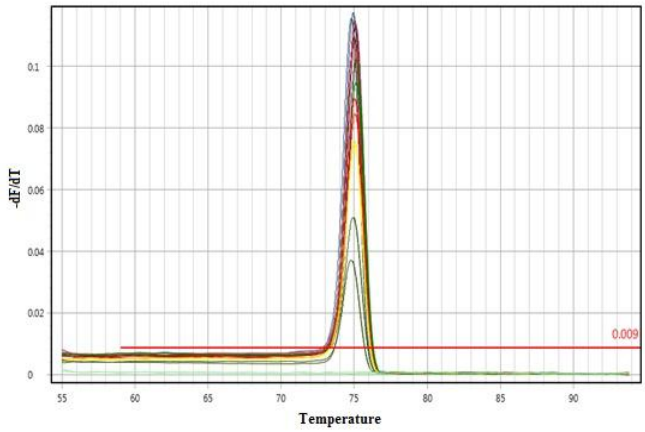


(C)

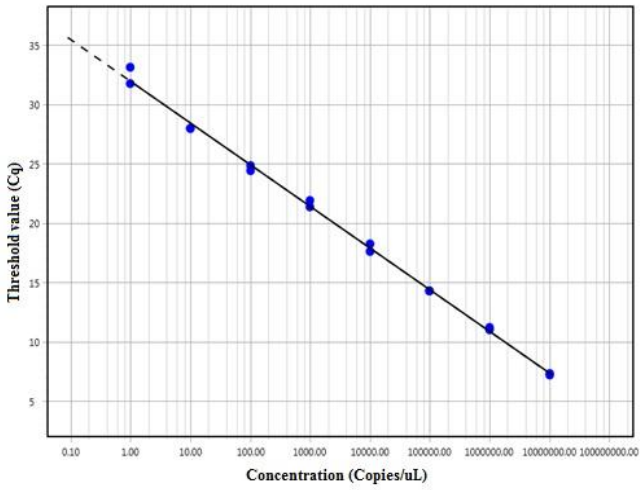
Figure 5.2. Real-time PCR amplification curve (A), derivative melt curve (B) and standard curve (C) for canine adenovirus-2 (CAdV-2) specific real-time PCR. Standard curve was prepared using eight 10-fold dilutions (10^7 to 10^0 copies/µL) of a linearized plasmid containing the target sequence, in duplicate. In graph A and B the dilutions (copies/µL) are denoted as red- 10^7, green- 10^6, dark blue- 10^5, orange- 10^4, violet- 10^3, light blue- 10^2, brown- 10^1 and blue- 10^0 and the red horizontal line is the threshold. Nuclease free water was used as a non-template control. The CAdV-2 real-time PCR had efficiency: 0.95 and R^2: 0.99.



(A)

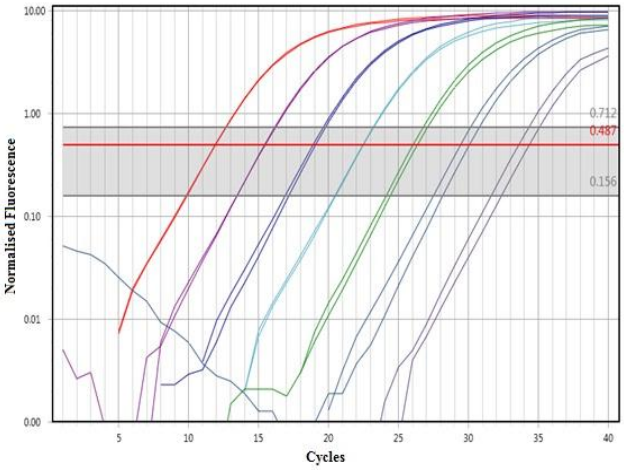


(B)

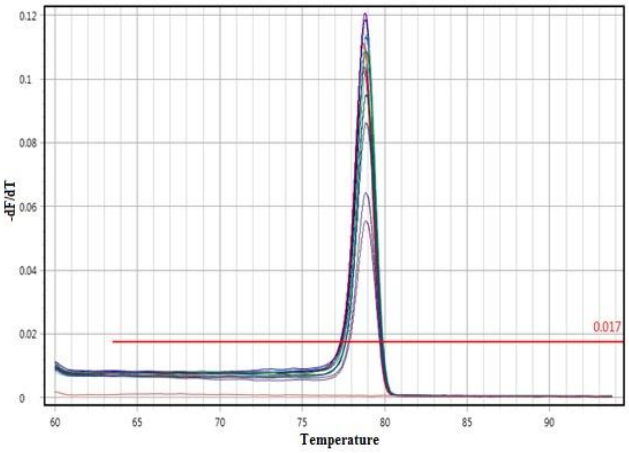


(C)

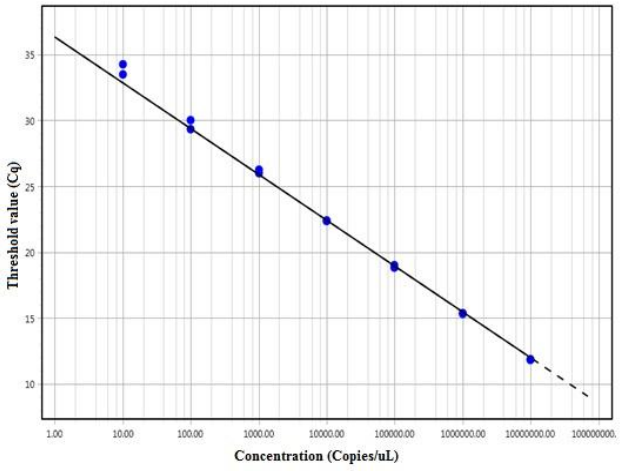
Figure 5.3. Real-time PCR amplification curve (A), derivative melt curve (B) and standard curve (C) for canine herpesvirus-1 (CHV-1) specific real-time PCR. Standard curve was prepared using eight 10-fold dilutions (10^7 to 10^0 copies/µL) of a linearized plasmid containing the target sequence, in duplicate. In graph A and B the dilutions (copies/µL) are denoted as dark blue- 10^7, violet- 10^6, brown- 10^5, pink- 10^4, green- 10^3, red- 10^2, yellow- 10^1 and black- 10^0 and the red horizontal line is the threshold. Nuclease free water was used as a non-template control. The CHV-1 real-time PCR had efficiency: 0.96 and R^2: 0.99.



(A)

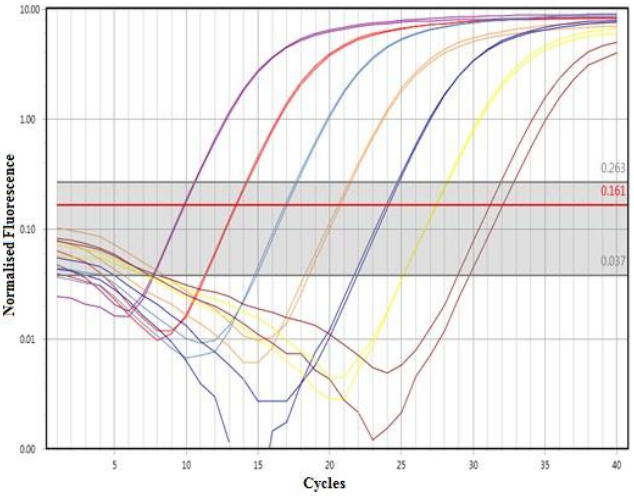


(B)

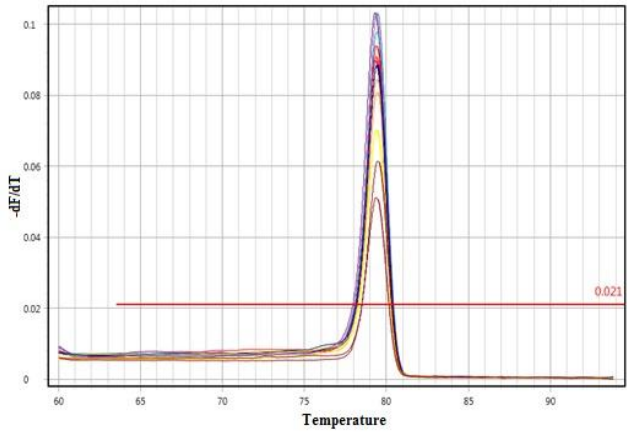


(C)

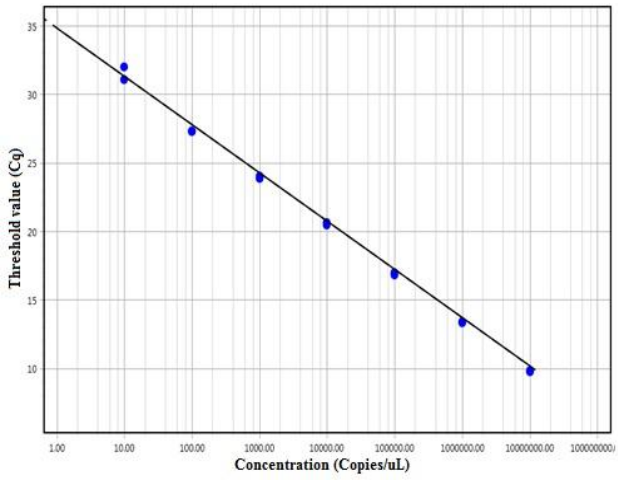
Figure 5.4. Real-time PCR amplification curve (A), derivative melt curve (B) and standard curve (C) for canine pneumovirus (CnPnV) specific real-time PCR. Standard curve was prepared using seven 10-fold dilutions (10^7 to 10^1 copies/µL) of a linearized plasmid containing the target sequence, in duplicate. In graph A and B the dilutions (copies/µL) are denoted as red- 10^7, violet- 10^6, dark blue- 10^5, light blue- 10^4, green- 10^3, blue- 10^2 and black- 10^1 and the red horizontal line is the threshold. Nuclease free water was used as a non-template control. The CnPnV real-time PCR had efficiency: 0.94 and R^2 0.99.



(A)



(B)



(C)

Figure 5.5. Real-time PCR amplification curve (A), derivative melt curve (B) and standard curve (C) for canine respiratory coronavirus (CRCoV) specific real-time PCR. Standard curve was prepared using seven 10-fold dilutions (10^7 to 10^1 copies/µL) of a linearized plasmid containing the target sequence, in duplicate. In graph A and B the dilutions (copies/µL) are denoted as violet- 10^7, red- 10^6, blue- 10^5, orange- 10^4, dark blue- 10^3, yellow- 10^2 and dark pink- 10^1 and the red horizontal line is the threshold. Nuclease free water was used as a non-template control. The CRCoV real-time PCR had efficiency 0.92 and R^2 0.99.

Table 5.5. Samples with dog number from (i) dogs with signs of ITB and, (ii) healthy dogs, positive for canine respiratory viruses, as determined by virus specific real-time PCR for canine parainfluenza virus (CPiV), canine adenovirus type-2 (CAdV-2), canine herpesvirus type 1 (CHV-1), canine respiratory coronavirus (CRCoV) and canine pneumovirus (CnPnV) are given below. The Cq values and copies/µL of sample cDNA for each virus present in the sample is calculated.

(i) Dogs with signs of ITB

Dog number	CnPnV		CRCoV		CHV-1		CAdV-2		CPiV	
	Cq value	copies per µL	Cq value	copies per µL	Cq value	copies per µL	Cq value	copies per µL	Cq value	copies per µL
ITB – 004	35.89	1.1	-		-		-		-	
ITB – 005	-		-		-		36.58	0.06	-	
ITB – 006	35.1	1.5	-		-		-		-	
ITB – 007	24.3	933	-		22.37	1080.8	-		-	
ITB – 015	-		-		-		31.4	4.6	-	
ITB – 020	36.08	0.41	-		-		-		-	
ITB – 022	-		-		-		36.23	0.08	-	
ITB – 025	32.78	3.63	-		-		-		-	
ITB – 026	32.94	3.16	-		-		-		30.8	25.92
ITB – 030	36.06	0.41	-		-		-		-	
ITB – 031	35.2	0.72	-		35.1	0.7	-		-	
ITB – 036	36.36	0.34	-		-		-		-	
ITB – 044	-		-		-		-		36.29	0.33
ITB – 046	-		Positive[$]		-		-		-	
ITB – 048	27.47	117	-		-		-		-	
ITB – 049	25.14	503.36	-		-		-		-	
ITB – 050	35.6	0.7	-		-		-		-	
ITB – 053	-		-		-		34.64	0.15	-	
ITB – 054	35.92	0.52	-		-		33.36	0.4	-	
ITB – 055	31.47	8.97	-		-		-		-	
ITB – 056	35.61	0.63	-		-		-		-	

[$] Cq value for this sample was not calculated by qPCR software due to high background, however sample positive for CRCoV was based on the melt curve analysis, gel electrophoresis and sequencing.

(ii) Healthy dogs

Dog number	CnPnV		CRCoV		CHV-1		CAdV-2		CPiV	
	Cq value	Copies per µL	Cq value	Copies per µL	Cq value	Copies per µL	Cq value	Copies per µL	Cq value	Copies per µL
HEAL – 003	36.3	0.41	-		-		-		-	
HEAL – 007	37.13	0.24	-		-		-		-	
HEAL – 009	36.48	0.37	-		-		-		-	
HEAL – 011	36.16	0.45	-		-		-		-	
HEAL – 018	35.09	0.9	-		-		-		-	
HEAL – 024	33.9	0.58	-		-		-		-	
HEAL – 028	34.48	0.39	-		-		-		-	
HEAL – 036	33.8	0.6	-		-		30.88	6.33	-	
HEAL – 037	34.77	0.33	-		-		32.7	1.57	-	
HEAL – 038	-		-		-		33.28	1.26	-	
HEAL – 040	33.59	0.72	-		-		-		-	
HEAL – 041	33.65	0.67	-		-		-		-	
HEAL – 042	32.17	1.74	-		-		-		-	
HEAL – 046	35.98	0.49	-		-		-		-	
HEAL – 048	34.70	1.10	-		-		-		-	

5.3.2. Detection of respiratory viruses

Infections with at least one respiratory virus were detected in 21/56 (37.5%) dogs with signs of ITB and in 15/60 (25%) healthy dogs (*p* 0.16, 95%CI 0.75-4.33). Viruses identified in dogs with signs of ITB included CPiV, CHV-1, CAdV-2, CnPnV and CRCoV; whereas only CnPnV and CAdV-2 were identified in healthy dogs. The rate of detection of CnPnV and CAdV-2 in dogs with signs of ITB compared to the healthy dogs was not statistically significant (p = 0.675 and 0.479 respectively, Table 5.6). However, the level of CnPnV shedding in dogs with signs of ITB was higher compared to the healthy dogs (p = 0.02, 95%CI 0.13-1.42; Fig. 5.6).

Table 5.6. Frequency of detection of canine respiratory viruses in oropharyngeal swab samples from dogs with signs of infectious tracheobronchitis (ITB) and from healthy dogs, as determined by virus specific real-time PCR for canine pneumovirus (CnPnV), canine adenovirus type-2 (CAdV-2), canine parainfluenza virus (CPiV), canine herpesvirus type 1 (CHV-1) and canine respiratory coronavirus (CRCoV).

Virus	Total number (%) of dogs positive (*n=116*)	Number (%) of dogs with signs of ITB (*n=56*)	Number (%) of healthy dogs (*n=60*)	*p* value
CnPnV	29 (25)	15 (26.78)	14 (23.33)	0.675 (95%CI 0.47-3.04)
CAdV-2	8 (6.8)	5 (8.92)	3 (5.00)	0.479 (95%CI 0.34-12.52)
CPiV	2 (1.7)	2 (3.57)	0	0.230 (95%CI 0.20-∞)
CHV-1	2 (1.7)	2 (3.57)	0	0.230 (95%CI 0.20-∞)
CRCoV	1 (0.8)	1 (1.78)	0	0.482 (95%CI 0.02-∞)

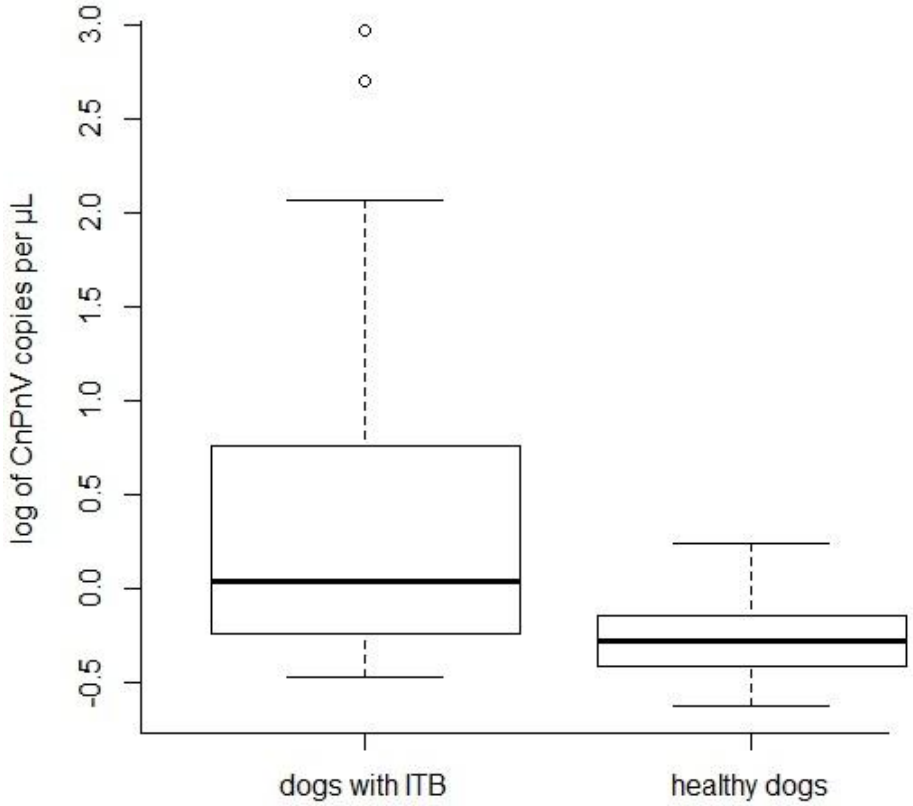


Figure 5.6. The box plot depicting the level of CnPnV shedding between dogs with signs of infectious tracheobronchitis (ITB) and healthy dogs. Y-axis shows log-transformed values of copies per µL of CnPnV obtained from dogs positive on real-time PCR.

Co-infection with more than one respiratory virus occurred in six dogs, out of which 4/56 (7.1%) dogs had signs of ITB and 2/60 (3.3%) were healthy dogs (Table 5.7).

Table 5.7. Detection of viral co-infections in dogs with signs of infectious tracheobronchitis (ITB) and in healthy dogs using virus specific real-time PCR for canine parainfluenza virus (CPiV), canine adenovirus type-2 (CAdV-2), canine herpesvirus type 1 (CHV-1), canine respiratory coronavirus (CRCoV) and canine pneumovirus (CnPnV).

Viruses	Number (%) of dogs with signs of ITB (*n = 56*)	Number (%) of healthy dogs (*n = 60*)
CnPnV and CHV-1	2 (3.5)	0
CnPnV and CAdV-2	1 (1.7)	2 (3.3)
CnPnV and CPiV	1 (1.7)	0
Two or more pathogens	4 (7.1)	2 (3.3)

No statistically significant difference was observed in the detection of respiratory viruses between dogs from private households and kenneled dogs (Table 5.8). Samples from 21/59 (35.5%) dogs from private households and 15/57 (26.3%) kenneled dogs were positive for one or more viruses (*p* 0.31, 95%CI 0.65-3.72). Among the dogs with ITB, 16/35 (45.7%) dogs from private households and 5/21 (23.8%) kenneled dogs were positive for at least one of the virus tested (Table 5.8). Among the healthy dogs, 5/24 (20.8%) from private households were positive for CnPnV, while 10/36 (27.7%) kenneled dogs were positive for either CAdV-2 or CnPnV or both (Table 5.8).

Table 5.8. Number of dogs from private households and kenneled dogs positive and negative for respiratory viruses tested.

Dogs	Number (%) of positive samples	Number (%) of negative samples	Total
Dogs with signs of ITB			
Private households	16 (45.7%)	19 (54.3%)	35
Kenneled dogs	5 (23.8%)	16 (76.2%)	21
Healthy dogs			
Private households	5 (20.8%)	19 (79.2%)	24
Kenneled dogs	10 (27.8%)	26 (72.2%)	36

When the analysis was performed comparing the prevalence of positive qPCR results for each virus among dogs from private households and kenneled dogs, no significant difference in the detection of viruses between the two groups were observed (Table 5.9).

Table 5.9. Frequency of detection of canine respiratory viruses in oropharyngeal swab samples of dogs from private households and kenneled dogs as determined by virus specific real-time PCR for canine pneumovirus (CnPnV), canine adenovirus type-2 (CAdV-2), canine parainfluenza virus (CPiV), canine herpesvirus type 1 (CHV-1) and canine respiratory coronavirus (CRCoV).

	Dogs from private households				Kenneled dogs			
Virus	**Total number (%) of dogs (*n= 59*)**	**Number (%) of dogs with signs of ITB (*n=35*)**	**Number (%) of healthy dogs (*n=24*)**	***p* value (95%CI)**	**Total number (%) of dogs (*n= 57*)**	**Number (%) of dogs with signs of ITB (*n=21*)**	**Number (%) of healthy dogs (*n=36*)**	***p* value (95%CI)**
CnPnV	15 (25.4)	10 (28.5)	5 (20.8)	0.55 (0.38-6.61)	14 (24.5)	5 (23.8)	9 (25)	1.00 (0.20-3.81)
CAdV-2	5 (8.4)	5 (14.2)	0	0.07 (0.65-∞)	3 (5.2)	0	3 (8.3)	0.28 (0-4.13)
CPiV	1 (1.6)	1 (2.8)	0	1.00 (0.01-∞)	1 (1.7)	1 (4.7)	0	0.36 (0.04-∞)
CHV-1	1 (1.6)	1 (2.8)	0	1.00 (0.01-∞)	1 (1.7)	1 (4.7)	0	0.36 (0.04-∞)
CRCoV	1 (1.6)	1 (2.8)	0	1.00 (0.01-∞)	0	0	0	-

5.4. Discussion

This real-time PCR study confirmed the presence of CPiV, CAdV-2, CHV-1, CRCoV and CnPnV in dogs in New Zealand.

In this study, a total of 21/56 (37.5%) dogs with signs of ITB were positive for atleast one of the canine respiratory viruses, with the most prevalent being CnPnV (26.78%) followed by CAdV-2 (8.92%), CPiV (3.57%), CHV-1 (3.57%) and CRCoV (1.78%). In the previous study by Sowman *et al.* (2013), neither CnPnV nor CRCoV were detected in New Zealand dogs with respiratory disease. Thus, the present study has widened our

understanding of the possible involvement of recently described pathogens (CnPnV and CRCoV) in canine respiratory disease in New Zealand.

CnPnV was found in 26.78% of dogs with signs of ITB, but was also found in 23.33% of healthy dogs. The association between the presence of CnPnV and canine respiratory disease was not statistically significant (p = 0.675), however, previous research work has demonstrated a strong association between CnPnV and respiratory disease in dogs (Mitchell *et al.*, 2013). It may be possible that CnPnV-positive healthy dogs were infected with CnPnV but may have remained asymptomatic at the time of sample collection. This was not ruled out as the information regarding exposure of healthy dogs to ITB-affected dogs was not obtained in the current study.

Although CnPnV was detected in the oropharyngeal swabs of both groups, the dogs with signs of ITB shed significantly more virus compared to healthy dogs (p = 0.020, Fig. 5.6). It was seen that three ITB affected dogs positive for CnPnV shed a high copy number of virus. But this does not prove the causality of CnPnV in canine ITB cases in the study population. It may be possible that the respiratory mucosal damage was primarily caused by other ITB pathogen, and the presence of CnPnV may have exacerbated the disease leading to an increased shedding in these dogs.

Most of the healthy dogs showed lower levels of CnPnV shedding in oropharyngeal swabs. The current study could not ruled out the possibility that PCR detection of CnPnV preceded clinical infection i.e. virus shedding may have occurred during the incubation period in these healthy dogs. Follow-up of these healthy dogs after sample collection could have helped to determine whether these dogs remained clinically healthy. Alternatively, the presence of virus in healthy dogs may also indicate true subclinical infection. This may imply that low virus levels tigger at most, a minor

inflammatory response without causing abnormal respiratory signs. In addition, it is also possible that CnPnV detected in the oropharyngeal swabs of these healthy dogs may have been inhaled or ingested and passed into the oropharyngeal region without replicating in these dogs. The clinical revelence of these findings remains unclear and should be considered in further investigations.

CRCoV was detected in only one out of 116 dogs (ITB-046, Table 5.5). The dog in question showed signs of ITB, and was from a private household (Table 5.9). The dog was not co-infected with any other canine respiratory virus evaluated, suggesting its possible involvement in canine ITB. The findings in the present study, although a relatively small number of dogs were tested, are consistent with those reported by Yachi and Mochizuki (2006) and Erles and Brownlie (2005) who also confirmed low molecular prevalence of CRCoV among dog populations in Japan and the UK. In all these studies, including the present study, the samples were collected from either oropharyngeal or tonsillar region which may have not been the ideal site for detection of CRCoV. It is possible that, the detection of CRCoV may have failed due to insufficient number of CRCoV infected cells present in these samples. Thus, obtaining bronchial lavage or nasal washings could have given better results, however, this was beyond the scope of the current study.

The presence of CAdV-2 was confirmed in this study, with an overall prevalence of 6.8% in dogs in New Zealand. In this study, due to the unavailability of field CAdV-2 culture, a vaccine CAdV-2 was used for the development of real-time PCR assay. Thus this PCR could not differentiate between the field and vaccine CAdV-2 virus. The present study, however, was conducted on the samples collected from dogs having no history of recent vaccination. Hence, CAdV-2 detected in the study population may represent a field virus, but requires further clarification.

The overall prevalence of CPiV was low (1.7%) and was detected only in dogs with signs of ITB. This finding is in contrast to those reported in other recent research studies that showed a high prevalence of CPiV in dogs with respiratory infections (Mochizuki *et al.*, 2008, Schulz *et al.*, 2014b, Decaro *et al.*, 2016, Joffe *et al.*, 2016). It is possible that extensive vaccination programs against CPiV among the New Zealand dog population may have reduced the prevalence of CPiV in dogs in New Zealand.

Only two dogs were positive for CHV-1 (1.7%), and both the dogs showed ITB signs and were also co-infected with CnPnV (Table 5.7). It is documented that CHV-1 is present in the tissues of healthy dogs and can be re-activated during the stress caused by other viral and bacterial infections in the course of the disease (Burr *et al.*, 1996). In the present study, it is possible that CHV-1 may have been re-activated due to ITB caused by either CnPnV or other bacterial pathogens.

There are several limitations in the present study. Most importantly, the samples tested for viral pathogens were collected from the oropharynx region. Hence, there is a possibility of not detecting viruses present in the other regions of respiratory tract (nasal region and trachea). Kaczorek *et al.* (2017) showed varying prevalence of respiratory viruses in the samples collected from tracheal lavage and swabs (obtained from nasal passage, laryngeal region and tracheal orifice) from the same dogs. Therefore, it is ideal to collect samples from different areas of respiratory tract to ensure appropriate detection of the causative agents involved in canine ITB whenever possible.

Secondly, variation in the timings of sample collection may have resulted in obtaining a low viral prevalence in canine ITB cases. The samples were collected from the dogs with duration ranging from 1 to 14 days (mean = 5.14) after the onset of clinical signs; and it is likely that in some dogs, casual virus may have cease to shed by the time of

sampling. It is also possible that viruses may have acted as a predisposing factor facilitating respiratory infection and the clinical signs may have arisen due to a secondary bacterial infection rather than the primary viral infection. Hence, the samples collected later after the onset of clinical disease may not have detected primary pathogen.

Thirdly, the present study did not evaluate the presence of bacteria such as *B. bronchiseptica*, *M. cynos* and *Streptococcus zooepidemicus* in the clinical samples. Thus bacterial involvement in dogs with ITB tested in the present study could not be established. Future investigation into canine respiratory disease should also consider detection of these pathogens. Furthermore, viral sequences of influenza C virus and CanPV were detected by shot-gun sequencing (Chapter 4). However, due to the unavailability of control viruses for the development of real-time PCR assays, individual samples could not be tested for the presence of these two viruses. Hence, the prevalence of influenza C virus and CanPV in the study population was not determined. Finally, the sample size used in the present study was small, and may have resulted in a biased or inaccurate estimate of true prevalence. Future studies should investigate large sample sizes from dogs housed in groups or from sheltered environments, and also from different locations to understand the true prevalence of respiratory pathogens among dogs in New Zealand.

In conclusion, the study highlights the presence of recently described viruses such as CRCoV and CnPnV in dogs with respiratory disease. This suggests that these viruses may have a role in the pathogenesis of respiratory disease in dogs in New Zealand.

In order to better understand the recently identified viruses (CRCoV and CnPnV), studies were undertaken for isolation (Chapter 6) and molecular characterization of

CnPnV (Chapter 7) and CRCoV (Chapter 8) circulating among dogs in New Zealand, thereby addressing the need for specific vaccines.

Chapter 6: VIRUS ISOLATION OF CRCoV and CnPnV

6.1. Introduction

CRCoV and CnPnV are two examples of recently discovered canine respiratory viruses. In Chapter 4, the first molecular evidence for the presence of these viruses in New Zealand was described. In order to perform further studies, *in vitro* isolation of these viruses were attempted in this chapter.

To date, isolation of CRCoV has only been successful in the HRT-18 cell line and its clone HRT-18G (Erles *et al.*, 2007, An *et al.*, 2010a). However, other cell lines including A-72, BHK-21, CHO, DH82, MDBK and MDCK failed to propagate CRCoV (Erles *et al.*, 2007). The CRCoV propagates on HRT-18 cell lines without producing visible CPE; and hence, other methods such as hemagglutination and PCR have to be applied to confirm its growth (Erles *et al.*, 2007).

There is limited literature available on *in vitro* growth requirements of CnPnV. So far, the virus has only been successfully isolated in A-72 cell lines (Renshaw *et al.*, 2010). However, other authors failed to isolate CnPnV from lung tissue of a dog positive for CnPnV on real-time PCR using the same cell line (Decaro *et al.*, 2014a). Hence, attempts should be made to isolate CnPnV on different cell lines until the ideal cell type has been established.

Sequence analysis of three regions located in N and L gene of CnPnV suggested that CnPnV is closely related to MPV (Renshaw *et al.*, 2010). CnPnV has also shown to efficiently replicate in the lung tissue of mice following experimental infection (Percopo *et al.*, 2011). This suggests that the cell lines that support the growth of MPV might also support growth of CnPnV. Hence, different cell lines such as mouse

monocyte-macrophage (RAW 264.7) cell line (established from a tumor induced by the Abelson murine leukemia virus) and Vero were used to isolate CnPnV.

In this chapter, attempts were undertaken to isolate CRCoV and CnPnV from the oropharyngeal swab samples of dogs that were positive for CRCoV and CnPnV based on real-time PCR (Chapter 5).

6.2. Materials and methods

6.2.1. Cell culture

The three cell lines routinely used in the study, HRT-18 cell lines for CRCoV isolation, and RAW 264.7 and Vero cell lines for CnPnV isolation, were commercially obtained from American Type Culture Collection (ATCC, Manassas, USA). Throughout the study, standard laboratory procedures were used for propagation and maintenance of cell lines (Freshney, 2005).

6.2.1.1. Media

The cell lines were grown in Advanced Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, Missouri, USA) supplemented with 2% v/v fetal bovine serum (FBS) (Gibco™ Life technologies, New York, USA), 1% v/v antibiotic solution (5000 U/mL of penicillin and 5000 µg/mL streptomycin) (Thermo Fisher, Waltham, USA), and 1% v/v glutamine (Gibco™ Life technologies, New York, USA). The maintenance medium where used, was the same as the growth medium, with the exception that it contained 1% v/v FBS.

6.2.1.2. Sub-culturing of cells

The cells were routinely sub-cultured twice weekly when they were 90% confluent. The cells were maintained in 75 cm^2 cell culture flasks (T75, Corning). The monolayers were washed thrice with warm PBS. Washed cells were trypsinized using 500 µL of

TrypLE Express (Gibco™ Life technologies, New York, USA) and incubated at 37 °C for 2-3 min. After cells detached from the flask, they were re-suspended in an appropriate volume of fresh pre-warmed growth media and seeded at 1:5 split ratio. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

6.2.1.3. Cryopreservation of cells

The cells, maintained in T75 flasks, were checked for confluence and absence of bacterial or fungal contamination. The cells were then washed with warm PBS thrice, trypsinized and re-suspended in 10 mL of the growth medium. The cells were pelleted by centrifugation at 300 × g for 10 min and re-suspended in the freezing medium (advanced DMEM with 20% v/v FBS and 10% v/v DMSO) at a concentration of 1 × 10^7 cells per mL. The cell suspension was dispensed into 1.5 mL cryo-vials (Greiner Bio-One, Kremsmünster, Austria), cooled slowly at 1 °C per min in a freezing container (BioCision CoolCell®, San Rafael, USA) in -80 °C freezer, before the vials were placed in liquid nitrogen for long term storage.

6.2.2. Sample processing for virus isolation

Samples were collected and processed as described in Section 3.1.5. Only samples from dogs with ITB positive for CRCoV or CnPnV by real-time PCR (Chapter 5) were used for virus isolation. These included one sample (sample ID: ITB-046) that was positive for CRCoV, and 15 samples (sample ID: ITB-004, -006, -007, -020, -025, -026, -030, -031, -036, -048, -049, -050, -054, -055 and -056) positive for CnPnV. Each aliquot (~500 µL) of oropharyngeal swab sample was thawed, filtered using 0.2 µM syringe filter (Membrane solutions®, Kent, USA) and dispensed into a new sterile Eppendorf tube.

6.2.3. Virus isolation

For virus isolation, separate 25 cm^2 culture flasks (Corning® Sigma-Aldrich, Missouri, USA) were seeded with HRT-18, RAW 264.7 and Vero cell line at 1×10^6 cells. The flasks were used for virus isolation when monolayers were approximately 70% confluent, usually following a day of seeding.

For CRCoV isolation, HRT-18 cell monolayers were washed once with plain advanced DMEM containing 1 µg/mL trypsin (Gibco™ Life technologies, New York, USA). Approximately 500 µL of oropharyngeal filtrate from sample ITB-046 was supplemented with trypsin to a final concentration of 1 µg/mL and absorbed onto the washed cell monolayer for 1 hr at 37 °C in 5% CO_2. After incubation, the inoculum was removed and the cells were maintained in 8 ml of the maintenance medium containing 1 µg/mL trypsin (Gibco™ Life technologies, New York, USA).

For isolation of CnPnV, the monolayers of RAW 264.7 and Vero cells were washed with plain advanced DMEM. Approximately 500 µL of oropharyngeal filtrate was absorbed onto inoculated to the washed cell monolayer for 1 hr at 37 °C in 5% CO_2. The inoculum was then removed and the cells were maintained in 8 mL of maintenance medium.

All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 5-6 days and observed twice daily for the presence of viral CPE. After 5-6 days the infected cell cultures were freeze-thawed thrice and 100 µL of cell lysates from first passage (P1) were transferred to the new T25 flask at the time of seeding (P2). The cultures were routinely passaged one more time in the similar fashion (P3). Additional passages were occasionally performed if results of confirmatory testing (section 6.2.4.) were

unclear. Un-inoculated controls of HRT-18, RAW 264.7 and Vero cells were maintained in parallel throughout the virus isolation procedure as negative controls.

6.2.4. Virus detection

CRCoV

The cell lysate from the P3 culture was used as a template for virus-specific qPCR. In addition, the P4 lysate was subjected to hemagglutination (HA) test. Cultures were considered positive for CRCoV if both qPCR and HA tests were positive.

CnPnV

The cell lysates from the P3 cultures were used as a template for virus-specific qPCR. Samples were considered positive for CnPnV when the P3 cell lysates were positive for CnPnV RNA by qPCR.

6.2.4.1. Viral nucleic acid extraction and qPCR

After freeze-thawing, 200 µl of infected cell lysates from P3 were used for viral nucleic acid extraction using High Pure Viral Nucleic acid Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). The extraction was carried out according to manufacturer's instructions. RNA was transcribed into cDNA using qSCRIPT™ (Quanta Bioscience, Gaithersburg, USA).

The qPCR assays for CRCoV and CnPnV were performed as described in Chapter 5.

6.2.4.2. Haemagglutination test for CRCoV

The cell lysates from P3 that were positive on qPCR were passaged further on HRT-18 cells. The freeze-thawed cell lysates from the forth passage (P4) were used for HA tests.

Preparation of red blood cells

Fresh chicken blood was collected and diluted 1:2 in Alsever's solution (Sigma-Aldrich, Missouri, USA). Red blood cells were pelleted by centrifugation at 1200 × g for 10 min

and washed three times in PBS. During each wash the cells were gently re-suspended in 10 mL of PBS and then pelleted by centrifugation at 2000 rpm for 10 min. Following the final wash, packed RBCs were used to prepare 1% RBC suspension in PBS. The remaining cells were mixed with Alsever's solution to the final concentration of 10% v/v for later use. Cells stored in Alsever's solution were kept at 4 °C for one week and then discarded.

HA procedure

The freeze thawed cell lysates from P4 passage were used for HA. Two-fold dilutions of cell lysates (50 µL volume) in PBS were made in a microtiter plate (V-bottom) in duplicates. Fifty microliters of 1% RBC suspension was then added to each well and the plate was incubated at 4 °C until HA was observed in control wells. The positive control consisted of bovine coronavirus. Negative controls consisted of cell lysate from non-inoculated HRT-18 cells. All samples that produced a clear pellet of sedimented RBC (button formation) were considered negative.

6.3. Results

6.3.1. Virus isolation

CRCoV

The P3 cell lysates of ITB-046 showed ambiguous results for the presence of CRCoV on qPCR, as no amplification curve was obtained, but the melting peak was similar to the ITB-046 sample (Fig 6.1). The P3 cell lysates were further passaged (P4) to confirm CRCoV growth using HA test. However, the P4 cell lysate of ITB-046 did not elicit hemagglutination with poultry RBCs, indicating a very low titer or failure of CRCoV to grow in the cell culture. The CRCoV qPCR performed on the P4 cell lysate of ITB-046 showed negative results.

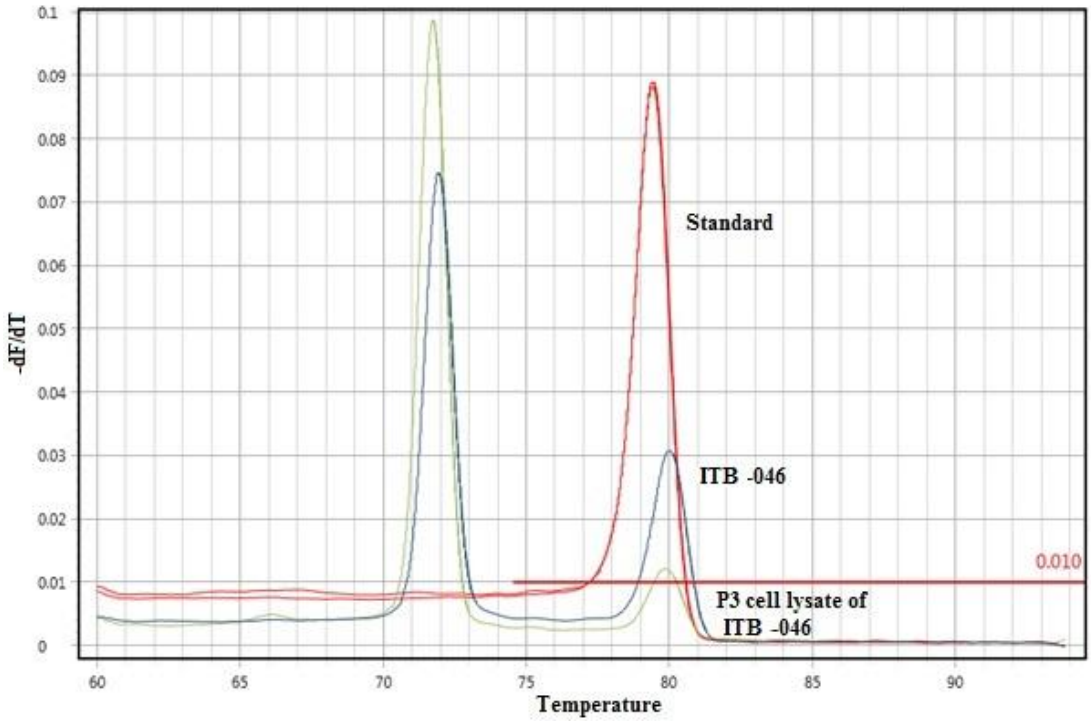


Figure 6.1. Canine respiratory coronavirus real-time PCR melting curve analysis of standard (10^5 dilution of plasmid), positive sample (ITB-046) and P3 cell lysate of ITB-046. The melting peak of the standard (red curve) was at 79.44 °C, ITB-046 (blue curve) at 79.98 °C and P3 cell lysate of ITB-046 (light green curve) at 79.90 °C.

CnPnV

The P3 cell lysates of ITB-004, -006, -007, -020, -025, -026, -030, -031, -036, -048, -049, -050, -054, -055 and -056 were negative for the presence of CnPnV based on qPCR.

6.4. Discussion

In this study, the attempts to isolate CRCoV from the oropharyngeal swab sample, which was positive for CRCoV RNA by qPCR, was unsuccessful. This is consistent with several previous researchers who were also unsuccessful in propagating CRCoV in HRT-18 cells from samples that were previously confirmed to be positive by PCR (Decaro *et al.*, 2007, Lorusso *et al.*, 2009, Lu *et al.*, 2017, Yachi and Mochizuki, 2006). This indicates that other factors may play a role in successful isolation of CRCoV *in vitro*. Optimal culture conditions are required for successful isolation of CRCoV which

may not have been used in the present study. Alternatively, it is possible that CRCoV may have become inactivated during the transportation or storage of oropharyngeal swab samples.

Our attempts to isolate CnPnV in RAW 264.7 and in Vero cell lines were also unsuccessful. RAW 264.7 cells have previously been successfully used for propagating MPV (Dyer *et al.*, 2007). However, failure of CnPnV to replicate in these cell lines may indicate that CnPnV lacks appropriate receptors on these cell lines. Alternatively, it is possible that CnPnV may have got inactivated during sample collection. However, that seems less plausible since several disparate samples were tested. So far, CnPnV has been successfully isolated only on A-72 cell lines (Renshaw *et al.*, 2010). Thus, future attempts to propagate New Zealand CnPnV should be carried out using the A-72 cell line.

Chapter 7: GENOTYPING OF CANINE PNEUMOVIRUS FROM NEW ZEALAND

7.1. Introduction

Canine pneumovirus is an enveloped, negative sense, single-stranded RNA virus classified within the family *Pneumoviridae*, subfamily *Pneumovirinae* and genus *Pneumovirus* (Renshaw *et al.*, 2010). CnPnV was first identified during a diagnostic investigation of acute infectious respiratory disease in dog breeding colonies in the USA (Renshaw *et al.*, 2011, Renshaw *et al.*, 2010). Since the initial report, the virus has been detected during an outbreak of canine respiratory disease in Italy (Decaro *et al.*, 2014b) as well as in kenneled dogs in the UK and the Republic of Ireland (Mitchell *et al.*, 2013).

The attachment (G) protein of pneumoviruses is responsible for viral attachment to the host cell receptor and is a major target for neutralizing antibodies (Ling and Pringle, 1989, Levine *et al.*, 1987). Two distinct groups (group A and B) of CnPnV have been described based on sequence variation in the G glycoprotein gene of several CnPnV sequences from the USA (Glineur *et al.*, 2013).

In Chapter 4, the first molecular evidence for the presence of CnPnV in New Zealand was described using shot-gun sequencing. The detection of CnPnV in 25% of 116 oropharyngeal swab samples (Chapter 5) suggests that CnPnV infections are common among dogs in New Zealand. In order to further understand the CnPnV circulating in dogs in New Zealand, the aim of the current study was to characterise New Zealand CnPnVs based on the complete G gene sequence.

7.2. Materials and methods

Amplification of glycoprotein G gene was attempted from all 15 samples from dogs showing signs of ITB that were positive for CnPnV on real-time PCR (Table 5.5, Chapter 5).

7.2.1. cDNA synthesis

The total nucleic acids from CnPnV positive samples were reverse transcribed using Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics GmbH, Basel, Switzerland). 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 min, 55 °C for 30 min and 85 °C for 5 min.

7.2.2. Amplification and sequencing of CnPnV G gene

The CnPnV G glycoprotein gene was amplified using overlapping primers previously described by Glineur *et al.* (2013) (Table 7.1).

Table 7.1. Primers used for sequencing the G glycoprotein gene of canine pneumovirus (Glineur *et al.*, 2013).

Primer	Primer sequence	Position on CnPnV Brne-17 (GenBank no. GU247050.2)	Product size
PVM-SH204	5'-CAATCACCCTCCACCAAGTTATG-3'	4307-4329	952
PVM-G662R	5'-AACCAGAAAGGAAGAAACAGAAGC-3'	5236-5259	
PVM-G547F	5'-AACATGGGCTTAAACAATGGTGG-3'	5121-5143	1063
PVM-F311R	5'-AGCCCCGAGACCAAGAATCAAAC-3'	6162-6184	

In order to amplify the target sequences, a high fidelity KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, USA) was used. 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 and 0.3 mM of each forward and reverse primer. The cycling conditions for reactions with primers PVM-SH204F and PVM-G662R were as follows: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 98 °C for 20 sec, annealing at 67 °C for 15 sec and extension at 72 °C for 1 min followed by final extension of 72 °C for 1 min. The cycling conditions for reactions with primers PVM-G547F and PVM-F311R were as above, except that the annealing temperature was 64 °C. PCR products were subjected to electrophoresis through a 1% ethidium bromide stained agarose gel (Axygen Inc., USA). The PCR bands were visualized using a GelDoc reader (Bio-Rad, Hercules, CA, USA) and the bands corresponding to the expected product size were cut from the gel, placed on the top of a 200 µL filtered pipette tip that had approximately half of its tip cut off and had been placed in a sterile microtube. The microtube was snap frozen in liquid nitrogen. DNA was recovered at the bottom of the microtube following centrifugation at 13,000 × g for 5 min at room temperature. The gel remnants trapped in the filter were discarded and the DNA-containing elute was stored at 4 °C for sequencing. The purified PCR products were sequenced directly using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster city, USA) on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster city, USA). Obtained sequences were assembled and analyzed using Geneious version 10.2.3. (Kearse *et al.*, 2012).

7.2.3. Phylogenetic analysis

The complete nucleotide and amino acid sequences of the G gene of New Zealand CnPnVs were aligned with 12 CnPnV and two MPV sequences using multiple sequence alignment in Geneious version 10.2.3. (Kearse *et al.*, 2012). The 12 CnPnV sequences from dogs, including CnPnV dog/Bari/100-12/ITA/2012 (KF015281.1), CnPnV

dog/Ane4/USA/2008 (HQ734815.1) and ten CnPnV sequences from the USA (KC495958.1-KC495967.1); and two from rodent species MPV-15 (AY729016.1) and MPV-J3666 (NC_006579.1) were then used for building a phylogenetic tree. Maximum likelihood analysis was performed using the PhyML package (Guindon *et al.*, 2010) based on TN93 substitution model (Tamura and Nei, 1993) in the Geneious version 10.2.3, with 100 bootstrap replicates.

7.3. Results

Out of the samples positive for CnPnV, glycoprotein G gene was amplified only from three samples that had > 10 copies of CnPnV per µL of template based on qPCR analysis (Table 5.5, Chapter 5). These samples were from following dogs: ITB-007, ITB-048 and ITB-049. The signalment and clinical history for these three dogs are presented in Appendix D.

7.3.1. Sequence analysis of the G gene

Three complete nucleotide sequences of the G gene of New Zealand CnPnV (CnPnV NZ-007, CnPnV NZ-048 and CnPnV NZ-049) were obtained and used for comparative study. The complete sequences of the G gene of CnPnV NZ-007, CnPnV NZ-048 and CnPnV NZ-049 are given in Appendix G. These sequences are submitted in the GenBank with accession number: MK121747 for CnPnV NZ-007, MK121748 for CnPnV NZ-048 and MK121749 for CnPnV NZ-049.

