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Pathogens associated with acute infectious canine tracheobronchitis in New Zealand

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ABSTRACT

Infectious canine tracheobronchitis (ICT) or canine infectious respiratory disease, commonly known as kennel cough, is an acute, highly contagious respiratory disease that affects the larynx, trachea, bronchi, and occasionally the parenchyma of the lower respiratory tract. Several pathogens have been implicated in ICT including viruses, bacteria and mycoplamsa. Little is known about the prevalence of canine respiratory pathogens in New Zealand. Hence, the aim of this study was to identify potential respiratory pathogens from dogs that are affected by ICT in New Zealand, and compare agents found in diseased dogs to those found in healthy dogs. In house (IH) qPCR assays were developed for the detection of canine adenovirus type 2 (CAdV-2), canine herpesvirus (CHV) and canine parainfluenza (CPIV).

A total of 96 dogs were sampled, including 47 healthy and 49 diseased dogs, which comprised three different groups of dogs: greyhounds, pet dogs, and working farm dogs. A questionnaire was included for each dog sampled. The samples collected were then subjected to the following tests: virus isolation, haemagglutination assay for CPIV, IH qPCR for CAdV-2 and CHV, as well as IDEXX RealPCR respiratory disease panel, and bovine respiratory coronavirus ELISA to detect antibody to canine respiratory coronavirus (CRCoV).

Based on IDEXX qPCR, CPIV (7.3%), *Bordetella bronchiseptica* (7.3%) and *Mycoplasma cynos* (17.0%) were the most common agents detected in samples from diseased dogs, whereas CAdV-2 (10.6%) was the most common pathogen amongst healthy dogs. Based on IH qPCR, CAdV-2 infection was very common among all dogs sampled, with 34/47 (72%) positive diseased dogs and 37/47 (78.6%) positive healthy dogs.

A total of 47/92 (51%) of dogs were positive for CRCoV antibodies, including 32/46 (69.6%) of diseased dogs and 14/46 (30.4%) of healthy dogs. In addition, acute serum samples from diseased dogs were significantly more likely to be positive for CRCoV antibodies compared to sera from healthy dogs (RR 5.22, CI 1.972, 14.115, p=0.0003).

The results of this study suggest that CRCoV, *M.cynos* and potentially CPIV may have a role in ICT in New Zealand, however further investigation is required to support these findings. In addition, if one excluded dogs positive for CAdV-2 (as there was no difference in levels of detection of this virus between healthy and diseased dogs), then only 13/47 (27.6%) of diseased dogs were positive for at least one agent via IDEXX and IH qPCR. This suggests that

other aetiological agents, not examined in this study, may have contributed to respiratory disease in sampled dogs. Techniques such as next generation sequencing may help to identify these pathogens.

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Abbreviations

bp: base pair

CAdV-2: Canine adenovirus type 2

CCoV: canine enteric coronavirus

CHV: canine herpesvirus

CIV: canine influenza

CMI: cell mediated

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CPE: cytopathic effect

CPIV: canine parainfluenza

CRCoV: canine respiratory coronavirus

FBS: fetal bovine serum

HA: haemagglutination assay

HI: haemagglutintion inhibition

HN: haemagglutinin/neuraminidase protein

ICT: Infectious canine tracheobronchitis

IFN: Interferon

Mda-5: melanoma differentiation-associated gene-5

MDCK: Madin Darby canine kidney

MHC: major histocompatibility complex

MRV: mammalian reovirus

NTC: non- template control

PCR: polymerase chain reaction

POI: Percentage of inhibition

qPCR: quantitative PCR

RBC: red blood cells

SH: small hydraulic protein

STAT: Signal transducer and activator of transcription

SV5: simian virus-5

TNF: tumour necrosis factor

VN: virus neutralising

VNT: Virus neutralisation test

Chapter 1: Pathogens associated with the kennel cough syndrome.

1.1: Introduction

Kennel cough, otherwise known as infectious canine tracheobronchitis (ICT) is an acute, highly contagious respiratory disease that affects the larynx, trachea, and bronchi (Buonavoglia and Martella 2007). The disease often occurs within groups of kennelled dogs such as greyhounds and rehoming centres. The viral pathogens that are most commonly implicated in ICT include canine parainfluenza (CPIV) and canine adenovirus type 2 (CAV-2) (Erles *et al.* 2004). It is common to vaccinate for both CAV-2 and CPIV worldwide, including New Zealand. Canine herpesvirus (CHV) has an uncertain role in ICT (Decaro *et al.* 2008), however it has been isolated from the upper respiratory tract both from experimentally and naturally infected dogs with respiratory disease (Karpas *et al.* 1968). Canine respiratory coronavirus (CRCoV) and canine influenza (CIV) have only been identified in outbreaks of ICT overseas in the last 10 years (Erles *et al.* 2007; Payungporn *et al.* 2008). The role of mammalian reovirus (MRV) in ICT is currently unknown, since limited studies have investigated its role in ICT (Buonavoglia and Martella 2007). Nearly all of these viruses individually cause either asymptomatic or mild infections, with the latter being characterised by a dry, hacking cough (Buonavoglia & Martella, 2007). However, in immune compromised or young dogs, disease may be more severe, and multiple pathogens may be involved. It is not possible to distinguish different types of viral or bacterial causes based soley on clinical signs of ICT. Hence, supportive therapy includes cough suppressants, mucolytics, bronchodilators and corticosteroids to decrease severity of clinical signs. (Thrusfield *et al.* 1991).