The multiple nucleotide sequence alignment of CnPnV NZ-007, CnPnV NZ-048 and CnPnV NZ-049 with murine pneumovirus MPV-15 (AY729016.1), MPV- J3666 (NC_006579.1); CnPnV sequences from the USA (KC495958.1-KC495967.1), CnPnV Ane4 (HQ734815.1) and Italian CnPnV Bari/100-12 (KF015281.1) is given in Appendix H. Similar to CnPnVs from the USA; all three CnPnVs from New Zealand

also displayed 18 additional N-terminal amino acids compared to MPV-J3666 (Fig. 7.1). Of the three CnPnV G amino acid sequences, both CnPnV NZ-048 and CnPnV NZ-049 had 100% nucleotide identity with each other, while CnPnV NZ-007 had 89.5% nucleotide identity with both CnPnV NZ-048 and 049 sequences (Table 7.2). The G gene of CnPnV NZ-007 was 1266 nt long encoding 421 aa; while the G gene of CnPnV NZ-048 and 049 were 1245 nt long each and encoded 414 aa, similar to other CnPnV sequences deposited in the GenBank, except for CnPnV 126724-10-4DC which was 419 aa long. Different length of the G protein sequence was due to changes in the stop codon usage. The G proteins of 414 and 421 residue were observed corresponding to the stop codon located at nt 1243 (UAA) and nt 1264 (UAA), respectively. CnPnV NZ-048 and CnPnV NZ-049 used stop codon located at nt 1243, while CnPnV NZ-007 used stop codon located at nt 1264.

Table 7.2. Nucleotide (nt) and amino acid (aa) sequence identity of G protein of canine pneumovirus (CnPnV) NZ-048, -049 and -007 with other canine pneumovirus and murine pneumovirus sequences. CnPnVs belonging to the same group are highlighted with the same color: group A CnPnVs in pink and group B CnPnVs in blue.

Sequences	CnPnV NZ-048 and CnPnV NZ-049		CnPnV NZ-007	
	nt identity (%)	aa identity (%)	nt identity (%)	aa identity (%)
CnPnV NZ-048, -049	-	-	90.92	89.37
CnPnV-Ane4	96.15	96.62	91.73	90.58
CnPnV Bari-100/12	97.83	96.86	91.89	90.58
CnPnV114378-10-29KY	97.99	97.10	91.41	90.10
CnPnV 91065-11MA	97.83	97.83	91.49	89.86
CnPnV 56706-09NYC	96.07	96.38	91.65	90.34
CnPnV 13505-11OH	95.67	95.89	91.65	90.58
CnPnV 142847-10NV	95.58	95.89	91.49	90.10
CnPnV 86842-09PA	91.81	91.06	95.42	95.89
CnPnV 109594-10KS	91.33	89.13	95.10	93.96
CnPnV 7963-11OK	91.08	88.41	95.50	94.93
CnPnV 110230-11TX	91.00	88.65	95.26	94.93
CnPnV 126724-10-4DC	90.01	88.89	96.84	96.66
CnPnV NZ-007	89.57	89.37	-	-
MPV-15	80.81	91.74	93.50	91.46
MPV-J3666	88.23	91.41	93.62	91.67

7.3.2. Phylogenetic analysis of the G gene

Based on phylogenetic analysis of the G gene, CnPnV NZ-007, CnPnV NZ-048 and CnPnV NZ-049 clustered with CnPnV sequences from other geographical regions (Fig. 7.2). The phylogenetic tree of CnPnV was branched into group A and group B. The CnPnV NZ-048 and CnPnV NZ-049 belonged to group A, while CnPnV NZ-007 belonged to group B.

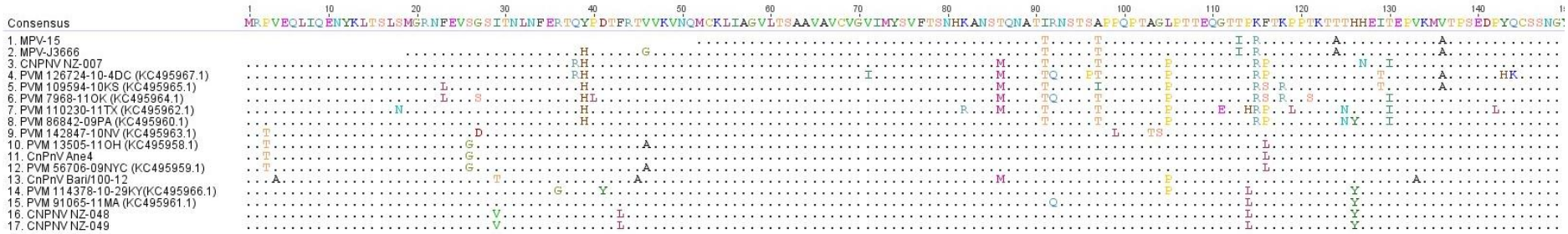


Figure 7.1. Comparison of N-termini of G protein sequences of canine pneumovirus (CnPnV) NZ-007, CnPnV NZ-048 and CnPnV NZ-049 with murine pneumovirus MPV-15 (AY729016.1), MPV- J3666 (NC_006579.1); CnPnV sequences from the USA (KC495958.1-KC495967.1), CnPnV Ane4 (HQ734815.1) and Italian CnPnV Bari/100-12 (KF015281.1). Dots represent 100 % identity with the consensus sequence.

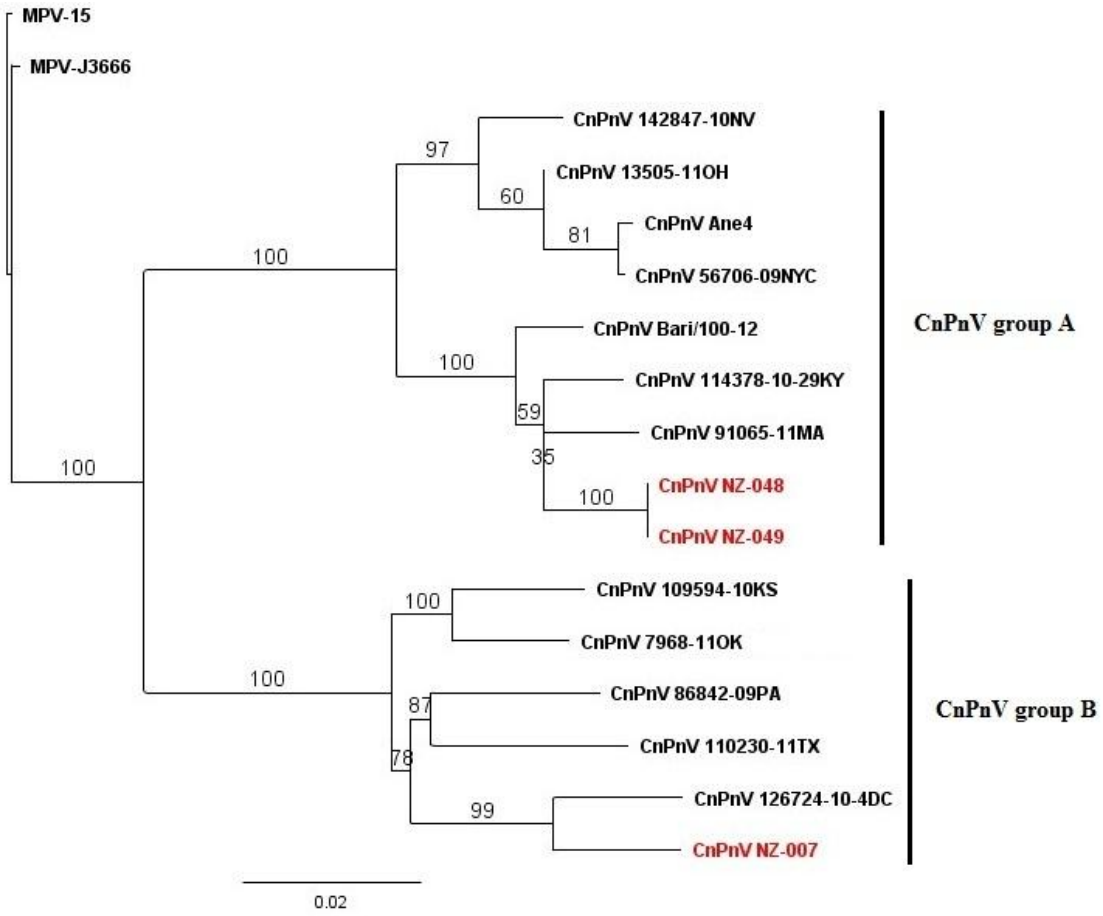


Figure 7.2. Phylogenetic analysis of nucleotide sequence (1245 nt) of the G gene of canine pneumovirus (CnPnV) NZ-007 (1266 nt), CnPnV NZ-048 and CnPnV NZ-049 with murine pneumovirus MPV-15 (AY729016.1) and MPV-J3666 (NC_006579.1), CnPnV Ane4 (HQ734815.1) and other ten CnPnV sequences from the USA (KC495958.1-KC495967.1) and Italian CnPnV Bari/100-12 (KF015281.1). The tree was inferred using the Maximum Likelihood method with a 100 bootstrap value, using PhyML package. Numbers at the nodes depict % of bootstrap support.

7.4. Discussion

The research work presented in this chapter describes the G gene sequence analysis of CnPnVs obtained from three New Zealand dogs. The aim of this study was to sequence the complete G gene from all the samples positive for CnPnV from dogs with signs of ITB. However, due to low copies of CnPnV template present in most of the samples, only three complete G gene sequences (CnPnV NZ-007, CnPnV NZ-048 and CnPnV NZ-049) were obtained for analysis.

Genetic analysis showed considerable amount of variations in the G nucleotide sequences of CnPnV NZ-007 and CnPnV NZ-048/049 which resulted in translation of different amino acids (Fig. 7.1). Similar genetic variations in the G protein sequence has been observed in other members of the *Pneumovirinae* subfamily (Johnson *et al.*, 1987). This suggests that CnPnV, like other pneumoviruses, has a tendency to acquire genetic mutations at a high rate.

The CnPnVs from New Zealand showed different length of predicted G protein due to different positions of the stop codon. Similar findings were also reported in the G gene sequences of hRSV (Bastien *et al.*, 2004). In hRSV, the G protein is responsible for neutralizing protective immunity (Rueda *et al.*, 1991). The role of G protein in CnPnV has not yet been clarified. Assuming that the G protein in CnPnV may have similar function as that of hRSV, the nucleotide variation leading to amino acid changes may have a selective advantage to the G protein changes in CnPnV.

In this study, phylogenetically distinct groups (group A and group B) were observed in the CnPnV sequences obtained from New Zealand dogs. These findings were consistent with the findings reported by Glineur *et al.* (2013). However, it is not clear whether or not any differences exist between the pathogenicity of CnPnV from groups A and B. Glineur *et al.* (2013) demonstrated that experimental inoculation of CnPnV 114378-10-29-KY-F (group A) and CnPnV 7968-11-OK (group B) in BALB/c mice produced similar pathogenesis with varying levels of cytokine response. Further studies are required to understand the pathogenicity of CnPnV from the two groups in detail.

Close similarity of CnPnV with the rodent viruses, MPV-15 and MPV-J3666 (Fig. 7.2), suggests a possibility that CnPnV may have evolved from rodent pneumoviruses. However, due to the limited number of CnPnV sequences available in the GenBank, it

cannot be ruled out whether CnPnV and MPV co-evolved independently or that both viruses had a common ancestor. This could not be understood in the present study, as it is unknown whether or not MPV is present in New Zealand.

In conclusion, group A and group B CnPnVs are present among New Zealand dog population. More sequences from different geographical regions are required to understand the genetic variability of CnPnV.

Chapter 8: GENETIC CHARACTERIZATION OF CANINE RESPIRATORY CORONAVIRUS DETECTED IN NEW ZEALAND

8.1. Introduction

Canine respiratory coronavirus is an enveloped positive sense, single-stranded RNA virus classified within *Betacoronavirus* genus in *Betacoronavirus 1* species (Erles *et al.*, 2003). Other viruses included in this species are BCoV, HCoV-OC43, equine coronavirus, human enteric coronavirus (HECoV) and porcine hemagglutinating encephalomyelitis virus (King *et al.*, 2011). Canine respiratory coronavirus was first detected in samples collected from dogs from a large re-homing center in the UK suffering from severe respiratory disease in 2003 (Erles *et al.*, 2003). Since then, the CRCoV has been identified in other geographical areas including Italy, Korea, Japan and China (Soma *et al.*, 2008, Lorusso *et al.*, 2009, An *et al.*, 2010a, Hong *et al.*, 2015).

The genomic organization of betacoronaviruses has already been described in detail in Chapter 2 (section 2.3.2.1). The 3' genomic region of viruses from *Betacoronavirus 1* species includes several structural protein genes: hemagglutinin esterase (HE), spike (S), envelope (E), membrane (M) and nucleocapsid (N) genes (Sturman and Holmes, 1983, Murphy *et al.*, 2012). In addition, interspersed among the structural genes are accessory genes; one coding for a 32.2 kDa protein located between the replicase gene and HE gene, two or more accessory genes located between the S gene and E genes, and one coding for internal (I) protein inserted within the N gene (Chapter 2, Fig. 2.1). To date, only two complete CRCoV genome sequences from dogs in Korea (Lim *et al.*, 2013) and China (Lu *et al.*, 2017); and entire 3' genomic region of CRCoV sequences from the UK (Erles *et al.*, 2003), Italy (Lorusso *et al.*, 2009) and Korea (An *et al.*, 2010a) are publically available.

Comparison of all available CRCoV sequences revealed a high genetic similarity at the regions coding for major structural and non-structural proteins, with the exception of the region between the S and E genes which appears to be variable (Erles *et al.*, 2007, Lorusso *et al.*, 2009, An *et al.*, 2010a). The accessory proteins predicted to be encoded in this region by different CRCoV genomes are shown in Figure 2.2 (Chapter 2). CRCoV genome from a dog with respiratory disease from one kennel in the UK (CRCoV-4182) was predicted to encode two accessory proteins, 8.8 kDa and 12.8 kDa in size (Erles *et al.*, 2007), while CRCoVs from another dog from the UK (CRCoV-G9142), Italy (CRCoV-240/05), China (CRCoV BJ232) and Korea (CRCoV-K9, -K37 and -K39), were predicted to encode three accessory proteins, 4.9 kDa, 2.7 kDa and 12.7 kDa in size, within the corresponding genomic region (Lorusso *et al.*, 2009, An *et al.*, 2010a, Lu *et al.*, 2017).

Although the presence of CRCoV in New Zealand dog population has been demonstrated based on serology (Knesl *et al.*, 2009), the virus has never been isolated in New Zealand and subsequently, no research has been carried out to characterise the CRCoVs circulating in this country. In the present study, CRCoV sequences were detected in the pooled oropharyngeal swab sample of dogs with signs of ITB using metagenomic shot-gun sequencing (Chapter 4). It appeared that CRCoV sequences detected by shot-gun sequencing originated from only one sample based on qPCR analysis of individual swab samples that contributed to the pool (Chapter 5).

The aim of the research presented in this chapter was to determine whether CRCoV identified in New Zealand was similar to CRCoVs from other geographical areas. In order to achieve this, the entire 3’ genomic end of New Zealand CRCoV was determined and analyzed including prediction of the coding regions and phylogeny.

8.2. Materials and methods

8.2.1. cDNA synthesis

Total nucleic acids extracted from the ITB-046 oropharyngeal swab sample using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Basel, Switzerland) (Chapter 5) comprised the starting material for the study. In order to obtain long cDNA fragments for amplification of large PCR products, RNA was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics GmbH, Basel, Switzerland). 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 $^{\circ}$C for 10 min, 55 $^{\circ}$C for 30 min and 85 $^{\circ}$C for 5 min.

8.2.2. Sequencing 3' genomic region of CRCoV

Complementary DNA from ITB-046 was used as a template in PCR reactions using previously published primers (Table 8.1). The overlapping PCR products were expected to span the entire 3' genomic region of CRCoV.

All PCR reactions were carried out using Platinum™ SuperFi™ Green PCR master mix (Invitrogen, Carlsbad, USA). The PCR reaction setup and cycling conditions were followed as per manufacturer's instructions. Each PCR reaction included 1 X master mix, 0.5 µM of each primer and 2 µL of cDNA in a total reaction volume of 10 µL. The cycling conditions were as follows: initial denaturation at 98 $^{\circ}$C for 30 sec, 30 cycles of denaturation at 98 $^{\circ}$C for 10 sec, annealing for 10 sec and extension at 72 $^{\circ}$C for 2 min followed by final extension at 72 $^{\circ}$C for 5 min. The T_m calculator (Thermo Fisher Scientific, Waltham, USA) was used to calculate the annealing temperatures for each PCR reactions (Table 8.1).

Table 8.1. Primers used for sequencing the 3' genomic end of CRCoV NZ-046/16 (Erles *et al.*, 2007, An *et al.*, 2010a). Position of primers given below is based on CRCoV-4182 (GenBank no. DQ682406.1).

Primer	Primer sequence	Position	Product size (bp)	Annealing temperature
NS32-1	5'-CATTTTGGACGGTTTTCTGC-3'	1-20	1272	60 °C
NS32-2	5'-ACATTGGTAGGAGGGTTATCA-3'	1272-1252		
HE-3	5'-TGCGTAAAGAGCTAGAAGAAGGTGA-3'	991-1015	1532	60 °C
HE-4	5'-AAAAGCCATTGGTAAGGAA-3'	2523-2505		
Sp-9	5'-CGGTCATAATTATTGTAGTTTTGT-3'	2400-2423	4200	-
Sp-10	5'-GGAGCCAATAAATCAAAGACGAAC-3'	6600-6577		
NSR-1	5'-GGGACTAGTTGTTTTAAGAAATG-3'	6487-6509	595	60 °C
NSR-2	5'-CAAGCATGACTAGGAACTCTAC-3'	7082-7061		
M-1	5'-AGAGTTCCTAGTCATGCTTGGTG-3'	7063-7085	1212	66 °C
M-2	5'-AGGACGCTCTACTACTGGATTGCTTAC-3'	8275-8249		
NP-1	5'-GTAATTACCGACTGCCATCAACC-3'	8153-8175	1662	68 °C
NP-2	5'-TGGTAACTTAACATGCTGGCTCTT-3'	9815-9792		
S1F	5'-GCTGCATGATGCTTAGACCA-3'	2455–2474	1069	63 °C
S1R	5'-TTAATGGAGAAGGCACCGAC-3'	3523–3504		
S2F	5'-AACGGTTACACTGTTCAGCC-3'	3415–3434	1378	60 °C
S2R	5'-TCGATCTACGACTTCGTCTT-3'	4792–4773		
S3F	5'-TTCACGACAGCTGCAACCTA-3'	4635–4654	1108	64 °C
S3R	5'- CTGAGCTTGCGCTTCAAGAG-5'	5742–5723		
S4F	5'- GCAGCAGCAGGTGTACCATT-3'	5440–5459	1134	60 °C
S4R	5'- GTCGTCATGTAAGGTTTTAATTAC-3'	6573–6550		

The PCR products were separated by electrophoresis through a 1% ethidium bromide stained agarose gel (Axygen Inc., USA). Each desired PCR product was cut out from the gel and placed into a 200 µL filtered pipette tip that had been cut at its tip and placed in a sterile Eppendorf tube. The gel fragments were then snap frozen in liquid nitrogen. DNA was recovered at the bottom of the Eppendorf tube following centrifugation at 13,000 × g for 5 min at room temperature. The gel remnants trapped in the filter were discarded and the DNA-containing elute was stored at 4 °C for sequencing. Eluted PCR products were directly sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster city, USA) on the ABI PRISM

310 genetic analyzer (Applied Biosystems, Foster city, USA), using a commercial sequencing facility (Massey Genome Service, Massey University, Palmerston North).

8.2.3. Sequence and phylogenetic analysis

Obtained sequences were assembled and analyzed using Geneious version 10.2.3. (Kearse *et al.*, 2012). The NCBI graphical analysis ORF Finder tool (Tatusov and Tatusov, 2011) was used to identify the number of ORFs in the CRCoV NZ-046/16 sequence. The transcription regulatory sequence (TRS) for each gene of CRCoV was predicted based on locations previously described (Erles *et al.*, 2007, Lorusso *et al.*, 2009, An *et al.*, 2010a).

The phylogenetic analysis was conducted using nucleotide sequences of HE, S, M and N genes of New Zealand CRCoV and the following sequences were retrieved from the GenBank: one sequence from the UK, CRCoV-4182 (DQ682406.1); one sequence from China, CRCoV-BJ232 (KX432213.1); one sequence from Italy CRCoV 240/05 (EU999954.1); three sequences from Korea, CRCoV K37 (EU983106.1), CRCoV K39 (EU983107.1) and CRCoV K9 (EU983105.1); and other betacoronaviruses including BCoV (NC_003045.1), BCoV-LY138 (AF058942.1), BCoV Mebus (U00735.2), BCoV Quebec (AF220295.1), HCoV-OC43 (NC_005147.1) and HECoV-4408 (L07748.1).

Nucleotide sequences were aligned using Geneious version 10.2.3 (Kearse *et al.*, 2012). Maximum likelihood phylogenetic trees were generated using the PhyML package (Guindon *et al.*, 2010) based on TN93 substitution model (Tamura and Nei, 1993) in the Geneious version 10.2.3, with 100 bootstrap replicates.

8.2.4. Analysis of predicted proteins

The predicted amino acid sequences of viral proteins encoded by CRCoV NZ-046/16 were aligned with amino acid sequences of corresponding proteins encoded by CRCoV-

4182, CRCoV 240/05, CRCoV K39, CRCoV K9, CRCoV K37 and CRCoV BJ232 for comparative analysis. Putative *N*-glycosylation sites were predicted by using the NetNGlyc 1.0 server, and *O*-glycosylation sites by using NetOGlyc 4.0 server (Steentoft *et al.*, 2013). Signal peptides in amino acid sequences were predicted using SignalP 4.1 server (Bendtsen *et al.*, 2004). The ExPASy ProtParam tool was used to calculate the molecular weight of amino acid sequences (Gasteiger *et al.*, 2005).

8.2.5. GenBank accession number

The partial sequence of CRCoV NZ-046/16 has been deposited in the GenBank under accession number MH249786.

8.3. Results

8.3.1. PCR amplification

Nine out of 10 PCR reactions resulted in amplification of the target CRCoV product. The PCR using Sp-9 and Sp-10 primers, flanking 4200 bp region of the S gene, did not result in successful amplification of the target region. So, the primers amplifying ~1 kb fragments of the spike gene (S1F/R, S2F/R, S3F/R and S4F/R) were used (Table 8.1). The PCR products obtained using S3F/R and S4F/R primers were sequenced successfully. Due to the limited availability of oropharyngeal swab sample from dog 046, the PCR products using S1F/R and S2F/R were not amplified and sequenced. Hence, the nucleotide sequence of a 5' fragment of the spike gene from CRCoV NZ-046/16 of about 1644 nt in size remained undetermined. The sequence coverage of CRCoV NZ-046/16 is shown in Appendix I.

8.3.2. Sequence analysis of 3'-genomic end of CRCoV

The graphical view of the sequenced region of CRCoV NZ-046/16 is shown in the Figure 8.1. The nucleotide sequence and sequence quality of CRCoV NZ-046/16 is presented in Appendix J and K.

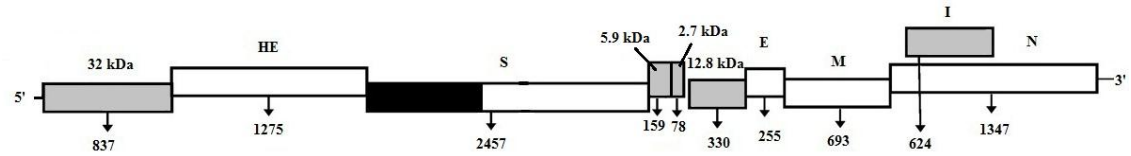


Figure 8.1. A graphical representation of the sequenced region of CRCoV NZ-046/16 including complete sequence of gene encoding 32 kDa, complete sequence of hemagglutinin-esterase (HE) gene, partial sequence of the spike (S) gene, genes encoding 5.9 kDa, 2.7 kDa and 12.8 kDa accessory proteins, complete sequence of envelope (E) gene, membrane (M) gene, nucleocapsid (N) gene and internal (I) gene. The accessory genes present in the sequence are shaded in grey color. The unsequenced region is colored in black. The nucleotide length is given below each gene.

The coding regions and TRS of each ORF are shown in the Table 8.2. The amino acid sequence alignment of CRCoV NZ-046/16 with CRCoV-4182, CRCoV 240/05 and CRCoV K39, CRCoV K9, CRCoV K37 and CRCoV BJ232 for the structural and accessory protein is shown in Appendix L.

The predicted accessory protein, encoded by ORF2a, had a molecular weight of 32.2 kDa. The potential TRS for ORF2a was present seven nucleotides prior to the AUG start codon (nt 301). Six synonymous and three non-synonymous nucleotide changes were found in comparison to CRCoV-4182. The 32 kDa protein was predicted to contain two *N*-glycosylation sites (aa 161 and 241).

The molecular weight of the predicted HE protein, encoded by ORF2b, was 47.7 kDa. The potential TRS for ORF2b was located nine nucleotides upstream of the AUG start codon (nt 1149). The HE protein was predicted to contain eight potential *N*-glycosylation sites (aa 54, 89, 104, 153, 236, 301, 316 and 358), similar to CRCoV 240-

05. A potential signal peptide of the CRCoV NZ-046/16 HE protein was identified at aa 1-18 with a predicted cleavage site between aa 18 and 19, similar to other CRCoVs (Erles *et al.*, 2007, Lorusso *et al.*, 2009, An *et al.*, 2010a).

A complete nucleotide sequence of the S gene was not sequenced in the present study. Hence, the S protein, encoded by ORF3, was not analyzed further.

Table 8.2. Coding regions and transcription regulatory sequences (TRS) of 32 kDa, hemagglutinin esterase (HE), partial spike (S), 5.9 kDa, 2.7 kDa, 12.8 kDa, envelope (E), membrane (M) and nucleocapsid (N) and internal (I) open reading frames of CRCoV NZ-046/16. The predicted length and molecular weight of each protein is given. The sequence was numbered based on mapping of PCR products to the CRCoV reference sequence (DQ642406.1), including the gap of predicted 1644 nt (Appendix J).

ORF	Protein product	Coding region[a]	Length (nt)	Length (aa)	TRS sequence	TRS position (nt)
2a	**32 kDa**	301-1137	837	278	CUAAAC	288-293
2b	**HE**	1149-2423	1275	424	CUAAAC	1134-1139
3	**S (partial)**	<4647-6530	2457	818	NA[b]	NA
4a	**5.9 kDa**	6520-6678	159	52	unknown	unknown
4b	**2.7 kDa**	6689-6766	78	25	unknown	unknown
5	**12.8 kDa**	6897-7226	330	109	CUAAAC	6819-6824
6	**E**	7213-7467	255	84	CCAAAC	7084-7089
7	**M**	7482-8174	693	230	CCAAAC	7473-7478
8a	**N**	8184-9530	1347	448	CUAAAC	8171-8176
8b	**I**	8245-8868	624	207	CUAAAC	8171-8176

[a] in CRCoV NZ 046/16,
[b]NA: not available

The predicted accessory protein, encoded by ORF5, had a molecular weight of 12.8 kDa. The potential TRS for ORF5 was located 72 nucleotides upstream of the AUG start codon (nt 6897). Three synonymous and three non-synonymous nucleotide substitutions were detected in CRCoV NZ-046/16 sequence in comparison to the gene encoding 12.8 kDa protein of CRCoV-4182.

The predicted E protein, encoded by ORF6, was the most conserved among all structural proteins analyzed and had a molecular weight of 9.5 kDa. The potential TRS for ORF6 was located 123 nucleotides upstream of the AUG start codon (nt 7213). The nucleotide sequence of the E gene of CRCoV NZ-046/16 was similar to CRCoV-4182. No glycosylation sites were found in the E protein of CRCoV NZ-046/16.

The predicted M protein, encoded by ORF7, had a molecular weight of 26.4 kDa. The potential TRS for ORF7 was located three nucleotides upstream of the AUG start codon (nt 7482). The M protein was predicted to contain one potential *N*-glycosylation site consistent with other CRCoVs, while no *O*-glycosylation site was found in CRCoV NZ-046/16. One synonymous and three non-synonymous nucleotide substitutions were found in CRCoV NZ-046/16 with respect to the M gene of CRCoV-4182.

The predicted N protein, encoded by ORF8a, had a molecular weight of 49.3 kDa. The potential TRS for ORF8a was located seven nucleotides upstream of the AUG start codon (nt 8184). Five synonymous and three non-synonymous nucleotide substitutions were observed in CRCoV NZ-046/16 with respect to the N gene of CRCoV-4182. The predicted internal I protein, encoded by ORF8b, had a molecular weight of 23.1 kDa. One synonymous and two non-synonymous nucleotide substitutions were observed in CRCoV NZ-046/16 with respect to the I gene of CRCoV-4182.

The nucleotide and amino acid sequence identity of structural and accessory protein of CRCoV NZ-046/16 to selected other betacoronaviruses is given in table 8.3.

Table 8.3. Nucleotide (top) and predicted amino acid sequence (bottom in bold) identity between genes encoding for structural and accessory proteins of CRCoV NZ-046/16 and other coronaviruses (CoV). The structural and accessory proteins compared included hemagglutinin esterase (HE), partial spike (S), envelope (E), membrane (M), nucleocapsid (N), 32 kDa, 12.8 kDa and internal (I) proteins.

CoV	GenBank number	32 kDa	HE	S (partial)	2.7 kDa	12.8 kDa	E	M	N	I
CRCoV	EU999954.1	99.4[a]	99.3	99.1	98.7	97.8	99.6	98.5	99.1	99.6
240-05		**99.2**[b]	**99.7**	**98.6**	**96.0**	**96.3**	**100**	**97.3**	**99.3**	**99.5**
CRCoV	DQ682406.1	98.9	99.0	98.9	NP[c]	98.1	100	99.4	99.4	99.5
4182		**98.9**	**99.2**	**98.2**		**97.2**	**100**	**98.7**	**99.3**	**99.0**
CRCoV	GQ918141.1	97.9	98.4	99.1	97.4	97.8	100	98.2	98.1	97.9
K9		**96.7**	**98.8**	**98.2**	**96.0**	**96.3**	**100**	**96.9**	**98.6**	**94.6**
CRCoV	GQ918142.1	97.9	97.8	99.1	97.4	97.5	100	97.6	97.6	97.4
K37		**96.7**	**97.4**	**98.6**	**96.0**	**96.3**	**100**	**96.9**	**97.9**	**93.2**
CRCoV	GQ918141.1	98.3	98.5	99.1	97.4	97.5	100	97.8	98.0	97.9
K39		**97.4**	**98.3**	**98.5**	**96.0**	**95.4**	**100**	**97.3**	**98.2**	**94.6**
CRCoV	KX432213.1	98.2	98.0	99.2	97.4	97.5	100	98.4	98.5	98.0
BJ232		**97.1**	**98.3**	**98.6**	**96.0**	**95.4**	**100**	**97.4**	**99.1**	**95.1**
BCoV	NC_003045.1	97.2	97.3	97.0	NP	96.9	99.6	97.1	97.2	97.4
		96.0	**96.9**	**96.5**		**95.4**	**98.8**	**96.0**	**97.7**	**93.7**
BCoV	U00735.2	97.9	97.8	97.4	NP	97.5	98.8	97.8	98.0	98.0
Mebus		**97.1**	**97.6**	**96.0**		**96.3**	**97.6**	**97.3**	**98.4**	**94.6**
BCoV	AF220295.1	97.3	97.8	97.4	NP	96.6	99.2	97.8	98.0	98.0
Quebec		**88.4**	**97.4**	**96.0**		**76.1**	**98.8**	**97.3**	**98.4**	**94.6**
BCoV	AF058942.1	98.8	97.8	97.8	NP	97.8	99.2	98.2	98.2	98.5
LY-138		**98.5**	**97.4**	**93.7**		**96.3**	**98.8**	**97.8**	**98.6**	**96.6**
HCoV	NC_005147.1	94.9	95.5	95.1	NP	94.2	98.4	95.2	96.3	96.5
OC43		**94.9**	**93.1**	**92.8**		**89.9**	**96.4**	**93.4**	**97.5**	**91.3**
HECoV	L07748.1	98.2	97.2	97.3	NP	96.6	99.6	97.5	98.0	97.9
4408		**96.7**	**97.1**	**96.3**		**95.4**	**98.8**	**96.5**	**98.6**	**94.6**

[a] nucleotide sequence identity
[b] amino acid sequence identity in bold
[c] NP: not present

8.3.3. Analysis of accessory genes located at the region between the S gene and the gene encoding 12.8 kDa accessory protein

Variations were observed in the region between ORF3 and ORF5 of CRCoV NZ-046/16. Nucleotide sequence alignment showed a single base deletion (nt 6648) in NZ-046/16 sequence compared to CRCoV 240/05, BJ232, K9, K37 and K39, and two nucleotide deletion (nt T, T) at the same site in the sequence of CRCoV-4182 (Fig. 8.2). This resulted in the absence of a stop codon and addition of eight amino acids to the accessory protein predicted to be encoded by ORF4a of CRCoV NZ-046/16 (5.9 kDa protein), instead of 4.9 kDa protein observed in CRCoV 240/05, BJ232, K9, K37 and K39 (Fig. 8.3). The 2.7 kDa accessory protein predicted to be encoded by ORF4b of

CRCoV NZ-046/16 displayed 96% amino acid identity with the corresponding protein of CRCoV 240/05, BJ232, K9, K37 and K39. The closest TRS (CCAAAC) was located 317 nucleotides upstream to the ORF4a start codon (nt 6520).

Five nucleotide deletion (TTTCT) was also observed after nt 6801 in CRCoV NZ-046/16 sequence, compared to CRCoV 240/05, BJ232, K9, K37 and K39 (Fig. 8.2).

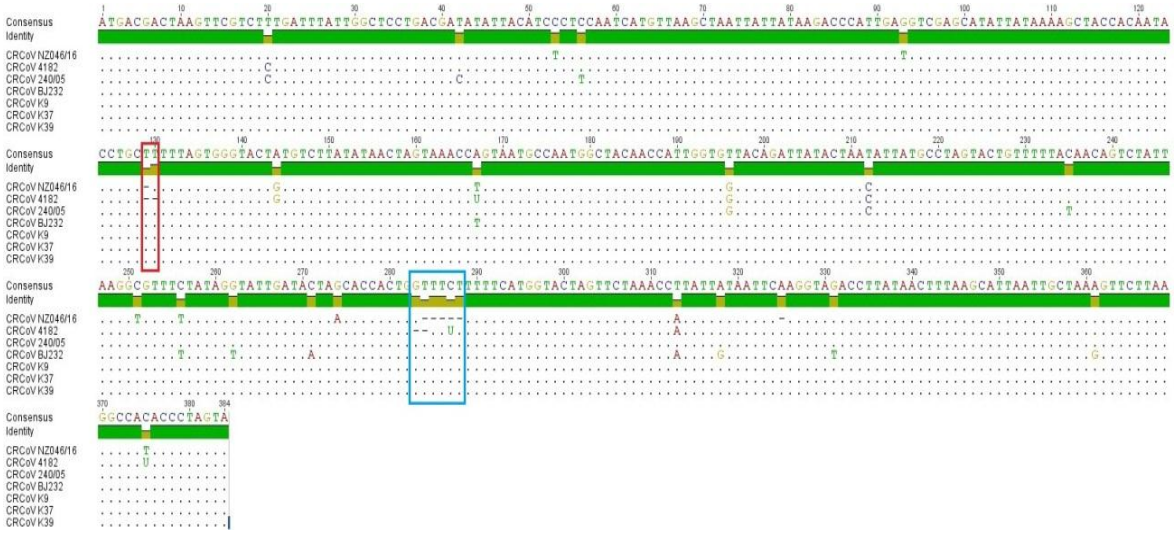


Figure 8.2. Multiple sequence alignment of a region between spike and 12.8 kDa gene of CRCoV NZ-046/16 with a corresponding region from sequences of CRCoV 4182 (DQ682406.1), CRCoV BJ232 (KX432213.1), CRCoV K37 (EU983106.1), CRCoV K39 (EU983107.1), CRCoV K9 (EU983105.1), CRCoV 240/05 (EU999954.1). Red box highlights the region of single base deletion in CRCoV NZ-046/16 sequences and two nucleotide deletions in CRCoV 4182. Blue box highlights the five nucleotide deletion (TTTCT) in CRCoV NZ-046/16.

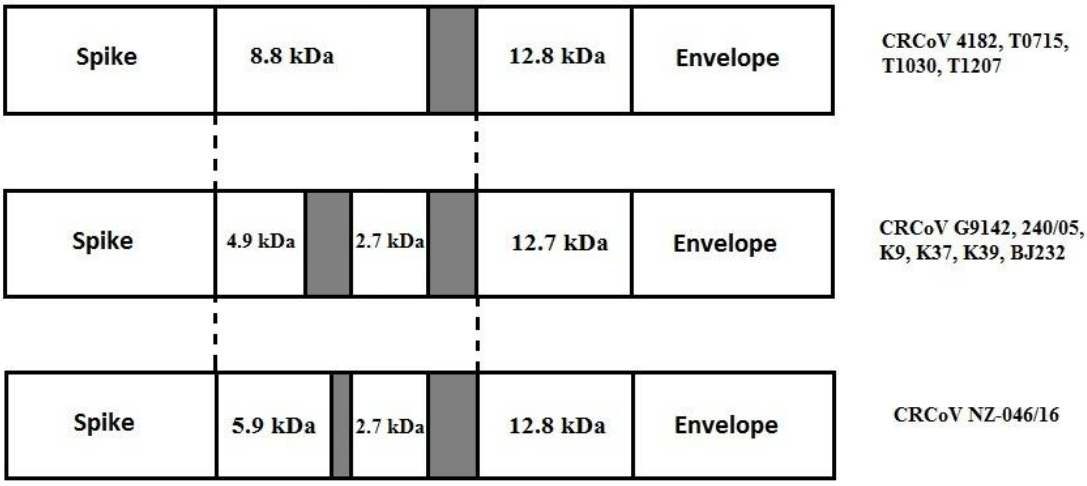


Figure 8.3. Comparative study of genomic organization of the region located between the spike and envelope genes of CRCoVs. Grey boxes represent non-coding sequences.

8.3.4. Phylogenetic analysis

Phylogenetic analysis based on the partial S gene sequence showed a strong support for separation between the branches containing CRCoVs and BCoVs (Fig. 8.4), while the phylogenetic analysis based on HE and M gene did not show a clear separation between CRCoVs and BCoVs (Fig. 8.5 and Fig. 8.6). The phylogenetic analysis based on the N gene showed two clusters of CRCoVs with Korean CRCoV sequences (K9, K37 and K39) branching with BCoVs; while CRCoV-4182, CRCoV 240/05, CRCoV BJ232 and CRCoV NZ-046/16 forming a separate branch (Fig. 8.7).

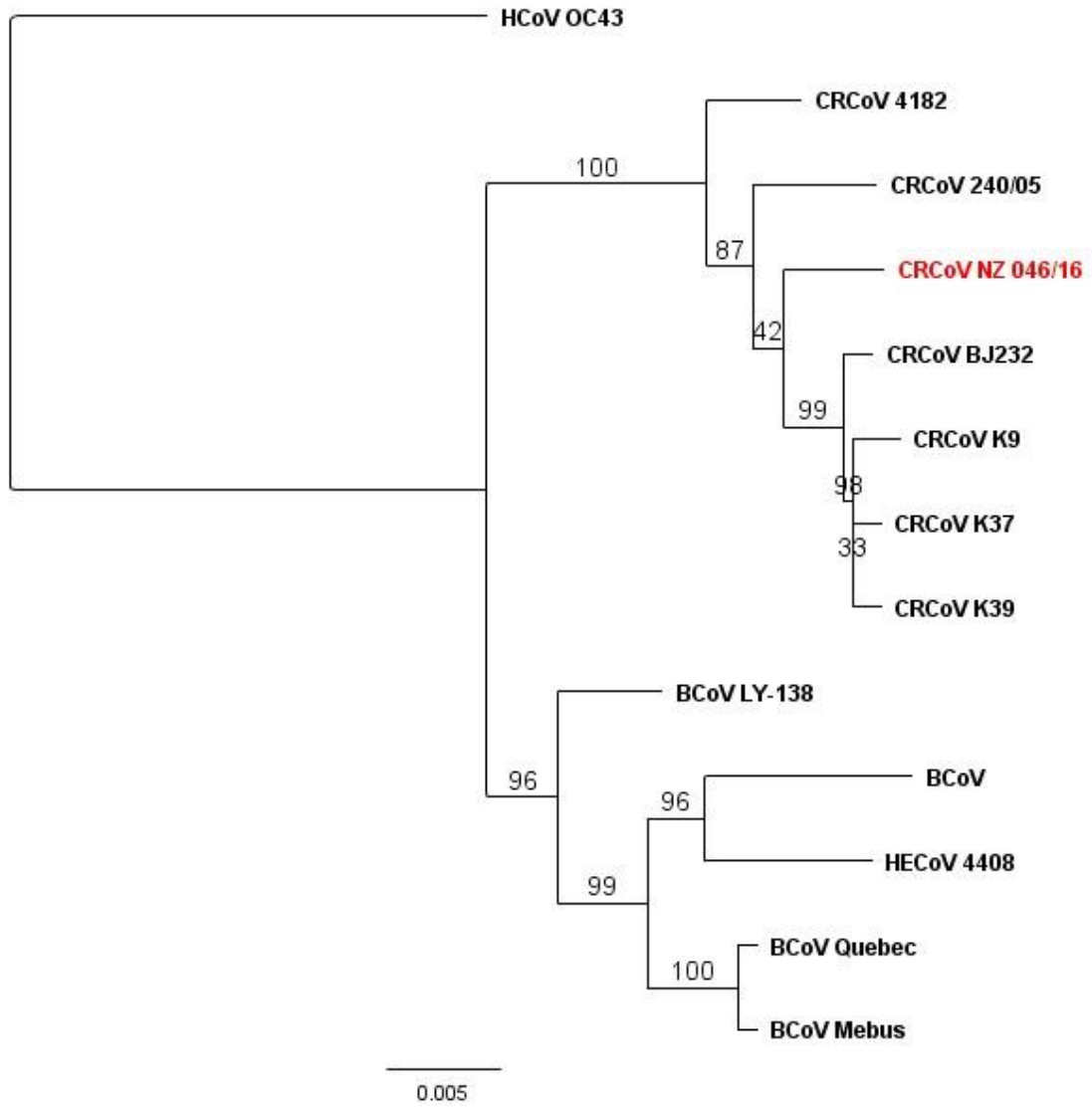


Figure 8.4. Phylogenetic tree obtained from multiple sequence alignment of the partial spike gene (2457 nt) of canine respiratory coronavirus (CRCoV) from New Zealand (CRCoV NZ-046/16) and other coronaviruses. For the analysis, the following sequences were used: CRCoV 4182 (UK) (DQ682406.1), CRCoV BJ232 (China) (KX432213.1), CRCoV 240/05 (Italy) (EU999954.1), three CRCoV from Korea: CRCoV K37 (EU983106.1), CRCoV K39 (EU983107.1), CRCoV K9 (EU983105.1); BCoV (NC_003045.1), BCoV-LY138 (AF058942.1), BCoV Mebus (U00735.2), BCoV Quebec (AF220295.1), HCoV OC43 (NC_005147.1), and HECoV 4408 (L07748.1). The tree was inferred using the Maximum Likelihood method with a 100 bootstrap value, using PhyML package. Numbers at the nodes depict % of bootstrap support.

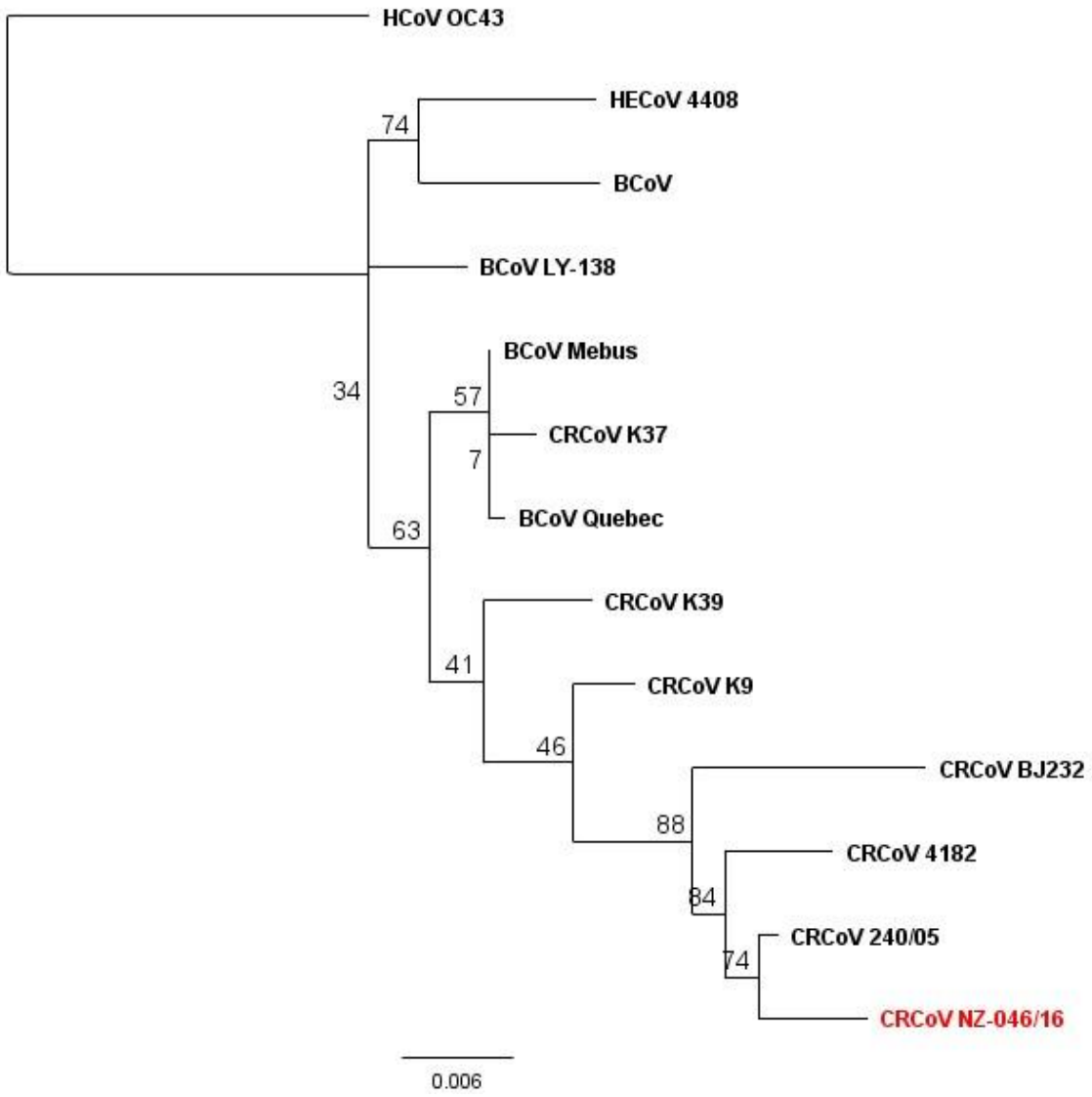


Figure 8.5. Phylogenetic tree obtained from multiple sequence alignment of complete hemagglutinin-esterase (HE) gene sequence of canine respiratory coronavirus (CRCoV) from New Zealand (CRCoV NZ-046/16) and other coronaviruses. For the analysis, the following sequences were used: CRCoV 4182 (UK) (DQ682406.1), CRCoV BJ232 (China) (KX432213.1), CRCoV 240/05 (Italy) (EU999954.1), three CRCoV from Korea: CRCoV K37 (EU983106.1), CRCoV K39 (EU983107.1), CRCoV K9 (EU983105.1); BCoV (NC_003045.1), BCoV-LY138 (AF058942.1), BCoV Mebus (U00735.2), BCoV Quebec (AF220295.1), HCoV OC43 (NC_005147.1), and HECoV 4408 (L07748.1). The tree was inferred using the Maximum Likelihood method with a 100 bootstrap value, using PhyML package. Numbers at the nodes depict % of bootstrap support.

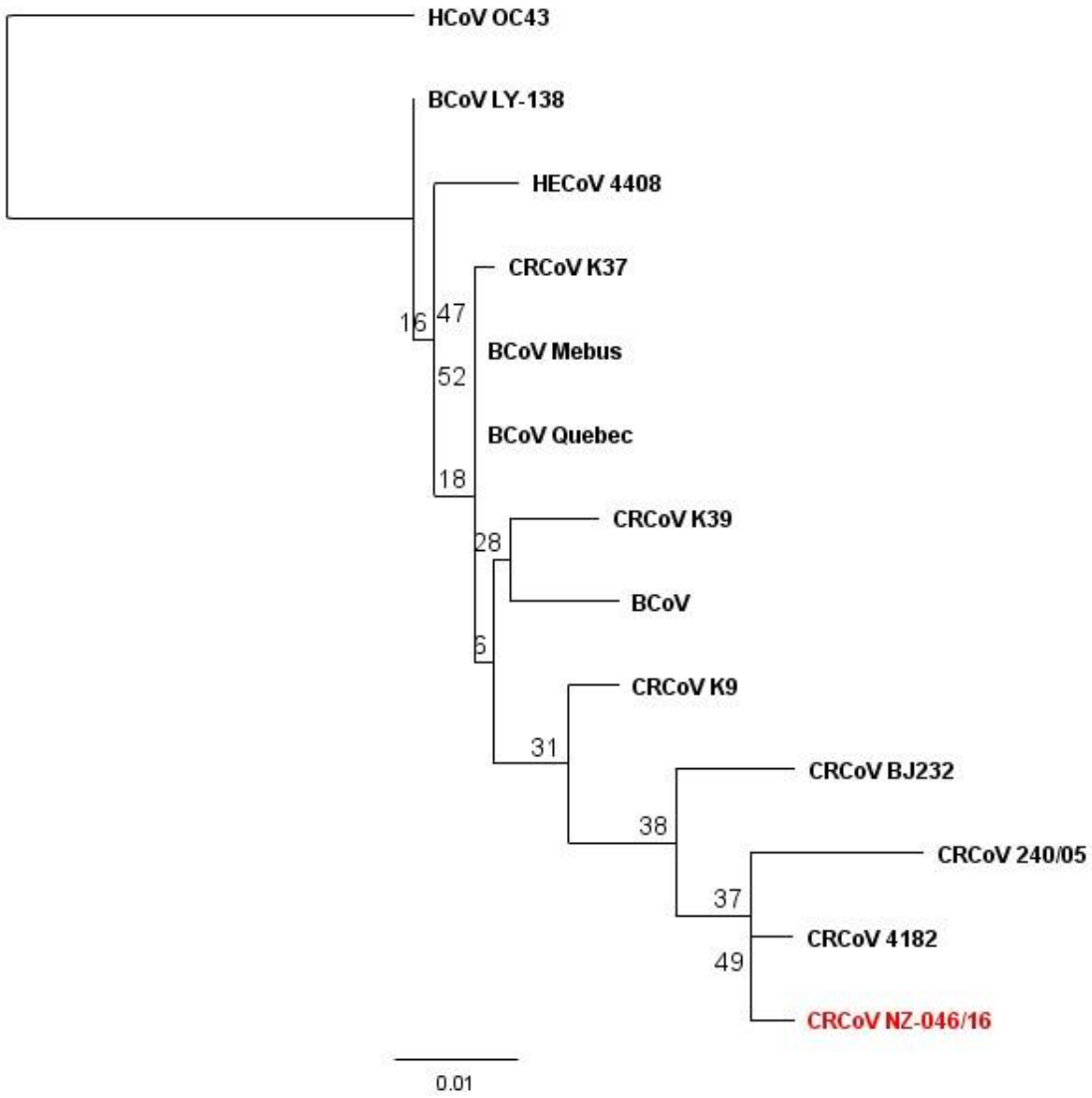


Figure 8.6. Phylogenetic tree obtained from multiple sequence alignment of complete membrane (M) gene sequence of canine respiratory coronavirus (CRCoV) from New Zealand (CRCoV NZ-046/16) and other coronaviruses. For the analysis, the following sequences were used: CRCoV 4182 (UK) (DQ682406.1), CRCoV BJ232 (China) (KX432213.1), CRCoV 240/05 (Italy) (EU999954.1), three CRCoV from Korea: CRCoV K37 (EU983106.1), CRCoV K39 (EU983107.1), CRCoV K9 (EU983105.1); BCoV (NC_003045.1), BCoV-LY138 (AF058942.1), BCoV Mebus (U00735.2), BCoV Quebec (AF220295.1), HCoV OC43 (NC_005147.1), and HECoV 4408 (L07748.1). The tree was inferred using the Maximum Likelihood method with a 100 bootstrap value, using PhyML package. Numbers at the nodes depict % of bootstrap support.

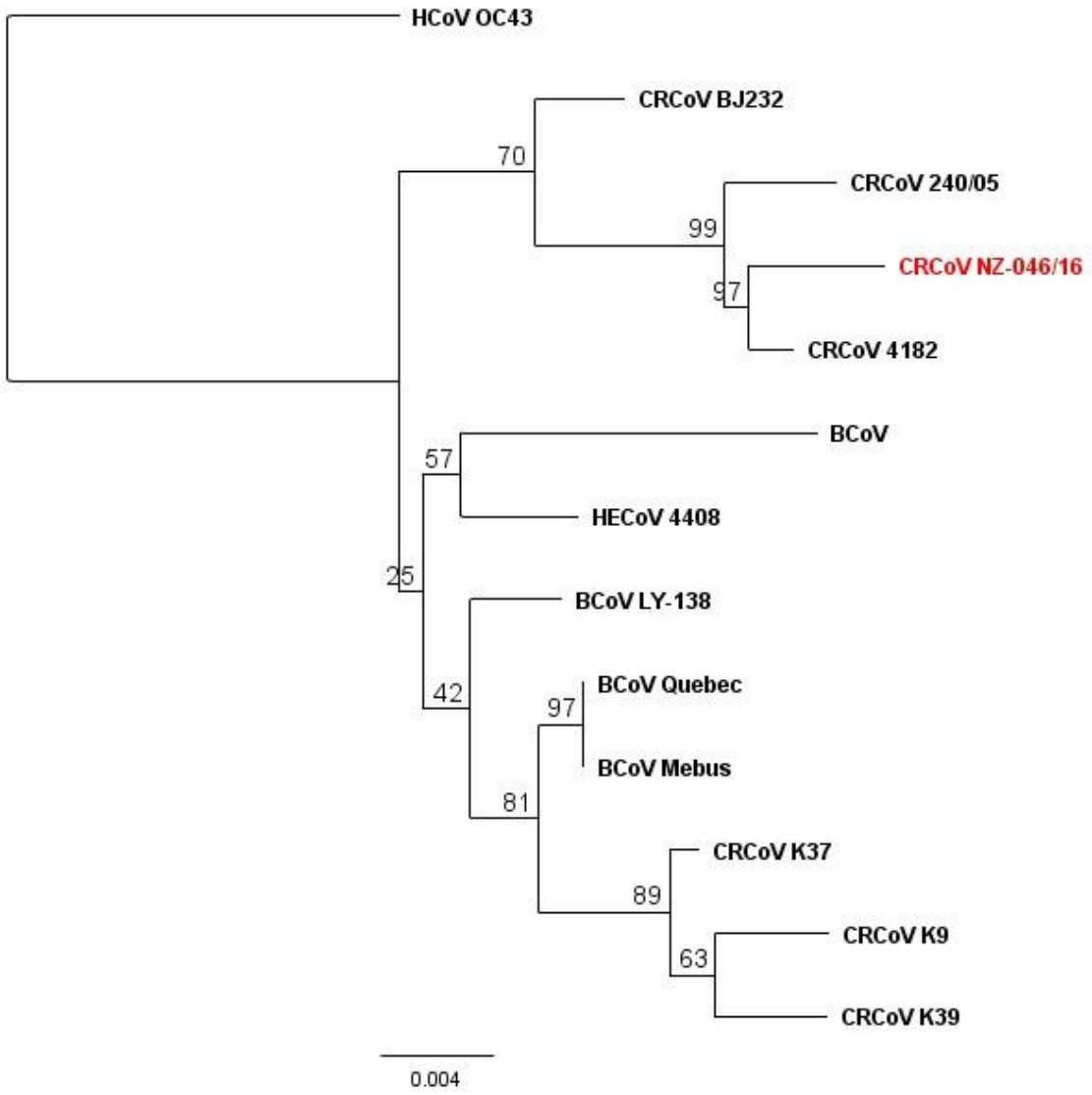


Figure 8.7. Phylogenetic tree obtained from multiple sequence alignment of complete nucleocapsid (N) gene sequence of canine respiratory coronavirus (CRCoV) from New Zealand (CRCoV NZ-046/16) and other coronaviruses. For the analysis, the following sequences were used: CRCoV 4182 (UK) (DQ682406.1), CRCoV BJ232 (China) (KX432213.1), CRCoV 240/05 (Italy) (EU999954.1), three CRCoV from Korea: CRCoV K37 (EU983106.1), CRCoV K39 (EU983107.1), CRCoV K9 (EU983105.1); BCoV (NC_003045.1), BCoV-LY138 (AF058942.1), BCoV Mebus (U00735.2), BCoV Quebec (AF220295.1), HCoV OC43 (NC_005147.1), and HECoV 4408 (L07748.1). The tree was inferred using the Maximum Likelihood method with a 100 bootstrap value, using PhyML package. Numbers at the nodes depict % of bootstrap support.

8.4. Discussion

This is the first study on molecular characterization of CRCoV from New Zealand. The presence of CRCoV in New Zealand has been previously demonstrated based on serological evidence (Knesl *et al.*, 2009). The virus itself, however, had not been

detected prior to this research. In this chapter, molecular characterization of a partial sequence of CRCoV NZ-046/16 is described. The sequence was obtained directly from an oropharyngeal swab sample of a dog with signs of ITB, thus it represents a field virus without any mutations that may have been introduced during adaptation to cell culture.

The structural genes present within the 3'-genomic region of CRCoV NZ-046/16 were highly conserved when compared with the corresponding genes of other CRCoVs (Table 8.3). The most striking variation was observed in the region between the S gene and the gene encoding a 12.8 kDa accessory protein. A deletion of single nucleotide was observed at this region in CRCoV NZ-046/16 sequence (Fig. 8.2) which led to predicted expression of 5.9 kDa and 2.7 kDa accessory proteins, as opposed to 4.9 kDa and 2.7 kDa accessory proteins typically present in other CRCoVs (BJ232, K9, K37, K39 and 240/05) (Fig. 8.3). In general, all RNA viruses have a tendency to undergo point mutations and/or recombination (Drake and Holland, 1999). Researchers have reported a high mutation rate of 4×10^{-4} nucleotide changes per site per year in coronaviruses (Sánchez *et al.*, 1992, Vijgen *et al.*, 2005, Bidokhti *et al.*, 2013). Considerable genetic variation has been found in the region between the S and E genes of betacoronaviruses (Abraham *et al.*, 1990b, Mounir and Talbot, 1993). For example, mouse hepatitis virus (MHV)-JHM codes a single 15 kDa protein (Skinner and Siddell, 1985); while MHV-A59 was predicted to encode two proteins, 2.2 kDa and 11.7 kDa in size (Weiss *et al.*, 1993) at the corresponding genomic region. The BCoV genome also contains two ORFs at this region encoding 4.9 kDa and 4.8 kDa proteins (Abraham *et al.*, 1990b), whereas in HCoV-OC43 a deletion of 290 nt sequence was observed in the corresponding region (Mounir and Talbot, 1993).

The presence of predicted 5.9 kDa protein in the CRCoV NZ-046/16 may possibly have arose during the transcription process where viral RNA polymerase may have skipped some nucleotides leading to deletions at the region. Similar genetic heterogeneity due to accumulation of mutations during viral replication has also been observed in other coronaviruses (Battilani *et al.*, 2003, Zhang *et al.*, 2007). The mutant CRCoV sequenced in the present study may indicate the presence of quasispecies in the sample.

Among the CRCoVs sequenced from dogs from two different kennels in the UK, point mutations leading to the predicted translation of 8.8 kDa protein were detected in CRCoV-4182 and other CRCoVs circulating among dogs within the same kennel; whereas, CRCoV-G9142 sequenced from dogs from another kennel encoded 4.9 kDa and 2.7 kDa proteins (Erles *et al.*, 2007), as did CRCoVs from China (CRCoV BJ232), Korea (CRCoV K9, K37, K39) and Italy (CRCoV 240/05) (Lorusso *et al.*, 2009, An *et al.*, 2010a, Lu *et al.*, 2017) (Fig. 8.2). Thus it appears that CRCoVs most commonly encode two accessory proteins, i. e. 4.9 kDa and 2.7 kDa, at the region between the S gene and the gene encoding 12.8 kDa protein.

There are no data available on the role of accessory genes present in the region between the S and E genes of CRCoV. Based on the studies on accessory genes of other coronaviruses, these genes are known to be dispensable for *in vitro* replication, but may play an important role in natural infections (de Haan *et al.*, 2002, Ortego *et al.*, 2003, Haijema *et al.*, 2004). Ortego *et al.* (2003) demonstrated that deletion of one accessory gene (gene 7) in transmissible gastroenteritis coronavirus was correlated with a decrease in virus virulence. Similarly, deletion of any of the two accessory genes (gene 3abc or 7ab) of feline infectious peritonitis virus produced mutants with an attenuated phenotype in a cat, and did not produce any signs of disease following experimental infection in cats (Haijema *et al.*, 2004). In MHV, experimental deletion of accessory

genes (gene 4 and 5a) had no effect on the viral growth *in vitro,* but produced mutants with attenuated phenotype upon infection in the natural host (de Haan *et al.*, 2002). Considering that the accessory proteins of CRCoV may have similar function, it would be intriguing to know whether the 5.9 kDa protein described in this study affects virulence of CRCoV during infection of the canine host.

The topology of the S gene indicated that all the CRCoVs were related to each other (Fig. 8.4), while topology of HE, M and N genes indicated that CRCoV NZ-046/16 was more closely related to CRCoV-4182, CRCoV 240/05 and CRCoV BJ232 compared to CRCoVs K9, K37 and K39 (Fig. 8.5, Fig. 8.6 and Fig. 8.7). This suggests that CRCoV NZ-046/16, CRCoV-4182, CRCoV 240/05 and CRCoV BJ232 viruses may have descended from a common ancestor. With respect to Korean CRCoVs, Lu *et al.* (2017) demonstrated that CRCoV K37 has arisen from recombination events between CRCoV and BCoV; however these recombination events were not observed in CRCoV NZ-046/16 based on the phylogenetic analysis of HE, M and N genes. This indicates that CRCoV NZ-046/16 and Korean CRCoVs (K9, K37 and K39) may have a different evolutionary pattern. Until now, only a few CRCoV from different geographical regions of Asia and Europe have been reported. Further studies are required to analyze more CRCoV sequences which will help to understand the origin and evolutionary pattern of CRCoV circulating worldwide.

In this study, the attempt was made to sequence the entire 3' genomic region of CRCoV from the oropharyngeal swab sample. However, due to limited sample availability of the source material and unsuccessful attempt to grow CRCoV on the cell culture (Chapter 6), the 5' end of the S gene fragment of CRCoV NZ-046/16 was not sequenced. This limitation could have been avoided by employing whole genome amplification of the sample prior to PCR.

In summary, this is the first study to conduct molecular characterization of CRCoV from New Zealand. The accessory proteins of 5.9 kDa, 2.7 kDa and 12.8 kDa in size were identified in CRCoV NZ-046/16 at the region between the S and E genes. Phylogenetically, CRCoV NZ-046/16 was more closely related to CRCoV-4182, CRCoV 240/05 and CRCoV BJ232 than to the CRCoV K9, K37 and K39.

The work in the current chapter characterised CRCoV sequence obtained from dog with ITB signs from New Zealand. In order to understand the seroprevalence of CRCoV and risk factors associated with the prevalence of CRCoV among New Zealand dogs, a serological survey of CRCoV was conducted in the next chapter (Chapter 9).

Chapter 9: A SEROLOGICAL SURVEY OF CANINE RESPIRATORY CORONAVIRUS IN THE NEW ZEALAND DOG POPULATION

9.1. Introduction

CRCoV is distinct from canine enteric coronavirus, showing only 68.5% sequence identity in the analyzed 215 nt segment of the polymerase gene (Erles *et al.,* 2003). Previous studies have shown that the antibodies raised to CRCoV are not cross-reactive with canine enteric coronavirus (Decaro *et al.*, 2007, Priestnall *et al.*, 2007). Similarly, vaccines against the enteric coronavirus do not elicit protection against CRCoV infection due to the antigenic dissimilarity (Erles *et al.*, 2003, Erles and Brownlie, 2008). In contrast, CRCoV has 98.8% and 98.4% sequence identity in the analyzed 215 nt segment of the polymerase gene to BCoV and HCoV-OC43, respectively (Erles *et al.*, 2003). A high sequence identity at the spike protein of BCoV and CRCoV has enabled the use of BCoV antigens for the detection of CRCoV antibodies (Priestnall *et al.*, 2006). Due to difficulty in isolating CRCoV *in vitro,* BCoV antigen has been used to detect CRCoV antibodies in canine sera in most of the seroprevalence studies (Priestnall *et al.*, 2006, Soma *et al.*, 2008). Seropositivity to CRCoV has been previously detected using an enzyme-linked immunosorbent assay (ELISA), a hemagglutination-inhibition test, an immunofluorescence assay or a virus neutralization test (An *et al.*, 2010b, Decaro *et al.*, 2007, Kaneshima *et al.*, 2006, Knesl *et al.*, 2009, Priestnall *et al.*, 2006). Out of these, ELISA was reported to have the highest specificity and sensitivity for serological diagnosis of CRCoV (Decaro *et al.*, 2007).

There are limited reports on serological studies of CRCoV among dogs in New Zealand (Knesl *et al.*, 2009, Sowman *et al.*, 2013). Knesl *et al.* (2009) reported 29% seroprevalence of CRCoV based on a single cross-sectional survey using a relatively small sample size (*n=251*). The population sampled in this study represented dogs from

only the central and lower North Island, and did not include dogs from other geographical regions of New Zealand.

Recently, the presence of CRCoV infection was serologically demonstrated in 47/94 (50%) dogs in New Zealand (Sowman *et al.*, 2018). The same study also revealed seroconversion to CRCoV in some dogs with signs of ITB. Although no conclusions regarding CRCoV aetiological involvement could be made due to the poor match between diseased and healthy dogs, the data suggested that CRCoV may be involved in ITB outbreaks among New Zealand dogs (Sowman *et al.*, 2018). Thus, further research into the epidemiology of CRCoV among New Zealand dogs would be beneficial. As such, the aim of the present study was to investigate seroprevalence of CRCoV in a large population of New Zealand dogs and to explore the association with respiratory signs, health status, age, sex, breed, month and region; thereby elucidating the epidemiology of CRCoV infections in New Zealand and generating further hypotheses for investigation of canine respiratory pathogens in New Zealand.

9.2. Materials and methods

9.2.1. Sample collection

A convenience sample of sera was obtained from a commercial veterinary laboratory (New Zealand Veterinary Pathology (NZVP) Ltd., New Zealand) on a monthly basis. Approximately 100 serum samples, representing 10% of monthly laboratory submissions, were randomly selected every month from March to December 2014. Only those samples with information on sex, age, region and clinical history were included in the study. Samples having incomplete information and duplicate sera having same label numbers were excluded from the study. In addition, a small number of samples collected from racing Greyhounds (*n=17*, including four from dogs with respiratory disease and 13 from healthy dogs), obtained from dogs used for molecular study in

previous chapters, were also included in the study. The serum samples were categorised into samples that came from dogs for which no abnormal respiratory signs were listed on the submission form ('no respiratory signs'), and dogs for which atleast one of the clinical signs commonly associated with respiratory disease (coughing, sneezing or nasal discharge) was listed on the submission form ('respiratory signs'). The sera were also categorised into 'healthy' and 'sick' groups, based on health status provided. Healthy group included sera from dogs of pre-anaesthetic work-up and from dogs testing for pre-mating progesterone levels; while the sick group included sera from dogs having other infectious or non-infectious diseases including abnormal respiratory signs. Sera were stored at -20 $^{\circ}$C until further use.

9.2.2. Detection of antibodies to CRCoV

Presence of CRCoV antibodies in canine sera was determined using a commercially available competitive ELISA with BCoV antigen (BIO K 392 - Monoscreen AbELISA Bovine coronavirus / Competition, Bio-X diagnostics, Rochefort, Belgium). The test was performed according to the manufacturer's instructions. The positive and negative controls provided with the commercial kit were used for the analysis. The results were assessed based on the optical density (OD) value at 450 nm. The seropositivity to CRCoV was calculated as percentage of inhibition (POI) according to the formula:

$POI = [(OD_{450}\ \text{negative} - OD_{450}\ \text{sample})/OD_{450}\ \text{negative}]*100.$

The cut-offs were determined based on the manufacturer's recommendations. The samples with POI $\geq$ 20 were considered positive for CRCoV antibody.

9.2.3. Statistical analysis

The risk factors considered for this study were month, age, sex, breed, geographical region, health status and presence of respiratory signs. The continuous variable age was

categorised using the following age groups: ≤ 2, 3-6, 7-10 and ≥ 11 years. Due to the large number of different breeds (*n = 161*), they were classified into three categories, a) working dogs (Huntaway and Heading dog) and Greyhounds, b) pet dogs and c) unknown dogs. Data was stratified by ten geographical regions, namely Auckland, Hawkes Bay, Manawatu, Marlborough/Canterbury, Nelson/Tasman, Northland, Otago, Taranaki, Waikato and Wellington (Fig. 9.2) to examine regional differences. For some analyses, geographical regions were categorised as South Island and North Island. Descriptive data were presented as proportions with 95% CI. The status of the sera with regards to the presence of CRCoV antibodies was considered a dichotomous variable (positive/negative). The levels of CRCoV antibodies represented by the POI values, in dogs showing 'no respiratory signs' and 'respiratory signs', were analysed as a continuous variable. Kruskal Wallis chi-squared test was used to determine whether there were any effects of respiratory signs, age, breed, gender, region and month on the prevalence of CRCoV antibodies. The mean POI for dogs of different age categories or health status were compared using one-way ANOVA and Tukey's -test.

A logistic regression model was created to analyse the risk factors associated with the seroprevalence of CRCoV. A preliminary univariate screening for all risk factors was performed to select those variables that were included in the final multivariate analysis. In the univariate analyses, 95% CI were calculated by the use of exact methods. All risk factors in the univariate analyses at a value of $p \leq 0.20$ were chosen to build a multivariable model by a stepwise backwards selection process retaining variables with a p value of ≤ 0.05. Data were analyzed using R statistical software (R v 3.1.0; R Development Core team, 2012; R Foundation for Statistical Computing, Vienna, Austria). The R package pROC was used to conduct logistic regression.

9.3. Results

9.3.1. Descriptive statistics

The total study population consisted of 1015 dogs. The details of dogs tested in this study are given in Appendix M. The histogram of age distribution of dogs included in this study is shown in Fig. 9.1. The study population consisted of 557 female and 458 male dogs. The majority of samples were from the North Island (*n=921*, 90.7%), with 94 (9.2%) samples obtained from the South Island (Fig. 9.2). The overall prevalence of CRCoV antibodies was 538/1015 (53.0 (95%CI 49.8-56.1)%), with a prevalence of 492/921 (53.4 (95%CI 50.1-56.6)%) in the North Island and 46/94 (48.9 (95%CI 38.5-59.3)%) in the South Island. The prevalence of CRCoV antibodies increased with age (p = 0.014) (Fig. 9.3). The prevalence of CRCoV antibodies was 116/223 (52.0 (95%CI 45.2-58.7)%) among working dogs, 411/770 (53.3 (95%CI 49.7-56.9)%) among pet dogs and 11/22 (50 (95%CI 30.7-69.2)%) among unknown dogs.

The majority of samples were collected for reasons other than respiratory infections. The prevalence of CRCoV antibodies in sera obtained from 'healthy group' was 69/133 (51.8 (95%CI 43.0-60.5)%), while the prevalence in the 'sick group' was 469/882 (53.1 (95%CI 49.8-56.5)%).

Out of the study population, abnormal respiratory signs (coughing, nasal discharge or sneezing) were reported in only 28/1015 (2.758%) dogs sampled. Among the dogs positive for CRCoV antibodies, the group of dogs with respiratory signs (*n=19*) showed higher CRCoV antibody levels compared to dogs without respiratory signs (*n=519*) (Fig. 9.4). In addition, among seropositive dogs, those with respiratory signs (*n=19*) tended to have higher mean POI (60.0 (95% CI=49.6–70.3)%) compared to all other seropositive dogs (*n=519*) (50.8 (95% CI=48.8–52.7)%, p = 0.082), but mean POI was

similar to that of seropositive healthy dogs (*n=69*) (55.2 (95% CI=49.5–61.0)%, *p* = 0.439).

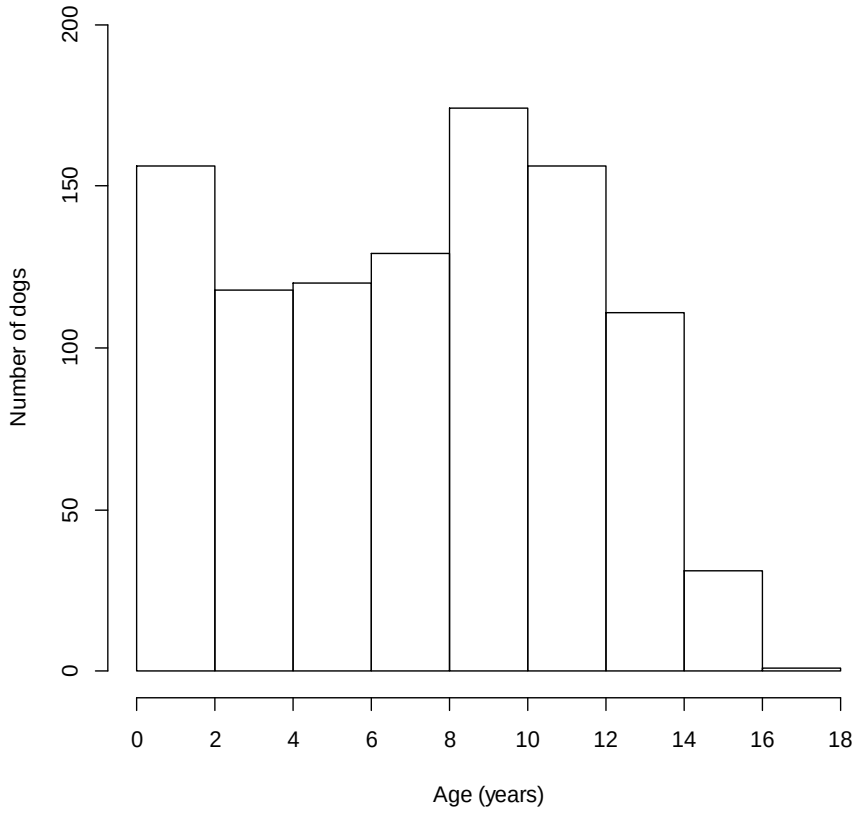


Figure 9.1. Histogram showing the age distribution of sampled population tested for antibody to canine respiratory coronavirus. A total of 1015 sera were collected within a 10 month period, which represented ~ 10 % of the samples submitted to New Zealand Veterinary Pathology laboratory within the sampling period.

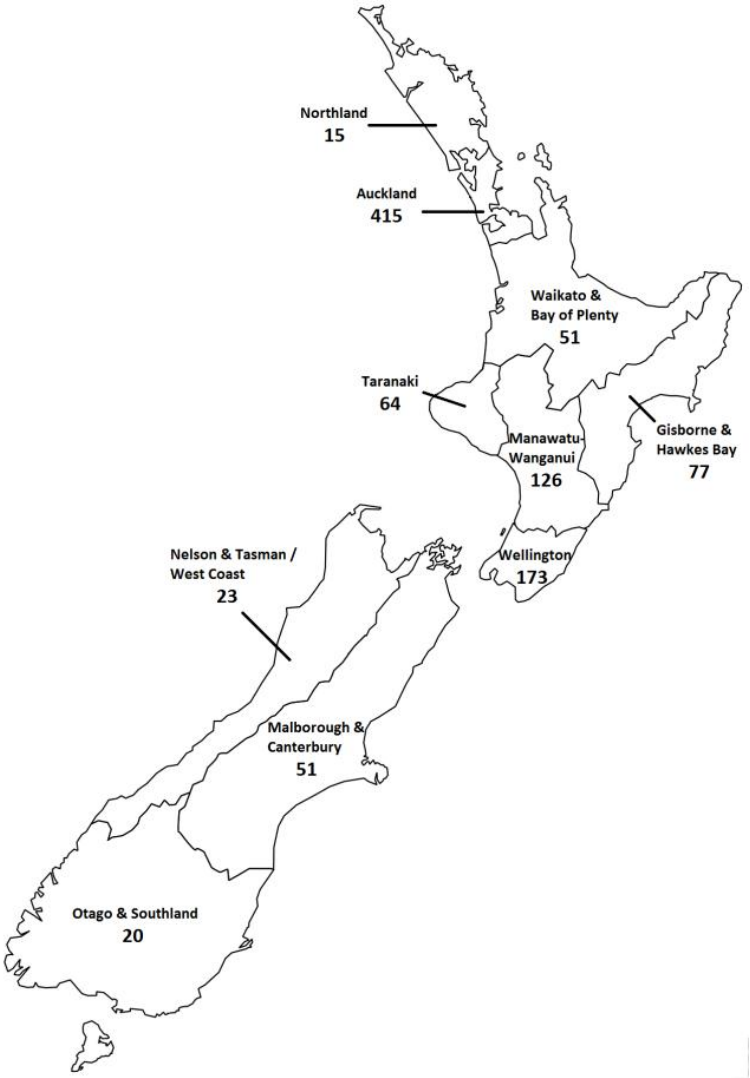


Figure 9.2. Geographical origins of samples included in a serological survey for canine respiratory coronavirus. The samples were randomly selected from submissions to the veterinary diagnostic laboratory between March and December 2014. The numbers indicate the number of canine sera tested from each region.

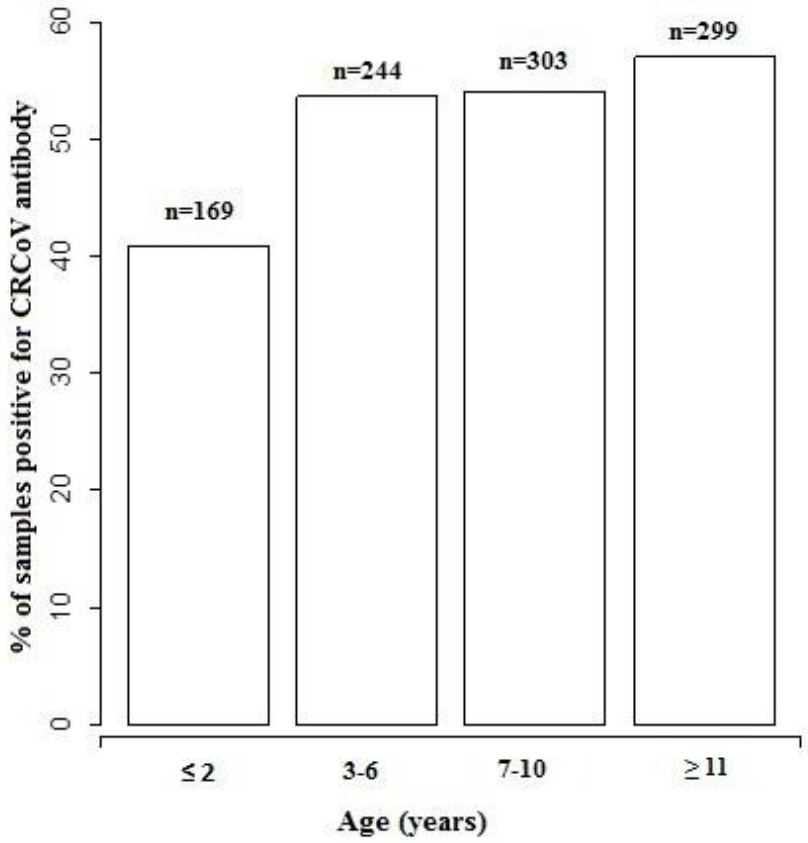


Figure 9.3. A bar graph representing the percent of canine sera positive for canine respiratory coronavirus (CRCoV) antibody stratified by age of dogs sampled. The number of serum samples tested within each age group is shown above the corresponding bar.

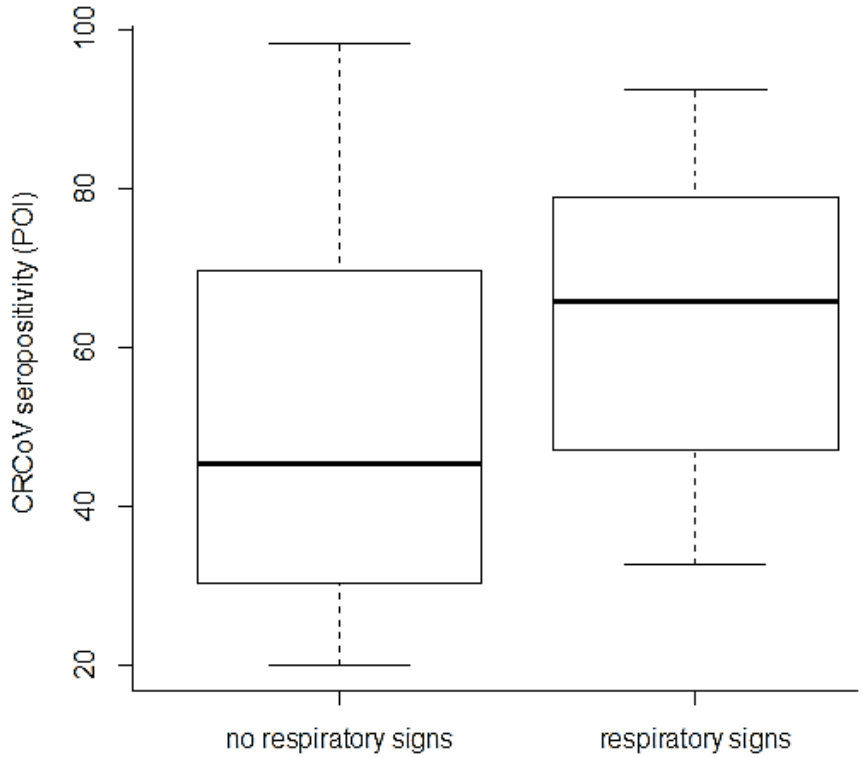


Figure 9.4. Boxplots depicting the level of seropositivity to canine respiratory coronavirus (CRCoV) in dogs with respiratory signs (*n = 19*) and without respiratory signs (*n = 519*) positive for CRCoV antibodies. The CRCoV seropositivity is expressed as percentage of inhibition (POI). The middle line in each box represents the median and whiskers represent minimum and maximum values of POI.

9.3.2. Association between presence of CRCoV antibody and putative risk factors

Univariate analyses revealed a significant association between the presence of CRCoV antibodies and sampling month, as well as age (Table 9.1). The CRCoV seroprevalence in July (OR 0.23, 95%CI 0.12-0.41) and August (OR 0.27, 95%CI 0.15-0.49) was lower than in the reference month, March (Table 9.2, Fig. 9.5). Dogs more than two years old were more likely to be positive for CRCoV antibodies, when compared with the reference group (≤ 2 years). There was no significant association found between the presence of CRCoV antibodies and the presence of respiratory signs (p = 0.110). Similarly, no significant association between the presence of CRCoV antibodies and geographical location (North vs South Island), health status, breed, or gender of the dogs sampled was observed.

Table 9.1. Univariate analysis of association between CRCoV seropositivity and variables examined: sampling month and geographical region, Island, age, breed, sex, health status and presence of abnormal respiratory signs based on analysis of 1015 sera collected between March and December 2014.

Risk factor	Category	Odds Ratio	*p* value	95% CI
Month	***March***	*Ref*		
	April	0.880	0.662	0.49-1.55
	May	0.724	0.259	0.41-1.26
	June	1.671	0.095	0.91-3.07
	July	0.234	< 0.001	0.12-0.41
	August	0.276	<0.001	0.15-0.49
	September	1.209	0.518	0.67-2.15
	October	0.564	0.046	0.31-0.98
	November	0.491	0.013	0.27-0.86
	December	0.747	0.313	0.42-1.31
Age (years)	***≤ 2***	*Ref*		
	3-6	1.626	0.015	1.09-2.42
	7-10	1.628	0.011	1.11-2.38
	≥ 11	1.843	0.001	1.26-2.70
Breed group	***Unknown***	*Ref*		
	Pet dogs	1.144	0.754	0.48-2.70
	Working dogs	1.084	0.857	0.44-2.63
Island	***North***	*Ref*		
	South	0.835	0.407	0.54-1.27
Sex	***Female***	*Ref*		
	Male	0.972	0.824	0.75-1.24
Abnormal respiratory signs	***No***	*Ref*		
	Yes	1.903	0.116	0.87-4.45
Health status	***Healthy***	*Ref*		
	Sick	1.05	0.780	0.73-1.51
Geographical region	***Auckland***	*Ref*		
	Hawkes bay	0.589	0.034	0.36-0.96
	Manawatu	0.530	0.002	0.35-0.79
	Marlborough-Cantebury	0.420	0.004	0.22-0.75
	Nelson/Tasman	0.920	0.847	0.39-2.20
	Northland	1.061	0.911	0.37-3.21
	Otago	1.651	0.313	0.64-4.74
	Taranaki	0.969	0.910	0.57-1.66
	Waikato	0.536	0.380	0.29-0.96
	Wellington	0.803	0.230	0.56-1.14

Table 9.2. Monthly variation in canine respiratory coronavirus (CRCoV) seroprevalence and signalment (sex, age and presence of abnormal respiratory signs) among dogs included in the study.

Sampling month in 2014	Sample size	Sex		Mean age (range) in years	% reporting respiratory signs[§]	Overall CRCoV seroprevalence (%)
		Female	Male			
March	100	55	45	7.08 (0.02-16)	4	63
April	100	65	35	7.82 (0.25-16)	3	60
May	105	55	50	7.55 (0.33-15)	6.6	55.2
June	100	71	29	7.99 (0.15-16)	3	74
July	105	50	55	7.61 (0.16-18)	0	28.5
August	100	47	53	7.39 (0.58-16)	1	32
September	104	54	50	7.58 (0.41-15)	0.9	67.3
October	100	51	49	8.13 (0.58-15)	1	49
November	101	55	46	7.28 (0.75-15)	4.9	45.5
December	100	54	46	7.84 (0.91-16)	3	56
TOTAL	**1015**	**557**	**458**	**7.627**	**2.74**	**53.0**

[§] Recorded as any of the following: nasal discharge, coughing or sneezing.

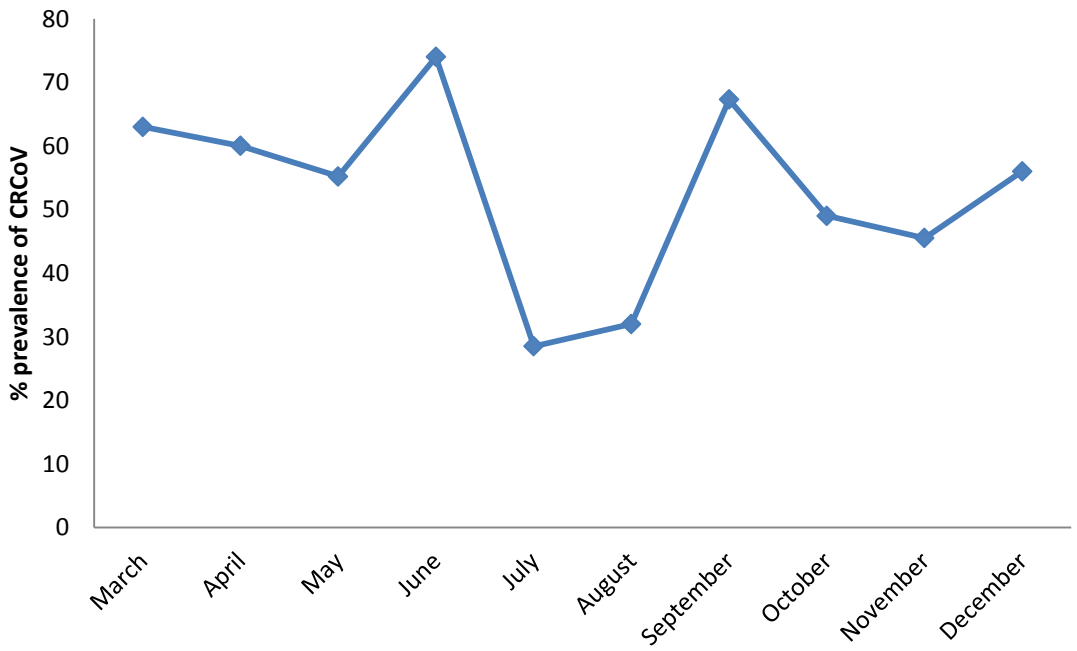


Figure 9.5. The plot showing monthly prevalence of CRCoV seropositive dogs (blue line) sampled during each month.

Four variables satisfied the criteria for inclusion in the initial multivariate model ($p \leq 0.2$), namely age, sampling month, geographical region and presence of abnormal respiratory signs. Only one variable, age, was significantly associated with seropositivity to CRCoV. The results of the final multivariate analysis are shown in Table 9.3.

Table 9.3. Multivariate analysis of the association between CRCoV seropositivity and the month of sampling, geographical region, presence of abnormal respiratory signs and age of dogs sampled based on analysis of serum samples (*n = 1015*) submitted to the veterinary diagnostic laboratory for unrelated testing between March and December 2014.

Risk factor	Category	Odds Ratio	*p* value	95% CI
Month	***March***	*Ref*		
	April	0.859	0.612	0.47-1.54
	May	0.673	0.177	0.37-1.19
	June	1.498	0.199	0.80-2.80
	July	0.218	<0.001	0.11-0.39
	August	0.248	<0.001	0.13-0.44
	September	1.158	0.626	0.63-2.10
	October	0.473	0.012	0.26-0.84
	November	0.450	0.007	0.25-0.80
	December	0.648	0.147	0.35-1.16
Age (years)	***≤ 2***	*Ref*		
	3-6	1.759	0.008	1.15-2.69
	7-10	1.787	0.005	1.19-2.69
	≥ 11	1.774	0.005	1.18-2.67
Abnormal respiratory signs	***No***	*Ref*		
	Yes	2.206	0.067	0.97-5.39
Geographical region	***Auckland***	*Ref*		
	Hawkes bay	0.634	0.087	0.37–1.06
	Manawatu	0.458	<0.001	0.29–0.70
	Marlborough-Cantebury	0.371	0.002	0.19–0.69
	Nelson/Tasman	0.836	0.693	0.34–2.08
	Northland	1.026	0.964	0.33–3.36
	Otago	1.597	0.366	0.60–4.76
	Taranaki	0.864	0.610	0.49–1.52
	Waikato	0.521	0.042	0.27–0.97
	Wellington	0.775	0.186	0.53–1.13

9.4. Discussion

The results of this study demonstrated a high prevalence (53%) of CRCoV antibody among New Zealand dogs. A similar seroprevalence to CRCoV was also reported in dogs from the USA (54.7%) and Canada (59.1%) (Priestnall *et al.*, 2006).

The present study showed a higher prevalence of CRCoV antibodies compared to the seroprevalence (29%) previously reported among dogs in New Zealand (Knesl *et al.*, 2009). Knesl *et al.* (2009) conducted a study on a single cross-sectional sample population which may have reflected the seroprevalence of CRCoV in that particular dog population during that time. The increased CRCoV antibody prevalence observed in the present study may be due to the large sample size tested from a wide geographical area of New Zealand. Alternatively, the serological assay used in the present study was different from that used by Knesl *et al.* (2009) which may have also influenced the results. Researchers have reported varying results in the detection of antibodies when different serological assays were compared. A study conducted by Lindenmayer *et al.* (1990) has demonstrated ELISA to be more sensitive compared to indirect immunofluroscent assay for diagnosis of Lyme disease in dogs.

The prevalence of CRCoV antibodies varied between different months (Table 9.2), and no clear seasonal pattern was observed (Fig. 9.5). Erles and Brownlie (2005) reported a peak rise in CRCoV antibodies during winter months (December and January) in a UK kennel having a history of respiratory disease outbreaks. This pattern of CRCoV antibody prevalence was not observed in the present study. A low prevalence to CRCoV antibodies was observed during late winter months (July and August) which may be attributed to reduced dog activity or reduced kenneling of dogs during these months.

The present study revealed an increase in the prevalence of CRCoV antibodies with age (p = 0.014), which is similar to earlier reports (Priestnall *et al.*, 2006, Knesl *et al.*, 2009). An increase in CRCoV antibody prevalence with age can be explained by cumulative increase in likelihood of exposure to CRCoV infection in older dogs with time.

The levels of CRCoV antibodies were higher in dogs with respiratory signs compared to the dogs without respiratory signs, although this difference was not statistically significant (Fig 9.4). There was, however, a big difference in the number of dogs between the two groups. Hence, the increased levels of CRCoV antibodies in 'respiratory signs' group may have simply reflected the poor match between the two groups. Other researchers (Erles *et al.*, 2003, Soma *et al.*, 2008) have shown a higher seropositivity to CRCoV in dogs with respiratory signs compared to healthy dogs, and therefore it is also possible that the results obtained represented a true finding. Another limitation of the present study was reliance on submission data for designation of 'respiratory signs' versus 'no respiratory signs' categories. Therefore, the former group may have included those dogs that were presented with abnormal respiratory signs due a non-infectious cause. Altogether, high levels of CRCoV antibodies observed in 'respiratory signs' group in the present study should be interpreted with caution. Field studies using similarly sized, age-matched groups of dogs would be needed to further support this observation.

The sampled population contained many different dog breeds (*n=161*) making the analysis of breed association with CRCoV seropositivity impractical. When the breeds were dichotomized into broad breed categories, no association with CRCoV seropositivity was observed (p = 0.900). This finding is consistent with a study published by An *et al.* (2010b) that showed no significant difference in CRCoV

seropositivity between the farm dog category (23/197) and pet dog category (39/286). This indicates that CRCoV infection is present among all breed groups. Future studies should include other risk factors including kenneling, dog activity (racing dogs and household dogs).

There was no positive association between the sex of the dog (male or female) and the presence of CRCoV antibodies (p = 0.823). This finding is consistent with results presented in other studies where no association between sex and seropositivity to CRCoV was found (Erles and Brownlie, 2005, Soma *et al.*, 2008).

In the present study, no statistically significant difference was observed between the presence of CRCoV antibodies and the health status (healthy versus sick) of sampled dogs (p = 0.780). The number of sera obtained from healthy dogs (*n=133*) were fewer compared to the sick dogs (*n=882*). As this grouping was based on the history provided in the submission form, some of the dogs with pre-anesthetic work-up may have other unspecified medical conditions that required anesthesia. The retrospective nature of the present study makes it impossible to rule out this possibility. Future studies should include prospective samples obtained from dogs with a thorough history in order to categorise samples based on health status.

In the present study, no statistically significant difference (p = 0.406) was detected in the prevalence of CRCoV antibodies between the North and South Islands. The number of sera obtained from the South Island (*n=94*) was lower than from the North Island (*n=921*). There are number of reasons for obtaining more samples from the North Island compared to the South Island. Firstly, the North Island is more populated compared to the South Island and so presumably there are more dogs living in the North Island compared to the South Island. Secondly, it is possible that veterinarians from the

South Island may prefer another veterinary pathology laboratory than the one utilized in the current study, which may have resulted in obtaining fewer serum samples from the South Island region than from the North Island.