Although this review will focus predominately on viral causes of ICT, several bacterial agents have also been associated with ICT. These include: *Bordetella bronchiseptica*, *Streptococcus equi* subsp. *zooepidemicus* and *Mycoplasma cynos. Bordetella bronchiseptica* is considered one of the most important pathogens in ICT (Keil and Fenwick 1998), however it has been isolated from both healthy and diseased dogs. *Streptococcus equi* subsp. *zooepidemicus* has been detected in samples from dogs with haemorrhagic pneumonia and other severe respiratory clinical signs (Posuwan and Payungporn 2011). *Mycoplasma cynos* is considered to be a part of the normal flora in the upper respiratory tract of dogs. Thus, although it has

been commonly isolated from the lower respiratory tract in association with ICT, its role in the disease process is undetermined (Rosendal 1972).

1.2: Canine Parainfluenza (CPIV)

1.2.1: General features

Canine parainfluenza is classified within the genus *Rubulavirus*, in the family *Paramyxoviridae* (Chatziandreou *et al.* 2004). Canine parainfluenza is closely related to Simian virus-5 (SV5) with 98.5% nucleotide sequence homology (Buonavoglia & Martella, 2007). The structure of CPIV includes a lipid envelope which is host derived, and single stranded, negative sense, RNA which is non-segmented. The genome encodes 6 genes in invariant order, as well as a unique 7th gene (small hydraulic (SH) protein involved in apoptosis). The SH protein is not essential for virus viability in cell culture (He *et al.* 1998), however it has been shown to prevent apoptosis, and parainfluenza with mutant versions of SH proteins showed accelerated CPE in MDCK cells (Ellis and Krakowka 2012). The haemagglutinin/neuraminidase (HN) protein can bind to many RBC from different species, including canine, cat, porcine, rat, sheep and human. The virus replicates in many different cell types *in vitro*, including canine, feline, bovine, pig, simian, mink and human cells (Ellis and Krakowka 2012). Two phenotypically different CPIV, designated CPI+ and CPI-, have been isolated from dogs. The CPI+ viruses show pronounced cytopathic effect (CPE) in Vero cells, with plaque and syncytial formation, and intracytoplasmic inclusion bodies (Crandell *et al.* 1968). The CPI- viruses show little CPE and decreased ability to haemadsorb RBC, as well as reduced HN and fusion (F) protein synthesis. The *in vitro* phenotypes were linked to *in vivo* characteristics, with a reduction in virulence observed for CPIV (-) viruses as compared with CPIV (+) viruses following experimental infection of ferrets (Baumgartner *et al.* 1991).

1.2.2: Replication

Canine parainfluenza attaches to host cells via the HN protein through binding to sialic acid residues. This interaction is favoured at a specific neutral extracellular pH and halide concentration (Ellis and Krakowka 2012). After binding, a conformational change in the F protein is likely to occur. This allows for fusion between the viral envelope and the host cell, as well as fusion between infected cells and neighbouring unaffected cells (Baumgartner *et al.* 1991). Both the HN and F proteins are required, since there is no significant syncytium

formation unless HN and F proteins are co expressed (Chancock *et al.* 2001). Once the viral nucleocapsid has penetrated the cell membrane, the virus replicates in the cytoplasm of the cell with the formation of intracytoplasmic inclusion bodies (Ellis and Krakowka 2012). Replication is aided by the L, N and P proteins. The L protein is a major polymerase subunit responsible for various catalytic steps in RNA synthesis (Chancock *et al.* 2001). Phosphorylation of the P protein at position 286 has been found to increase viral RNA synthesis and hence promote viral growth (Sun *et al.* 2011). After viral proteins accumulate, a complete positive sense anti genome strand is made, which acts as a template for synthesis of the negative sense genomic RNA. The N protein also mediates interactions with the M protein to ensure incorporation of viral RNA into virions (Schmitt *et al.* 2010). The M protein plays an important role in maturation and assembly of the virions, which are then released from the cell via budding (Maclachlan and Dubovi 2010).

The V protein has several effects which aide the spread of virions from cell to cell. Firstly, the V protein inhibits production of IFN, which typically acts to limit viral replication and spread by activating IFN stimulated genes (Gale and Sen 2009). To achieve this, the V protein can bind to and block melanoma differentiation-associated gene-5 (mda-5) function. This in turn inhibits activation of NF-κβ and IRF-3, which are required to stimulate IFN-β signalling (Childs *et al.* 2007). Hence, the V protein helps prevent apoptosis of infected cells (Poole et al., 2002). Secondly, V proteins can also target STAT proteins for degradation, which in turn prevents the activation of interferon (Andrejeva *et al.* 2002).