Similar to the results obtained in this study, other studies found regional differences in CRCoV seroprevalence, as can be exemplified by differences between various regions in the United States of America and in the United Kingdom. It has been suggested that the CRCoV seroprevalence may be higher in areas with higher density of humans, and therefore presumably canine, populations (Priestnall *et al.* 2006). However, CRCoV seroprevalence was similar in some of the less densely populated regions of the South Island compared with the more densely populated region of Auckland. On the other hand, regional differences were apparent, as seroprevalence in dogs from Auckland was higher in comparison to dogs from the Hawkes Bay, Manawatu, Marlborough and Waikato regions. One possible reason for these differences is sample size and associated selection bias. In addition, population density in most parts of New Zealand, possibly with the exception of Auckland, is not uniform. Hence, it may have been of interest to stratify the samples by the size of town/city, in addition to the geographical region. This was not performed as we did not have access to addresses of the submitters beyond the region classification. Overall this data suggest that the epidemiology of CRCoV in New Zealand is similar to that observed overseas, particularly in Europe and in the United States of America.

Convenience sera tested in the present study were obtained from submissions to a pathology laboratory for various diagnostic purposes, which may have introduced a selection bias. The grouping of breeds into broad categories based on presumed activity may have caused misclassification bias in this study. It is possible that some breeds that are typically used as farm working dogs may have been kept as pet dogs, while some

dogs that were classified as pets based on their breeds may have been used as working dogs.

In conclusion, a high proportion of the study population was seropositive to CRCoV indicating that CRCoV infections are highly prevalent among dogs in New Zealand. The results presented in this study suggest that CRCoV infections in dogs occur throughout the year.

Similar serological studies can help in understanding the epidemiology of CnPnV in New Zealand. Future research should develop serological assay and conduct seroprevalence studies for CnPnV among dogs in New Zealand.

Part of this chapter was presented at an international conference:

More G.D.; Dunowska M.; Acke E. and Cave N. J. Seroprevalence of a group 2 coronavirus in New Zealand dogs. *Poster presented at 8th Australasian virology society meeting*; (**2015)** Dec 6-9; Crowne Plaza Hunter Valley, NSW, Australia.

Chapter 10: FINAL DISCUSSION

Infectious tracheobronchitis is an important disease among dog populations worldwide. The disease is caused by involvement of bacteria and viruses acting alone or synergistically. The present research was focused on determining the viral causes of canine ITB in New Zealand. The main aim of this research was to detect traditional and recently described canine respiratory viruses that are associated with respiratory disease among dogs in New Zealand. Healthy dogs and dogs with signs of ITB from private households and from the kenneled environment were investigated for the presence of canine respiratory viral nucleic acids in the oropharyngeal swabs. Molecular characterization of CRCoV and CnPnV, and attempts to isolate these viruses were also undertaken. Additionally, a seroepidemiological survey of CRCoV was conducted on dog sera obtained from diagnostic laboratory submissions.

The results presented in this thesis indicate that most of the currently known canine respiratory viruses circulate among dogs in New Zealand. The known canine respiratory viruses detected in the present study include CPiV, CAdV-2, CHV-1, CRCoV and CnPnV. The CRCoV and CnPnV viruses, which have not been investigated earlier in canine ITB outbreaks in New Zealand, were also present among dogs in New Zealand. This suggests that involvement of newer ITB pathogens can provide a possible explanation for the development of recent ITB infections among dogs in New Zealand. Hence, a thorough investigation for all canine respiratory pathogens, including CnPnV and CRCoV, in future canine ITB outbreaks will shed more light on the causative agents involved.

CnPnV infections appear to be common among dogs in New Zealand. The results obtained in the molecular survey showed a high prevalence (25%) of CnPnV in the

study population. CnPnV is associated with mild to moderate signs of respiratory disease in dogs (Renshaw *et al.*, 2010). But the present study could not prove a significant association between the presence of CnPnV and respiratory disease in dogs, as some healthy dogs were also positive for CnPnV. This association could have been clearly understood by sampling paired sera (acute and convalescent sera) from acutely infected dogs and determining seroconversion to CnPnV. However, due to the unavailability of virus culture, it was not possible to develop an in-house serological assay for the detection of CnPnV antibodies; and thereby conduct a serological survey of CnPnV in dogs in New Zealand.

CRCoV infections also appeared to be common among New Zealand dogs based on the results obtained from serological survey (Chapter 9). There was a discrepancy in the serological and molecular prevalence of CRCoV in different dog populations in New Zealand. It is possible that CRCoV are detected in ITB-affected dogs during the early stages of infection, before the clinical signs of respiratory disease were obvious. In this study, oropharyngeal swabs were collected from dogs that showed visible clinical signs of respiratory disease. This may have resulted in low molecular detection of CRCoV in dogs with signs of ITB. On the other hand, antibodies to CRCoV persists in the blood stream for longer duration after the clinical or subclinical infections. This can be one explanation for obtaining a high seroprevalence of CRCoV in dogs in New Zealand.

Questions remain as to how recently described viruses, CRCoV and CnPnV, entered New Zealand. Perhaps these viruses were always present among dogs in New Zealand. Or, these viruses may have been introduced to New Zealand from dogs that have migrated from countries with the presence of these viruses. The detection of CRCoV and CnPnV has been reported in most developed countries such as USA, UK and Europe (Erles *et al.*, 2003, Renshaw *et al.*, 2010, Mitchell *et al.*, 2013, Schulz *et al.*,

2014b). Introduction of these viruses to New Zealand via dog migration from developed countries is possible since investigation for recently described pathogens such as CRCoV and CnPnV has never been undertaken in New Zealand before. The phylogenetic analysis of CRCoV and CnPnV, in the present study, also revealed that these viruses were closely related to respective viruses from other countries indicating possible virus entry in New Zealand via dog migration. However, more studies are required to confirm this statement. Future research should include serological testing of archived samples to trace back if these viruses were circulating in New Zealand before their discovery.

New Zealand biosecurity undertakes a strict surveillance for most of the important canine pathogens such as rabies virus, CDV and CIV, thus keeping New Zealand free from these pathogens. A previous serological survey could not demonstrate the presence of CIV antibodies in dogs in New Zealand (Knesl *et al.*, 2009). This indicates that CIV is either absent or has a very low prevalence in New Zealand. In the present study, CIV viral sequences were also not detected by shot-gun sequencing. Despite of the absence of CIV, veterinarians practicing in New Zealand should always consider CIV as a differential diagnosis for canine ITB outbreaks.

The prevalence of canine respiratory viruses (CPiV, CAdV-2, CHV-1, CRCoV and CnPnV) in dogs with ITB, in the present study, was 37.5%. This suggests that most of the dogs with signs of ITB did not show presence of known canine respiratory viruses. It is possible that these dogs may have developed clinical signs of respiratory disease due to bacterial pathogens. Involvement of *B. bronchiseptica, S. zooepidemicus* and *M. cynos* in canine respiratory diseases has already been discussed by other researchers (Wright *et al.*, 1973, Chalker *et al.*, 2003, Chalker *et al.*, 2004). In the present study, attempts were not made to detect any bacteria, and hence bacterial involvement in the

dogs with ITB was not clarified. Future investigations on canine respiratory outbreaks should include screening for these bacteria to determine their involvement in canine ITB.

The shot-gun sequencing approach detected several microbial sequences in the pooled sample of oropharyngeal swabs. Apart from the known canine respiratory viral sequences, other viral sequences such as canine picornavirus and influenza C viruses were also identified in the pooled sample of dogs with signs of ITB. Since very few of these viral sequences were detected in the diseased dogs, their causative role in canine ITB was not clarified. However, mere identification of these viral sequences in diseased dogs does not necessarily indicate their causative role in the development of signs of respiratory disease. It is possible that their presence may be due to the favorable environment created by the causative agents of canine ITB.

Among the canine respiratory viruses detected, CnPnV, CHV-1 and CRCoV were detected by both shot-gun sequencing and qPCR, while CPiV and CAdV-2 were only detected by qPCR. There are several reasons why shot-gun sequencing may have failed to identify CPiV and CAdV-2 viruses. Firstly, these viral sequences may have been lost during sample processing for shot-gun sequencing. On qPCR, CPiV and CAdV-2 were detected at a high Cq value indicating low levels of these viruses in the individual samples. Secondly, pooling of samples may have underestimated the detection of some viral sequences using shot-gun sequencing; and deep sequencing of individual samples would have given better results. Sequencing of individual samples using shot-gun approach was beyond the scope of the current study.

In the present study, although several canine respiratory viruses were detected in the dogs with signs of ITB, the causative relationship between the virus and disease could

not be established. According to the Koch's postulates, the basic concepts for disease causation are as follows (Rivers, 1937):

- The microorganism occurs in every case of the disease.
- It is not found in healthy individuals.
- The microorganism can be isolated from the body and repeatedly grown in a pure culture, and can induce the disease anew.
- The microorganism must be then re-isolated from experimentally infected host.

Based on these postulates, the respiratory viruses identified in this survey could not fulfil the pathogen-disease causation due to various reasons. Firstly, some of the canine respiratory viruses were not exclusively detected in dogs with signs of ITB but were also present in healthy dogs. Secondly, CnPnV and CRCoV were not isolated from experimental studies. However, with our current understanding of viral diseases it has become apparent that the Koch's postulates are too stringent to be applied to qualify viruses for disease causation, for the following reasons, i) viruses are often found in mild infections which show little resemblance to a fully developed disease and are found in a proportion of apparently healthy animals, ii) viruses are seldom recovered from every case of the disease investigated, iii) viruses are difficult to grow *in vitro* when knowledge about their growth requirements is limited or unknown, iv) ethical considerations and difficulty in finding an experimental animal (model) which is susceptible to viral infection, and v) viruses may not cause disease without other independent factors (Robinson, 1958). Considering these limitations to prove viral aetiology of a disease, it is possible that the viruses (CnPnV and CRCoV) detected in this study may have a role in canine ITB in New Zealand.

It is clear that, molecular diagnostic techniques have impressively changed our ability to accurately diagnose respiratory infections caused by known, recently discovered, and

unknown pathogens (Wang *et al.*, 2002, Lam *et al.*, 2007, Capobianchi *et al.*, 2013). Despite these advances, detection of a pathogen in a clinical sample is not sufficient to make a diagnosis. Thus, instead of focusing on pathogen detection, identification of host gene expression signatures can help to differentiate infected dogs from asymptomatic healthy dogs. Ramilo and Mejías (2009) proposed that different pathogens elicit a distinct host immune response and that information is readily available in circulating leucocytes. Hence, a combined approach of pathogen detection and identifying host gene expression will help in developing biomarkers of the disease and improve our understanding on disease pathogenesis (Ramilo and Mejías, 2009). This approach will also help to understand whether the pathogen detected in a clinical sample plays a causative role in canine ITB or simply reflects asymptomatic shedding. Thus, focusing on a broader picture in the future investigation of canine ITB will help to better understand the disease.

More comprehensive studies are required to prove a causal association between a microbial agent and a disease. One such study includes performing inoculation of healthy animals with the infectious agent, to reproduce clinical disease (Rivers, 1937, Robinson, 1958). These studies can have a high ethical cost, and are usually expensive. Also, the clinical outcome of such studies can be complicated by various factors including host genetics, passive immunity due to maternally acquired antibodies and cross-protection by prior infections with related and less pathogenic microbial agents. Furthermore, experimental studies may not always prove disease causation if certain microbial agents produce only mild or no clinical signs alone, and cause significant disease when co-infected with other pathogens. For example, porcine circovirus type-2 causes mild clinical signs of postweaning multisystemic wasting syndrome in colostrum

deprived pigs; but co-infection with porcine circovirus type 2 and other pathogens, like porcine parvovirus, results in more severe disease (Kennedy *et al.*, 2000).

Another way to prove disease causation is by detecting the presence of antibodies against an agent and the time of their appearance in serum (Rivers, 1937). Association of onset of clinical signs with IgM detection and a rise in IgG levels will help support viral pathogenicity. Also, conducting a longitudinal study in a large study population over a period of time can help in determining the causative agent involved. Mitchell *et al.* (2013) reported a significant increase in CnPnV seroprevalence in dogs after 21 days' stay in a kennel. The researchers in this study not only detected the presence of CnPnV antibodies, but also the rapid transmission to immunologically naïve dogs. The dogs also showed increased chance of clinical signs of respiratory infection with increased length of stay in kennel.

The current prophylaxis for canine ITB is based on the use of vaccines against *B. bronchiseptica*, CPiV, CDV, and CAdV-2. According to the recent World Small Animal Veterinary Association Congress (WSAVA) guidelines (Day *et al.*, 2016), only those vaccines conferring protection against viral infections caused by CDV, CPV, CAdV-1 and CAdV-2 are considered in core vaccines; while the kennel cough vaccines, that provides protection against infections caused by *B. bronchiseptica* and CPiV, are considered optional (Day *et al.*, 2016). With the increasing pathogenic role of different viral and bacterial ITB agents, future vaccines should reflect the multifactorial aetiology of canine ITB. Currently, vaccines against CIV infection have been licensed only in the USA and are used in the dog population in USA (Deshpande *et al.*, 2009, Larson *et al.*, 2011). Development of a vaccine against CRCoV is underway. Whether the detection of new respiratory viruses such as CnPnV, CBoV and CnNPHV also requires development of new prophylactic measures is an issue to be addressed.

Recommendations for future research

Acute infectious tracheobronchitis is undoubtedly far more complex and multifactorial in aetiology than anticipated earlier. Veterinarians and veterinary pathologists in New Zealand should remain vigilant to the potential of new and re-emerging pathogens involved in animal diseases including canine ITB.

CnPnV was frequently detected in the study population. Further studies are required to determine the seroprevalence and the risk factors associated with CnPnV infections; thereby elucidating the epidemiology of CnPnV infections in dogs in New Zealand.

Molecular characterization of CRCoV NZ-046/16 revealed the presence of two accessory proteins (5.9 kDa and 2.7 kDa) encoded between the spike protein and 12.8 kDa protein, not present in other CRCoVs. It remains to be determined if the accessory proteins of CRCoV have any role in natural infections, similar to other coronaviruses. If so, further manipulation of the TRS region and deletion of these accessory genes would facilitate the development of an attenuated CRCoV which may have potential for vaccine development.

Canine ITB is often caused by co-infections with two or more respiratory pathogens. Thus, understanding the complex dependencies between different respiratory pathogens and the host would enable better understanding of pathogenicity of the disease. This will in turn allow development of better control and preventive strategies.

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Appendix

A. Letter distributed to veterinarians

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

Dear Colleagues,

We are asking for your help in conducting '**A study on respiratory pathogens responsible for kennel cough outbreaks in New Zealand dogs**'. There are new and re-emerging pathogens which are now known to be associated with infectious respiratory outbreaks in dogs around the world and vaccines are being developed for those pathogens. Currently, very little is known about the cause of respiratory outbreaks in New Zealand dogs.

The aim of the research project is to screen for the possible pathogens responsible for causing kennel cough in New Zealand. It may be that the pathogens existing in New Zealand are same as those currently recognised elsewhere in the world, however the possibility remains that new pathogens are present that have not yet been identified. If this is the case, this could have far reaching implications in terms of future prevention of kennel cough in New Zealand.

The Study
Over the next 12-18 months, we propose to screen pathogens shed in healthy as well as dogs with respiratory disease. To do this, we require a blood sample and oropharyngeal swabs from both healthy dogs and dogs with respiratory disease. We will then test these samples for the presence of a number of infectious organisms. We wish to obtain samples from a wide geographic distribution as possible and so, on bended knee, we ask for your help and support in this study. We are distributing sample packs and questionnaire forms to private practices nationwide. We wish to obtain 200 samples from healthy and dogs with respiratory disease, but will merrily and gratefully receive more than that.

Should you choose to contribute to this study, we would require the following:

1 Oropharyngeal swabs in Viral transport media (virology) and Amies transport media(bacteriology)
2 A clotted blood sample in plain tube (acute and convalescent from dogs with respiratory disease)
3 A completed questionnaire form

If you have any comments or questions, please do not hesitate to contact me. In anticipation of your participation, we sincerely thank you for helping out with this project.

Yours sincerely,

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B. Questionnaires

Questionnaire for pet owners

Date: …….. Case number…….
Pet name, owner: ……
Breed: ……
Age: ……..
Gender: ☐ Male ☐ Female Neutered: ☐ Yes ☐ No

Reason for presenting: ☐ Neutering ☐ Vaccination/Health Check ☐ Dental
If other, please specify ……

Diet: ☐ Raw meat ☐ Commercial ☐ Other
My dog is mostly: ☐ Indoors ☐ Outdoors ☐ Both
☐ Town ☐ Rural

My dog is in contact with animals:
☐ Dogs ☐ Cats ☐ Livestock ☐ Horses
How frequent is contact?..... Any illness?.....

	Yes	**No**	**Don't know**	**If yes**
Does your dog receive yearly vaccination?	[ ]	[ ]	[ ]	Date of last vaccination: Vaccine name:
Do you vaccinate your pet for kennel cough?	[ ]	[ ]	[ ]	Date of last vaccination: Vaccine name: Intranasal or skin:
Does the pet have any history of respiratory illness?	[ ]	[ ]	[ ]	Date and duration:
Was your dog in kennels within last 6 months?	[ ]	[ ]	[ ]	
Was your dog obtained from rescue shelter within last 6 months?	[ ]	[ ]	[ ]	
Did your dog attend any shows in last 6 months?	[ ]	[ ]	[ ]	Type:
Any other illness in last 6 months? Describe illness and treatment given in comments	[ ]	[ ]	[ ]	Type, treatment and duration:

Address: ……
Phone: ……
I agree to be contacted: ☐ Yes ☐ No

This is not a diagnostic service that will allow identification of all the possible organisms in a time.
I am the owner of the above pet and give consent for nasal and throat swabs, and a blood sample to be collected.
Signature:

Questionnaire for clinicians

Dog name: ……..
Age: ……..
Gender: ……
Case number/Practice ID:……..
☐ Client-owned dogs ☐ Kenneled dog

Clinical signs:
Duration of respiratory signs: ……

Clinical signs	**Yes**	**No**	*If yes* ***please indicate the appropriate***
Cough	[ ]	[ ]	*Type* [ ] Soft [ ] Moist/productive [ ] Harsh *Frequency* [ ] Occasional [ ] Frequent [ ] Paroxysms [ ] tracheal pinch only (Occasional ≤ 1 x daily , frequent ≥ 1x daily)
Nasal discharge	[ ]	[ ]	*Type* [ ] Serous [ ] Mucoid [ ] Purulent [ ] Haemorrhagic
Altered respiration	[ ]	[ ]	[ ] Tachypnoea [ ] Dyspnoea
Sneezing	[ ]	[ ]	
Lymphadenopathy	[ ]	[ ]	Lymph nodes:
Pyrexia	[ ]	[ ]	Temperature:

If any diagnostic tests performed, which tests and briefly describe results:
……
……

Any other signs present ……

Treatment administered and duration:
……

Veterinarian's Signature

C. Instructions for Sampling and Transport

We would like you to collect one swab from each dog. The swab should be collected from the tonsillar region of the oropharynx.

Virology samples:

The pack includes 5 x 1mL UTM transport medium tubes **(Red top)** and 10 x virological swabs.

For sampling:

1. Insert the swab deep into the mouth and rub it against the oropharyngeal.

2. Replace the swab into a tube of UTM transport medium all the way to the bottom of the tube.
3. Holding the swab shaft close to the rim of the tube, break the applicator shaft at the colored breakpoint indication line. **Hold the tube opening away from your face.**
4. Store the sample in the fridge until the courier pick up time.

Labelling of samples:

Please label each sample with:

- The **name** of the dog
- The **date** of the sample
- The corresponding **number**
- Where the sample was taken (e.g. throat/ nasal cavity)

The corresponding number will be found on the top right corner of the questionnaire and consent forms. **Please make sure the number on the questionnaire matches the number on the consent form and label.**

Transportation of samples:

Once samples are collected, they must be transported **immediately via overnight courier** to the address provided. Courier packs, labels and ice packs are provided**. Please use the supplied ice packs** to transport samples.

Please contact us if you have any questions or concerns, or you require more sampling equipment.

D. Detailed list of dogs with respiratory disease and healthy dogs sampled for molecular survey

i) Dogs with respiratory disease sampled for the survey with detailed history of respiratory signs (cough, nasal discharge, sneezing, pyrexia, altered respiration and lymphadenopathy).

Dog number	Dog breed	Dog type	Age (yrs)	Sex	Duration	Cough	Cough type	Cough frequency	Nasal discharge	Sneezing	Altered respiration	Pyrexia	Lymphadenopathy
ITB - 001	Bischon	pet	6	Male	10	Yes	Harsh	Frequent	No	No	No	Yes	No
ITB - 002	Labrador	pet	3	Male	1	Yes	Harsh	Frequent	No	No	No	No	No
ITB - 003	Greyhound	kennel	1.25	Male	7	Yes	Soft	Occasional	No	No	No	No	No
ITB - 004	Greyhound	kennel	2.6	Female	7	Yes	Soft	Frequent	No	No	No	No	No
ITB – 005	Cross	pet	8.98	Female	4	Yes	Harsh		No	No	No	No	No
ITB – 006	Blue terrier	pet	11	Male	14	No	No	No	No	No	No	No	No
ITB – 007	Shih tsu	pet	4	Male	3	Yes	Soft	Occasional	Serous	Yes	No	No	No
ITB – 008	Terrier	pet		Female	4	Yes	Moist/ productive	Occasional	No	Yes	No	No	No
ITB – 009	G. Pointer	pet	2.5	Male	7	Yes	Moist/ productive	Frequent	No	No	No	No	No
ITB – 010	Greyhound	kennel	2	Male	4	Yes	Soft	Frequent	No	No	No	No	No
ITB – 011	Greyhound	kennel	2	Male	3	Yes	Soft	Frequent	No	No	No	No	No
ITB – 012	Pug	pet	8	Male	5	Yes	Harsh	Frequent	No	Yes	No	No	No
ITB – 013	Maltese	pet	8	Male	8	Yes			No	No	No	No	No
ITB – 014	Labrador	pet	1.5	Female	3	Yes	Soft	Occasional	No	No	No	No	No
ITB – 015	Labrador	pet		Male	1	Yes	Soft	Occasional	No	No	No	No	No
ITB – 016	Poodle	pet	1	Male	3	Yes	Soft	Occasional	No	No	No	No	No
ITB – 017	Labrador	pet	1.5	Male	3	Yes	Soft	Occasional	No	No	No	No	No
ITB – 018	Poodle	pet	1.66	Female	5	No	No	No	No	No	No	No	Yes
ITB – 019	Farm dog	pet	4	Male	14	No	No	No	No	No	No	No	No
ITB – 020	Jack Russel terrier	pet	1.75	Female	1	Yes	Harsh	Occasional	No	No	No	No	No
ITB – 021	Lhasa Apso	pet	10.91	Female	2	Yes	Harsh	Frequent	No	Yes	No	No	Yes

ITB – 022	Maltese	pet	5	Male	2	Yes	Moist/ productive	Frequent	No	No	No	No	Yes
ITB - 023	Huntaway	kennel	2	Male	2	Yes	Moist/ productive	Frequent	No	No	No	No	No
ITB - 024	Heading	kennel	4	Female	2	Yes	Moist/ productive	Frequent	No	No	No	No	Yes
ITB - 025	Huntaway	kennel	4	Male	5	Yes	Moist/ productive	Frequent	No	No	No	No	No
ITB - 026	Heading	kennel	3.5	Female	5	Yes	Moist/ productive	Frequent	No	No	No	No	No
ITB - 027	Heading	kennel	3.5	Male	5	Yes	Moist/ productive	Frequent	No	No	No	No	No
ITB - 028	Huntaway	kennel	1.33	Female	7	Yes	Moist/ productive	Frequent	Yes	No	No	No	Yes
ITB - 029	Huntaway	kennel	2	Female	7	Yes	Moist/ productive	Frequent	No	No	No	No	Yes
ITB - 030	Huntaway	kennel	2	Female	7	Yes	Moist/ productive	Frequent	No	No	No	No	Yes
ITB - 031	Heading	kennel	2	Female	7	Yes	Moist/ productive	Frequent	Yes	No	No	No	Yes
ITB - 032	Bull terrier	pet	1.75	Female	1	Yes	Moist/ productive	Frequent	No	No	No	No	Yes
ITB - 033	Huntaway	kennel	3	Male	2	Yes	Moist/ productive	Frequent	No	Yes	No	No	Yes
ITB - 034	Huntaway	kennel	3	Male	2	Yes	Moist/ productive	Frequent	No	No	No	No	No
ITB - 035	Heading	kennel	4	Male	7	Yes	Moist/ productive	Frequent	No	No	No	No	No
ITB - 036	Pug	pet	5	Female	6	Yes	Harsh	Frequent	No	No	No	No	Yes
ITB - 037	Jack Russel terrier	pet	4	Male	7	Yes	Harsh	Frequent	No	No	No	No	Yes
ITB - 038	Labrador	pet	3	Female	14	Yes	Soft	Frequent	No	No	No	No	No
ITB - 039	Labrador	pet	4	Female	14	Yes	Harsh	Frequent	No	No	No	No	No
ITB - 040	Fox terrier	pet	13	Female	6	Yes	Harsh	Frequent	No	Yes	No	No	No
ITB - 041	Greyhound	kennel		Male	11	Yes	Soft	Frequent	No	No	No	No	No
ITB - 042	Greyhound	kennel		Female	10	Yes	Soft	Frequent	No	No	No	No	No
ITB - 043	Greyhound	kennel		Female	11	Yes	Soft	Frequent	No	No	No	No	No
ITB - 044	Labrador	pet	4.75	Male	3	Yes	Harsh	Frequent	Yes	No	Yes	No	No

ITB - 045	Greyhound	kennel	15	Male	2	Yes	Soft	Occasional	No	No	Yes	No	Yes
ITB - 046	Jack Russel terrier	pet	6	Male	2	Yes	Harsh	Frequent	Yes	No	No	No	Yes
ITB - 047	ND	pet		Male	6	Yes			No	No	No	No	No
ITB - 048	Bichon	pet	1.5	Male	4	Yes	Soft	Frequent	No	No	No	No	No
ITB - 049	Bichon	pet	6	Male	2	Yes	Moist/ productive	Frequent	No	Yes	No	No	No
ITB - 050	Maltese	pet	6	Female	9	Yes	Harsh	Frequent	No	Yes	No	No	No
ITB - 051	Bichon	pet	9	Male	2	Yes	Harsh	Frequent	No	Yes	No	No	No
ITB - 052	Greyhound	kennel	5	Female	7	Yes	Moist/ productive	Frequent	Yes	No	No	No	No
ITB - 053	Labrador	pet	8	Female	1	Yes		Frequent	Yes	No	No	No	No
ITB - 054	German shepherd	pet	1.5	Male	7	No	No	No	No	Yes	No	No	No
ITB - 055	German shepherd	pet	1.5	Male	2	No	No	No	No	Yes	No	No	No
ITB - 056	Labrador	pet	1	Female	7	Yes	Moist/ productive	Frequent	No	No	No	No	No

ii) List of healthy dogs sampled for molecular survey

Dog number	Dog breed	dog type	Age (yrs)	Sex
HEAL - 001	Greyhound	kennel	1.25	Male
HEAL - 002	Greyhound	kennel	1.25	Male
HEAL - 003	Greyhound	kennel	1.33	Male
HEAL - 004	Greyhound	kennel	1.25	Female
HEAL - 005	Greyhound	kennel	1.25	Female
HEAL - 006	Greyhound	kennel	1.25	Male
HEAL - 007	Greyhound	kennel	1.25	Female
HEAL - 008	Greyhound	kennel	1.25	Male
HEAL - 009	Greyhound	kennel	3	Female
HEAL - 010	Greyhound	kennel	3	Male
HEAL - 011	Greyhound	kennel	3	Female
HEAL - 012	Greyhound	kennel	3	Male
HEAL - 013	Greyhound	kennel	3	Female
HEAL - 014	Labrador	kennel	0.66	Female
HEAL - 015	Labrador	kennel	0.58	Female
HEAL - 016	Labrador	kennel	1.16	Male
HEAL - 017	Labrador	kennel	1.16	Male
HEAL - 018	Labrador	kennel	1.5	Female
HEAL - 019	Labrador	pet	2	Female
HEAL - 020	Labrador	pet	1	Male
HEAL - 021	Greyhound	kennel	0.5	Female
HEAL - 022	Greyhound	kennel	10	Female
HEAL - 023	Greyhound	kennel	10	Female
HEAL - 024	Greyhound	kennel	1.33	Male
HEAL - 025	ND	pet	10	Female
HEAL - 026	Greyhound	kennel	10.33	Male
HEAL - 027	ND	pet		Male
HEAL - 028	Greyhound	kennel	3	Female
HEAL - 029	Greyhound	kennel	0.83	Female
HEAL - 030	Greyhound	kennel	4	Male
HEAL - 031	Greyhound	kennel	7.5	Female
HEAL - 032	Greyhound	kennel	4.5	Male
HEAL - 033	Greyhound	kennel	0.66	Female
HEAL - 034	Greyhound	kennel	14.5	Female
HEAL - 035	Greyhound	kennel	2	Male
HEAL - 036	Greyhound	kennel	0.75	Female
HEAL - 037	Greyhound	kennel	0.5	Female
HEAL - 038	Huntaway	kennel	2	Female
HEAL - 039	Huntaway	kennel	6	Female
HEAL - 040	Huntaway	kennel	10	Female
HEAL - 041	Poodle	pet	10.5	Female

HEAL - 042	Labrador	pet	9	Female
HEAL - 043	Labrador	pet	10	Female
HEAL - 044	Labrador	pet	13	Female
HEAL - 045	ND	pet	2.6	Female
HEAL - 046	ND	pet	1.2	Female
HEAL - 047	Bichon	pet	0.5	Male
HEAL - 048	Maltese	pet	0.11	Female
HEAL - 049	Bichon	pet		Male
HEAL - 050	Labrador	pet	12	Male
HEAL - 051	Labrador	pet	1.2	Female
HEAL - 052	German shephard	pet	6	Male
HEAL - 053	German shephard	pet	1	Male
HEAL - 054	Labrador	pet	0.5	Male
HEAL - 055	Jack Russel terrier	pet	2	Female
HEAL - 056	Labrador	pet	6	Male
HEAL - 057	Fox terrier	pet	4	Female
HEAL - 058	ND	pet	4	Male
HEAL - 059	Poodle	pet	13	Female
HEAL - 060	ND	pet	1.6	Male

E. Sequencing run summary report

Run1

Level	**Yield Total (G)**	**Projected Total Yield (G)**	**Aligned (%)**	**Error Rate (%)**	**Intensity Cycle 1**	**% >= Q30**	
Read 1	5.9	5.9	0.64	1.82	132	79.8	
Read 2	0.1	0.1	0	0	569	55	
Read 3	5.9	5.9	0.62	2.13	109	66	
Total	11.98397	11.98397	0.633362	1.975143	269.869	72.68833	
Read 1							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	1631 +/- 16	79.25 +/- 1.51	0.097 / 0.071	29.94	23.73	79.8
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
6 +/-	250	0.64 +/- 0.01	1.82 +/- 0.13	0.15 +/- 0.02	0.23 +/- 0.13	0.28 +/- 0.10	132 +/- 9
Read 2							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	1631 +/- 16	79.25 +/- 1.51	0.000 / 0.000	29.94	23.73	55
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
0 +/-	0	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	569 +/- 53
Read 3							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	1631 +/- 16	79.25 +/- 1.51	0.098 / 0.072	29.94	23.73	66
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
6 +/-	250	0.62 +/- 0.01	2.13 +/- 0.13	0.30 +/- 0.03	0.37 +/- 0.03	0.42 +/- 0.03	109 +/- 8

Run 2

Level	Yield Total (G)	Projected Total Yield (G)	Aligned (%)	Error Rate (%)	Intensity Cycle 1	% >= Q30	
Read 1	4.2	4.2	1.44	1.68	232	85.2	
Read 2	0.1	0.1	0	0	1016	92.2	
Read 3	4.2	4.2	1.42	1.72	203	75.9	
Total	8.384823	8.384823	1.433686	1.699901	483.7976	80.71847	
Read 1							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	990 +/- 18	89.18 +/- 1.36	0.087 / 0.083	18.62	16.6	85.2
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
4 +/-	250	1.44 +/- 0.02	1.68 +/- 0.10	0.11 +/- 0.01	0.16 +/- 0.02	0.21 +/- 0.03	232 +/- 11
Read 2							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	990 +/- 18	89.18 +/- 1.36	0.000 / 0.000	18.62	16.6	92.2
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
0 +/-	0	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	1016 +/- 74
Read 3							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	990 +/- 18	89.18 +/- 1.36	0.094 / 0.072	18.62	16.6	75.9
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
4 +/-	250	1.42 +/- 0.02	1.72 +/- 0.10	0.16 +/- 0.02	0.23 +/- 0.03	0.27 +/- 0.03	203 +/- 9

Run 3

Level	**Yield Total (G)**	**Projected Total Yield (G)**	**Aligned (%)**	**Error Rate (%)**	**Intensity Cycle 1**	**% >= Q30**	
Read 1	3.7	3.7	0.61	1.46	223	85	
Read 2	0.1	0.1	0	0	976	92.9	
Read 3	3.7	3.7	0.6	1.47	200	76.5	
Total	7.467937	7.467937	0.607547	1.46489	466.2738	80.90778	
Read 1							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	865 +/- 16	90.71 +/- 2.26	0.085 / 0.084	16.31	14.79	85
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
4 +/-	250	0.61 +/- 0.01	1.46 +/- 0.12	0.11 +/- 0.02	0.16 +/- 0.03	0.21 +/- 0.04	223 +/- 6
Read 2							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	865 +/- 16	90.71 +/- 2.26	0.000 / 0.000	16.31	14.79	92.9
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
0 +/-	0	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	976 +/- 42
Read 3							
Lane	**Tiles**	**Density (K/mm2)**	**Clusters PF (%)**	**Phas/Prephas (%)**	**Reads (M)**	**Reads PF (M)**	**% >= Q30**
1	28	865 +/- 16	90.71 +/- 2.26	0.088 / 0.072	16.31	14.79	76.5
Yield(G)	**Cycles Err Rated**	**Aligned (%)**	**Error Rate (%)**	**Error Rate 35 cycle (%)**	**Error Rate 75 cycle (%)**	**Error Rate 100 cycle (%)**	**Intensity Cycle 1**
4 +/-	250	0.60 +/- 0.01	1.47 +/- 0.13	0.17 +/- 0.02	0.24 +/- 0.13	0.27 +/- 0.10	200 +/- 9

Run summary report definitions

READ 1: First sequence read.

READ 2: Index read or second read for a non-indexed run.

READ 3: Second read for an indexed run.

The following metrics are displayed in the top table, split out by read and total:

Yield Total—The number of bases sequenced, in gigabases.

Projected Total Yield—The projected number of bases sequenced at the end of the run, in gigabases.

Yield Perfect—The number of bases in reads that align perfectly, in gigabases, as determined by a spiked in PhiX control sample. If no PhiX control sample is run in the lane, this chart is not available.

Yield <=3 errors—The number of bases in reads that align with 3 errors or less, in gigabases, as determined by a spiked in PhiX control sample. If no PhiX control sample is run in the lane, this chart is not available**.**

Aligned—The percentage of the sample that aligned to the PhiX genome.

% Perfect [Num Cycles]—The percentage of bases in reads that align perfectly, as determined by a spiked in PhiX control sample, at the cycle indicated in the brackets. If no PhiX control sample is run in the lane, this chart is not available.

% <=3 errors [Num Cycles]—The percentage of bases in reads that align with 3 errors or less, as determined by a spiked in PhiX control sample, at the indicated cycle. If no PhiX control sample is run in the lane, this chart is not available.

Error Rate—The calculated error rate, as determined by the PhiX alignment.

Intensity Cycle 1—The average of the four intensities (one per channel or base type) measured at the first cycle averaged over filtered clusters.

% Intensity Cycle 20—The corresponding intensity statistic at cycle 20 as a percentage of that at the first cycle.

%**Q>=30**—The percentage of bases with a quality score of 30 or higher, respectively. This chart is generated after the 25th cycle, and the values represent the current cycle.

The following metrics are available in the Read tables, split out by lane:

Tiles—The number of tiles per lane.

Density—The density of clusters (in thousands per mm2) detected by image analysis.

Clusters PF—The percentage of clusters passing filtering.
Phas./Prephas.—The value used by RTA for the percentage of molecules in a cluster for which sequencing falls behind (phasing) or jumps ahead (prephasing) the current cycle within a read.

Reads—The number of clusters (in millions).

Reads PF—The number of clusters (in millions) passing filtering.

%**Q>=30**—The percentage of bases with a quality score of 30 or higher, respectively. This chart is generated after the 25th cycle, and the values represent the current cycle.

Cycles Err Rated—The number of cycles that have been error rated starting at cycle 1.

Aligned—The percentage of the sample that aligned to the PhiX genome.

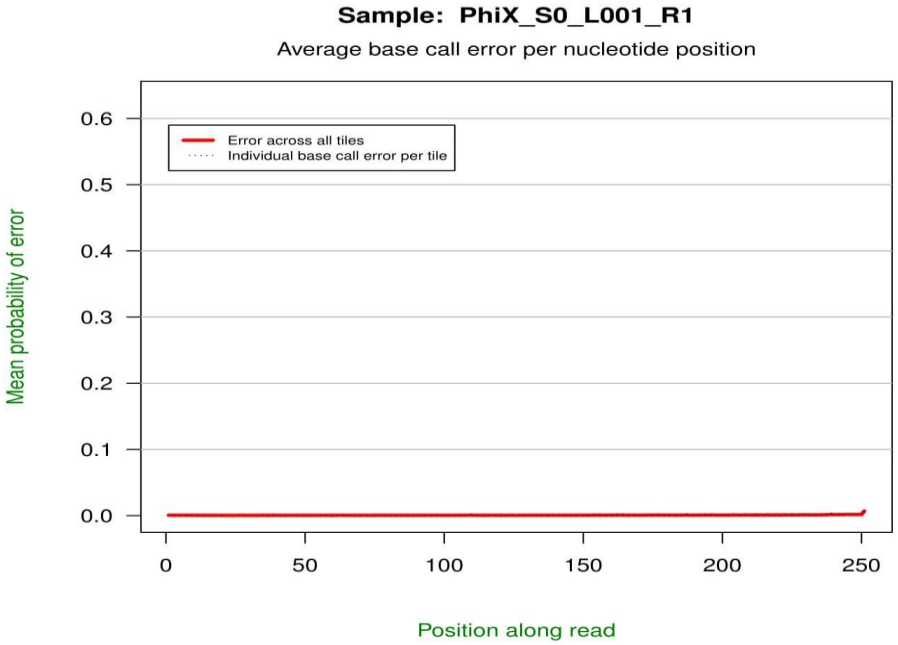
Error Rate—The calculated error rate, as determined by the PhiX alignment. Subsequent columns display the error rate for cycles 1–35, 1–75, and 1–100.

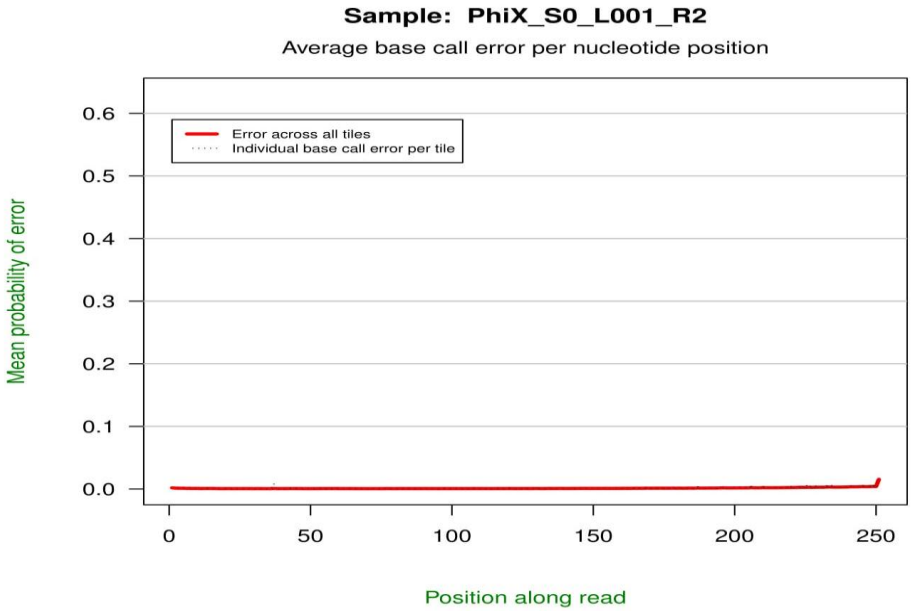
Intensity Cycle 1—The average of the four intensities (one per channel or base type) measured at the first cycle averaged over filtered clusters.

%Intensity Cycle 20—The corresponding intensity statistic at cycle 20 as a percentage of that at the first cycle.

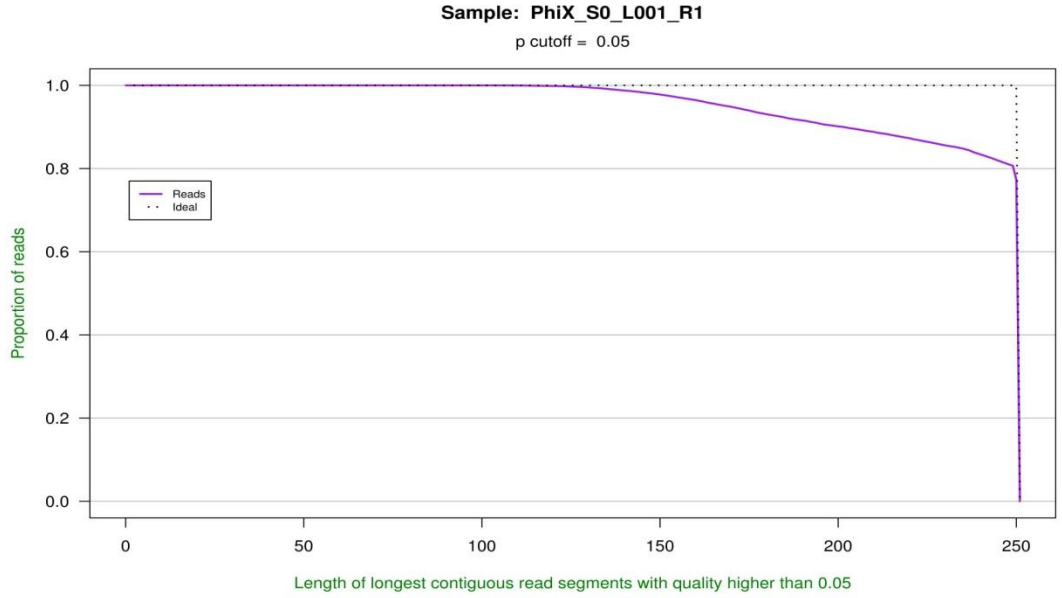
F. Phix quality check for all sequences in read 1 (r1) and read 2 (r2).

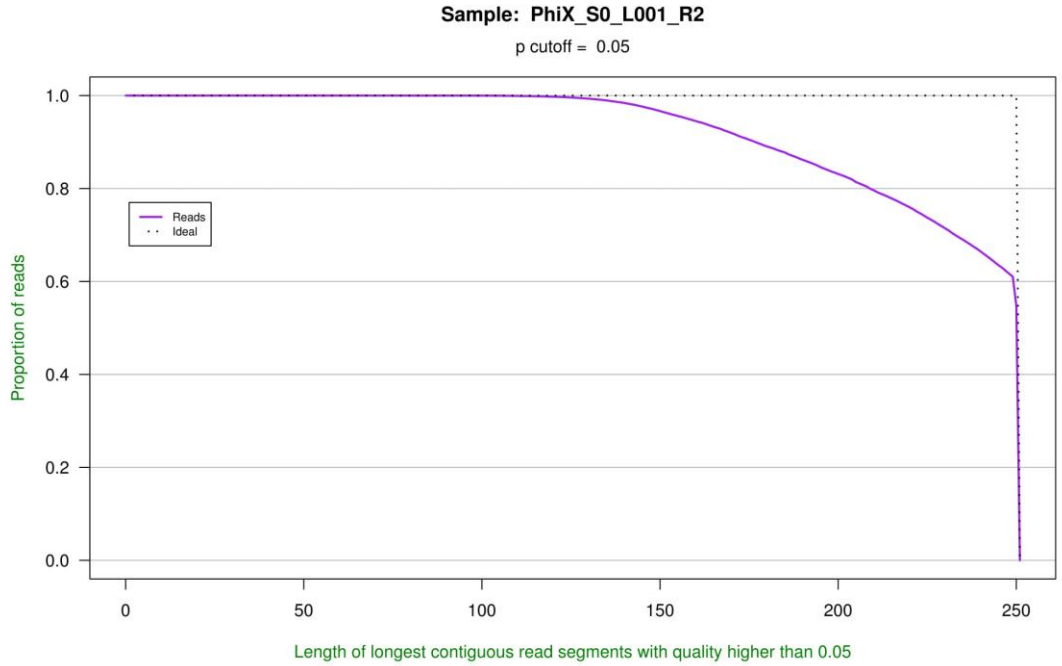
I. The qualities for individual tiles are shown as thin lines and the average by a red dot. Data is reported in terms of error probabilities, rather than the NGS equivalent of Phred scores that some other tools use.



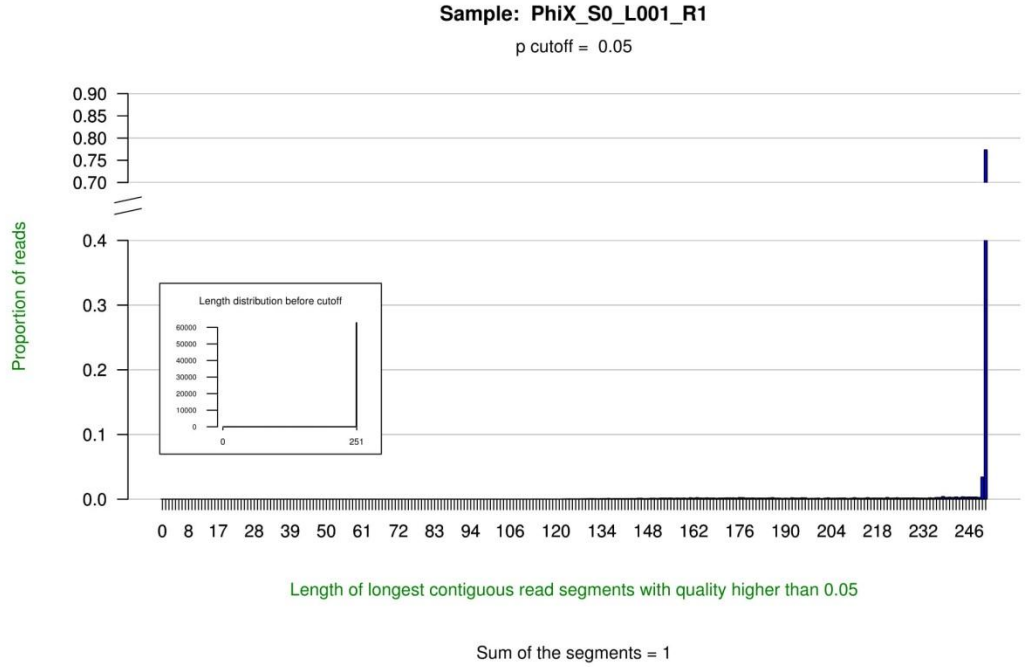


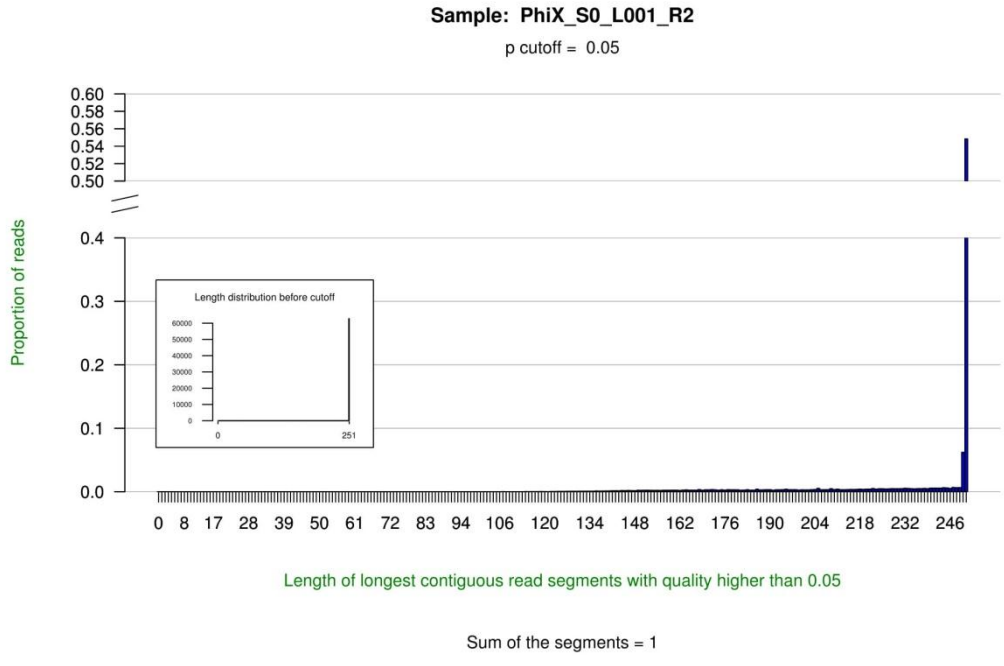
II. A line graph showing a cumulative frequency of trimmed read lengths, with the perfect result shown as a dotted line. This graph shows the fraction of data at a given length or longer.



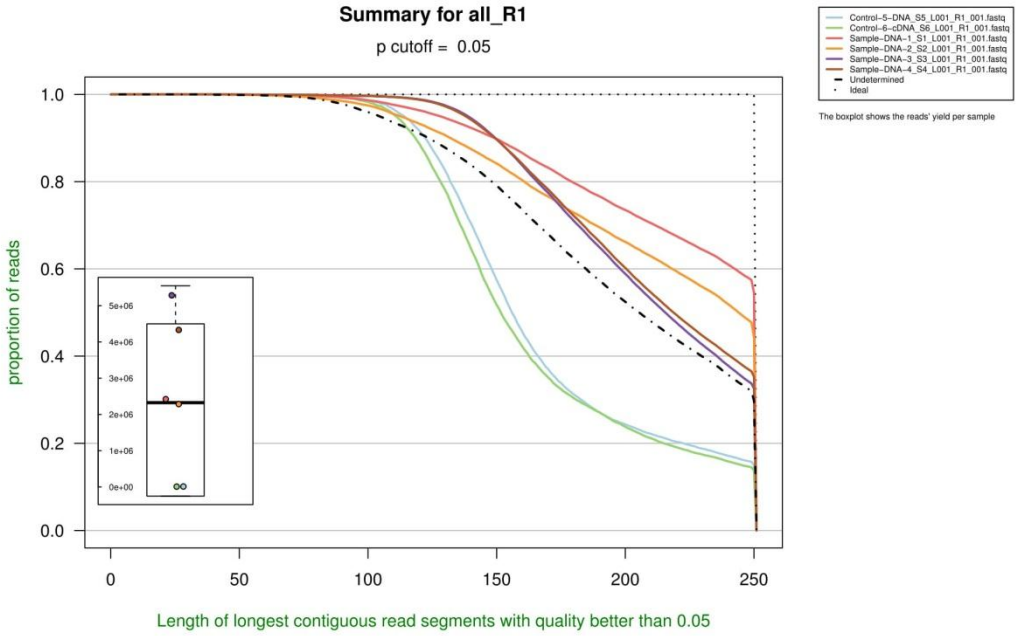


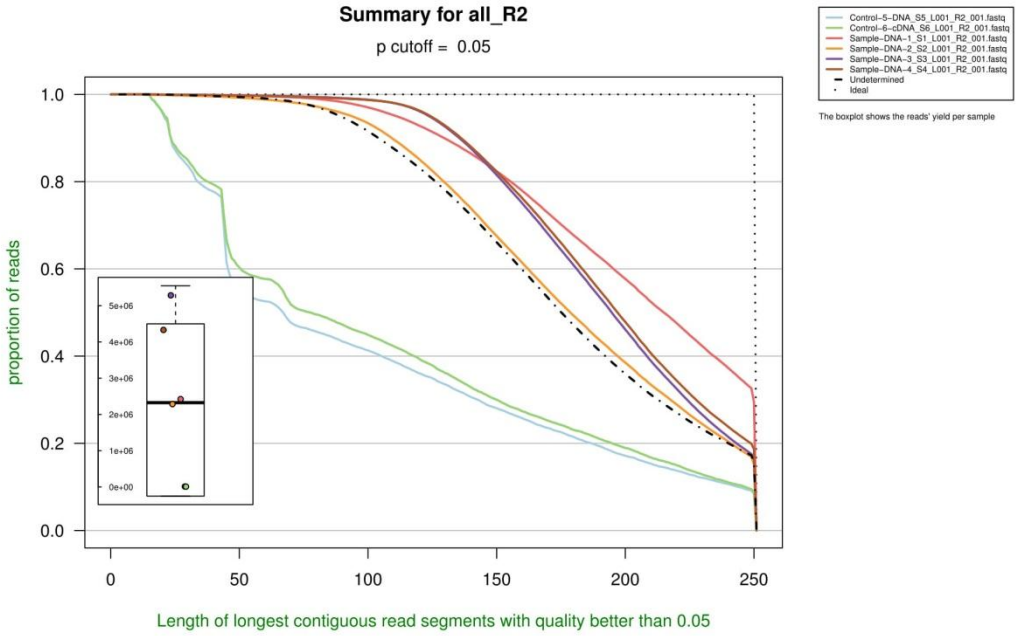
III. Distribution of sequences from the read 1 (R1) and 2 (R2) using a histogram plot. The default value is 0.05, approximately equal to a Phred score of ~13.





IV. Cummulative plots across all samples (NGS1-4 submitted as sample DNA 1-4) and control 5-6 in read 1 (R1) and read 2 (R2) for sequence quality using solexaQA.





F. Buffers and solutions

ALSEVER'S SOLUTION

Tri-sodium Citrate	8.0 g
Citric acid	0.55 g
NaCl	4.2 g
Glucose	20.5 g

Make up to 1000 mL with distilled H_2O, adjust to pH 6.0 and sterilize by autoclaving.

FREEZING MIXTURE

Plain media	7 mL
Fetal bovine serum (20 %)	2 mL
DMSO (syringe filtered) (10 %)	1 mL

Make up to 10 mL of freezing mixture solution.

GEL LOADING BUFFER

1 × TBE
50% Glycerol
0.005% Bromophenol Blue
0.025% Xylene Cyanol

GLUTAMAX™ SUPPLEMENT (GIBCO BRL)

200 µM in 0.85% NaCl

PHOSPATE BUFFERED SALINE, pH 7.0 (PBS)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

Make up to 1000 mL with distilled H_2O, adjust to pH 7.0 and sterilize by autoclaving.

PROTEINASE K STOCK SOLUTION (10 µg/µL)

Proteinase K	2.6 mg
Distilled H_2O	260.0 µL

SODIUM ACETATE 0.3 M

Sodium acetate	26.61 g
Distilled H2O	80 mL

Adjust to pH 5.2 and make up to 100 mL with distilled H_2O and sterilize by autoclaving.

TBE ELECTROPHORESIS BUFFER: STOCK SOLUTION (10x)

Trizma base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g
Distilled H_2O	Up to 1000 mL

TISSUE CULTURE MATERIAL

Nunc EasYFlasks™ 25 cm^3, 75 cm^3 and 150 cm^3 polysterene tissue culture flasks

G. CnPnV complete G gene sequences from New Zealand

a) CnPnV NZ-007 (1266 nt)

ATGAGACCTGTAGAGCAGCTCATACAAGAGAACTACAAGTTAACTTCACTTAGTATGGGAAGGAACTTTG
AAGTGAGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCGGCATCCTGACACGTTTAGGACTGTTGT
AAAGGTGAACCAAATGTGTAAGCTTATCGCGGGTGTGCTTACAAGTGCTGCTGTGGCAGTTTGTGTGGGG
GTCATAATGTATTCTGTATTCACATCAAACCACAAGGCCAACTCCATGCAGAATGCCACAACCCGGAACA
GCACATCCACACCTCCTCAACCAACCGCCGGTCCGCCCACCACAGAGCAAGGGACCACCCCCAGACCCAC
CAAACCTCCCACCAAGACCACCACCCACAACGAGATCATAGAGCCTGTCAAAATGGTAACACCTTCAGAG
GATCCTTACCAATGCTCCAGTAATGGTTATTTGGACCGACCTGATCTACCTGAAAATTTCAAACTCGTAT
TGGATGTTTTATGCAAGCCTCCAGGTCCTGAACACCACAACACTAGCTGTTATGAGAAACATGAAATCAA
CCCAGGAAGTGTTTGCCCTGATTTTGTAACAATGAAGGCAAACATGGGCTTAAACAATGGTGGTGGGGAG
GAAGCTGCACCTTATATAGAGGTTACCACCCTTTCTATGTACTCCAACAAAAGAGCAATGTGTGTCCACA
ATGGGTGTGATCAAGGCTTCTGTTTCTTCCTATCTGGTTTAAGTACTGATCAGGAGAGAGCTGTGCTAGA
GCTTGGAGGTCAACAGGCTATCATGGAGTTGCATTATGATTCCTACTGGAGACACTATTGGAGTAACTCT
AATTGTGTTGTTCCCAGAACAAACTGCAACCTGACAGACCAAGCTGTGATTTTGTTCCCTAGGTTTAACA
ACAAGAATCAGTCTCAGTGCACCACCTGTGCAGATTCAGTTGGCTTAGACAACAAATTTTATCTCACATG
TGATGGGCTTTTAAGAAACCTCCCTCTAGTTGGACTACCCAGCTTAAGTCCTCAAGCTCACAAAGCTGCA
CACATGCAGACCATAGGCACCACTGCAGCACCAACATCAGAGACGAGACTCTCAACCCCCGCACCCAGGG
GGTCCAAACCTCTTAGTCGGAAGAAGAGAGCTTTATGTGGTGTAAACCCAAACAGAGAACCCAAGCCAAC
AATGCCTTATTGGTGTCCCATGCTCCAATTATTTCCAAGGAGGTCCAATTCCCAAGTGACTAATTCCCTG
AATTAA

b) CnPnV NZ-048 (1245 nt)

ATGAGACCTGTGGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGAAGGAACTTTG
AAGTGAGTGGCAGCGTTACTAATTTGAACTTTGAGAGAACTCAGTATCCTGACACATTGAGGACTGTTGT
AAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTCACAAGTGCTGCTGTGGCAGTTTGTGTGGGG
GTCATAATGTATTCTGTATTCACATCAAATCACAAGGCCAACTCCACGCAGAATGCCACAATCCGGAACA
GCACATCCGCCCCTCCCCAACCAACCGCCGGCCTGCCCACCACAGAGCAAGGGACCACCCTTAAATTCAC
CAAACCCCCCACGAAAACTACCACCTACCATGAGATCACAGAGCCTGTCAAAATGGTTACACCCTCAGAG
GATCCCTACCAATGCTCCAGCAACGGCTACCTAGACCGACCTGACCTACCTGAAGATTTCAAACTCGTAT
TGGATGTTATATGCAAGCCTCCAGGTTCCGAACATCACAGCACTACCTGTTATGAGAAACGTGAGATCAA
CTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCAAACATGGGCTTAAACAATGGTGGAGGGGAG
GAAGCTGCACCTTACATAGAGGTTATCACCCTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCATA
ATGGGTGTGATCAGGGCTTCTGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCTGTGCTAGA
GCTTGGAGGTCAACAAGCTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGCAACTCT
AATTGTGCTGTTCCCAGAACAAACTGCAACTTGACAGACCAAACTGTGATTTTGTTTCCTAGTTTCAACA
ACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGATAACAAATTTTATCTCACATG
TGATGGGCTTTCAAGAAACCTCCCTCTAGTTGGACTACCCAGCCTAAGTCCTCAAGCCCACAAAGCTGCA
CTCAAACAATCCACAGGCACCACCACAGCATCAACACCAGAAACAAGGAACCCAACCCCTGCACCCAGGA
GGTCCAAACCTCTCAGTCGGAAGAAAAGAGCTTTATGTGGAGTAGACTCAAGCAGAGAACCCAAACCAAC
AATGCCTTATTGGTGTCCTATGCTCCAATTATTTCCAAGGAGATCTAATTCTTAA

c) CnPnV NZ-049 (1245 nt)

ATGAGACCTGTGGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGAAGGAACTTTG
AAGTGAGTGGCAGCGTTACTAATTTGAACTTTGAGAGAACTCAGTATCCTGACACATTGAGGACTGTTGT
AAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTCACAAGTGCTGCTGTGGCAGTTTGTGTGGGG
GTCATAATGTATTCTGTATTCACATCAAATCACAAGGCCAACTCCACGCAGAATGCCACAATCCGGAACA
GCACATCCGCCCCTCCCCAACCAACCGCCGGCCTGCCCACCACAGAGCAAGGGACCACCCTTAAATTCAC
CAAACCCCCCACGAAAACTACCACCTACCATGAGATCACAGAGCCTGTCAAAATGGTTACACCCTCAGAG
GATCCCTACCAATGCTCCAGCAACGGCTACCTAGACCGACCTGACCTACCTGAAGATTTCAAACTCGTAT
TGGATGTTATATGCAAGCCTCCAGGTTCCGAACATCACAGCACTACCTGTTATGAGAAACGTGAGATCAA
CTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCAAACATGGGCTTAAACAATGGTGGAGGGGAG
GAAGCTGCACCTTACATAGAGGTTATCACCCTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCATA
ATGGGTGTGATCAGGGCTTCTGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCTGTGCTAGA

GCTTGGAGGTCAACAAGCTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGCAACTCT
AATTGTGCTGTTCCCAGAACAAACTGCAACTTGACAGACCAAACTGTGATTTTGTTTCCTAGTTTCAACA
ACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGATAACAAATTTTATCTCACATG
TGATGGGCTTTCAAGAAACCTCCCTCTAGTTGGACTACCCAGCCTAAGTCCTCAAGCCCACAAAGCTGCA
CTCAAACAATCCACAGGCACCACCACAGCATCAACACCAGAAACAAGGAACCCAACCCCTGCACCCAGGA
GGTCCAAACCTCTCAGTCGGAAGAAAAGAGCTTTATGTGGAGTAGACTCAAGCAGAGAACCCAAACCAAC
AATGCCTTATTGGTGTCCTATGCTCCAATTATTTCCAAGGAGATCTAATTCTTAA

H. Multiple nucleotide sequence alignment of G protein sequences of CnPnV NZ-007, CnPnV NZ-048 and CnPnV NZ-049 with murine pneumovirus MPV-15 (AY729016.1), MPV- J3666 (NC_006579.1); CnPnV sequences from the USA (KC495958.1-KC495967.1), CnPnV Ane4 (HQ734815.1) and Italian CnPnV Bari/100-12 (KF015281.1).

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                      1        10        20        30        40        50        60
                      |        |         |         |         |         |         |
            MPV-15    ------------------------------------------------------------
          MPV-J3666   ------------------------------------------------------------AUGGGA
      CnPnV NZ-007    ATGAGACCTGTAGAGCAGCTCATACAAGAGAACTACAAGTTAACTTCACTTAGTATGGGA
 CnPnV 126724-10-4DC  ATGAGACCTGTAGAGCAGCTCATACAAGAGAACTACAAGTTAACTTCACTTAGTATGGGA
  CnPnV 110230-11TX   ATGAGACCTGTTGAGCAGCTCATACAAGAGAACTACAAGTTAACTTCACTTAATATGGGA
    CnPnV 86842-09PA  ATGAGACCTGTTGAGCAGCTCATACAAGAGAACTATAAGTTAACTTCACTTAGTATGGGA
    CnPnV 7968-11OK   ATGAGACCTGTAGAGCAGCTCATACAAGAGAACTACAAGTTAACTTCACTTAGTATGGGA
   CnPnV 109594-10KS  ATGAGACCTGTAGAGCAGCTCATACAAGAGAACTACAAGTTAACTTCACTTAGTATGGGA
  CnPnV 142847-10NV   ATGAGAACTGTAGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGA
    CnPnV 13505-11OH  ATGAGAACTGTAGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGA
   CnPnV 56706-09NYC  ATGAGAACTGTAGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGA
          CnPnV Ane4  AUGAGAACUGUAGAGCAGCUCAUACAAGAGAACUACAAGUUGACUUCACUUAGUAUGGGA
        CnPnV NZ-049  ATGAGACCTGTGGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGA
        CnPnV NZ-048  ATGAGACCTGTGGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGA
   CnPnV 91065-11MA   ATGAGACCTGTAGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGA
  CnPnV Bari/100-12   AUGAGACCUGCGGAGCAGCUCAUACAAGAGAACUACAAGUUGACUUCACUUAGUAUGGGA
CnPnV 114378-10-29KY  ATGAGACCTGTGGAGCAGCTCATACAAGAGAACTACAAGTTGACTTCACTTAGTATGGGA

            MPV-15    ------------------------------------------------------------
          MPV-J3666   AGGAACUUUGAAGUGAGUGGCAGCAUUACCAAUUUGAACUUUGAGAGAACUCAGCAUCCU
      CnPnV NZ-007    AGGAACTTTGAAGTGAGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCGGCATCCT
 CnPnV 126724-10-4DC  AGGAACTTTGAAGTGAGTGGCAGCATTACCAATTTGAACTTTGAGAGAACTCGGCATCCT
  CnPnV 110230-11TX   AGGAACTTTGAAGTGAGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCAGCATCCT
    CnPnV 86842-09PA  AGGAACTTTGAAGTGAGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCAGCATCCT
    CnPnV 7968-11OK   AGGAACCTTGAAGTGAGTAGCAGCATTACTAATTTGAACTTTGAGAGAACTCAGCATCTT
   CnPnV 109594-10KS  AGAAACCTTGAAGTGAGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCAGCATCCT
  CnPnV 142847-10NV   AGGAACTTTGAAGTGAGTGACAGCATTACTAATTTGAACTTTGAGAGAACTCAGTATCCT
    CnPnV 13505-11OH  AGGAACTTTGAAGTGGGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCAGTATCCT
   CnPnV 56706-09NYC  AGGAACTTTGAAGTGGGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCAGTATCCT
          CnPnV Ane4  AGGAACUUUGAAGUGGGUGGCAGCAUUACUAAUUUGAACUUUGAGAGAACUCAGUAUCCU
        CnPnV NZ-049  AGGAACTTTGAAGTGAGTGGCAGCGTTACTAATTTGAACTTTGAGAGAACTCAGTATCCT
        CnPnV NZ-048  AGGAACTTTGAAGTGAGTGGCAGCGTTACTAATTTGAACTTTGAGAGAACTCAGTATCCT
   CnPnV 91065-11MA   AGGAACTTTGAAGTGAGTGGCAGCATTACTAATTTGAACTTTGAGAGAACTCAGTATCCT
  CnPnV Bari/100-12   AGGAACUUUGAAGUGAGUGGCAGCACUACUAAUUUGAACUUUGAGAGAACUCAGUAUCCU
CnPnV 114378-10-29KY  AGGAACTTTGAAGTGAGTGGCAGCATTACTAATTTGAACTTTGAGGGAACTCAGTATCCT

            MPV-15    ----------------------------------AUGUGUAAGCUUAUUGCAGGUGUGCUC
          MPV-J3666   GACACAUUUAGGACUGGUGUAAAAGUGAACCAAAUGUGUAAGCUUAUUGCAGGUGUGCUC
      CnPnV NZ-007    GACACGTTTAGGACTGTTGTAAAGGTGAACCAAATGTGTAAGCTTATCGCGGGTGTGCTT
 CnPnV 126724-10-4DC  GACACGTTTAGGACTGTTGTAAAGGTGAACCAAATGTGTAAGCTTATTGCGGGTGTGCTT
  CnPnV 110230-11TX   GACACATTTAGGACTGTTGTAAAGGTGAACCAAATGTGTAAGCTTATTGCGGGTGTGCTT
    CnPnV 86842-09PA  GACACATTCAGGACTGTTGTAAAGGTGAACCAAATGTGTAAGCTTATTGCGGGTGTGCTT
    CnPnV 7968-11OK   GACACATTTAGGACTGTTGTAAAAGTGAACCAGATGTGTAAGCTTATTGCGGGTGTGCTT
   CnPnV 109594-10KS  GACACATTTAGGACTGTTGTAAAAGTGAACCAGATGTGTAAGCTTATTGCGGGTGTTCTT
  CnPnV 142847-10NV   GACACATTTAGGACTGTTGTAAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTC
    CnPnV 13505-11OH  GACACATTTAGGACTGCTGTAAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTC
   CnPnV 56706-09NYC  GACACATTTAGGACTGCTGTAAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTC
          CnPnV Ane4  GACACAUUUAGGACUGUUGUAAAAGUGAACCAAAUGUGUAAGCUUAUUGCAGGUGUGCUC
        CnPnV NZ-049  GACACATTGAGGACTGTTGTAAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTC
        CnPnV NZ-048  GACACATTGAGGACTGTTGTAAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTC
   CnPnV 91065-11MA   GACACATTTAGGACTGTTGTAAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTC
  CnPnV Bari/100-12   GACACAUUUAGGGCUGUUGUAAAAGUGAACCAAAUGUGUAAGCUUAUUGCAGGUGUGCUC
CnPnV 114378-10-29KY  TACACATTTAGGACTGTTGTAAAAGTGAACCAAATGTGTAAGCTTATTGCAGGTGTGCTC

            MPV-15    ACAAGUGCUGCUGUGGCAGUUUGUGUGGGGGUCAUAAUGUAUUCUGUUUUCACAUCAAAC
          MPV-J3666   ACAAGUGCUGCUGUGGCAGUUUGUGUGGGGGUCAUAAUGUAUUCUGUUUUCACAUCAAAC
      CnPnV NZ-007    ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAC
 CnPnV 126724-10-4DC  ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGATCATAATGTATTCTGTATTCACATCAAAC
  CnPnV 110230-11TX   ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTGTTCACATCAAAC
    CnPnV 86842-09PA  ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTTATAATGTATTCTGTATTTACATCAAAC
    CnPnV 7968-11OK   ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAC
   CnPnV 109594-10KS  ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAC
  CnPnV 142847-10NV   ACTAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAT
    CnPnV 13505-11OH  ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAT
   CnPnV 56706-09NYC  ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAT
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          CnPnV Ane4   ACAAGUGCUGCUGUGGGCAGUUUGUGUGGGGGGUCAUAAUGUAUUCUGUAUUCACAUCAAAU
        CnPnV NZ-049   ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAT
        CnPnV NZ-048   ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAT
   CnPnV 91065-11MA    ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAC
  CnPnV Bari/100-12    ACAAGUGCUGCUGUGGGCAGUUUGUGUGGGGGUCAUAAUGUAUUCUGUAUUCACAUCAAAU
CnPnV 114378-10-29KY   ACAAGTGCTGCTGTGGCAGTTTGTGTGGGGGTCATAATGTATTCTGTATTCACATCAAAT

             MPV-15    CACAAGGCCAACUCCACGCAGAAUGCCACGACCCGGAACAGCACAUCCACCCCUCCCCAA
           MPV-J3666   CACAAGGCCAACUCCACGCAGAAUGCCACGACCCGGAACAGCACAUCCACCCCUCCCCAA
      CnPnV NZ-007     CACAAGGCCAACTCCATGCAGAATGCCACAACCCGGAACAGCACATCCACACCTCCTCAA
 CnPnV 126724-10-4DC   CACAAGGCCAACTCTATGCAGAATGCCACAACCCAGAACAGCACACCCACACCTCCTCAA
  CnPnV 110230-11TX    CACAGAGCCAACTCCATGCAGAACGCCACGACCCGGAACAGCACATCCACCCCTCCTCAA
    CnPnV 86842-09PA   CACAAGGCCAACTCCACGCAGAACGCCACGACCCGGAACAGCACATCCACCCCCCCTCAA
    CnPnV 7968-11OK    CACAAGGCCAACTCCATGCAGAATGCCACGACCCAGAACAGCACATCCACCCCTCCTCAA
   CnPnV 109594-10KS   CACAAGGCCAACTCCATGCAGAACGCCACGACCCGGAACAGCACATCCATCCCCTCCTCAA
  CnPnV 142847-10NV    CACAAGGCCAACTCCACGCAGAACGCCACAATCCGGAACAGCACATCCGCCCCTCTCCAA
    CnPnV 13505-11OH   CACAAGGCCAACTCCACGCAGAACGCCACAATCCGGAACAGCACATCYGCCCCTCCCCAA
   CnPnV 56706-09NYC   CACAAGGCCAACTCCACGCAGAACGCCACAATCCGGAACAGCACATCTGCCCCTCCCCAA
          CnPnV Ane4   CACAAGGCCAACUCCACGCAGAACGCCACAAUCCGGAACAGCACAUCCGCCCCUCCCCAA
        CnPnV NZ-049   CACAAGGCCAACTCCACGCAGAATGCCACAATCCGGAACAGCACATCCGCCCCTCCCCAA
        CnPnV NZ-048   CACAAGGCCAACTCCACGCAGAATGCCACAATCCGGAACAGCACATCCGCCCCTCCCCAA
   CnPnV 91065-11MA    CACAAGGCCAACTCCACGCAGAATGCCACAATCCAGAACAGCACATCCGCCCCTCCCCAA
  CnPnV Bari/100-12    CACAAGGCCAACUCCAUGCAGAAUGCCACAAUCCGGAACAGCACAUCCGCCCCUCCCCAA
CnPnV 114378-10-29KY   CACAAGGCCAACTCCACGCAGAACGCCACAATCCGGAACAGCACATCCGCCCCTCCCCAA

             MPV-15    CCAACCGCCGGUCUGCCCACCACAGAGCAAGGGACCAUCCCCAGAUUCACCAAACCCCCC
           MPV-J3666   CCAACCGCCGGUCUGCCCACCACAGAGCAAGGGACCAUCCCCAGAUUCACCAAACCCCCC
      CnPnV NZ-007     CCAACCGCCGGTCCGCCCACCACAGAGCAAGGGACCACCCCCAGACCCACCAAACCTCCC
 CnPnV 126724-10-4DC   CCAACCGCCGGTCCGCCCACCACAGAGCAAGGGACCACCCCCAGACCCACCAAACCCCCC
  CnPnV 110230-11TX    CCAACCGCCGGTCCGCCCACCACAGAGCAAGAGACCACCCACAGACCCACCAAACTCCCT
    CnPnV 86842-09PA   CCAACCGCCGGTCCACCCACCACAGAGCAGGGGACCACCCCCAGACCCACCAAACCCCCC
    CnPnV 7968-11OK    CCAACCGCCGGTCCACCCACCACAGAGCAAGGGACCACCCCCAGATCCACCAGACCCCCC
   CnPnV 109594-10KS   CCAACCGCCGGTCCACCCACCACAGAGCAAGGGACCACCCCCAGATCCACCAGACCCCCC
  CnPnV 142847-10NV    CCAACCACCAGCCTGCCCACCACAGAGCAAGGGACCACCCCCAAATTCACCAAACCCCCC
    CnPnV 13505-11OH   CCAACCGCCGGYCTGCCCACCACAGAGCAAGGGACCACCCCCAAACTCACCAAACCCCCC
   CnPnV 56706-09NYC   CCAACCGCCGGCCTGCCCACCACAGAGCAAGGGACCACCCCCAAACTCACCAAACCCCCC
          CnPnV Ane4   CCAACCGCCGGCCUGCCCACCACAGAGCAAGGGACCACCCCCAAACUCACCAAACCCCCC
        CnPnV NZ-049   CCAACCGCCGGCCTGCCCACCACAGAGCAAGGGACCACCCTTAAATTCACCAAACCCCCC
        CnPnV NZ-048   CCAACCGCCGGCCTGCCCACCACAGAGCAAGGGACCACCCTTAAATTCACCAAACCCCCC
   CnPnV 91065-11MA    CCAACCGCCGGCCTGCCCACCACAGAGCAAGGGACCACCCTTAAATTCACCAAACCCCCC
  CnPnV Bari/100-12    CCAACCGCCGGCCCGCCCACCACAGAGCAAGGGACCACCCCCAAAUUCACCAAACCCCCC
CnPnV 114378-10-29KY   CCAACCGCCGGCCCGCCCACCACAGAGCAAGGGACCACCCTTAAATTCACCAAACCCCCC

             MPV-15    ACCAAAACCGCCACCCACCAUGAGAUCACAGAGCCCGUCAAAAUGGCAACACCUUCAGAG
           MPV-J3666   ACCAAAACCGCCACCCACCAUGAGAUCACAGAGCCCGUCAAAAUGGCAACACCUUCAGAG
      CnPnV NZ-007     ACCAAGACCACCACCCACAACGAGATCATAGAGCCTGTCAAAATGGTAACACCTTCAGAG
 CnPnV 126724-10-4DC   ACCAAGACCACCACCCACCACGAGACCACAGAGCCTGTCAAAATGGCAACACCCTCAGAG
  CnPnV 110230-11TX    ACCAAGACCACAAACCACCATGAGATCATAGAGCCTGTCAAAATGGTAACACCTTCAGAG
    CnPnV 86842-09PA   ACCAAGACCACCAACTATCATGAGATCATAGAGCCTGTCAAAATGGTAACACCTTCAGAG
    CnPnV 7968-11OK    TCCAAGACCACCACCCACCATGAGATCATAGAGCCTGTCAAAATGGTAACACCTTCAGAG
   CnPnV 109594-10KS   ACCAAGACCACCACCCACCACGAGACCACAGAGCCTGTCAAAATGGCAACACCCTCAGAG
  CnPnV 142847-10NV    ACCAAAACCACCACCCACCATGAGATCACAGAGCCTGTCAAAATGGTAACACCCTCAGAG
    CnPnV 13505-11OH   ACCAAAACCACCACCCACCATGAGATCACAGAGCCTGTCAAAATGGTGACACCCTCAGAG
   CnPnV 56706-09NYC   ACCAAAACCACCACCCACCATGAGATCACAGAGCCTGTCAAAATGGTGACACCCTCAGAG
          CnPnV Ane4   ACCAAAACCACCACCCACCAUGAGAUCACAGAGCCUGUCAAAAUGGUGACACCCUCAGAG
        CnPnV NZ-049   ACGAAAACTACCACCTACCATGAGATCACAGAGCCTGTCAAAATGGTTACACCCTCAGAG
        CnPnV NZ-048   ACGAAAACTACCACCTACCATGAGATCACAGAGCCTGTCAAAATGGTTACACCCTCAGAG
   CnPnV 91065-11MA    ACGAAAACTACCACCTACCATGAGATCACAGAACCTGTCAAAATGGTAACACCCTCAGAG
  CnPnV Bari/100-12    ACGAAAACCACCACCCACCAUGAGAUCACAGAGCCCGCCAAAAUGGUAACACCCUCAGAG
CnPnV 114378-10-29KY   ACGAAAACTACCACCTACCATGAGATCACAGAGCCTGTCAAAATGGTAACACCCTCAGAG

             MPV-15    GAUCCCUACCAAUGCUCCAGCAAUGGUUAUUUGGACCGACCUGAUUUACCUGAAAAUUUC
           MPV-J3666   GAUCCCUACCAAUGCUCCAGCAAUGGUUAUUUGGACCGACCUGAUUUACCUGAAAAUUUC
      CnPnV NZ-007     GATCCTTACCAATGCTCCAGTAATGGTTATTTGGACCGACCTGATCTACCTGAAAATTTC
 CnPnV 126724-10-4DC   GATCCCCACAAATGCTCCAGCAATGGTTATTTGGACCGACCTGATTTACCTGAAAATTTC
  CnPnV 110230-11TX    GATCTCTACCAATGCTCCAGTAATGGTTATTTGGACCGACCTGATCTACCTGAAAATTTC
    CnPnV 86842-09PA   GATCCCTACCAATGCTCCAGTAATGGTTATTTAGACCGACCTGATCTACCTGAAGATTTC
    CnPnV 7968-11OK    GATCCCTACCAATGCTCCAGTAATGGTTATTTGGACCGACCTGATCTACCTGAAAATTTC
   CnPnV 109594-10KS   GATCCCTACCAATGCTCCAGTAATGGTTATTTGGACCGACCTGATCTACCTGAAAATTTC
  CnPnV 142847-10NV    GATCCTTACCAATGCTCCAGCAATGGTTATTTAGACCGACCTGATTTACCTGAAGATTTC
    CnPnV 13505-11OH   GATCCCTACCAATGCTCCAGCAATGGTTATTTAGACCGACCTGATTTACCTGAAGATTTC
   CnPnV 56706-09NYC   GATCCCTACCAATGCTCCAGCAATGGTTATTTAGACCGACCTGATTTACCTGAAGATTTC
          CnPnV Ane4   GAUCCCUACCAAUGCUCCAGCAAUGGUUAUUUAGACCGACCUGAUUUACCUGAAGAUUUC
        CnPnV NZ-049   GATCCCTACCAATGCTCCAGCAACGGCTACCTAGACCGACCTGACCTACCTGAAGATTTC
        CnPnV NZ-048   GATCCCTACCAATGCTCCAGCAACGGCTACCTAGACCGACCTGACCTACCTGAAGATTTC
   CnPnV 91065-11MA    GATCCCTACCAATGCTCCAGCAATGGCTACCTAGACCGACCTGACCTACCTGAAGATTTC
  CnPnV Bari/100-12    GAUCCCUACCAAUGCUCCAGCAAUGGCUACCUAGACCGACCUGACCUACCUGAAGAUUUC
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CnPnV 114378-10-29KY   GATCCTTACCAATGCTCCAGCAATGGCTACCTAGACCGACCTGACCTACCTGAAGATTTC

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          MPV-J3666    AAACUCGUAUUGGAUGUUAUAUGCAAGCCUCCAGGUCCUGAACAUCACAACACCAGCUGU
      CnPnV NZ-007     AAACTCGTATTGGATGTTTTATGCAAGCCTCCAGGTCCTGAACACCACAACACTAGCTGT
 CnPnV 126724-10-4DC   AAACTCATATTGGATGTTTTATGCAAGCCTCCAGGTCCTGAACATCACAACACCAGCTGT
  CnPnV 110230-11TX    AAACCCGTATTGGATATTTTATGCAAGCCTCCAGGTCCTGAACATCACAACACTAGCTGT
    CnPnV 86842-09PA   AAACTCGTATTGGATGTTTTATGCAAGCCTCCAGGTCCTGAACATCACAACACCAGCTGT
    CnPnV 7968-11OK    AAACTCGTATTGGATGTTTTATGCAAGCCTCCAGGTCCTGAACATCACAACACCAGCTGT
   CnPnV 109594-10KS   AAACCCGTATTGGATGTTTTATGCAAGCCTCCAGGTCCTGAACATCACAGCACCAGCTGT
  CnPnV 142847-10NV    AAACTCGTATTGGATGTTCTATGCAAGCCCCCAGGTCCTGAACATCACAGCACCAACTGT
    CnPnV 13505-11OH   AAGCTCGTATTGGATGTTCTATGCAAGCCTCCAGGTCCTGAACATCACAGCACCAACTGT
   CnPnV 56706-09NYC   AAGCTCGTATTGGATGTTCTATGCAAGCCTCCAGGTCCTGAACATCACAGCACCAACTGT
          CnPnV Ane4   AAGCUCGUAUUGGAUGUUCUAUGCAAGCCUCCAGGUCCUGAACAUCACAGCACCAACUGU
        CnPnV NZ-049   AAACTCGTATTGGATGTTATATGCAAGCCTCCAGGTTCCGAACATCACAGCACTACCTGT
        CnPnV NZ-048   AAACTCGTATTGGATGTTATATGCAAGCCTCCAGGTTCCGAACATCACAGCACTACCTGT
   CnPnV 91065-11MA    AAACTTGTATTGGATGTTATATGCAAGCCTCCAGGTCCCGAACATCACAGCACCAACTGT
  CnPnV Bari/100-12    AAACUCGUAUUGGAUGUUAUAUGCAAGCCUCCAGGUCCCGAACAUCACAGCACCAACUGU
CnPnV 114378-10-29KY   AAACTCGTGTTGGATGTTATATGCAAGCCTCCAGGTCCCGAACATCACAGCACCAACTGT

            MPV-15     UAUGAGAAACGUGAAAUCAACCCAGGAAGUGUUUGCCCUGAUCUUGUAACAAUGAAGGCA
          MPV-J3666    UAUGAGAAACGUGAAAUCAACCCAGGAAGUGUUUGCCCUGAUCUUGUAACAAUGAAGGCA
      CnPnV NZ-007     TATGAGAAACATGAAATCAACCCAGGAAGTGTTTGCCCTGATTTTGTAACAATGAAGGCA
 CnPnV 126724-10-4DC   TATGAGAAACATGAAATCAACCCAGGAAGTGTTTGCCCGGATTTTGTAACAATGAAGGCA
  CnPnV 110230-11TX    TATGAGAAGCATGAAATCAACCCAGGAAGTGTTTGCCCTGATTTTGTAACAATGAAGGCA
    CnPnV 86842-09PA   TATGAGAAACATGAAATCAACCCAGGAAGTGTTTGCCCTGATTTTGTAACAATGAAGGCA
    CnPnV 7968-11OK    TATGAGAAACATGAAATCAACCCAGGAAGTGTTTGCCCTGATTTTGTAACAATGAAGGCA
   CnPnV 109594-10KS   TATGAGAAACATGAAATCAACCCAGGAAGTGTTTGCCCTGATTTTGTAACAATGAAGGCA
  CnPnV 142847-10NV    TATGAGAAACGTGAGATCAACTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCT
    CnPnV 13505-11OH   TATGAGAAACGTGAGATCAACTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCA
   CnPnV 56706-09NYC   TATGAGAAACGTGAGATCAACTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCA
          CnPnV Ane4   UAUGAGAAACGUGAGAUCAACUUAGGAAGUGUUUGCCCUGAUCUUGUAACAAUGAAGGCA
        CnPnV NZ-049   TATGAGAAACGTGAGATCAACTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCA
        CnPnV NZ-048   TATGAGAAACGTGAGATCAACTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCA
   CnPnV 91065-11MA    TATGAGAAACGTGAGATCAACTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCA
  CnPnV Bari/100-12    UAUGAGAAACGUGAGAUCAACUUAGGAAGUGUUUGCCCUGAUCUUGUAACAAUGAAGGCA
CnPnV 114378-10-29KY   TATGAGAAACATGAGATCAACTTAGGAAGTGTTTGCCCTGATCTTGTAACAATGAAGGCA

            MPV-15     AACAUGGGCUUAAACAAUGGUGGUGGGGAGGAUGCUGCACCUUAUAUAGAGGUUACCACC
          MPV-J3666    AACAUGGGCUUAAACAAUGGUGGUGGGGAGGAUGCUGCACCUUAUAUAGAGGUUACCACC
      CnPnV NZ-007     AACATGGGCTTAAACAATGGTGGTGGGGAGGAAGCTGCACCTTATATAGAGGTTACCACC
 CnPnV 126724-10-4DC   AACATGGGCTTAAACAATGGTGGTGGGGAGGAAGCTGCACCTTATATAGAGGTTACCACC
  CnPnV 110230-11TX    AACATGGGCTTAAACAATGGTGGTGGGGAGGAAGCTGCACCTTACATAGAGGTTACCACC
    CnPnV 86842-09PA   AACATGGGCTTAAACAATGGTGGTGGGGAGGAAGCTGCACCTTATATAGAGGTTACCACC
    CnPnV 7968-11OK    AACATGGGCTTAAACAATGGTGGTGGGGAGGAAGCTGCACCTTATATAGAAGTTACCACC
   CnPnV 109594-10KS   AACATGGGCTTAAACAATGGTGGTGGGGAAGAAGCTGCACCTTATATAGAGGTTACCACC
  CnPnV 142847-10NV    AACATGGGCTTAAACAATGGTGGTGGGGAAGAAGCTGCACCTTATATAGAGGTTATCACC
    CnPnV 13505-11OH   AACATGGGCTTAAACAATGGTGGTGGGGAAGAAGCTGCACCTTATATAGAGGTTATCACC
   CnPnV 56706-09NYC   AACATGGGCTTAAACAATGGTGGTGGGGAAGAAGCTGCACCTTATATAGAGGTTATCACC
          CnPnV Ane4   AACAUGGGCUUAAACAAUGGUGGUGGAGAAGAAGCUGCACCUUAUAUAGAGGUUAUCACC
        CnPnV NZ-049   AACATGGGCTTAAACAATGGTGGAGGGGAGGAAGCTGCACCTTACATAGAGGTTATCACC
        CnPnV NZ-048   AACATGGGCTTAAACAATGGTGGAGGGGAGGAAGCTGCACCTTACATAGAGGTTATCACC
   CnPnV 91065-11MA    AACATGGGCTTAAACAATGGTGGAGGGGAGGAAGCTGCACCTTATATAGAGGTTATCACC
  CnPnV Bari/100-12    AACAUGGGCUUAAACAAUGGUGGAGGGGAGGAAGCUGCACCUUAUAUAGAGGUUAUCACC
CnPnV 114378-10-29KY   AACATGGGCTTAAACAATGGTGGAGGGGAGGAAGCTGCACCTTATATAGAGGTTATCACC

            MPV-15     CUUUCUACGUACUCCAACAAAAGGGCAAUGUGUGUCCACAAUGGGUGUGAUCAAGGCUUC
          MPV-J3666    CUUUCUACGUACUCCAACAAAAGGGCAAUGUGUGUCCACAAUGGGUGUGAUCAAGGCUUC
      CnPnV NZ-007     CTTTCTATGTACTCCAACAAAAGAGCAATGTGTGTCCACAATGGGTGTGATCAAGGCTTC
 CnPnV 126724-10-4DC   CTTTCTATGTACTCCAACAAAAGAGCAATGTGTGTCCACAATGGGTGTGACCAAGGCTTC
  CnPnV 110230-11TX    CTTTCTATGTACTCCAACAAAAGAGCAATGTGTGTCCACAATGGGTGTGACCAAGGCTTC
    CnPnV 86842-09PA   CTTTCTATGTACTCCAACAAAAGAGCAATGTGTGTCCACAATGGGTGTGACCAAGGCTTC
    CnPnV 7968-11OK    CTTACTATGTACTCCAACAAAAGAGCAATGTGTGTCCACAATGGGTGTGACCAAGGCTTC
   CnPnV 109594-10KS   CTTACTATGTACTCCAACAAAAGAGCAATGTGTGTCCACAATGGGTGTGACCAAGGCTTC
  CnPnV 142847-10NV    CTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCACAATGGATGTGATCAGGGCTTC
    CnPnV 13505-11OH   CTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCACAATGGGTGTGATCAGGGCTTC
   CnPnV 56706-09NYC   CTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCACAATGGGTGTGATCAGGGCTTC
          CnPnV Ane4   CUUUCUACGUACUCCAACAAAAGGGCAAUGUGUGUCCACAAUGGGUGUGAUCAGGGCUUC
        CnPnV NZ-049   CTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCATAATGGGTGTGATCAGGGCTTC
        CnPnV NZ-048   CTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCATAATGGGTGTGATCAGGGCTTC
   CnPnV 91065-11MA    CTTTCTACGTACTCCAACAAAAGGGCAATGTGTATCCACAATGGGTGTGATCAGGGCTTT
  CnPnV Bari/100-12    CUUUCUACGUACUCCAACAAAAGGGCAAUGUGUGUCCAUAAUGGGUGUGAUCAGGGCUUC
CnPnV 114378-10-29KY   CTTTCTACGTACTCCAACAAAAGGGCAATGTGTGTCCATAATGGGTGTGATCAGGGCTTC

            MPV-15     UGUUUCUUCCUUUCUGGUUUAAGCACUGAUCAGGAGAGAGCUGUGCUAGAGCUUGGAGGU
          MPV-J3666    UGUUUCUUCCUUUCUGGUUUAAGCACUGAUCAGGAGAGAGCUGUGCUAGAGCUUGGAGGU
      CnPnV NZ-007     TGTTTCTTCCTATCTGGTTTAAGTACTGATCAGGAGAGAGCTGTGCTAGAGCTTGGAGGT
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 CnPnV 126724-10-4DC   TGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGGAGAGAGCTGTGCTAGAGCTTGGAGGT
  CnPnV 110230-11TX    TGTTTCTTCCTATCTGGTCTAAGCACTGATCAGGAGAGAGCTGTGCTAGAGCTTGGAGGT
    CnPnV 86842-09PA   TGTTTTTTCCTATCTGGTTTAAGTACTGATCAGGAGAGGGCTGTGCTAGAGCTTGGAGGT
    CnPnV 7968-11OK    TGTTTCTTCCTATCTGGTTTAAGCACTGATCAGGAGAGAGCTGTGCTAGAGCTTGGAGGT
   CnPnV 109594-10KS   TGTTTCTTCCTATCTGGTTTAAGCACTGATCAGGAGAGAGCTGTGCTAGAGCTTGGAGGT
  CnPnV 142847-10NV    TGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCTGTGCTAGAGCTTGGAGGT
    CnPnV 13505-11OH   TGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCCGTGCTAGAGCTTGGAGGT
   CnPnV 56706-09NYC   TGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCCGTGCTAGAGCTTGGAGGT
          CnPnV Ane4   UGUUUCUUCCUUUCUGGUUUAAGCACUGAUCAGAAGAGAGCCGUGCUAGAGCUUGGAGGU
        CnPnV NZ-049   TGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCTGTGCTAGAGCTTGGAGGT
        CnPnV NZ-048   TGTTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCTGTGCTAGAGCTTGGAGGT
   CnPnV 91065-11MA    TGTTTCTTTCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCTGTGCTAGAGCTTGGAGGT
  CnPnV Bari/100-12    UGUUUCUUCCUUUCUGGUUUAAGCACUGAUCAGAAGAGAGCUGUGCUAGAGCUUGGAGGC
CnPnV 114378-10-29KY   TGCTTCTTCCTTTCTGGTTTAAGCACTGATCAGAAGAGAGCTGTGCTAGAGCTTGGAGGT

             MPV-15    CAACAGGCUAUCAUGGAGUUGCAUUAUGAUUCCUACUGGAAACACUAUUGGAGUAACUCU
           MPV-J3666   CAACAGGCUAUCAUGGAGUUGCAUUAUGAUUCCUACUGGAAACACUAUUGGAGUAACUCU
       CnPnV NZ-007    CAACAGGCTATCATGGAGTTGCATTATGATTCCTACTGGAGACACTATTGGAGTAACTCT
 CnPnV 126724-10-4DC   CAACAGGCTATCATGGAGTTGCATTATGACTCCTACTGGAAACACTATTGGAGTAACTCT
  CnPnV 110230-11TX    CAACAGGCTATCATGGAGTTGCACCATGACTCCTACTGGAAACACTATTGGAGTAACTCC
    CnPnV 86842-09PA   CAACAGGCTATCATGGAGTTGCATTATGACTCTTACTGGAAACACTATTGGAGTAACTCT
    CnPnV 7968-11OK    CAACAGGCTATCATGGAGTTGCATTACGACTCCTACTGGAAACACTATTGGAGTAACTCT
   CnPnV 109594-10KS   CAACAGGCTATCATGGAGTTGCATTATGACTCATACTGGAAACACTATTGGAGTAACTCA
  CnPnV 142847-10NV    CAACAAGCTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGTAACTCT
    CnPnV 13505-11OH   CAACAAGTTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGTAACTCT
   CnPnV 56706-09NYC   CAACAAGTTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGTAACTCT
          CnPnV Ane4   CAACAAGUUAUCAUGGAAUUGCAUUAUGAUUCCUAUUGGAAACACUAUUGGAGUAACUCU
        CnPnV NZ-049   CAACAAGCTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGCAACTCT
        CnPnV NZ-048   CAACAAGCTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGCAACTCT
   CnPnV 91065-11MA    CAACAAGCTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGTAACTCT
  CnPnV Bari/100-12    CAACAAGCUAUCAUGGAAUUGCAUUAUGAUUCCUAUUGGAAACACUAUUGGAGCAACUCU
CnPnV 114378-10-29KY   CAACAAGCTATCATGGAATTGCATTATGATTCCTATTGGAAACACTATTGGAGCAACTTT

             MPV-15    AAUUGUGUUGUUCCCAGAACAAACUGCAACCUGACAGACCAAACUGAGAUUUUGUUUCCU
           MPV-J3666   AAUUGUGUUGUUCCCAGAACAAACUGCAACCUGACAGACCAAACUGAGAUUUUGUUUCCU
       CnPnV NZ-007    AATTGTGTTGTTCCCAGAACAAACTGCAACCTGACAGACCAAGCTGTGATTTTGTTCCCT
 CnPnV 126724-10-4DC   AATTGTGTTGTTCCCAGAACAAACTGCAACCTGACAGACCAAACTGTGATTTTGTTCCCT
  CnPnV 110230-11TX    AACTGTGTTGTTCCCAGAACAAACTGCAATCTGACAGACCAAACTGTGATTTTGTTTCCT
    CnPnV 86842-09PA   AATTGTGTTGTTCCCAGAACAAGCTGCAACCTGACAGACCAAACTGTGATTTTGTTTCCT
    CnPnV 7968-11OK    AATTGTGTTGTTCCCAGAACAAACTGCAATCTGACAGACCAAACTGTGACTCTGTTTCCT
   CnPnV 109594-10KS   AATTGTGTTGTTCCCAGAACAAACTGCAATCTGACAGACCAAACTGTGATTTTGTTTCCT
  CnPnV 142847-10NV    AATTGTATTGTTCCCAGAACAAACTGCAACCTGACAGACCAAACTGTGATTTTGTTTCCT
    CnPnV 13505-11OH   AATTGTGTTGTTCCCAGAACAAACTGCAACCTGACAGACCAAACTGTGATTTTGTTTCCT
   CnPnV 56706-09NYC   AATTGTGTTGTTCCCAGAACAAACTGCAACCTGACAGACCAAACTGTGATTTTGTTTCCT
          CnPnV Ane4   AAUUGUGUUGUUCCCAGAACAAACUGCAACCUGACAGACCAAACUGUGAUUUUGUUUCCU
        CnPnV NZ-049   AATTGTGCTGTTCCCAGAACAAACTGCAACTTGACAGACCAAACTGTGATTTTGTTTCCT
        CnPnV NZ-048   AATTGTGCTGTTCCCAGAACAAACTGCAACTTGACAGACCAAACTGTGATTTTGTTTCCT
   CnPnV 91065-11MA    AATTGTGTTGTTCCCAGAACAAACTGCAACCTGACAGACCAAACTGTGATTTTGTTTCCT
  CnPnV Bari/100-12    AAUUGUGUUGUUCCCAGAACAAACUGCAACCUGACAGACCAAACUGUGAUUUUGUUUCCU
CnPnV 114378-10-29KY   AATTGTGTTGTTCCCAGAACAAACTGCAACCTGACAGACCAAACTGTGATTTTGTTTCCT

             MPV-15    AGGUUUAACAACAAGAAUCAGUCUGAGUGUACCACCUGUGCAGAUUCAGCUGGCCUAGAU
           MPV-J3666   AGGUUUAACAACAAGAAUCAGUCUCAGUGUACCACCUGUGCAGAUUCAGCUGGCCUAGAU
       CnPnV NZ-007    AGGTTTAACAACAAGAATCAGTCTCAGTGCACCACCTGTGCAGATTCAGTTGGCTTAGAC
 CnPnV 126724-10-4DC   AGGTTTAACAACAAGAATCAGTCTCAGTGCACCACCTGTGCAGATTCAGCTGGCCTAGAT
  CnPnV 110230-11TX    AGGTTTAACAACAAAAATCAGTCTCAGTGCACCACCTGTGCAGATTCAGCTGGCCTAGAT
    CnPnV 86842-09PA   AGGTTTAACAACAAGAATCAGTCTCAGTGCACCACCTGTGCAGATTCAGCTGGCCTAGAT
    CnPnV 7968-11OK    AGGTTTAACAACAAGAATCAGTCTCAGTGCACCACCTGTGCAGATTCAGTTGGCCTAGAT
   CnPnV 109594-10KS   AGGTTTAACAACAAGAATCAGTCTCAGTGCATCACCTGTGCAGATTCAGCTGGCCTAGAT
  CnPnV 142847-10NV    AGTTTTAACAACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCAGGCCTAGAT
    CnPnV 13505-11OH   AGTTTTAACAACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGAT
   CnPnV 56706-09NYC   AGTTTTAACAACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGAT
          CnPnV Ane4   AGUUUUAACAACAAGAAUCAGUCUCAGUGUACCACCUGUGCAGACUCAGCUGGCCUAGAU
        CnPnV NZ-049   AGTTTCAACAACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGAT
        CnPnV NZ-048   AGTTTCAACAACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGAT
   CnPnV 91065-11MA    AGTTTTAACAACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGAT
  CnPnV Bari/100-12    AGUUUUAACAACAAGAAUCAGUCUCAGUGUACCACCUGUGCAGACUCAGCUGGCUUAGAU
CnPnV 114378-10-29KY   AGTTTTAACAACAAGAATCAGTCTCAGTGTACCACCTGTGCAGACTCAGCTGGCCTAGAT

             MPV-15    AACAAAUUUUAUCUCACAUGUGAUGGGCUUUUAAGAACCCUCCCUCUAGUUGGACUACCC
           MPV-J3666   AACAAAUUUUAUCUCACAUGUGAUGGGCUUUUAAGAACCCUCCCUCUAGUUGGACUACCC
       CnPnV NZ-007    AACAAATTTTATCTCACATGTGATGGGCTTTTAAGAAACCTCCCTCTAGTTGGACTACCC
 CnPnV 126724-10-4DC   AACAAATTTTATCTCACATGTGATGGGCTTTTAAGAAACCTCCCTCTAGTTGGACTACCC
  CnPnV 110230-11TX    AACAAATTTTATCTCACATGTGATGGGCTTTTAAGAAACCTCCCTCTAGTTGGACTACCC
    CnPnV 86842-09PA   AACAAATTTTATCTCACATGTGATGGGCTTTTAAGAAACCTCCCTCTAGTTGGACTACCC
    CnPnV 7968-11OK    AATAAATTTTATCTCACATGTGATGGGCTTCTAAGAAACCTCCCTCTAGTTGGACTACCC
   CnPnV 109594-10KS   AACAAATTTTATCTCACATGTGATGGGCTTCTAAGAAACCTCCCTCTAGTTGGACTACCC
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  CnPnV 142847-10NV    AACAAATTTTATCTCACATGTGATGGGCTTTTAAGAAACCTCCCTCTAGTTGGACTACCC
    CnPnV 13505-11OH   AACAAATTTTATCTCACATGTGATGGGCTTTTAAGAAACCTTCCTCTAGTTGGACTACCC
   CnPnV 56706-09NYC   AACAAATTTTATCTCACATGTGATGGGCTTTCAAGAAACCTTCCTCTAGTTGGACTACCC
          CnPnV Ane4   AACAAAUUUUAUCUCACAUGUGAUGGGCUUUCAAGAAACCUUCCUCUAGUUGGACUACCC
        CnPnV NZ-049   AACAAATTTTATCTCACATGTGATGGGCTTTCAAGAAACCTCCCTCTAGTTGGACTACCC
        CnPnV NZ-048   AACAAATTTTATCTCACATGTGATGGGCTTTCAAGAAACCTCCCTCTAGTTGGACTACCC
   CnPnV 91065-11MA    AACAAATTTTATCTCACATGTGATGGGCTTTCAAGAAACCTCCCTCTAGTTGGACTACCC
  CnPnV Bari/100-12    AACAAAUUUUAUCUCACAUGUGAUGGGCUUUCAAGAAACCUCCCUCUAGUUGGACUACCC
CnPnV 114378-10-29KY   AACAAATTTTACCTCACATGTGATGGGCTTTCAAGAAACCTCCCTCTAGTTGGACTACCC

             MPV-15    AGCCUAAGUCCUCAGGCUUACAAAGCUGUACCCACACAAACUACAGGCACCACCACGGCA
           MPV-J3666   AGCCUAAGUCCUCAGGCUUACAAAGCUGUACCCACACAAACUACAGGCACCACCACGGCA
       CnPnV NZ-007    AGCTTAAGTCCTCAAGCTCACAAAGCTGCACACATGCAGACCATAGGCACCACTGCAGCA
 CnPnV 126724-10-4DC   AGCTTAAGTCCTCAAGCTCACAAAGCTGCACACATGCAGACCACAGGCACCACTGCAGCA
  CnPnV 110230-11TX    AGCCTAAGTCCTCAAGCTCACAAAGCTGCACACACGCAGGCCTCAGGCACCACCGCAGCA
    CnPnV 86842-09PA   AGCCTAAGTCCTCAAGCCCACAAAGCTGCACACACGCAGGCCACAGGCACCACCGCAGCA
    CnPnV 7968-11OK    AGCCTAAGTCCTCAGACTCACAAAGCTGCACACACGCAGGCCACAGGCACCACCGCAGCA
   CnPnV 109594-10KS   AGCCTAAGTCCTCAGACTTACAAAGCTGCACACACGCAGGCCACAGGCACCACCGCAGCA
  CnPnV 142847-10NV    AGCCTAAGTCCTCAGGCCCACAAGGCTGCACTCAAACAATCCACAGGCACCACCACGGCA
    CnPnV 13505-11OH   AGCCTAAGTCCTCAGGCCCACAAGGCTGCACTCAAACAATCCACAGGCACCACCACGGCA
   CnPnV 56706-09NYC   AGCCTAAGTCCTCAAGCCCACAAAGCTGCACTCAAACAATCCACAGGCACCACCACGGCA
          CnPnV Ane4   AGCCUAAGUCCUCAAGCCCACAAAGCUGCACUCAAACAAUCCACAGGCACCACCACGGCA
        CnPnV NZ-049   AGCCTAAGTCCTCAAGCCCACAAAGCTGCACTCAAACAATCCACAGGCACCACCACAGCA
        CnPnV NZ-048   AGCCTAAGTCCTCAAGCCCACAAAGCTGCACTCAAACAATCCACAGGCACCACCACAGCA
   CnPnV 91065-11MA    AGCCTAAGTCCTCAAGCCCACAAAGCTGCACTCAAACAATCCACAGGCACCACCACAGCA
  CnPnV Bari/100-12    AGCCUAAGUCCUCAAGCCCACAAAGCUGCACUCAAACAAUCCACAGGCACCACCACAGCA
CnPnV 114378-10-29KY   AGCCTAAGTCCTCAAGCCCACAAAGCTGAACTCAAACAATCCACAGGCACCACCACAGCA

             MPV-15    CCAACAUCAGAGACGAGGCACCCAACCCCUGCACCCAGGAGGUCCAAACCUCUCAGUCGG
           MPV-J3666   CCAACAUCAGAGUCGAGGCACCCAACCCCUGCACCCAGGAGGUCCAAACCUCUCAGUCGG
       CnPnV NZ-007    CCAACATCAGAGACGAGACTCTCAACCCCCGCACCCAGGGGGTCCAAACCTCTTAGTCGG
 CnPnV 126724-10-4DC   CCAACATCAGAGACGAGGCTCTCAACCCCCGCACCCAGGGGGTCCAAACCTCTTAGTCGG
  CnPnV 110230-11TX    CCAACACCAGAGACGAGGCTCCCAACCCCCGCACCCAGGGGGTTCAAACCTCTCAGTCGG
    CnPnV 86842-09PA   CCAACACCAGAGACGAGGCTCCCAACTCCCACACCTAGGGGGTCCAAACCTCTCAGTCGG
    CnPnV 7968-11OK    CCAACACCAGAGACGAGGCTCCCAACCCCCGTACCCAAGGGGTCCAAACCTCTTAGTCGA
   CnPnV 109594-10KS   CCAACACCAGAGACGAGGCTCCCAACCCCCACACCCAGGGGGTCCAAACCTTTCAGTCGG
  CnPnV 142847-10NV    CCAACACCTGAGACGAGGAACCCAACCCCTGCACCCAGGGAGTCCAAACCTCTCAGTCGG
    CnPnV 13505-11OH   CCAACACCTGAGACGAGGAACCCAACCCCTGCACCCAGGGAGTCCAAACCTCTCAGTCGG
   CnPnV 56706-09NYC   CCAACACCGGAGACAAGGAACCCAACCCCCGCACCTAGGAGGTCCAAACCTCTCAGTCGG
          CnPnV Ane4   CCAACACCGGAGACAAGGAACCCAACCCCCGCACCUAGGAGGUCCAAACCUCUCAGUCGG
        CnPnV NZ-049   TCAACACCAGAAACAAGGAACCCAACCCCTGCACCCAGGAGGTCCAAACCTCTCAGTCGG
        CnPnV NZ-048   TCAACACCAGAAACAAGGAACCCAACCCCTGCACCCAGGAGGTCCAAACCTCTCAGTCGG
   CnPnV 91065-11MA    CCAACACCAGAGACAAGGAACCCAACCCCTGCGCCCAGGAGGTCCAAACCTCTCAGTCGG
  CnPnV Bari/100-12    CCAACACCAGAGACAAGGAACCCAACCCCUGCACCCAGGAGGUCCAAACCUCUCAGUCGG
CnPnV 114378-10-29KY   CCAACACCAGAGACAAGGAACCCAACCCCTGCACCCAGGAGGTCCAAACCTCTCAGTCGG

             MPV-15    AAGAAGAGAGCUUUAUGUGGUGUAGACUCAAGCAGAGAACCCAAACCAACAAUGCCUUAC
           MPV-J3666   AAGAAGAGAGCUUUAUGUGGUGUAGACUCAAGCAGAGAACCCAAACCAACAAUGCCUUAC
       CnPnV NZ-007    AAGAAGAGAGCTTTATGTGGTGTAAACCCAAACAGAGAACCCAAGCCAACAATGCCTTAT
 CnPnV 126724-10-4DC   AAGAAGAGAGCTTTATGTGGTGTAAACCCAAACAGAGAACCCAAGCCAACAATGCCTTAT
  CnPnV 110230-11TX    AAGAAGAGAGCTTTATGTGGTGTAAACCCAAACAGAGAACCCAAACCAACAATGCCTTAT
    CnPnV 86842-09PA   AAGAAGAGAGCTTTATGTGGTGTAAACCCAAACAGAGAACCCAAACCAACAATGCCTTAT
    CnPnV 7968-11OK    AAGAAGAGAGCTTTATGTGGTGTAAACCCAAACAGAGAACCCAAACCAACAATGCCTTAT
   CnPnV 109594-10KS   AAGAAGAGAGCTTTATGTGGTGTAAACCCAAACAGAGAACCCAAACCAACAATGCCTTAT
  CnPnV 142847-10NV    AAGAAAAGAGCTTTATGTGGAGTAGGCTCAAGCAGAGAACCCAAACCAACAATGCCCTAT
    CnPnV 13505-11OH   AAGAAAAGAGCTTTATGTGGAGTAGGCTCAAGCAGAGAACCCAAACCAACAATGCCCTAT
   CnPnV 56706-09NYC   AAGAAAAGAGCTTTATGTGGAGTAGGCTCAAGCAGAGAACCCAAACCAACAATGCCCTAT
          CnPnV Ane4   AAGAAAAGAGCUUUAUGUGGAGUAGGCUCAAGCAGAGAACCCAAACCAACAAUGCCCUAU
        CnPnV NZ-049   AAGAAAAGAGCTTTATGTGGAGTAGACTCAAGCAGAGAACCCAAACCAACAATGCCTTAT
        CnPnV NZ-048   AAGAAAAGAGCTTTATGTGGAGTAGACTCAAGCAGAGAACCCAAACCAACAATGCCTTAT
   CnPnV 91065-11MA    AAGAAAAGAGCTTTATGTGGGGTAGACTCAAGCGGAGAACCCAAACCAACAATGCCTTAT
  CnPnV Bari/100-12    AAGAAAAGAGCUUUAUGUGGAGUAGACUCAAGCAGAGAACCCAAACCAACAAUGCCUUAU
CnPnV 114378-10-29KY   AAGAAAAGAGCTTTATGTGGAGTAGACTCAAGCAGAGAACCCAAACCAACAATGCCTTAT

             MPV-15    UGGUGUCCUAUGCUCCAAUUAUUUCCAAGGAGGUCUAAUUCUUAA----------------
           MPV-J3666   UGGUGUCCUAUGCUCCAAUUAUUUCCAAGGAGGUCUAAUUCUUAA----------------
       CnPnV NZ-007    TGGTGTCCCATGCTCCAATTATTTCCAAGGAGGTCCAATTCCCAAGTGACTAATTCCCTG
 CnPnV 126724-10-4DC   TGGTGTCCCATGCTCCAATTATTTCCAAGGAGGTCCAATTCCCAAGTGACCAATTCC---
  CnPnV 110230-11TX    TGGTGTCCTATGCTCCAATTATTTCCAAGGAGGTCCAACTCTTAA----------------
    CnPnV 86842-09PA   TGGTGTCCTCTGCTCCAATTATTTCCAAGGAGGTCTAATTCTTAA----------------
    CnPnV 7968-11OK    TGGTGCCCTATGCTCCAATTATTTCCAAGGAGGTCTAATTCTTAA----------------
   CnPnV 109594-10KS   TGGTGTCCTATGCTCCAATTATTTCCAAGGAGGTCTAATTCTTAA----------------
  CnPnV 142847-10NV    TGGTGTCCTATGCTCCAGTTATTTCCAAGGAGGTCTAATTCTTAA----------------
    CnPnV 13505-11OH   TGGTGTCCTATGCTCCAGTTATTTCCAAGGAGGTCTAATTCTTGA----------------
   CnPnV 56706-09NYC   TGGTGTCCTATGCTCCAATTATTTCCAAGGAGGTCTAATTCTTGA----------------
          CnPnV Ane4   UGGUGUCCUAUGCUCCAAUUAUUUCCAAGGAGGUCUAAUUCUUGA----------------
        CnPnV NZ-049   TGGTGTCCTATGCTCCAATTATTTCCAAGGAGATCTAATTCTTAA----------------
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   CnPnV Bari/100-12   UGGUGUCCUAUGCUCCAAUUAUUUCCAAGGAGGUCUAAUUCUUAA---------------
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             MPV-15    ------
           MPV-J3666   ------
       CnPnV NZ-007    AATTAA
 CnPnV 126724-10-4DC   ---TGA
  CnPnV 110230-11TX    ------
    CnPnV 86842-09PA   ------
    CnPnV 7968-11OK    ------
   CnPnV 109594-10KS   ------
  CnPnV 142847-10NV    ------
    CnPnV 13505-11OH   ------
   CnPnV 56706-09NYC   ------
          CnPnV Ane4   ------
        CnPnV NZ-049   ------
        CnPnV NZ-048   ------
    CnPnV 91065-11MA   ------
   CnPnV Bari/100-12   ------
CnPnV 114378-10-29KY   ------
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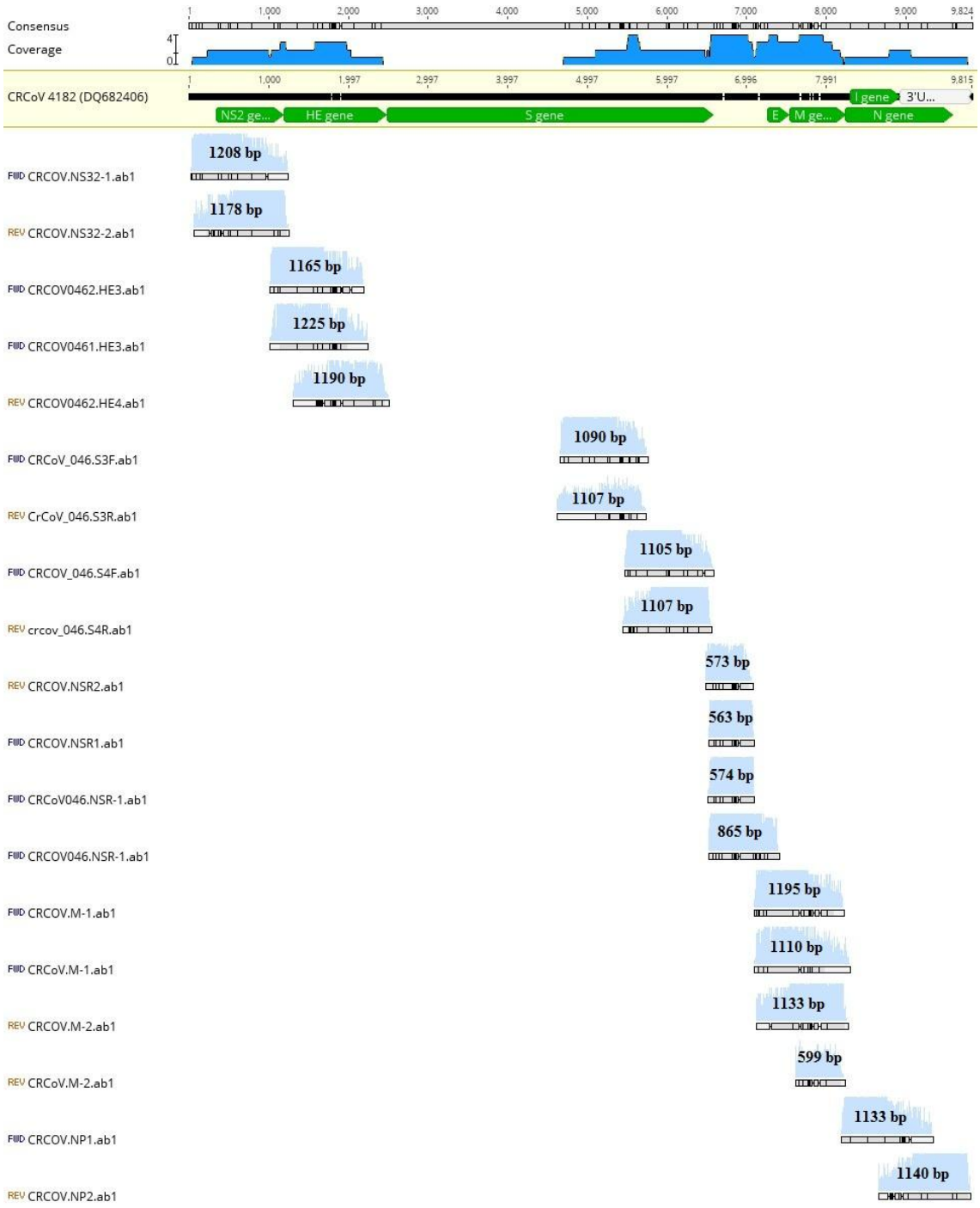
I. Sequence coverage of NZ 046/16 with respect to CRCoV 4182



J. CRCoV NZ-046/16 sequence

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>CRCoV NZ-046/16

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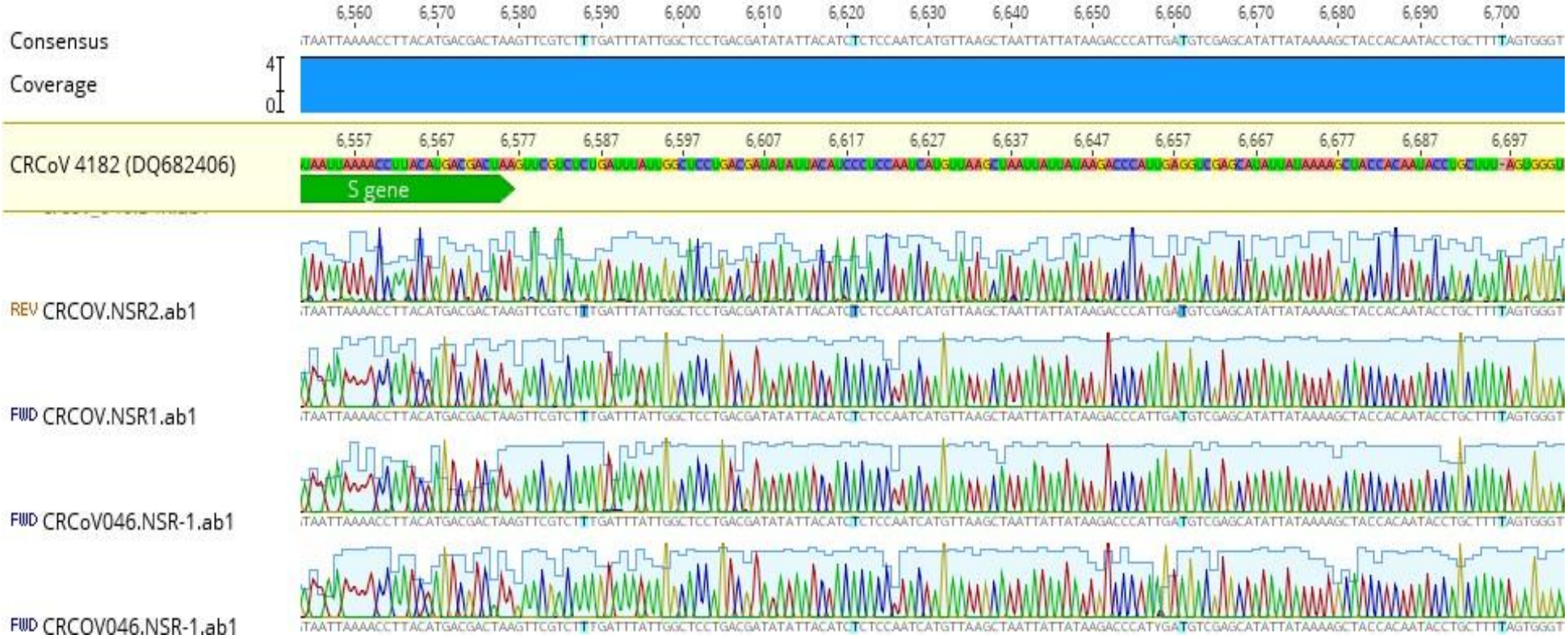
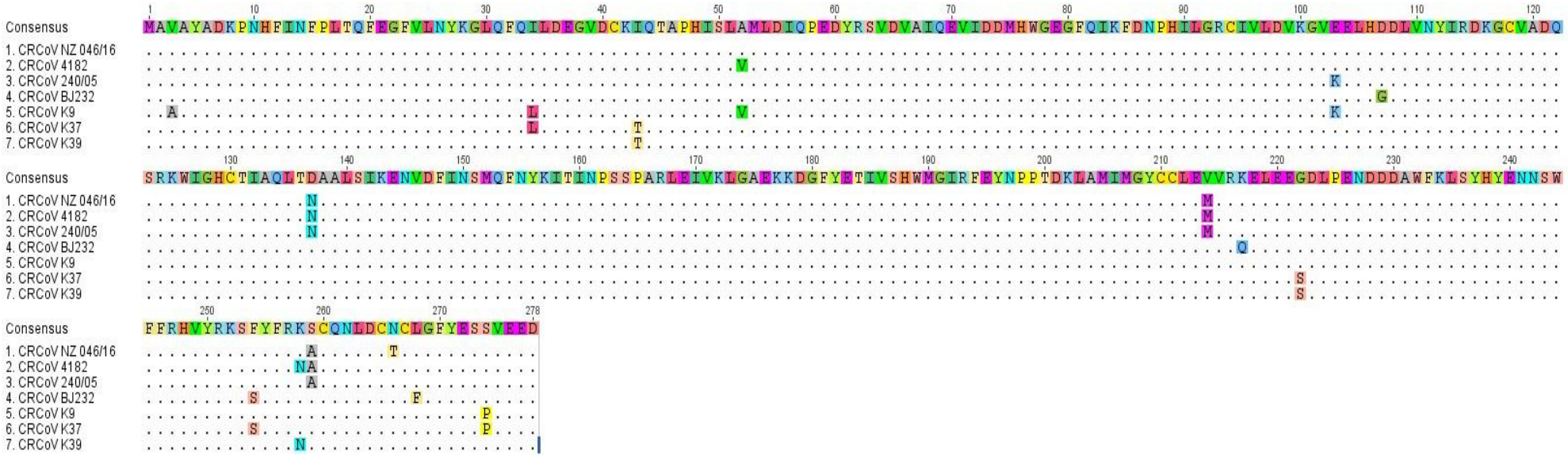
K. Sequence quality of CRCoV NZ 046/16 between the spike and 12.8 kDa protein gene

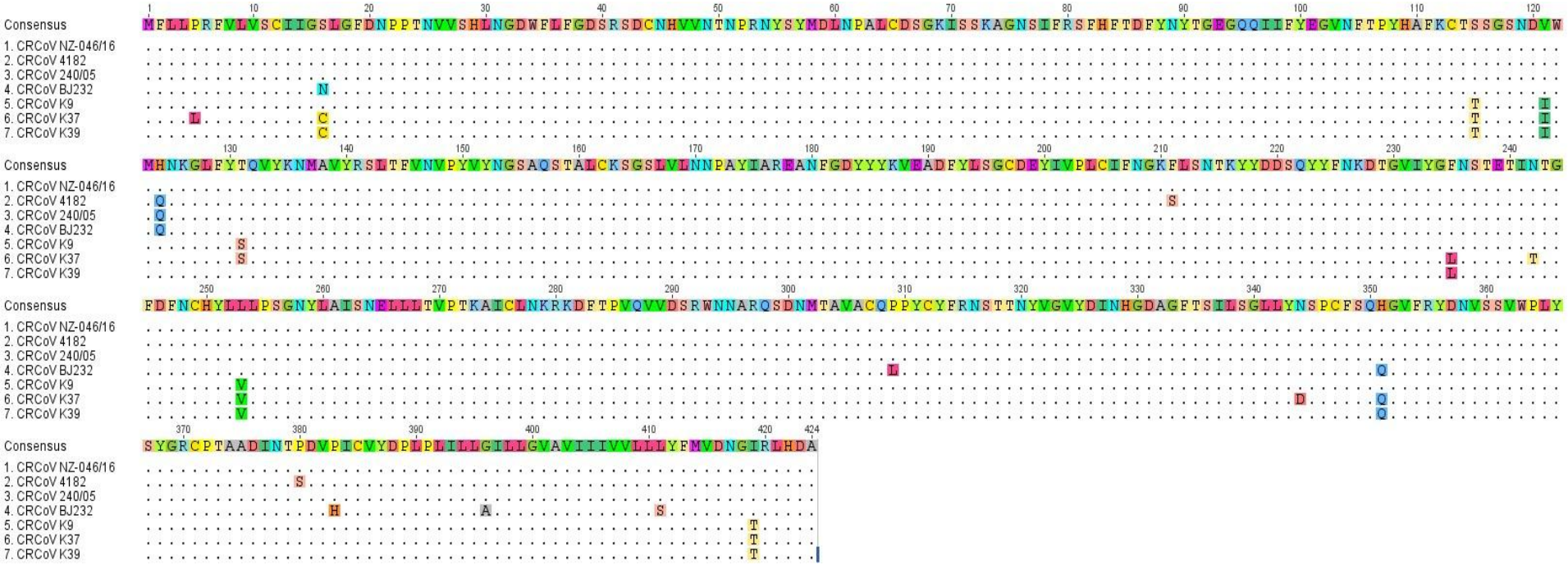


L. Multiple nucleotide sequence alignment of structural and non-structural proteins of CRCoV.

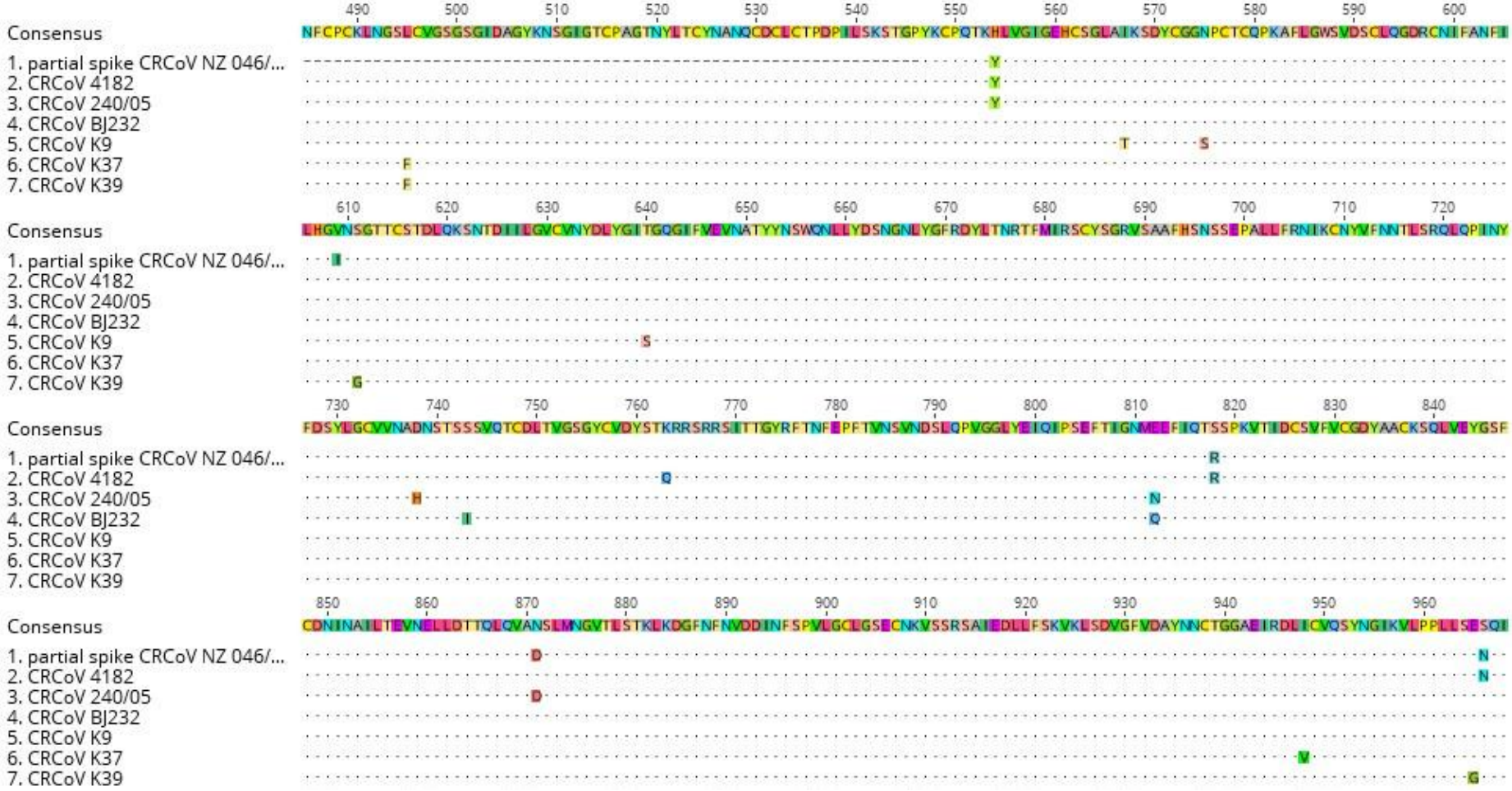
a) CRCoV NZ 046/16 32 kDa protein alignment with other CRCoVs.

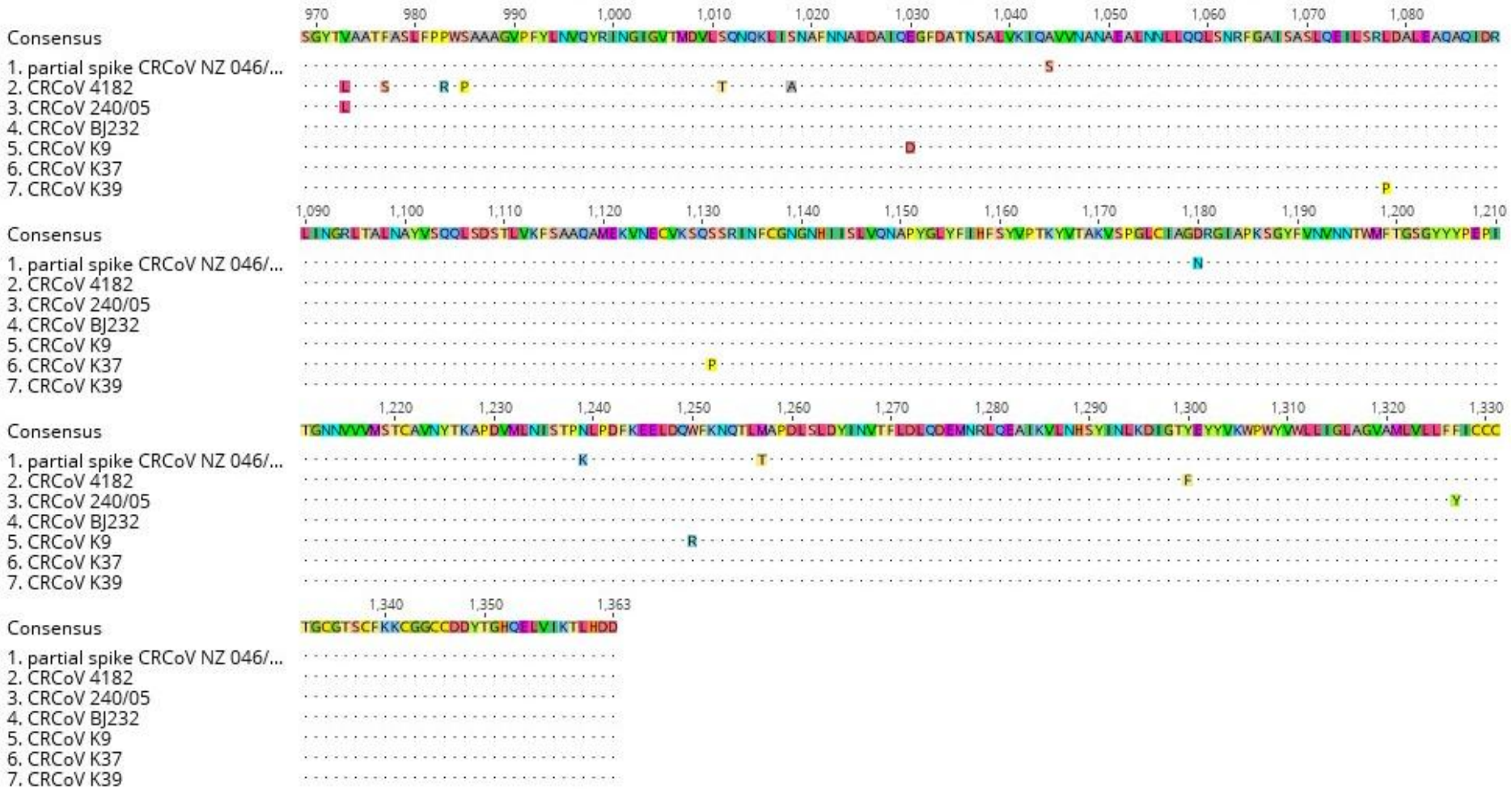


b) CRCoV NZ 046/16 HE protein alignment with other CRCoVs.



c) CRCoV NZ 046/16 partial spike protein alignment with other CRCoVs.

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Consensus
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2. CRCoV 4182
3. CRCoV 240/05
4. CRCoV BJ232
5. CRCoV K9
6. CRCoV K37
7. CRCoV K39
1,090 1,100 1,110 1,120 1,130 1,140 1,150 1,160 1,170 1,180 1,190 1,200 1,210
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3. CRCoV 240/05
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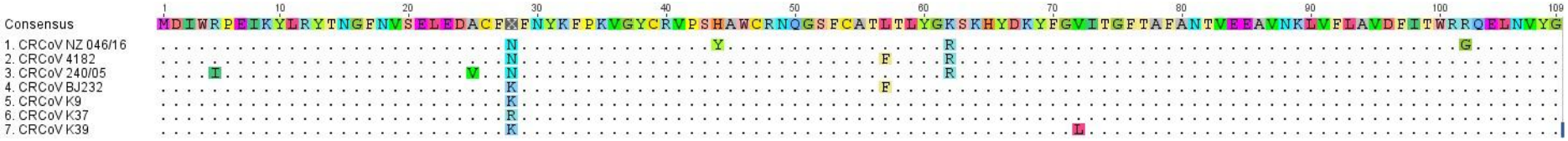
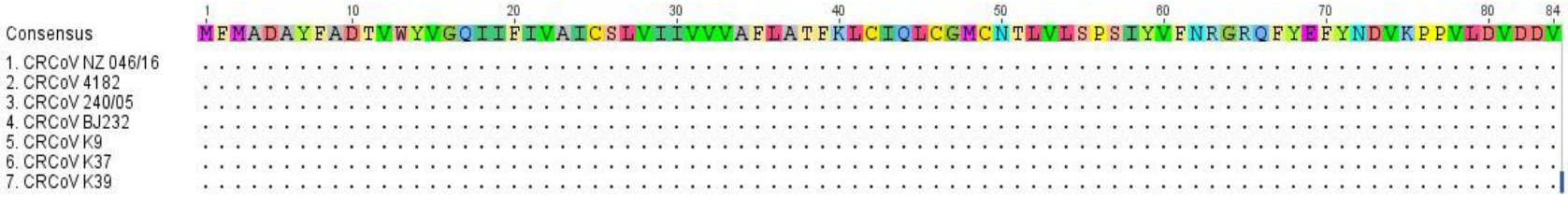
6. CRCoV K37
7. CRCoV K39

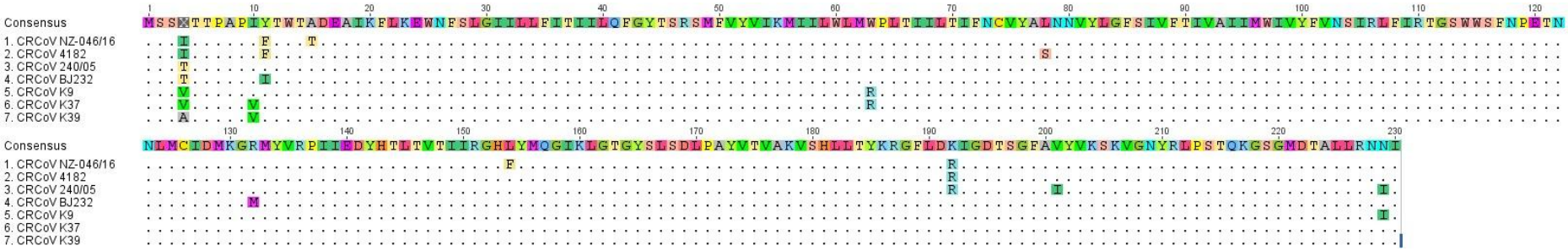
d) CRCoV NZ 046/16 12.8 kDa protein alignment with other CRCoVs.



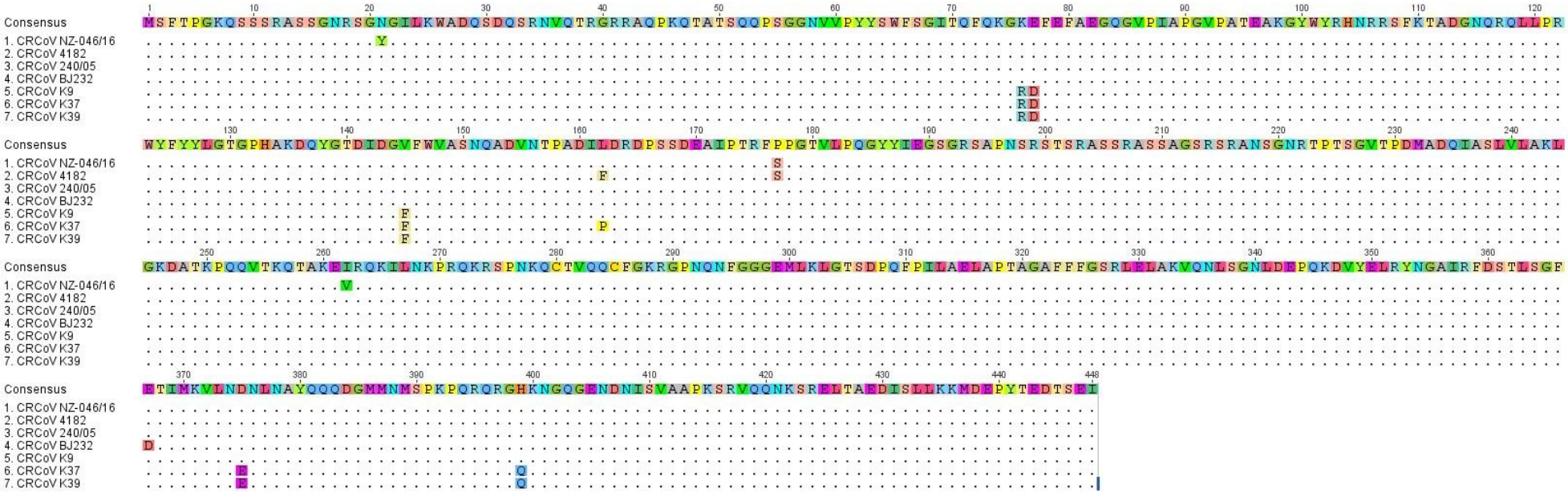
e) CRCoV NZ 046/16 E protein alignment with other CRCoVs



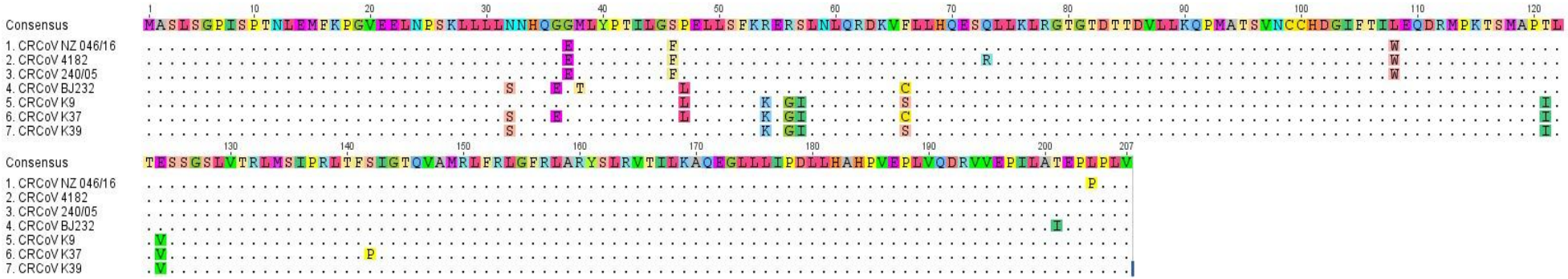
f) CRCoV NZ 046/16 M protein alignment with other CRCoVs



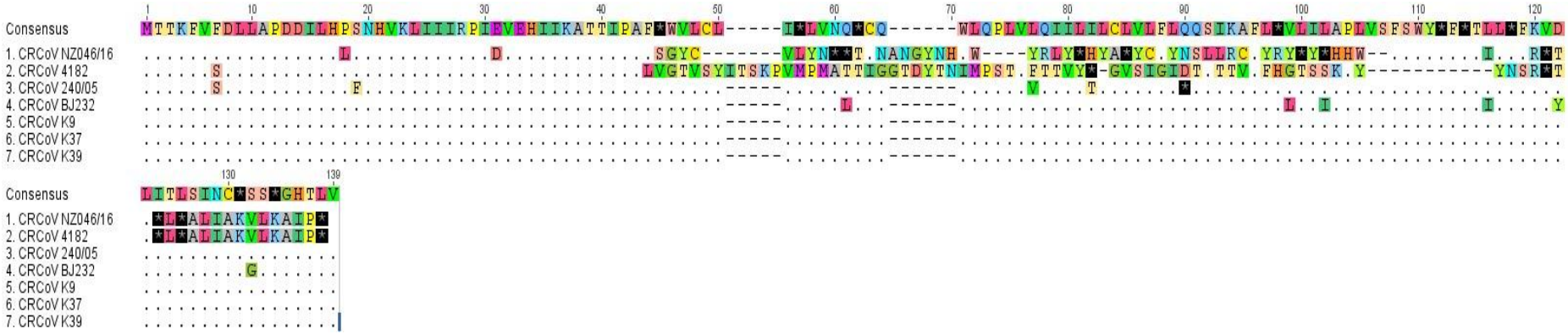
g) CRCoV NZ 046/16 N protein alignment with other CRCoVs



h) CRCoV NZ 046/16 I protein alignment with other CRCoVs



i) Alignment of the predicted amino acid sequence of the region between spike and 12.8 kDa protein of CRCoV NZ-046/16 in comparison with other CRCoV.



M. Details of serum samples used for CRCoV serology.

Accession number	Breed	Age	Sex	Island	Month	POI	Respiratory signs	CRCoV antibody positive/negative	Healthy /Sick
A14001681	Pet dogs	12	Male	North Island	Mar	92	no	Positive	Healthy
H14006470	Working dog	3	Female	North Island	Mar	68.64198	no	Positive	Healthy
H14006521	Pet dogs	12	Male	North Island	Mar	11.16049	no	Negative	Healthy
H14006895	Pet dogs	8	Female	North Island	Mar	46.33911	no	Positive	Healthy
H14008053	Working dog	1	Female	North Island	Mar	27.07129	no	Positive	Healthy
P14003965	Unknown	2	Male	North Island	Mar	13.19846	no	Negative	Healthy
P14003968	Working dog	4	Male	North Island	Mar	15.31792	no	Negative	Healthy
P14003971	Unknown	1	Male	North Island	Mar	11.27168	no	Negative	Healthy
P14003972	Unknown	2	Male	North Island	Mar	19.55684	no	Negative	Healthy
P14003974	Unknown	3	Male	North Island	Mar	28.61272	no	Positive	Healthy
P14003975	Unknown	4	Male	North Island	Mar	19.36416	no	Negative	Healthy
P14004667	Pet dogs	4	Female	North Island	Mar	0	no	Negative	Healthy
P14004990	Pet dogs	4	Female	North Island	Mar	22.44701	no	Positive	Healthy
P14005478	Working dog	9	Male	North Island	Mar	23.98844	no	Positive	Healthy
P14005498	Pet dogs	3	Female	North Island	Mar	58.76686	no	Positive	Healthy
P14005742	Pet dogs	11	Female	North Island	Mar	37.09056	no	Positive	Healthy
P14005838	Pet dogs	4	Female	North Island	Mar	79.28709	no	Positive	Healthy
P14005944	Pet dogs	4	Female	North Island	Mar	84.58574	no	Positive	Healthy
P14006060	Pet dogs	2	Female	North Island	Mar	41.1368	no	Positive	Healthy
A14001538	Pet dogs	9	Female	North Island	Mar	62.07407	no	Positive	Sick
A14001551	Working dog	2	Female	North Island	Mar	95.85185	no	Positive	Sick
A14001573	Pet dogs	8	Female	North Island	Mar	34.81481	no	Positive	Sick

A14001588	Pet dogs	11	Female	North Island	Mar	28.14815	no	Positive	Sick
A14001630	Working dog	2	Female	North Island	Mar	93.48148	no	Positive	Sick
A14001699	Pet dogs	11	Female	North Island	Mar	86.07407	no	Positive	Sick
A14001723	Pet dogs	15	Female	North Island	Mar	34.12346	no	Positive	Sick
A14001761	Pet dogs	9	Female	North Island	Mar	12.88889	no	Negative	Sick
A14001768	Working dog	15	Female	North Island	Mar	30.41975	no	Positive	Sick
A14001795	Pet dogs	12	Male	North Island	Mar	11.30864	no	Negative	Sick
A14001816	Working dog	11	Female	North Island	Mar	8.493827	no	Negative	Sick
A14001854	Pet dogs	12	Female	North Island	Mar	13.7284	no	Negative	Sick
A14001857	Pet dogs	10	Female	North Island	Mar	39.16049	no	Positive	Sick
A14001858	Pet dogs	14	Female	North Island	Mar	53.82716	no	Positive	Sick
A14001881	Pet dogs	9	Male	North Island	Mar	1.82716	no	Negative	Sick
A14001992	Pet dogs	14	Female	North Island	Mar	11.20988	no	Negative	Sick
A14002154	Pet dogs	6	Male	North Island	Mar	24.74074	no	Positive	Sick
A14002170	Pet dogs	4	Male	North Island	Mar	27.90123	no	Positive	Sick
A14002182	Pet dogs	7	Male	North Island	Mar	93.23457	no	Positive	Sick
A14002197	Working dog	10	Female	North Island	Mar	17.33333	no	Negative	Sick
A14002241	Pet dogs	13	Female	North Island	Mar	22.07407	no	Positive	Sick
A14002287	Pet dogs	0.4	Female	North Island	Mar	87.95062	no	Positive	Sick
A14002347	Pet dogs	15	Female	North Island	Mar	12.69136	no	Negative	Sick
H14006389	Pet dogs	12	Female	North Island	Mar	30.46914	no	Positive	Sick
H14006449	Pet dogs	12	Female	North Island	Mar	53.38272	no	Positive	Sick
H14006476	Working dog	3	Female	North Island	Mar	15.85185	no	Negative	Sick
H14006603	Pet dogs	4	Female	North Island	Mar	29.23457	no	Positive	Sick
H14006628	Working dog	9	Female	North Island	Mar	0	no	Negative	Sick
H14006708	Pet dogs	4	Male	North Island	Mar	0	yes	Negative	Sick
H14006994	Working dog	13	Male	North Island	Mar	20.71291	no	Positive	Sick

H14007000	Pet dogs	4	Male	North Island	Mar	24.3738	yes	Positive	Sick
H14007561	Pet dogs	1	Female	North Island	Mar	20.03854	no	Positive	Sick
H14007569	Working dog	5	Male	North Island	Mar	53.9499	no	Positive	Sick
H14007590	Pet dogs	9	Male	South Island	Mar	9.344894	no	Negative	Sick
H14007627	Working dog	4	Female	North Island	Mar	0	no	Negative	Sick
H14007674	Working dog	9	Female	North Island	Mar	39.11368	no	Positive	Sick
H14008056	Pet dogs	1	Male	North Island	Mar	32.36994	no	Positive	Sick
H14008130	Pet dogs	0.02	Male	North Island	Mar	40.75145	no	Positive	Sick
H14008158	Working dog	3	Female	North Island	Mar	25.43353	no	Positive	Sick
H14008235	Pet dogs	10	Male	North Island	Mar	5.973025	no	Negative	Sick
H14008249	Working dog	1	Male	North Island	Mar	28.42004	no	Positive	Sick
H14008461	Pet dogs	12	Female	North Island	Mar	28.51638	no	Positive	Sick
H14008483	Pet dogs	11	Male	North Island	Mar	35.93449	no	Positive	Sick
H14008768	Pet dogs	10	Male	South Island	Mar	56.84008	no	Positive	Sick
P14003896	Unknown	4	Female	North Island	Mar	26.78227	no	Positive	Sick
P14003897	Pet dogs	6	Female	North Island	Mar	88.34297	no	Positive	Sick
P14003958	Pet dogs	11	Male	North Island	Mar	29.09441	no	Positive	Sick
P14004154	Pet dogs	5	Female	North Island	Mar	36.7052	no	Positive	Sick
P14004251	Pet dogs	15	Male	North Island	Mar	57.41811	no	Positive	Sick
P14004311	Pet dogs	13	Male	North Island	Mar	30.25048	no	Positive	Sick
P14004313	Pet dogs	16	Male	South Island	Mar	0	no	Negative	Sick
P14004316	Pet dogs	14	Female	North Island	Mar	9.82659	no	Negative	Sick
P14004324	Pet dogs	1	Male	South Island	Mar	75.81888	no	Positive	Sick
P14004347	Pet dogs	2	Female	North Island	Mar	50.77071	no	Positive	Sick
P14004352	Pet dogs	14	Female	North Island	Mar	7.418112	no	Negative	Sick
P14004365	Working dog	10	Male	North Island	Mar	5.684008	no	Negative	Sick
P14004416	Pet dogs	11	Male	North Island	Mar	45.85742	yes	Positive	Sick

P14004471	Pet dogs	5	Female	North Island	Mar	80.44316	no	Positive	Sick
P14004525	Pet dogs	7	Female	North Island	Mar	86.51252	no	Positive	Sick
P14004537	Pet dogs	8	Female	North Island	Mar	0	no	Negative	Sick
P14004543	Pet dogs	10	Male	South Island	Mar	0	no	Negative	Sick
P14004561	Pet dogs	4	Male	North Island	Mar	3.468208	no	Negative	Sick
P14004572	Working dog	0.9	Male	North Island	Mar	0	no	Negative	Sick
P14004576	Pet dogs	0.5	Male	North Island	Mar	13.10212	no	Negative	Sick
P14004661	Working dog	9	Female	North Island	Mar	0.096339	no	Negative	Sick
P14004690	Pet dogs	2	Male	North Island	Mar	84.58574	yes	Positive	Sick
P14004752	Pet dogs	2	Female	North Island	Mar	0	no	Negative	Sick
P14004843	Pet dogs	1	Male	South Island	Mar	7.032755	no	Negative	Sick
P14004915	Pet dogs	10	Male	North Island	Mar	40.07707	no	Positive	Sick
P14004969	Working dog	6	Female	North Island	Mar	9.344894	no	Negative	Sick
P14005155	Pet dogs	6	Female	North Island	Mar	19.74952	no	Negative	Sick
P14005156	Pet dogs	3	Female	North Island	Mar	26.1079	no	Positive	Sick
P14005193	Pet dogs	0.8	Male	North Island	Mar	13.87283	no	Negative	Sick
P14005492	Working dog	3	Female	North Island	Mar	11.46435	no	Negative	Sick
P14005515	Working dog	14	Male	South Island	Mar	60.11561	no	Positive	Sick
P14005521	Pet dogs	9	Female	North Island	Mar	28.51638	no	Positive	Sick
P14005584	Pet dogs	6	Male	South Island	Mar	33.71869	no	Positive	Sick
P14005744	Pet dogs	8	Female	North Island	Mar	38.72832	no	Positive	Sick
P14005843	Working dog	5	Male	North Island	Mar	70.42389	no	Positive	Sick
P14005959	Pet dogs	6	Male	North Island	Mar	86.51252	no	Positive	Sick
P14006142	Pet dogs	11	Male	North Island	Mar	28.90173	no	Positive	Sick
A14002403	Working dog	2	Male	North Island	Apr	41.87621	no	Positive	Healthy
A14003076	Pet dogs	14	Female	North Island	Apr	46.7118	no	Positive	Healthy
H14009526	Working dog	2	Female	North Island	Apr	21.66344	no	Positive	Healthy

H14010432	Working dog	6	Female	North Island	Apr	0	no	Negative	Healthy
H14010651	Pet dogs	13	Male	North Island	Apr	34.91296	no	Positive	Healthy
H14010685	Pet dogs	2	Female	North Island	Apr	92.74662	no	Positive	Healthy
H14011233	Pet dogs	5	Female	North Island	Apr	80.07737	no	Positive	Healthy
H14011491	Pet dogs	2	Female	North Island	Apr	92.74662	no	Positive	Healthy
P14007050	Working dog	9	Female	North Island	Apr	37.13733	no	Positive	Healthy
P14007574	Pet dogs	6	Female	North Island	Apr	34.91296	no	Positive	Healthy
P14007694	Pet dogs	2	Female	North Island	Apr	0	no	Negative	Healthy
P14008011	Working dog	2	Female	North Island	Apr	6.673114	no	Negative	Healthy
P14008155	Pet dogs	2	Female	North Island	Apr	66.40502	no	Positive	Healthy
P14008262	Pet dogs	2	Female	North Island	Apr	34.22292	no	Positive	Healthy
A14002386	Pet dogs	3	Female	North Island	Apr	15.95745	no	Negative	Sick
A14002400	Pet dogs	15	Female	North Island	Apr	11.70213	no	Negative	Sick
A14002404	Pet dogs	11	Female	North Island	Apr	21.85687	no	Positive	Sick
A14002412	Pet dogs	5	Female	North Island	Apr	69.24565	no	Positive	Sick
A14002418	Working dog	2	Female	North Island	Apr	78.52998	no	Positive	Sick
A14002433	Pet dogs	14	Female	North Island	Apr	57.73694	no	Positive	Sick
A14002449	Working dog	4	Male	North Island	Apr	0	no	Negative	Sick
A14002479	Pet dogs	7	Female	North Island	Apr	44.48743	no	Positive	Sick
A14002500	Pet dogs	10	Female	North Island	Apr	58.41393	no	Positive	Sick
A14002508	Pet dogs	5	Female	North Island	Apr	94.77756	no	Positive	Sick
A14002510	Working dog	15	Male	North Island	Apr	4.158607	no	Negative	Sick
A14002571	Pet dogs	14	Female	North Island	Apr	44.68085	no	Positive	Sick
A14002579	Pet dogs	12	Female	North Island	Apr	82.7853	no	Positive	Sick
A14002583	Pet dogs	13	Female	North Island	Apr	22.63056	no	Positive	Sick
A14002589	Pet dogs	4	Female	North Island	Apr	35.39652	no	Positive	Sick
A14002622	Pet dogs	14	Female	North Island	Apr	46.13153	no	Positive	Sick

A14002640	Pet dogs	0.25	Male	North Island	Apr	0	no	Negative	Sick
A14002789	Working dog	2	Female	North Island	Apr	12.95938	no	Negative	Sick
A14002826	Pet dogs	4	Male	North Island	Apr	71.66344	no	Positive	Sick
A14002909	Working dog	3	Female	North Island	Apr	0	no	Negative	Sick
A14002953	Pet dogs	6	Female	North Island	Apr	0	no	Negative	Sick
A14002957	Pet dogs	5	Female	North Island	Apr	82.49516	no	Positive	Sick
A14002960	Working dog	13	Female	North Island	Apr	50.96712	no	Positive	Sick
A14002985	Pet dogs	16	Male	North Island	Apr	56.76983	no	Positive	Sick
A14002988	Pet dogs	13	Male	North Island	Apr	22.24371	no	Positive	Sick
A14002999	Working dog	0.5	Male	North Island	Apr	14.11992	no	Negative	Sick
A14003024	Working dog	12	Male	North Island	Apr	15.18375	no	Negative	Sick
A14003059	Pet dogs	9	Male	North Island	Apr	93.13346	no	Positive	Sick
A14003066	Pet dogs	14	Female	North Island	Apr	74.85493	no	Positive	Sick
A14003075	Pet dogs	1	Female	North Island	Apr	9.574468	no	Negative	Sick
A14003097	Pet dogs	11	Female	North Island	Apr	54.54545	no	Positive	Sick
A14003120	Pet dogs	12	Male	North Island	Apr	22.43714	no	Positive	Sick
A14003138	Pet dogs	12	Female	North Island	Apr	45.0677	no	Positive	Sick
A14003147	Working dog	10	Male	North Island	Apr	69.53578	no	Positive	Sick
A14003151	Pet dogs	10	Female	North Island	Apr	21.95358	no	Positive	Sick
H14009362	Pet dogs	12	Male	North Island	Apr	44.00387	no	Positive	Sick
H14009648	Pet dogs	8	Male	North Island	Apr	59.76789	no	Positive	Sick
H14010433	Unknown	10	Male	North Island	Apr	32.59188	no	Positive	Sick
H14010454	Pet dogs	4	Female	South Island	Apr	3.384913	no	Negative	Sick
H14010519	Pet dogs	3	Female	North Island	Apr	23.50097	no	Positive	Sick
H14011470	Pet dogs	2	Female	South Island	Apr	32.30174	no	Positive	Sick
P14006552	Pet dogs	5	Female	North Island	Apr	0	no	Negative	Sick
P14006613	Pet dogs	9	Male	North Island	Apr	22.9207	no	Positive	Sick

P14006621	Pet dogs	5	Female	North Island	Apr	3.384913	no	Negative	Sick
P14006825	Pet dogs	5	Female	North Island	Apr	12.08897	no	Negative	Sick
P14006844	Working dog	14	Female	North Island	Apr	22.9207	no	Positive	Sick
P14006915	Working dog	9	Female	North Island	Apr	78.23985	no	Positive	Sick
P14006971	Pet dogs	4	Male	North Island	Apr	0	no	Negative	Sick
P14006972	Pet dogs	7	Male	North Island	Apr	24.95164	no	Positive	Sick
P14007032	Pet dogs	10	Male	North Island	Apr	0	yes	Negative	Sick
P14007112	Working dog	10	Male	North Island	Apr	5.029014	no	Negative	Sick
P14007131	Pet dogs	12	Male	North Island	Apr	46.80851	yes	Positive	Sick
P14007136	Working dog	15	Female	North Island	Apr	65.86074	no	Positive	Sick
P14007165	Pet dogs	1	Female	North Island	Apr	8.027079	no	Negative	Sick
P14007213	Pet dogs	9	Female	North Island	Apr	51.6441	no	Positive	Sick
P14007284	Pet dogs	1	Male	North Island	Apr	0	no	Negative	Sick
P14007291	Pet dogs	14	Female	North Island	Apr	0	no	Negative	Sick
P14007300	Pet dogs	14	Female	North Island	Apr	32.10832	no	Positive	Sick
P14007324	Pet dogs	9	Male	North Island	Apr	0	no	Negative	Sick
P14007400	Pet dogs	7	Female	North Island	Apr	49.80658	no	Positive	Sick
P14007436	Pet dogs	14	Female	North Island	Apr	24.75822	no	Positive	Sick
P14007456	Unknown	13	Male	North Island	Apr	5.609284	no	Negative	Sick
P14007470	Working dog	13	Male	North Island	Apr	0.096712	no	Negative	Sick
P14007471	Working dog	11	Female	North Island	Apr	8.123791	no	Negative	Sick
P14007475	Pet dogs	1	Male	North Island	Apr	67.79497	no	Positive	Sick
P14007486	Pet dogs	11	Male	North Island	Apr	21.17988	no	Positive	Sick
P14007528	Pet dogs	14	Female	South Island	Apr	0	no	Negative	Sick
P14007545	Pet dogs	5	Male	North Island	Apr	63.05609	no	Positive	Sick
P14007650	Pet dogs	8	Male	South Island	Apr	20.30948	no	Positive	Sick
P14007697	Pet dogs	1	Female	North Island	Apr	19.6325	no	Negative	Sick

P14007698	Pet dogs	7	Female	North Island	Apr	51.74081	no	Positive	Sick
P14007701	Pet dogs	13	Female	North Island	Apr	29.11025	no	Positive	Sick
P14007710	Pet dogs	0.5	Male	North Island	Apr	92.35977	yes	Positive	Sick
P14007714	Pet dogs	12	Male	North Island	Apr	52.99807	no	Positive	Sick
P14007720	Pet dogs	6	Female	North Island	Apr	19.72921	no	Negative	Sick
P14007721	Pet dogs	8	Male	North Island	Apr	18.56867	no	Negative	Sick
P14007750	Working dog	9	Female	South Island	Apr	0	no	Negative	Sick
P14007758	Pet dogs	5	Female	South Island	Apr	0	no	Negative	Sick
P14007790	Pet dogs	8	Female	North Island	Apr	5.89942	no	Negative	Sick
P14007825	Pet dogs	4	Male	North Island	Apr	18.18182	no	Negative	Sick
P14007832	Pet dogs	2	Male	North Island	Apr	0	no	Negative	Sick
P14007876	Pet dogs	8	Female	North Island	Apr	16.73114	no	Negative	Sick
P14008112	Pet dogs	10	Female	South Island	Apr	18.05338	no	Negative	Sick
P14008120	Pet dogs	10	Female	North Island	Apr	4.945055	no	Negative	Sick
P14008172	Pet dogs	7	Female	North Island	Apr	54.78807	no	Positive	Sick
P14008185	Working dog	12	Female	North Island	Apr	78.96389	no	Positive	Sick
A14003509	Pet dogs	11	Male	North Island	May	11.31186	no	Negative	Healthy
A14003566	Pet dogs	0.5	Female	North Island	May	1.88531	no	Negative	Healthy
A14003686	Pet dogs	2	Female	North Island	May	0	no	Negative	Healthy
A14003987	Working dog	11	Female	North Island	May	93.32286	no	Positive	Healthy
H14013092	Pet dogs	5	Female	North Island	May	82.95365	no	Positive	Healthy
H14016683	Working dog	4	Female	North Island	May	4.634721	no	Negative	Healthy
HEAL - 001	Working dog	1.25	Male	North Island	May	0	no	Negative	Healthy
HEAL - 002	Working dog	1.25	Male	North Island	May	0	no	Negative	Healthy
HEAL - 003	Working dog	1.33	Male	North Island	May	0	no	Negative	Healthy
P14008760	Pet dogs	14	Female	North Island	May	18.8531	no	Negative	Healthy
P14009227	Pet dogs	1	Female	North Island	May	32.44305	no	Positive	Healthy

P14010552	Working dog	2	Female	North Island	May	26.78712	no	Positive	Healthy
P14010746	Pet dogs	2	Female	North Island	May	0	no	Negative	Healthy
A14003223	Pet dogs	13	Male	North Island	May	0	no	Negative	Sick
A14003224	Pet dogs	7	Male	North Island	May	0	no	Negative	Sick
A14003359	Working dog	3	Male	North Island	May	94.89395	no	Positive	Sick
A14003365	Pet dogs	9	Female	North Island	May	16.81068	no	Negative	Sick
A14003369	Pet dogs	6	Female	North Island	May	75.09819	no	Positive	Sick
A14003376	Pet dogs	10	Male	North Island	May	74.3912	no	Positive	Sick
A14003383	Working dog	5	Male	North Island	May	61.74391	no	Positive	Sick
A14003436	Pet dogs	6	Male	North Island	May	5.498822	no	Negative	Sick
A14003440	Working dog	13	Male	North Island	May	80.12569	no	Positive	Sick
A14003446	Pet dogs	14	Male	North Island	May	47.28987	yes	Positive	Sick
A14003457	Pet dogs	9	Male	North Island	May	16.02514	yes	Negative	Sick
A14003581	Pet dogs	15	Male	North Island	May	15.3967	no	Negative	Sick
A14003597	Working dog	12	Male	North Island	May	76.9835	no	Positive	Sick
A14003653	Working dog	5	Female	North Island	May	30.40063	no	Positive	Sick
A14003677	Pet dogs	10	Female	North Island	May	0	no	Negative	Sick
A14003691	Working dog	2	Female	North Island	May	4.713276	no	Negative	Sick
A14003698	Pet dogs	10	Female	North Island	May	7.541241	no	Negative	Sick
A14003778	Pet dogs	12	Female	North Island	May	0	no	Negative	Sick
A14003782	Pet dogs	9	Female	North Island	May	78.86881	no	Positive	Sick
A14003784	Working dog	4	Female	North Island	May	11.70463	no	Negative	Sick
A14003794	Working dog	0.58	Male	North Island	May	32.83582	no	Positive	Sick
A14003825	Pet dogs	14	Female	North Island	May	65.59309	no	Positive	Sick
A14003841	Pet dogs	12	Male	North Island	May	14.61115	no	Negative	Sick
A14003874	Unknown	10	Male	North Island	May	10.99764	no	Negative	Sick
A14003891	Pet dogs	9	Male	North Island	May	95.60094	no	Positive	Sick

A14003894	Pet dogs	10	Female	North Island	May	69.83504	no	Positive	Sick
A14003895	Pet dogs	12	Male	North Island	May	76.9835	no	Positive	Sick
A14003903	Pet dogs	15	Female	North Island	May	54.51689	no	Positive	Sick
A14003918	Pet dogs	14	Female	North Island	May	79.65436	no	Positive	Sick
A14003957	Working dog	6	Male	North Island	May	84.13197	no	Positive	Sick
A14003974	Pet dogs	10	Female	North Island	May	88.68814	no	Positive	Sick
A14003996	Pet dogs	5	Female	North Island	May	10.36921	no	Negative	Sick
A14004018	Pet dogs	10	Male	North Island	May	54.28123	no	Positive	Sick
A14004025	Pet dogs	9	Female	North Island	May	20.18853	no	Positive	Sick
A14004073	Pet dogs	3	Male	North Island	May	80.59701	no	Positive	Sick
A14004165	Working dog	12	Male	North Island	May	70.46347	no	Positive	Sick
A14004166	Pet dogs	12	Female	North Island	May	56.08798	no	Positive	Sick
CIRD - 003	Working dog	1.25	Male	North Island	May	0	yes	Negative	Sick
CIRD – 004	Working dog	2	Female	North Island	May	13.28443	yes	Negative	Sick
H14012774	Pet dogs	2	Female	North Island	May	0	no	Negative	Sick
H14012861	Pet dogs	3	Female	North Island	May	37.23488	no	Positive	Sick
H14013037	Pet dogs	1	Female	South Island	May	11.23331	no	Negative	Sick
H14013107	Pet dogs	10	Female	North Island	May	20.73841	no	Positive	Sick
H14014497	Pet dogs	7	Male	North Island	May	18.22467	no	Negative	Sick
H14014499	Pet dogs	13	Female	North Island	May	7.148468	no	Negative	Sick
P14008624	Pet dogs	15	Female	North Island	May	47.83975	no	Positive	Sick
P14008634	Pet dogs	1	Male	North Island	May	15.31815	no	Negative	Sick
P14008667	Working dog	0.9	Female	South Island	May	17.59623	no	Negative	Sick
P14009032	Pet dogs	9	Male	North Island	May	61.35114	no	Positive	Sick
P14009055	Working dog	11	Female	North Island	May	14.68971	no	Negative	Sick
P14009158	Pet dogs	1	Male	North Island	May	0.785546	no	Negative	Sick
P14009202	Working dog	4	Female	North Island	May	44.93323	no	Positive	Sick

P14009229	Working dog	0.33	Male	North Island	May	6.598586	no	Negative	Sick
P14009239	Unknown	13	Male	North Island	May	59.54438	no	Positive	Sick
P14009362	Pet dogs	10	Female	North Island	May	32.20738	no	Positive	Sick
P14009468	Pet dogs	12	Male	North Island	May	58.75884	no	Positive	Sick
P14009523	Pet dogs	9	Female	South Island	May	34.32836	no	Positive	Sick
P14009540	Pet dogs	14	Female	South Island	May	80.75412	no	Positive	Sick
P14009545	Pet dogs	5	Female	North Island	May	9.426551	no	Negative	Sick
P14009632	Working dog	7	Female	North Island	May	2.199529	no	Negative	Sick
P14009674	Unknown	2	Male	North Island	May	91.67321	yes	Positive	Sick
P14009689	Working dog	1	Female	North Island	May	41.39827	no	Positive	Sick
P14009697	Pet dogs	10	Female	North Island	May	16.96779	no	Negative	Sick
P14009713	Pet dogs	12	Male	North Island	May	37.62765	yes	Positive	Sick
P14009733	Pet dogs	6	Male	North Island	May	13.51139	no	Negative	Sick
P14009742	Pet dogs	9	Female	South Island	May	0	no	Negative	Sick
P14009791	Pet dogs	6	Male	North Island	May	82.56088	no	Positive	Sick
P14009815	Pet dogs	9	Female	South Island	May	17.59623	no	Negative	Sick
P14009946	Working dog	8	Male	South Island	May	81.22545	no	Positive	Sick
P14009996	Working dog	7	Male	North Island	May	54.04556	no	Positive	Sick
P14010052	Pet dogs	6	Male	South Island	May	24.50903	no	Positive	Sick
P14010053	Pet dogs	6	Male	North Island	May	56.71642	no	Positive	Sick
P14010077	Working dog	2	Female	North Island	May	8.483896	no	Negative	Sick
P14010188	Working dog	9	Male	North Island	May	0	no	Negative	Sick
P14010229	Pet dogs	14	Female	South Island	May	26.15868	no	Positive	Sick
P14010230	Pet dogs	11	Male	North Island	May	33.46426	no	Positive	Sick
P14010311	Pet dogs	15	Female	North Island	May	47.28987	no	Positive	Sick
P14010439	Pet dogs	5	Female	North Island	May	68.42105	no	Positive	Sick
P14010512	Pet dogs	11	Female	South Island	May	35.58523	no	Positive	Sick

P14010520	Pet dogs	8	Male	North Island	May	34.32836	no	Positive	Sick
P14010530	Pet dogs	9	Male	North Island	May	46.66143	no	Positive	Sick
P14010561	Pet dogs	5	Male	South Island	May	29.45797	no	Positive	Sick
P14010577	Pet dogs	3	Male	North Island	May	3.692066	no	Negative	Sick
P14010600	Pet dogs	1	Female	North Island	May	45.09034	no	Positive	Sick
P14010633	Pet dogs	11	Male	North Island	May	75.56952	yes	Positive	Sick
P14010653	Working dog	7	Female	North Island	May	91.20189	no	Positive	Sick
P14010663	Working dog	12	Female	North Island	May	54.59544	no	Positive	Sick
P14010664	Pet dogs	7	Male	North Island	May	51.99667	no	Positive	Sick
P14010679	Pet dogs	7	Male	South Island	May	13.14476	no	Negative	Sick
P14010840	Pet dogs	14	Female	North Island	May	16.05657	no	Negative	Sick
P14010867	Working dog	5	Male	North Island	May	0.665557	no	Negative	Sick
P14010877	Pet dogs	9	Female	North Island	May	13.97671	no	Negative	Sick
A14004313	Working dog	5	Male	North Island	Jun	77.98408	no	Positive	Healthy
H14017406	Pet dogs	1	Female	North Island	Jun	83.95225	no	Positive	Healthy
H14017508	Pet dogs	2	Female	North Island	Jun	87.66578	no	Positive	Healthy
H14019000	Pet dogs	9	Female	North Island	Jun	0	no	Negative	Healthy
P14011296	Pet dogs	2	Female	North Island	Jun	7.95756	no	Negative	Healthy
P14011313	Pet dogs	5	Female	North Island	Jun	58.687	no	Positive	Healthy
P14011314	Working dog	2	Female	North Island	Jun	42.17507	no	Positive	Healthy
P14011493	Pet dogs	2	Female	North Island	Jun	19.62865	no	Negative	Healthy
P14011507	Working dog	6	Female	North Island	Jun	65.78249	no	Positive	Healthy
P14011765	Pet dogs	3	Female	North Island	Jun	13.99204	no	Negative	Healthy
P14011766	Working dog	3	Female	North Island	Jun	81.83024	no	Positive	Healthy
P14012102	Pet dogs	3	Female	North Island	Jun	25.26525	no	Positive	Healthy
P14012170	Working dog	3	Female	North Island	Jun	24.20424	no	Positive	Healthy
P14012503	Pet dogs	5	Female	North Island	Jun	66.51194	no	Positive	Healthy

P14012772	Pet dogs	11	Female	North Island	Jun	0	no	Negative	Healthy
A14004202	Pet dogs	9	Female	North Island	Jun	12.00265	no	Negative	Sick
A14004236	Pet dogs	6	Male	North Island	Jun	60.27851	no	Positive	Sick
A14004242	Pet dogs	10	Male	North Island	Jun	55.03979	no	Positive	Sick
A14004250	Pet dogs	14	Female	North Island	Jun	69.96021	no	Positive	Sick
A14004282	Pet dogs	12	Male	North Island	Jun	92.24138	no	Positive	Sick
A14004290	Pet dogs	7	Female	North Island	Jun	65.11936	no	Positive	Sick
A14004299	Pet dogs	10	Male	North Island	Jun	80.90186	no	Positive	Sick
A14004309	Pet dogs	14	Female	North Island	Jun	24.60212	no	Positive	Sick
A14004327	Working dog	12	Male	North Island	Jun	22.87798	no	Positive	Sick
A14004336	Pet dogs	10	Female	North Island	Jun	64.45623	no	Positive	Sick
A14004364	Pet dogs	7	Female	North Island	Jun	48.80637	no	Positive	Sick
A14004367	Pet dogs	13	Female	North Island	Jun	55.90186	no	Positive	Sick
A14004373	Pet dogs	13	Female	North Island	Jun	20.88859	no	Positive	Sick
A14004390	Pet dogs	8	Male	North Island	Jun	37.59947	no	Positive	Sick
A14004438	Pet dogs	7	Male	North Island	Jun	33.1565	no	Positive	Sick
A14004466	Pet dogs	10	Female	North Island	Jun	6.962865	no	Negative	Sick
A14004475	Working dog	2	Female	North Island	Jun	15.84881	no	Negative	Sick
A14004479	Working dog	9	Female	North Island	Jun	11.7374	no	Negative	Sick
A14004500	Working dog	10	Female	North Island	Jun	25.46419	no	Positive	Sick
A14004517	Pet dogs	11	Male	North Island	Jun	22.74536	no	Positive	Sick
A14004752	Working dog	15	Female	North Island	Jun	13.99204	no	Negative	Sick
A14004754	Pet dogs	1	Female	North Island	Jun	11.47215	no	Negative	Sick
A14004759	Pet dogs	14	Female	North Island	Jun	0	no	Negative	Sick
A14004760	Pet dogs	1	Male	North Island	Jun	15.38462	no	Negative	Sick
A14004808	Pet dogs	12	Female	North Island	Jun	90.45093	no	Positive	Sick
A14004830	Pet dogs	4	Male	North Island	Jun	24.66844	no	Positive	Sick

A14004843	Pet dogs	1	Male	North Island	Jun	72.21485	yes	Positive	Sick
A14004852	Pet dogs	7	Male	North Island	Jun	41.57825	no	Positive	Sick
A14004874	Pet dogs	7	Female	North Island	Jun	81.29973	no	Positive	Sick
A14004930	Working dog	9	Female	North Island	Jun	31.6313	no	Positive	Sick
A14004938	Pet dogs	11	Female	North Island	Jun	12.53316	no	Negative	Sick
A14004943	Pet dogs	8	Female	North Island	Jun	74.40318	no	Positive	Sick
A14004975	Pet dogs	8	Female	North Island	Jun	24.86737	no	Positive	Sick
A14004990	Pet dogs	11	Female	North Island	Jun	59.01857	no	Positive	Sick
A14005011	Pet dogs	8	Male	North Island	Jun	34.28382	no	Positive	Sick
A14005043	Unknown	2	Female	North Island	Jun	45.68966	no	Positive	Sick
A14005044	Pet dogs	10	Female	North Island	Jun	52.38727	no	Positive	Sick
H14017244	Pet dogs	16	Male	North Island	Jun	76.79045	no	Positive	Sick
H14017261	Pet dogs	9	Male	South Island	Jun	14.92042	no	Negative	Sick
H14017262	Working dog	12	Male	South Island	Jun	0	no	Negative	Sick
H14017271	Pet dogs	8	Female	North Island	Jun	73.20955	no	Positive	Sick
H14017501	Pet dogs	4	Female	North Island	Jun	64.05836	no	Positive	Sick
H14017502	Pet dogs	14	Male	North Island	Jun	62.2679	no	Positive	Sick
H14017637	Pet dogs	9	Female	South Island	Jun	8.289125	no	Negative	Sick
P14010975	Pet dogs	4	Male	North Island	Jun	33.81963	no	Positive	Sick
P14011035	Pet dogs	10	Female	North Island	Jun	57.02918	no	Positive	Sick
P14011087	Pet dogs	6	Male	North Island	Jun	23.20955	no	Positive	Sick
P14011147	Working dog	2	Male	North Island	Jun	26.45889	no	Positive	Sick
P14011148	Pet dogs	3	Male	North Island	Jun	54.11141	no	Positive	Sick
P14011196	Pet dogs	5	Female	North Island	Jun	35.94164	no	Positive	Sick
P14011198	Pet dogs	8	Female	North Island	Jun	33.95225	no	Positive	Sick
P14011322	Pet dogs	12	Male	North Island	Jun	38.19629	no	Positive	Sick
P14011347	Pet dogs	9	Male	North Island	Jun	67.30769	yes	Positive	Sick

P14011363	Pet dogs	8	Female	North Island	Jun	23.4748	no	Positive	Sick
P14011432	Pet dogs	8	Female	South Island	Jun	11.07427	no	Negative	Sick
P14011479	Working dog	8	Female	North Island	Jun	30.1061	no	Positive	Sick
P14011495	Pet dogs	12	Male	North Island	Jun	47.9443	no	Positive	Sick
P14011575	Pet dogs	11	Female	North Island	Jun	52.58621	no	Positive	Sick
P14011621	Pet dogs	9	Female	North Island	Jun	53.77984	no	Positive	Sick
P14011627	Working dog	13	Female	South Island	Jun	29.97347	no	Positive	Sick
P14011652	Pet dogs	11	Female	South Island	Jun	68.10345	no	Positive	Sick
P14011730	Pet dogs	13	Female	South Island	Jun	36.47215	no	Positive	Sick
P14011762	Pet dogs	13	Female	North Island	Jun	89.19098	no	Positive	Sick
P14011842	Pet dogs	8	Female	North Island	Jun	64.38992	yes	Positive	Sick
P14011867	Pet dogs	13	Male	North Island	Jun	26.92308	no	Positive	Sick
P14011890	Unknown	12	Female	North Island	Jun	39.52255	no	Positive	Sick
P14011977	Pet dogs	11	Male	North Island	Jun	34.21751	no	Positive	Sick
P14012013	Working dog	13	Female	South Island	Jun	50.72944	no	Positive	Sick
P14012056	Pet dogs	5	Female	North Island	Jun	17.8382	no	Negative	Sick
P14012120	Pet dogs	6	Female	North Island	Jun	23.87268	no	Positive	Sick
P14012191	Pet dogs	0.15	Female	North Island	Jun	45.49072	no	Positive	Sick
P14012197	Working dog	4	Female	North Island	Jun	36.53846	no	Positive	Sick
P14012220	Pet dogs	11	Female	North Island	Jun	20.68966	no	Positive	Sick
P14012228	Pet dogs	4	Female	North Island	Jun	17.37401	no	Negative	Sick
P14012412	Pet dogs	13	Female	North Island	Jun	71.4191	no	Positive	Sick
P14012413	Pet dogs	9	Female	North Island	Jun	12.00265	no	Negative	Sick
P14012439	Pet dogs	11	Female	South Island	Jun	5.636605	no	Negative	Sick
P14012448	Pet dogs	14	Male	North Island	Jun	0	no	Negative	Sick
P14012521	Pet dogs	3	Female	North Island	Jun	56.96286	no	Positive	Sick
P14012526	Pet dogs	16	Female	North Island	Jun	36.14058	no	Positive	Sick

P14012591	Working dog	2	Female	South Island	Jun	1.988636	no	Negative	Sick
P14012621	Pet dogs	6	Female	North Island	Jun	0	no	Negative	Sick
P14012790	Pet dogs	7	Male	North Island	Jun	7.03125	no	Negative	Sick
P14012843	Pet dogs	5	Female	North Island	Jun	74.0767	no	Positive	Sick
P14012878	Working dog	4	Female	South Island	Jun	30.53977	no	Positive	Sick
A14005379	Pet dogs	12	Male	North Island	Jul	0	no	Negative	Healthy
A14005452	Pet dogs	13	Female	North Island	Jul	0	no	Negative	Healthy
A14005470	Unknown	12	Male	North Island	Jul	30.22847	no	Positive	Healthy
A14005770	Working dog	12	Male	North Island	Jul	52.19684	no	Positive	Healthy
H14021425	Working dog	1.5	Female	North Island	Jul	0	no	Negative	Healthy
H14021979	Pet dogs	3	Female	North Island	Jul	0	no	Negative	Healthy
H14021983	Pet dogs	6	Female	North Island	Jul	80.1406	no	Positive	Healthy
HEAL - 004	Working dog	1.25	Female	North Island	Jul	0	no	Negative	Healthy
HEAL - 005	Working dog	1.25	Female	North Island	Jul	0	no	Negative	Healthy
HEAL - 006	Working dog	1.25	Male	North Island	Jul	0	no	Negative	Healthy
HEAL - 007	Working dog	1.25	Female	North Island	Jul	0	no	Negative	Healthy
HEAL - 008	Working dog	1.25	Male	North Island	Jul	0	no	Negative	Healthy
P14013035	Pet dogs	6	Female	North Island	Jul	0	no	Negative	Healthy
P14013198	Pet dogs	2	Female	North Island	Jul	0	no	Negative	Healthy
P14013260	Pet dogs	2	Female	North Island	Jul	86.11599	no	Positive	Healthy
P14013261	Pet dogs	2	Female	North Island	Jul	0	no	Negative	Healthy
P14013272	Pet dogs	6	Female	North Island	Jul	78.8225	no	Positive	Healthy
P14013367	Pet dogs	1.5	Female	North Island	Jul	11.42355	no	Negative	Healthy
P14013422	Pet dogs	2	Female	North Island	Jul	0	no	Negative	Healthy
P14013589	Unknown	13	Female	North Island	Jul	23.11072	no	Positive	Healthy
P14013621	Pet dogs	7	Female	South Island	Jul	0	no	Negative	Healthy
P14013692	Pet dogs	3	Female	North Island	Jul	0	no	Negative	Healthy

P14013754	Pet dogs	4	Female	North Island	Jul	90.94903	no	Positive	Healthy
P14014183	Unknown	2	Female	North Island	Jul	12.97872	no	Negative	Healthy
P14014294	Pet dogs	6	Female	North Island	Jul	19.57447	no	Negative	Healthy
A14005175	Pet dogs	12	Male	North Island	Jul	0	no	Negative	Sick
A14005245	Pet dogs	6	Female	North Island	Jul	10.19332	no	Negative	Sick
A14005296	Pet dogs	12	Female	North Island	Jul	0	no	Negative	Sick
A14005298	Pet dogs	11	Male	North Island	Jul	93.93673	no	Positive	Sick
A14005332	Working dog	4	Male	North Island	Jul	96.04569	no	Positive	Sick
A14005338	Pet dogs	11	Male	North Island	Jul	5.975395	no	Negative	Sick
A14005339	Pet dogs	7	Male	North Island	Jul	0	no	Negative	Sick
A14005372	Pet dogs	1	Male	North Island	Jul	9.226714	no	Negative	Sick
A14005373	Working dog	0.61	Male	North Island	Jul	0	no	Negative	Sick
A14005404	Pet dogs	4	Male	North Island	Jul	0	no	Negative	Sick
A14005419	Working dog	12	Male	North Island	Jul	71.00176	no	Positive	Sick
A14005429	Pet dogs	14	Female	North Island	Jul	0	no	Negative	Sick
A14005474	Pet dogs	9	Female	North Island	Jul	91.91564	no	Positive	Sick
A14005477	Working dog	8	Male	North Island	Jul	52.89982	no	Positive	Sick
A14005485	Pet dogs	12	Male	North Island	Jul	17.83831	no	Negative	Sick
A14005503	Pet dogs	13	Male	North Island	Jul	31.19508	no	Positive	Sick
A14005520	Pet dogs	10	Male	North Island	Jul	20.38664	no	Positive	Sick
A14005546	Pet dogs	4	Male	North Island	Jul	28.20738	no	Positive	Sick
A14005600	Working dog	11	Male	North Island	Jul	0	no	Negative	Sick
A14005624	Pet dogs	10	Male	North Island	Jul	54.13005	no	Positive	Sick
A14005672	Pet dogs	6	Male	North Island	Jul	0	no	Negative	Sick
A14005676	Pet dogs	4	Male	North Island	Jul	0	no	Negative	Sick
A14005703	Pet dogs	12	Female	North Island	Jul	0	no	Negative	Sick
A14005784	Pet dogs	7	Male	North Island	Jul	88.13708	no	Positive	Sick

A14005799	Pet dogs	15	Female	North Island	Jul	3.690685	no	Negative	Sick
A14005812	Pet dogs	6	Female	North Island	Jul	83.65554	no	Positive	Sick
A14005818	Pet dogs	11	Male	North Island	Jul	0	no	Negative	Sick
A14005822	Working dog	7	Male	North Island	Jul	0	no	Negative	Sick
A14005843	Pet dogs	7	Female	North Island	Jul	64.85062	no	Positive	Sick
A14005886	Pet dogs	8	Female	North Island	Jul	0	no	Negative	Sick
A14005903	Pet dogs	1	Female	North Island	Jul	0	no	Negative	Sick
A14005907	Pet dogs	8	Male	North Island	Jul	0	no	Negative	Sick
A14005910	Pet dogs	10	Female	North Island	Jul	0	no	Negative	Sick
A14006030	Working dog	9	Male	North Island	Jul	0	no	Negative	Sick
A14006052	Working dog	3	Male	North Island	Jul	0	no	Negative	Sick
A14006072	Pet dogs	11	Male	North Island	Jul	0	no	Negative	Sick
A14006098	Working dog	18	Male	North Island	Jul	6.85413	no	Negative	Sick
H14021055	Pet dogs	8	Male	North Island	Jul	0	no	Negative	Sick
H14021605	Pet dogs	10	Female	North Island	Jul	0	no	Negative	Sick
H14021653	Pet dogs	3	Male	North Island	Jul	0	no	Negative	Sick
H14021735	Pet dogs	5	Male	North Island	Jul	0	no	Negative	Sick
H14022014	Unknown	2	Female	North Island	Jul	0	no	Negative	Sick
H14022455	Pet dogs	4	Male	North Island	Jul	0	no	Negative	Sick
H14022584	Working dog	10	Female	North Island	Jul	0	no	Negative	Sick
P14013004	Pet dogs	15	Female	North Island	Jul	0	no	Negative	Sick
P14013006	Pet dogs	10	Male	North Island	Jul	0	no	Negative	Sick
P14013048	Pet dogs	14	Male	North Island	Jul	0	no	Negative	Sick
P14013050	Pet dogs	9	Male	North Island	Jul	0	no	Negative	Sick
P14013084	Pet dogs	5	Female	South Island	Jul	0	no	Negative	Sick
P14013114	Pet dogs	12	Female	North Island	Jul	0	no	Negative	Sick
P14013117	Pet dogs	3	Male	North Island	Jul	25.92267	no	Positive	Sick

P14013122	Pet dogs	2	Male	North Island	Jul	88.92794	no	Positive	Sick
P14013151	Pet dogs	9	Male	North Island	Jul	0	no	Negative	Sick
P14013155	Pet dogs	12	Female	North Island	Jul	37.52197	no	Positive	Sick
P14013169	Pet dogs	2	Male	North Island	Jul	0	no	Negative	Sick
P14013172	Pet dogs	11	Male	North Island	Jul	0	no	Negative	Sick
P14013183	Pet dogs	14	Female	South Island	Jul	83.39192	no	Positive	Sick
P14013204	Pet dogs	4	Female	North Island	Jul	0	no	Negative	Sick
P14013262	Pet dogs	11	Female	North Island	Jul	0	no	Negative	Sick
P14013270	Pet dogs	12	Male	North Island	Jul	0	no	Negative	Sick
P14013311	Pet dogs	7	Male	North Island	Jul	50.26362	no	Positive	Sick
P14013326	Pet dogs	13	Female	North Island	Jul	8.435852	no	Negative	Sick
P14013334	Pet dogs	7	Male	South Island	Jul	0	no	Negative	Sick
P14013377	Pet dogs	11	Female	North Island	Jul	48.76977	no	Positive	Sick
P14013395	Pet dogs	13	Male	North Island	Jul	0	no	Negative	Sick
P14013451	Pet dogs	12	Male	South Island	Jul	0	no	Negative	Sick
P14013476	Pet dogs	9	Male	North Island	Jul	43.67311	no	Positive	Sick
P14013508	Pet dogs	9	Male	North Island	Jul	80.57996	no	Positive	Sick
P14013517	Pet dogs	8	Female	North Island	Jul	0	no	Negative	Sick
P14013534	Pet dogs	9	Male	North Island	Jul	0	no	Negative	Sick
P14013547	Pet dogs	9	Male	North Island	Jul	83.39192	no	Positive	Sick
P14013671	Pet dogs	9	Female	North Island	Jul	0	no	Negative	Sick
P14013708	Pet dogs	15	Male	North Island	Jul	6.678383	no	Negative	Sick
P14013713	Pet dogs	8	Male	North Island	Jul	0	no	Negative	Sick
P14013733	Pet dogs	8	Female	North Island	Jul	0	no	Negative	Sick
P14013742	Pet dogs	7	Male	North Island	Jul	0	no	Negative	Sick
P14013766	Pet dogs	7	Female	North Island	Jul	9.095745	no	Negative	Sick
P14013828	Working dog	10	Female	North Island	Jul	70.37234	no	Positive	Sick

P14014222	Working dog	0.16	Female	South Island	Jul	21.17021	no	Positive	Sick
P14014238	Working dog	5	Female	North Island	Jul	19.14894	no	Negative	Sick
A14006277	Working dog	3	Female	North Island	Aug	0	no	Negative	Healthy
A14006474	Pet dogs	4	Female	North Island	Aug	53.76429	no	Positive	Healthy
A14006702	Pet dogs	14	Female	North Island	Aug	0	no	Negative	Healthy
A14006961	Pet dogs	0.58	Female	North Island	Aug	0	no	Negative	Healthy
P14014880	Working dog	3	Female	North Island	Aug	31.37564	no	Positive	Healthy
P14015326	Pet dogs	5	Female	North Island	Aug	92.39259	no	Positive	Healthy
P14015862	Pet dogs	2	Female	North Island	Aug	95.11234	no	Positive	Healthy
P14015891	Pet dogs	3	Female	North Island	Aug	0	no	Negative	Healthy
A14006247	Pet dogs	13	Male	North Island	Aug	5.242412	no	Negative	Sick
A14006263	Pet dogs	6	Male	North Island	Aug	12.02207	no	Negative	Sick
A14006301	Pet dogs	8	Male	North Island	Aug	9.381159	no	Negative	Sick
A14006303	Pet dogs	12	Male	North Island	Aug	6.188412	no	Negative	Sick
A14006385	Working dog	10	Male	North Island	Aug	24.08356	no	Positive	Sick
A14006447	Working dog	4	Male	North Island	Aug	39.1013	no	Positive	Sick
A14006476	Working dog	0.83	Male	North Island	Aug	10.99724	no	Negative	Sick
A14006481	Pet dogs	2	Female	North Island	Aug	3.98108	no	Negative	Sick
A14006483	Pet dogs	12	Female	North Island	Aug	10.60307	no	Negative	Sick
A14006487	Working dog	10	Male	North Island	Aug	13.32282	no	Negative	Sick
A14006499	Working dog	16	Male	North Island	Aug	12.61332	no	Negative	Sick
A14006518	Pet dogs	14	Female	North Island	Aug	95.86125	no	Positive	Sick
A14006534	Pet dogs	0.66	Male	North Island	Aug	12.65274	no	Negative	Sick
A14006537	Pet dogs	7	Male	North Island	Aug	65.23453	no	Positive	Sick
A14006582	Pet dogs	10	Male	North Island	Aug	93.37801	no	Positive	Sick
A14006600	Pet dogs	10	Male	North Island	Aug	8.632243	no	Negative	Sick
A14006604	Pet dogs	10	Male	North Island	Aug	34.52897	no	Positive	Sick

A14006620	Pet dogs	0.75	Male	North Island	Aug	77.7296	no	Positive	Sick
A14006654	Pet dogs	9	Male	North Island	Aug	69.92511	no	Positive	Sick
A14006674	Pet dogs	1	Male	North Island	Aug	13.87466	no	Negative	Sick
A14006701	Pet dogs	11	Female	North Island	Aug	0	no	Negative	Sick
A14006710	Pet dogs	10	Female	North Island	Aug	22.30981	no	Positive	Sick
A14006750	Pet dogs	13	Female	North Island	Aug	60.85928	no	Positive	Sick
A14006790	Working dog	13	Male	North Island	Aug	38.11588	yes	Positive	Sick
A14006814	Working dog	2	Male	North Island	Aug	7.60741	no	Negative	Sick
A14006823	Pet dogs	6	Female	North Island	Aug	0	no	Negative	Sick
A14006831	Pet dogs	9	Male	North Island	Aug	0	no	Negative	Sick
A14006844	Pet dogs	13	Female	North Island	Aug	10.05124	no	Negative	Sick
A14006847	Pet dogs	7	Female	North Island	Aug	0	no	Negative	Sick
A14006855	Pet dogs	9	Female	North Island	Aug	0	no	Negative	Sick
A14006860	Working dog	10	Male	North Island	Aug	0	no	Negative	Sick
A14006878	Pet dogs	1	Male	North Island	Aug	8.868743	no	Negative	Sick
A14006920	Pet dogs	4	Female	North Island	Aug	95.9795	no	Positive	Sick
A14006944	Pet dogs	14	Female	North Island	Aug	0	no	Negative	Sick
A14006953	Pet dogs	4	Male	North Island	Aug	0	no	Negative	Sick
A14006956	Unknown	12	Female	North Island	Aug	51.63579	no	Positive	Sick
A14007189	Pet dogs	10	Female	North Island	Aug	0	no	Negative	Sick
A14007190	Pet dogs	5	Female	North Island	Aug	0	no	Negative	Sick
A14007194	Pet dogs	9	Male	North Island	Aug	24.47773	no	Positive	Sick
A14007208	Pet dogs	7	Male	North Island	Aug	16.1214	no	Negative	Sick
A14007223	Pet dogs	13	Male	North Island	Aug	8.671659	no	Negative	Sick
H14025703	Pet dogs	1	Male	North Island	Aug	13.00749	no	Negative	Sick
H14025798	Pet dogs	11	Male	South Island	Aug	17.65865	no	Negative	Sick
H14026027	Pet dogs	12	Female	South Island	Aug	3.389831	no	Negative	Sick

H14027241	Pet dogs	4	Male	North Island	Aug	2.837998	no	Negative	Sick
H14028462	Pet dogs	3	Male	South Island	Aug	20.18132	no	Positive	Sick
P14014428	Pet dogs	14	Female	South Island	Aug	40.16555	no	Positive	Sick
P14014621	Working dog	3	Male	North Island	Aug	3.902247	no	Negative	Sick
P14014742	Pet dogs	2	Female	South Island	Aug	0	no	Negative	Sick
P14014793	Pet dogs	12	Female	North Island	Aug	37.09105	no	Positive	Sick
P14014848	Pet dogs	13	Male	North Island	Aug	8.513993	no	Negative	Sick
P14014915	Working dog	9	Male	North Island	Aug	9.341742	no	Negative	Sick
P14014958	Working dog	10	Female	South Island	Aug	84.54868	no	Positive	Sick
P14015131	Pet dogs	8	Female	North Island	Aug	13.71699	no	Negative	Sick
P14015217	Working dog	2	Female	North Island	Aug	13.51991	no	Negative	Sick
P14015289	Pet dogs	8	Male	North Island	Aug	18.76232	no	Negative	Sick
P14015302	Pet dogs	3	Male	North Island	Aug	31.80922	no	Positive	Sick
P14015327	Pet dogs	2	Female	North Island	Aug	3.62633	no	Negative	Sick
P14015331	Pet dogs	8	Female	North Island	Aug	33.54356	no	Positive	Sick
P14015351	Pet dogs	9	Female	North Island	Aug	0	no	Negative	Sick
P14015412	Pet dogs	12	Female	North Island	Aug	5.321246	no	Negative	Sick
P14015422	Pet dogs	2	Female	North Island	Aug	11.35199	no	Negative	Sick
P14015436	Pet dogs	12	Male	North Island	Aug	5.439495	no	Negative	Sick
P14015476	Pet dogs	10	Male	South Island	Aug	13.99291	no	Negative	Sick
P14015490	Pet dogs	14	Male	North Island	Aug	6.976744	no	Negative	Sick
P14015581	Pet dogs	2	Female	North Island	Aug	1.655499	no	Negative	Sick
P14015684	Pet dogs	8	Female	North Island	Aug	13.87466	no	Negative	Sick
P14015811	Pet dogs	1	Male	South Island	Aug	8.908159	no	Negative	Sick
P14015818	Pet dogs	11	Male	South Island	Aug	3.902247	no	Negative	Sick
P14015828	Pet dogs	8	Male	South Island	Aug	0	no	Negative	Sick
P14016079	Pet dogs	16	Female	North Island	Aug	2.128498	no	Negative	Sick

P14016132	Pet dogs	8	Male	North Island	Aug	79.06977	no	Positive	Sick
P14016155	Pet dogs	6	Male	North Island	Aug	6.070162	no	Negative	Sick
P14016180	Pet dogs	13	Female	North Island	Aug	0	no	Negative	Sick
P14016200	Pet dogs	2	Male	North Island	Aug	8.238076	no	Negative	Sick
P14016423	Pet dogs	5	Female	North Island	Aug	8.43516	no	Negative	Sick
P14016524	Pet dogs	8	Male	North Island	Aug	22.15215	no	Positive	Sick
P14016622	Working dog	6	Female	North Island	Aug	15.29365	no	Negative	Sick
P14016643	Pet dogs	5	Male	North Island	Aug	10.95782	no	Negative	Sick
P14016716	Working dog	3	Male	North Island	Aug	0	no	Negative	Sick
P14016760	Pet dogs	10	Male	North Island	Aug	22.66456	no	Positive	Sick
P14016785	Pet dogs	10	Male	North Island	Aug	0	no	Negative	Sick
P14016813	Pet dogs	8	Female	North Island	Aug	64.05203	no	Positive	Sick
P14016865	Pet dogs	10	Male	North Island	Aug	0	no	Negative	Sick
P14016885	Pet dogs	9	Male	North Island	Aug	0	no	Negative	Sick
P14016892	Pet dogs	5	Female	North Island	Aug	6.070162	no	Negative	Sick
P14017114	Working dog	10	Female	North Island	Aug	30.10638	no	Positive	Sick
P14017127	Working dog	1	Female	North Island	Aug	0	no	Negative	Sick
P14017139	Pet dogs	9	Male	North Island	Aug	21.75532	no	Positive	Sick
P14017311	Working dog	1	Male	North Island	Aug	79.14894	no	Positive	Sick
P14017320	Pet dogs	4	Female	North Island	Aug	7.978723	no	Negative	Sick
P14017478	Pet dogs	3	Female	North Island	Aug	92.55319	no	Positive	Sick
A14007544	Pet dogs	0.5	Male	North Island	Sep	14.84043	no	Negative	Healthy
A14007999	Pet dogs	10	Male	North Island	Sep	22.07447	no	Positive	Healthy
A14008018	Pet dogs	2	Female	North Island	Sep	19.84043	no	Negative	Healthy
A14008209	Pet dogs	4	Female	North Island	Sep	16.2234	no	Negative	Healthy
HEAL - 009	Working dog	3	Female	North Island	Sep	45.7458	no	Positive	Healthy
HEAL - 010	Working dog	3	Male	North Island	Sep	44.90546	no	Positive	Healthy

HEAL - 011	Working dog	3	Female	North Island	Sep	47.53151	no	Positive	Healthy
HEAL - 012	Working dog	3	Male	North Island	Sep	58.40336	no	Positive	Healthy
HEAL - 013	Working dog	3	Female	North Island	Sep	51.15546	no	Positive	Healthy
P14017635	Pet dogs	5	Female	North Island	Sep	8.979809	no	Negative	Healthy
P14018166	Pet dogs	6	Female	North Island	Sep	75.29224	no	Positive	Healthy
P14018172	Working dog	2	Female	North Island	Sep	21.25399	no	Positive	Healthy
P14018401	Working dog	5	Female	North Island	Sep	10.99894	no	Negative	Healthy
P14018805	Pet dogs	3	Female	North Island	Sep	5.207226	no	Negative	Healthy
A14007232	Pet dogs	2	Female	North Island	Sep	32.07447	no	Positive	Sick
A14007235	Pet dogs	11	Male	North Island	Sep	19.20213	no	Negative	Sick
A14007240	Pet dogs	3	Male	North Island	Sep	96.91489	no	Positive	Sick
A14007252	Pet dogs	7	Female	North Island	Sep	77.02128	no	Positive	Sick
A14007280	Pet dogs	6	Female	North Island	Sep	21.38298	no	Positive	Sick
A14007368	Pet dogs	2	Female	North Island	Sep	28.98936	no	Positive	Sick
A14007371	Pet dogs	0.75	Female	North Island	Sep	60.37234	no	Positive	Sick
A14007383	Pet dogs	14	Female	North Island	Sep	37.81915	no	Positive	Sick
A14007393	Pet dogs	7	Female	North Island	Sep	27.28723	no	Positive	Sick
A14007396	Pet dogs	14	Female	North Island	Sep	46.01064	no	Positive	Sick
A14007447	Pet dogs	11	Male	North Island	Sep	70.53191	no	Positive	Sick
A14007508	Pet dogs	13	Male	North Island	Sep	78.08511	no	Positive	Sick
A14007511	Working dog	5	Female	North Island	Sep	90.6383	no	Positive	Sick
A14007526	Pet dogs	14	Female	North Island	Sep	22.81915	no	Positive	Sick
A14007541	Pet dogs	8	Male	North Island	Sep	51.2234	no	Positive	Sick
A14007553	Pet dogs	9	Female	North Island	Sep	69.78723	no	Positive	Sick
A14007635	Pet dogs	10	Male	North Island	Sep	53.61702	no	Positive	Sick
A14007646	Working dog	11	Male	North Island	Sep	65.79787	no	Positive	Sick
A14007653	Working dog	12	Female	North Island	Sep	92.55319	no	Positive	Sick

A14007714	Pet dogs	7	Male	North Island	Sep	91.91489	no	Positive	Sick
A14007735	Pet dogs	2	Male	North Island	Sep	55.58511	no	Positive	Sick
A14007753	Working dog	12	Female	North Island	Sep	21.48936	no	Positive	Sick
A14007892	Pet dogs	7	Male	North Island	Sep	52.76596	no	Positive	Sick
A14007942	Pet dogs	13	Male	North Island	Sep	81.01064	no	Positive	Sick
A14007948	Pet dogs	1	Male	North Island	Sep	2.446809	no	Negative	Sick
A14007957	Pet dogs	0.41	Female	North Island	Sep	6.170213	no	Negative	Sick
A14007984	Pet dogs	6	Male	North Island	Sep	86.75532	no	Positive	Sick
A14007986	Working dog	4	Female	North Island	Sep	92.65957	no	Positive	Sick
A14008000	Working dog	6	Female	North Island	Sep	35.31915	no	Positive	Sick
A14008048	Pet dogs	6	Female	North Island	Sep	62.18085	no	Positive	Sick
A14008067	Working dog	0.91	Male	North Island	Sep	25	no	Positive	Sick
A14008110	Pet dogs	4	Male	North Island	Sep	40.42553	no	Positive	Sick
A14008117	Pet dogs	11	Female	North Island	Sep	9.255319	no	Negative	Sick
A14008134	Pet dogs	9	Male	North Island	Sep	79.94681	no	Positive	Sick
A14008173	Pet dogs	8	Female	North Island	Sep	41.11702	no	Positive	Sick
A14008189	Pet dogs	7	Female	North Island	Sep	12.07447	no	Negative	Sick
A14008218	Pet dogs	13	Female	North Island	Sep	22.28723	no	Positive	Sick
A14008253	Pet dogs	10	Male	North Island	Sep	75.10638	no	Positive	Sick
A14008258	Pet dogs	15	Female	North Island	Sep	52.07447	no	Positive	Sick
A14008264	Pet dogs	14	Female	North Island	Sep	44.52128	no	Positive	Sick
A14008267	Pet dogs	1	Male	North Island	Sep	71.59574	no	Positive	Sick
A14008282	Pet dogs	0.83	Male	North Island	Sep	58.82979	no	Positive	Sick
A14008306	Pet dogs	12	Female	North Island	Sep	28.82979	no	Positive	Sick
A14008307	Pet dogs	8	Female	North Island	Sep	54.52128	no	Positive	Sick
A14008315	Working dog	12	Male	North Island	Sep	4.893617	no	Negative	Sick
P14017656	Pet dogs	6	Male	North Island	Sep	76.03613	no	Positive	Sick

P14017709	Pet dogs	2	Male	North Island	Sep	28.2678	no	Positive	Sick
P14017710	Working dog	0.58	Male	North Island	Sep	27.57705	no	Positive	Sick
P14017760	Working dog	1	Female	South Island	Sep	0	yes	Negative	Sick
P14017765	Pet dogs	13	Male	North Island	Sep	7.06695	no	Negative	Sick
P14017767	Working dog	7	Female	North Island	Sep	17.58767	no	Negative	Sick
P14017799	Working dog	1	Female	North Island	Sep	30.5526	no	Positive	Sick
P14017830	Pet dogs	4	Male	North Island	Sep	15.09033	no	Negative	Sick
P14017883	Pet dogs	7	Male	North Island	Sep	64.77152	no	Positive	Sick
P14017888	Working dog	10	Male	North Island	Sep	9.989373	no	Negative	Sick
P14017947	Pet dogs	11	Female	North Island	Sep	40.59511	no	Positive	Sick
P14017990	Working dog	11	Male	North Island	Sep	34.05951	no	Positive	Sick
P14018002	Pet dogs	15	Male	North Island	Sep	38.04463	no	Positive	Sick
P14018012	Pet dogs	15	Female	North Island	Sep	8.607864	no	Negative	Sick
P14018020	Pet dogs	12	Female	South Island	Sep	27.09883	no	Positive	Sick
P14018079	Pet dogs	6	Male	South Island	Sep	84.11265	no	Positive	Sick
P14018179	Pet dogs	14	Female	North Island	Sep	32.89054	no	Positive	Sick
P14018237	Pet dogs	6	Male	North Island	Sep	0	no	Negative	Sick
P14018269	Unknown	13	Female	North Island	Sep	38.04463	no	Positive	Sick
P14018290	Pet dogs	9	Female	South Island	Sep	41.1796	no	Positive	Sick
P14018300	Working dog	7	Male	South Island	Sep	0	no	Negative	Sick
P14018355	Working dog	14	Male	North Island	Sep	0	no	Negative	Sick
P14018364	Working dog	15	Male	North Island	Sep	0	no	Negative	Sick
P14018395	Pet dogs	6	Male	North Island	Sep	0	no	Negative	Sick
P14018403	Working dog	9	Female	North Island	Sep	59.56429	no	Positive	Sick
P14018408	Pet dogs	11	Female	North Island	Sep	1.115834	no	Negative	Sick
P14018521	Pet dogs	14	Male	South Island	Sep	2.072264	no	Negative	Sick
P14018552	Pet dogs	13	Male	North Island	Sep	0	no	Negative	Sick

P14018576	Working dog	8	Female	South Island	Sep	24.49522	no	Positive	Sick
P14018644	Working dog	13	Female	North Island	Sep	0.584485	no	Negative	Sick
P14018678	Pet dogs	5	Male	South Island	Sep	37.46015	no	Positive	Sick
P14018708	Pet dogs	12	Male	North Island	Sep	57.65143	no	Positive	Sick
P14018786	Pet dogs	9	Female	North Island	Sep	23.43252	no	Positive	Sick
P14018787	Pet dogs	8	Female	North Island	Sep	63.39001	no	Positive	Sick
P14018813	Pet dogs	6	Male	North Island	Sep	72.47609	no	Positive	Sick
P14018841	Pet dogs	6	Male	North Island	Sep	0	no	Negative	Sick
P14018852	Pet dogs	6	Male	North Island	Sep	4.729012	no	Negative	Sick
P14018858	Pet dogs	3	Male	North Island	Sep	82.04038	no	Positive	Sick
P14018878	Working dog	9	Male	North Island	Sep	0	no	Negative	Sick
P14019218	Pet dogs	10	Female	North Island	Sep	43.41126	no	Positive	Sick
P14020046	Pet dogs	3	Female	North Island	Sep	9.829968	no	Negative	Sick
P14020049	Pet dogs	12	Female	North Island	Sep	17.322	no	Negative	Sick
P14020098	Working dog	12	Female	North Island	Sep	14.61211	no	Negative	Sick
P14020099	Pet dogs	0.66	Male	North Island	Sep	0	no	Negative	Sick
P14020256	Pet dogs	11	Male	North Island	Sep	73.43252	no	Positive	Sick
A14008873	Pet dogs	8	Female	North Island	Oct	52.81615	no	Positive	Healthy
H14036842	Pet dogs	10	Female	North Island	Oct	6.565126	no	Negative	Healthy
P14021198	Working dog	3	Female	North Island	Oct	9.978992	no	Negative	Healthy
P14021216	Pet dogs	2	Female	North Island	Oct	11.65966	no	Negative	Healthy
P14021698	Pet dogs	1	Female	North Island	Oct	27.46849	no	Positive	Healthy
A14008387	Pet dogs	1	Male	North Island	Oct	0	no	Negative	Sick
A14008400	Pet dogs	11	Male	North Island	Oct	0	no	Negative	Sick
A14008421	Pet dogs	1	Male	North Island	Oct	23.96387	no	Positive	Sick
A14008426	Pet dogs	14	Male	North Island	Oct	0.850159	no	Negative	Sick
A14008427	Pet dogs	14	Female	North Island	Oct	0	no	Negative	Sick

A14008453	Pet dogs	2	Female	North Island	Oct	41.87035	no	Positive	Sick
A14008455	Pet dogs	11	Female	North Island	Oct	0	no	Negative	Sick
A14008456	Pet dogs	6	Male	North Island	Oct	20.13815	no	Positive	Sick
A14008461	Pet dogs	4	Male	North Island	Oct	4.729012	no	Negative	Sick
A14008463	Working dog	12	Female	North Island	Oct	1.594049	no	Negative	Sick
A14008472	Pet dogs	9	Female	North Island	Oct	38.41658	no	Positive	Sick
A14008489	Pet dogs	10	Male	North Island	Oct	1.434644	no	Negative	Sick
A14008492	Pet dogs	0.58	Male	North Island	Oct	81.66844	no	Positive	Sick
A14008497	Pet dogs	2	Male	North Island	Oct	54.72901	no	Positive	Sick
A14008507	Pet dogs	12	Male	North Island	Oct	58.76727	yes	Positive	Sick
A14008530	Pet dogs	1	Female	North Island	Oct	64.50584	no	Positive	Sick
A14008551	Pet dogs	1	Male	North Island	Oct	41.65781	no	Positive	Sick
A14008588	Pet dogs	8	Female	North Island	Oct	7.5983	no	Negative	Sick
A14008597	Pet dogs	4	Female	North Island	Oct	2.656748	no	Negative	Sick
A14008647	Pet dogs	7	Female	North Island	Oct	3.400638	no	Negative	Sick
A14008662	Pet dogs	8	Female	North Island	Oct	81.72157	no	Positive	Sick
A14008674	Pet dogs	11	Male	North Island	Oct	21.99787	no	Positive	Sick
A14008679	Pet dogs	13	Male	North Island	Oct	42.77365	no	Positive	Sick
A14008684	Unknown	11	Male	North Island	Oct	2.125399	no	Negative	Sick
A14008717	Pet dogs	10	Male	North Island	Oct	5.154091	no	Negative	Sick
A14008746	Pet dogs	13	Female	North Island	Oct	31.4559	no	Positive	Sick
A14008752	Pet dogs	4	Female	North Island	Oct	0	no	Negative	Sick
A14008793	Working dog	8	Male	North Island	Oct	0	no	Negative	Sick
A14008807	Pet dogs	14	Male	North Island	Oct	0	no	Negative	Sick
A14008817	Working dog	4	Female	North Island	Oct	91.55154	no	Positive	Sick
A14008831	Pet dogs	7	Male	North Island	Oct	11.47715	no	Negative	Sick
A14008832	Pet dogs	10	Female	North Island	Oct	27.78959	no	Positive	Sick

A14008845	Pet dogs	0.58	Female	North Island	Oct	37.03507	no	Positive	Sick
A14008854	Pet dogs	7	Female	North Island	Oct	0	no	Negative	Sick
A14008858	Pet dogs	11	Female	North Island	Oct	50.37194	no	Positive	Sick
A14008868	Pet dogs	5	Male	North Island	Oct	91.49841	no	Positive	Sick
A14008871	Pet dogs	11	Male	North Island	Oct	22.21041	no	Positive	Sick
A14008912	Pet dogs	12	Male	North Island	Oct	24.017	no	Positive	Sick
A14008974	Pet dogs	4	Female	North Island	Oct	13.86823	no	Negative	Sick
A14008988	Pet dogs	9	Male	North Island	Oct	0	no	Negative	Sick
A14009017	Pet dogs	9	Male	North Island	Oct	2.125399	no	Negative	Sick
A14009033	Pet dogs	15	Female	North Island	Oct	17.42827	no	Negative	Sick
A14009137	Pet dogs	0.58	Female	North Island	Oct	0	no	Negative	Sick
H14034471	Pet dogs	12	Male	South Island	Oct	20.85084	no	Positive	Sick
H14035693	Pet dogs	8	Male	North Island	Oct	1.470588	no	Negative	Sick
H14035906	Working dog	4	Female	North Island	Oct	7.615546	no	Negative	Sick
H14035979	Pet dogs	10	Male	North Island	Oct	4.14916	no	Negative	Sick
H14036204	Pet dogs	2	Male	North Island	Oct	8.193277	no	Negative	Sick
H14038451	Pet dogs	4	Female	South Island	Oct	2.678571	no	Negative	Sick
P14020363	Pet dogs	5	Male	North Island	Oct	42.96218	no	Positive	Sick
P14020493	Pet dogs	3	Female	North Island	Oct	25.68277	no	Positive	Sick
P14020546	Pet dogs	8	Male	North Island	Oct	14.12815	no	Negative	Sick
P14020570	Pet dogs	12	Female	South Island	Oct	21.48109	no	Positive	Sick
P14020589	Pet dogs	4	Male	North Island	Oct	1.05042	no	Negative	Sick
P14020619	Pet dogs	6	Female	North Island	Oct	18.06723	no	Negative	Sick
P14020634	Pet dogs	6	Female	North Island	Oct	12.07983	no	Negative	Sick
P14020915	Pet dogs	12	Female	North Island	Oct	28.09874	no	Positive	Sick
P14020981	Working dog	13	Male	North Island	Oct	12.71008	no	Negative	Sick
P14021002	Pet dogs	9	Female	North Island	Oct	27.94118	no	Positive	Sick

P14021006	Pet dogs	14	Female	North Island	Oct	32.77311	no	Positive	Sick
P14021007	Pet dogs	9	Female	North Island	Oct	9.243697	no	Negative	Sick
P14021025	Pet dogs	10	Female	South Island	Oct	92.85714	no	Positive	Sick
P14021117	Working dog	6	Female	North Island	Oct	27.2584	no	Positive	Sick
P14021122	Pet dogs	8	Female	North Island	Oct	34.87395	no	Positive	Sick
P14021211	Pet dogs	11	Female	North Island	Oct	41.17647	no	Positive	Sick
P14021234	Pet dogs	8	Male	North Island	Oct	17.54202	no	Negative	Sick
P14021275	Pet dogs	7	Female	South Island	Oct	55.35714	no	Positive	Sick
P14021380	Pet dogs	13	Male	South Island	Oct	28.93908	no	Positive	Sick
P14021392	Pet dogs	12	Female	South Island	Oct	45.58824	no	Positive	Sick
P14021476	Working dog	11	Male	North Island	Oct	0.630252	no	Negative	Sick
P14021492	Working dog	6	Male	North Island	Oct	33.82353	no	Positive	Sick
P14021611	Pet dogs	12	Male	North Island	Oct	27.46849	no	Positive	Sick
P14021642	Pet dogs	10	Male	North Island	Oct	27.04832	no	Positive	Sick
P14021646	Pet dogs	1	Male	North Island	Oct	71.63866	no	Positive	Sick
P14021681	Working dog	8	Male	North Island	Oct	62.92017	no	Positive	Sick
P14021723	Working dog	4	Male	North Island	Oct	9.716387	no	Negative	Sick
P14021732	Working dog	13	Male	North Island	Oct	27.52101	no	Positive	Sick
P14021737	Pet dogs	13	Female	North Island	Oct	14.39076	no	Negative	Sick
P14022112	Pet dogs	11	Male	North Island	Oct	28.72899	no	Positive	Sick
P14022122	Working dog	4	Female	North Island	Oct	4.621849	no	Negative	Sick
P14022235	Pet dogs	7	Female	North Island	Oct	14.39076	no	Negative	Sick
P14022277	Working dog	9	Male	North Island	Oct	12.44748	no	Negative	Sick
P14022299	Pet dogs	14	Male	North Island	Oct	19.27521	no	Negative	Sick
P14022347	Pet dogs	11	Male	South Island	Oct	56.03992	no	Positive	Sick
P14022354	Pet dogs	9	Female	North Island	Oct	22.0063	no	Positive	Sick
P14022379	Working dog	13	Female	North Island	Oct	9.663866	no	Negative	Sick

P14022498	Pet dogs	12	Male	North Island	Oct	66.33403	no	Positive	Sick
P14022503	Pet dogs	11	Female	North Island	Oct	33.98109	no	Positive	Sick
P14022684	Pet dogs	15	Female	North Island	Oct	10.87185	no	Negative	Sick
P14022755	Pet dogs	6	Male	South Island	Oct	44.90546	no	Positive	Sick
P14022788	Working dog	6	Female	North Island	Oct	16.80672	no	Negative	Sick
P14022804	Pet dogs	10	Male	North Island	Oct	2.993697	no	Negative	Sick
P14022817	Pet dogs	13	Female	North Island	Oct	4.516807	no	Negative	Sick
P14022852	Working dog	8	Male	North Island	Oct	27.10084	no	Positive	Sick
P14022875	Pet dogs	9	Female	North Island	Oct	0	no	Negative	Sick
A14009478	Pet dogs	8	Female	North Island	Nov	6.198347	no	Negative	Healthy
A14010169	Pet dogs	7	Male	North Island	Nov	3.896104	no	Negative	Healthy
H14039823	Pet dogs	1	Female	North Island	Nov	87.60331	no	Positive	Healthy
H14040612	Pet dogs	3	Female	North Island	Nov	26.21015	no	Positive	Healthy
H14040613	Pet dogs	5	Female	North Island	Nov	36.42267	no	Positive	Healthy
P14023370	Pet dogs	1	Male	North Island	Nov	7.142857	no	Negative	Healthy
P14024101	Working dog	5	Female	North Island	Nov	66.2928	no	Positive	Healthy
P14024237	Working dog	5	Female	North Island	Nov	67.65053	no	Positive	Healthy
A14009392	Working dog	11	Male	North Island	Nov	40.08264	no	Positive	Sick
A14009402	Pet dogs	11	Female	North Island	Nov	15.28926	no	Negative	Sick
A14009425	Pet dogs	7	Male	North Island	Nov	39.37426	no	Positive	Sick
A14009445	Pet dogs	4	Female	North Island	Nov	9.090909	no	Negative	Sick
A14009454	Working dog	12	Male	North Island	Nov	9.799292	no	Negative	Sick
A14009460	Pet dogs	8	Female	North Island	Nov	24.20307	no	Positive	Sick
A14009470	Pet dogs	9	Female	North Island	Nov	11.62928	no	Negative	Sick
A14009503	Pet dogs	7	Male	North Island	Nov	42.50295	no	Positive	Sick
A14009505	Working dog	12	Male	North Island	Nov	61.33412	no	Positive	Sick
A14009541	Pet dogs	6	Female	North Island	Nov	10.97993	no	Negative	Sick

A14009559	Pet dogs	9	Female	North Island	Nov	4.0732	no	Negative	Sick
A14009562	Pet dogs	9	Male	North Island	Nov	25.5608	no	Positive	Sick
A14009612	Working dog	4	Female	North Island	Nov	98.05195	no	Positive	Sick
A14009710	Working dog	5	Female	North Island	Nov	69.48052	no	Positive	Sick
A14009713	Working dog	5	Female	North Island	Nov	98.05195	no	Positive	Sick
A14009715	Working dog	12	Male	North Island	Nov	13.6954	no	Negative	Sick
A14009726	Pet dogs	13	Male	North Island	Nov	0	no	Negative	Sick
A14009728	Pet dogs	3	Male	North Island	Nov	10.21251	no	Negative	Sick
A14009743	Pet dogs	9	Male	North Island	Nov	58.38253	no	Positive	Sick
A14009747	Pet dogs	4	Female	North Island	Nov	15.82054	no	Negative	Sick
A14009752	Pet dogs	8	Female	North Island	Nov	41.91263	no	Positive	Sick
A14009756	Pet dogs	5	Female	North Island	Nov	16.64699	no	Negative	Sick
A14009803	Pet dogs	2	Female	North Island	Nov	18.83117	no	Negative	Sick
A14009858	Pet dogs	8	Female	North Island	Nov	77.50885	no	Positive	Sick
A14009879	Pet dogs	15	Male	North Island	Nov	18.12279	no	Negative	Sick
A14009914	Pet dogs	10	Male	North Island	Nov	31.16883	no	Positive	Sick
A14009927	Unknown	1	Male	North Island	Nov	5.844156	no	Negative	Sick
A14009940	Working dog	9	Male	North Island	Nov	30.69658	no	Positive	Sick
A14009995	Pet dogs	13	Male	North Island	Nov	48.17001	no	Positive	Sick
A14010022	Pet dogs	11	Female	North Island	Nov	77.92208	no	Positive	Sick
A14010037	Pet dogs	4	Male	North Island	Nov	0	no	Negative	Sick
A14010040	Working dog	10	Female	North Island	Nov	24.02597	no	Positive	Sick
A14010181	Pet dogs	5	Male	North Island	Nov	27.27273	no	Positive	Sick
A14010187	Pet dogs	7	Male	North Island	Nov	29.69303	no	Positive	Sick
A14010197	Pet dogs	0.75	Female	North Island	Nov	40.85006	no	Positive	Sick
A14010200	Pet dogs	10	Male	North Island	Nov	39.84652	no	Positive	Sick
A14010209	Pet dogs	13	Male	North Island	Nov	27.56789	no	Positive	Sick

A14010215	Pet dogs	2	Female	North Island	Nov	55.84416	no	Positive	Sick
A14010239	Pet dogs	4	Female	North Island	Nov	6.788666	no	Negative	Sick
A14010262	Pet dogs	8	Female	North Island	Nov	11.74734	no	Negative	Sick
A14010278	Pet dogs	7	Female	North Island	Nov	26.44628	no	Positive	Sick
A14010287	Pet dogs	9	Female	North Island	Nov	11.33412	no	Negative	Sick
CIRD - 010	Working dog	2	Male	North Island	Nov	44.90546	yes	Positive	Sick
CIRD - 011	Working dog	2	Male	North Island	Nov	47.53151	yes	Positive	Sick
H14039732	Working dog	13	Female	North Island	Nov	12.63282	no	Negative	Sick
H14039960	Working dog	4	Male	South Island	Nov	37.89847	no	Positive	Sick
H14039970	Pet dogs	12	Female	South Island	Nov	0.590319	no	Negative	Sick
H14040067	Pet dogs	9	Female	North Island	Nov	0	no	Negative	Sick
H14040200	Pet dogs	7	Male	North Island	Nov	15.64345	no	Negative	Sick
H14040554	Pet dogs	10	Male	North Island	Nov	0	no	Negative	Sick
H14041210	Pet dogs	5	Male	North Island	Nov	8.618654	no	Negative	Sick
P14023268	Pet dogs	5	Female	North Island	Nov	4.604486	no	Negative	Sick
P14023300	Pet dogs	10	Female	North Island	Nov	0.649351	yes	Negative	Sick
P14023332	Working dog	12	Female	South Island	Nov	10.03542	no	Negative	Sick
P14023397	Working dog	10	Female	North Island	Nov	8.323495	no	Negative	Sick
P14023450	Pet dogs	8	Female	South Island	Nov	12.45573	no	Negative	Sick
P14023516	Working dog	6	Male	North Island	Nov	41.49941	no	Positive	Sick
P14023554	Pet dogs	12	Female	North Island	Nov	41.8536	no	Positive	Sick
P14023556	Working dog	11	Female	South Island	Nov	6.316411	no	Negative	Sick
P14023568	Pet dogs	7	Female	South Island	Nov	20.01181	no	Positive	Sick
P14023619	Working dog	4	Female	North Island	Nov	1.062574	no	Negative	Sick
P14023746	Pet dogs	10	Female	North Island	Nov	21.54664	no	Positive	Sick
P14023748	Pet dogs	13	Female	North Island	Nov	2.715466	no	Negative	Sick
P14023812	Pet dogs	12	Female	North Island	Nov	21.90083	no	Positive	Sick

P14023895	Pet dogs	7	Male	North Island	Nov	2.479339	no	Negative	Sick
P14023919	Pet dogs	4	Male	North Island	Nov	5.903188	no	Negative	Sick
P14023943	Pet dogs	12	Male	North Island	Nov	11.92444	no	Negative	Sick
P14024012	Pet dogs	10	Female	South Island	Nov	2.243211	no	Negative	Sick
P14024034	Pet dogs	1	Male	North Island	Nov	6.90673	no	Negative	Sick
P14024060	Pet dogs	9	Female	North Island	Nov	2.892562	no	Negative	Sick
P14024070	Working dog	12	Female	North Island	Nov	35.65525	no	Positive	Sick
P14024100	Pet dogs	9	Male	North Island	Nov	5.608028	yes	Negative	Sick
P14024133	Pet dogs	11	Female	North Island	Nov	18.83117	no	Negative	Sick
P14024179	Pet dogs	4	Male	North Island	Nov	58.85478	no	Positive	Sick
P14024206	Pet dogs	4	Male	North Island	Nov	29.8111	no	Positive	Sick
P14024210	Pet dogs	9	Female	North Island	Nov	1.534829	no	Negative	Sick
P14024217	Working dog	4	Female	South Island	Nov	6.021251	no	Negative	Sick
P14024305	Pet dogs	2	Male	North Island	Nov	0	no	Negative	Sick
P14024375	Pet dogs	9	Male	North Island	Nov	34.94687	no	Positive	Sick
P14024480	Pet dogs	3	Male	North Island	Nov	2.83353	no	Negative	Sick
P14024553	Pet dogs	12	Male	North Island	Nov	15.11216	no	Negative	Sick
P14024588	Pet dogs	2	Female	North Island	Nov	0	no	Negative	Sick
P14024602	Pet dogs	10	Male	North Island	Nov	53.95514	no	Positive	Sick
P14024631	Pet dogs	15	Male	North Island	Nov	4.663518	no	Negative	Sick
P14024634	Pet dogs	1	Male	North Island	Nov	0.590319	no	Negative	Sick
P14024696	Working dog	3	Female	North Island	Nov	46.34002	no	Positive	Sick
P14024731	Pet dogs	1	Female	North Island	Nov	15.17119	no	Negative	Sick
P14024797	Pet dogs	6	Female	North Island	Nov	2.007084	no	Negative	Sick
P14024837	Pet dogs	12	Male	North Island	Nov	11.55462	no	Negative	Sick
P14024845	Pet dogs	5	Female	North Island	Nov	65.54622	no	Positive	Sick
P14024909	Pet dogs	6	Female	North Island	Nov	58.40336	no	Positive	Sick

P14025128	Pet dogs	1	Male	North Island	Nov	11.86975	no	Negative	Sick
P14025143	Working dog	11	Male	North Island	Nov	76.47059	yes	Positive	Sick
A14010350	Pet dogs	6	Female	North Island	Dec	16.93925	no	Negative	Healthy
A14010627	Working dog	11	Female	North Island	Dec	58.70327	no	Positive	Healthy
A14010849	Pet dogs	9	Male	North Island	Dec	15.71262	no	Negative	Healthy
A14011168	Pet dogs	6	Male	North Island	Dec	3.971963	no	Negative	Healthy
P14025196	Pet dogs	3	Female	North Island	Dec	3.154206	no	Negative	Healthy
P14025612	Working dog	0.91	Female	North Island	Dec	2.920561	no	Negative	Healthy
P14025711	Pet dogs	5	Female	North Island	Dec	85.57243	no	Positive	Healthy
P14026492	Working dog	8	Female	North Island	Dec	43.86682	no	Positive	Healthy
P14026628	Pet dogs	2	Female	North Island	Dec	40.24533	no	Positive	Healthy
P14026855	Pet dogs	2	Female	North Island	Dec	62.38318	no	Positive	Healthy
P14026886	Pet dogs	1	Female	North Island	Dec	14.65336	no	Negative	Healthy
P14027053	Pet dogs	3	Female	South Island	Dec	33.19328	no	Positive	Healthy
A14010334	Working dog	2	Female	North Island	Dec	84.28738	no	Positive	Sick
A14010403	Working dog	13	Female	North Island	Dec	11.33178	no	Negative	Sick
A14010407	Pet dogs	14	Male	North Island	Dec	46.78738	no	Positive	Sick
A14010446	Pet dogs	10	Male	North Island	Dec	67.05607	no	Positive	Sick
A14010464	Pet dogs	1	Female	North Island	Dec	76.0514	no	Positive	Sick
A14010508	Pet dogs	8	Female	North Island	Dec	95.56075	no	Positive	Sick
A14010510	Pet dogs	11	Male	North Island	Dec	3.329439	no	Negative	Sick
A14010522	Pet dogs	12	Female	North Island	Dec	12.90888	no	Negative	Sick
A14010590	Pet dogs	13	Female	North Island	Dec	32.59346	no	Positive	Sick
A14010621	Pet dogs	7	Female	North Island	Dec	3.796729	no	Negative	Sick
A14010629	Working dog	12	Male	North Island	Dec	10.57243	no	Negative	Sick
A14010631	Pet dogs	8	Male	North Island	Dec	27.21963	no	Positive	Sick
A14010635	Pet dogs	2	Female	North Island	Dec	0.233645	no	Negative	Sick

A14010660	Working dog	11	Female	North Island	Dec	10.9229	no	Negative	Sick
A14010758	Pet dogs	8	Male	North Island	Dec	44.04206	no	Positive	Sick
A14010786	Pet dogs	7	Male	North Island	Dec	53.56308	no	Positive	Sick
A14010808	Pet dogs	10	Male	North Island	Dec	81.95093	no	Positive	Sick
A14010846	Working dog	16	Male	North Island	Dec	34.46262	no	Positive	Sick
A14010871	Pet dogs	2	Male	North Island	Dec	90.1285	no	Positive	Sick
A14010902	Pet dogs	13	Female	North Island	Dec	12.73364	no	Negative	Sick
A14010904	Pet dogs	15	Female	North Island	Dec	3.679907	no	Negative	Sick
A14010911	Pet dogs	4	Male	North Island	Dec	64.01869	no	Positive	Sick
A14010919	Pet dogs	9	Male	North Island	Dec	0	no	Negative	Sick
A14010940	Pet dogs	1	Male	North Island	Dec	0	no	Negative	Sick
A14010941	Pet dogs	8	Female	North Island	Dec	0	no	Negative	Sick
A14011001	Pet dogs	2	Male	North Island	Dec	17.1729	no	Negative	Sick
A14011012	Pet dogs	9	Male	North Island	Dec	40.07009	no	Positive	Sick
A14011027	Working dog	5	Male	North Island	Dec	65.71262	no	Positive	Sick
A14011045	Pet dogs	6	Female	North Island	Dec	41.17991	no	Positive	Sick
A14011134	Pet dogs	13	Female	North Island	Dec	28.09579	no	Positive	Sick
A14011148	Pet dogs	13	Female	North Island	Dec	30.08178	no	Positive	Sick
A14011167	Pet dogs	3	Male	North Island	Dec	12.32477	no	Negative	Sick
A14011172	Working dog	5	Female	North Island	Dec	20.61916	no	Positive	Sick
A14011176	Pet dogs	7	Male	North Island	Dec	91.17991	yes	Positive	Sick
A14011186	Pet dogs	11	Male	North Island	Dec	75.70093	no	Positive	Sick
A14011194	Pet dogs	11	Male	North Island	Dec	39.36916	no	Positive	Sick
A14011226	Pet dogs	10	Male	North Island	Dec	24.53271	no	Positive	Sick
A14011230	Pet dogs	13	Female	North Island	Dec	41.82243	no	Positive	Sick
A14011239	Pet dogs	13	Female	North Island	Dec	23.89019	no	Positive	Sick
H14043609	Working dog	7	Female	North Island	Dec	4.147196	no	Negative	Sick

H14043623	Pet dogs	8	Female	South Island	Dec	18.92523	no	Negative	Sick
H14045221	Pet dogs	5	Male	North Island	Dec	75.93458	no	Positive	Sick
H14045425	Pet dogs	10	Male	North Island	Dec	22.31308	no	Positive	Sick
H14045585	Pet dogs	10	Female	North Island	Dec	46.02804	no	Positive	Sick
H14046049	Pet dogs	3	Male	South Island	Dec	34.92991	no	Positive	Sick
H14046442	Pet dogs	10	Female	North Island	Dec	34.69626	no	Positive	Sick
P14025234	Pet dogs	1	Female	South Island	Dec	4.147196	no	Negative	Sick
P14025309	Pet dogs	12	Female	North Island	Dec	14.6028	no	Negative	Sick
P14025333	Working dog	10	Female	North Island	Dec	0	no	Negative	Sick
P14025400	Pet dogs	10	Female	North Island	Dec	4.672897	no	Negative	Sick
P14025443	Pet dogs	5	Female	North Island	Dec	1.635514	no	Negative	Sick
P14025464	Pet dogs	10	Male	South Island	Dec	8.820093	no	Negative	Sick
P14025493	Pet dogs	3	Female	North Island	Dec	77.92056	no	Positive	Sick
P14025512	Pet dogs	5	Female	North Island	Dec	6.71729	no	Negative	Sick
P14025584	Pet dogs	9	Female	North Island	Dec	44.8014	no	Positive	Sick
P14025610	Pet dogs	12	Female	North Island	Dec	10.80607	no	Negative	Sick
P14025638	Pet dogs	5	Male	North Island	Dec	6.483645	no	Negative	Sick
P14025680	Pet dogs	11	Male	North Island	Dec	32.65187	no	Positive	Sick
P14025684	Pet dogs	5	Female	North Island	Dec	12.73364	no	Negative	Sick
P14025720	Working dog	10	Male	North Island	Dec	15.94626	no	Negative	Sick
P14025750	Pet dogs	8	Female	North Island	Dec	8.761682	no	Negative	Sick
P14025846	Pet dogs	10	Male	South Island	Dec	78.67991	no	Positive	Sick
P14025926	Working dog	8	Female	North Island	Dec	12.96729	no	Negative	Sick
P14025996	Working dog	7	Female	North Island	Dec	23.77336	no	Positive	Sick
P14026029	Pet dogs	2	Male	South Island	Dec	1.401869	no	Negative	Sick
P14026114	Pet dogs	4	Male	South Island	Dec	71.67056	no	Positive	Sick
P14026146	Working dog	12	Female	North Island	Dec	32.59346	yes	Positive	Sick

P14026188	Unknown	12	Male	North Island	Dec	4.731308	no	Negative	Sick
P14026280	Pet dogs	5	Male	North Island	Dec	42.23131	no	Positive	Sick
P14026370	Pet dogs	8	Male	South Island	Dec	12.67523	no	Negative	Sick
P14026372	Pet dogs	10	Female	South Island	Dec	4.030374	no	Negative	Sick
P14026414	Pet dogs	8	Male	South Island	Dec	84.46262	no	Positive	Sick
P14026494	Working dog	10	Male	North Island	Dec	45.56075	no	Positive	Sick
P14026546	Pet dogs	3	Male	South Island	Dec	25.58411	no	Positive	Sick
P14026569	Working dog	3	Male	North Island	Dec	22.66355	no	Positive	Sick
P14026570	Pet dogs	11	Female	North Island	Dec	66.1215	no	Positive	Sick
P14026601	Pet dogs	8	Male	North Island	Dec	10.63084	yes	Negative	Sick
P14026660	Pet dogs	10	Male	North Island	Dec	43.34112	no	Positive	Sick
P14026672	Pet dogs	9	Male	North Island	Dec	34.92991	no	Positive	Sick
P14026695	Pet dogs	4	Female	North Island	Dec	17.40654	no	Negative	Sick
P14026705	Pet dogs	9	Female	South Island	Dec	14.19393	no	Negative	Sick
P14026722	Pet dogs	13	Female	North Island	Dec	36.03972	no	Positive	Sick
P14026748	Pet dogs	11	Male	South Island	Dec	62.03271	no	Positive	Sick
P14026825	Pet dogs	7	Female	North Island	Dec	43.63318	no	Positive	Sick
P14026865	Pet dogs	5	Female	North Island	Dec	11.55462	no	Negative	Sick
P14027000	Pet dogs	11	Female	North Island	Dec	43.85504	no	Positive	Sick
P14027078	Pet dogs	7	Male	South Island	Dec	45.16807	no	Positive	Sick
P14027101	Pet dogs	13	Male	North Island	Dec	12.23739	no	Negative	Sick