1.2.3: Pathogenesis and Clinical signs

In vivo, CPIV infects mainly cells of the upper respiratory tract, but has also been detected in liver, kidney, intestinal tissues and cerebrospinal fluid (Baumgartner *et al.* 1991). The incubation period for CPIV is typically between 7-9 days in experimentally infected dogs (Wagener *et al.* 1984). Some suggested the incubation period may be shorter (mean of 4.5 days), however this was based on a small sample size (*n*=13) (Thrusfield *et al.* 1991). Clinical signs of CPIV infection include a dry hacking cough which is typical of ICT, and this generally lasts between 2 to 6 days. Other signs include fever, nasal discharge, pharyngitis, tonsillitis, lethargy, inappetance and occasionally pneumonia when secondary bacterial infections are present (Buonavoglia and Martella 2007).

1.2.4: Immune response

Haemagglutination inhibition (HI) and virus neutralising (VN) antibodies are detectable 7 days after infection, peak around day 21, and then persist at this level for at least 42 days (Binn *et al.* 1968). Antibodies are raised against both the F and HN protein, although inoculation of vaccinia-F and HN recombinant viruses into hamsters showed that the level of neutralising antibodies raised against the vaccinia-F virus was far greater than the vaccinia–HN virus. However, protection against SV5 was equal or poorer for animals inoculated with the vaccinia-F virus (Paterson *et al.* 1987). This suggested that HN may have a role in stimulating cell mediated immunity, although, currently, there is no evidence to support this theory. Despite the lack of studies into the CMI response to CPIV infection, it has been theorised that CD8 lymphocytes may confer short lived, incomplete protection against reinfection (Ellis and Krakowka 2012). In one study, depletion of CD8+ T cells from the immune splenocytes reduced the mice's ability to clear SV5 following experimental infection, whereas depletion of CD4+ cells had little effect on the clearance of the virus. Hence, this demonstrated that clearance of the virus was mediated by CD8+ cells (Young *et al.* 1990). In addition to this, the presence of neutralising antibodies had no effect on the rate of virus clearance (Young *et al.* 1990). Hence, it can be hypothesised that neutralising antibodies may help to limit the spread of CPIV, whereas CD8+ (and to some extent CD4+) may aide clearance of the virus from the respiratory tract.

1.2.5: Diagnosis

Diagnosis of CPIV includes virus isolation from the nasopharynx, usually in canine kidney cells (Erles *et al.* 2004). Serological diagnosis is often performed using either the virus neutralisation test (VNT) or the HI (Binn *et al.* 1967; Crandell *et al.* 1968; Rosenberg *et al.* 1971). Both of these methods can be used to test acute and convalescent sera to show a fourfold increase in the virus specific antibody titre in order to detect recent infection (Crandell *et al.* 1968). Enzyme linked immunoassay (ELISA) has been used by some authors (for example Erles, Dubovi, Brooks, & Brownlie, 2004). It is more rapid than VNT or HIA but it is also more expensive. Reverse transcription (RT) PCR to detect genomic RNA has also been developed (Erles *et al.* 2004). With the exception of the IDEXX canine respiratory disese panel, no real time quantitative PCR (qPCR) has been optimised specifically for CPIV.

However, conventional primers for CPIV (targetted at the nucleoprotein region) have been published previously (Kraus *et al.* 2008).

1.2.6: Vaccination

Because of the limited treatment options, it is best to try to prevent CPIV disease by vaccination. There are many different types of vaccines available. For CPIV, the core vaccination is an injectable vaccine containing CPIV, CAV-2, Canine distemper (CDV) and canine parvovirus (CPV) (Vanguard plus 5) (Mouzin *et al.* 2004). For ICT specifically, three vaccines are commonly used: Nobivac KC, Bronchi-shield and Canigen KC. Both Nobivac and Bronchi-shield are intranasal vaccines against *B.Bronchiseptica* and CPIV (MSDAnimalHealth 2017; Vetmedica 2018), however Bronchi-Shield also includes CAdV-2 antigen. Canigen KC is a killed adjuvanted, injectable vaccine against *B. Bronchisepctica* and CPIV (Virbac 2017). These vaccines alone rarely provide protection against infection, although they help to reduce the severity of the disease. For Vanguard plus 5, the serological response to CPIV can still be present for up to 48 months after vaccination (Mouzin *et al.* 2004)). Hence, although annual boosters are recommended, serological response to against CPIV after vaccination may last for much longer. It is hypothesised that the modified live intranasal vaccine may be more effective than injectable vaccines, since it stimulates local immunity, providing protection against infection at the portal of entry (Jacobs *et al.* 2005).

1.2.7: Epidemiology

CPIV is considered one of the most important viral pathogens in ICT, since it has been commonly isolated from ICT-affected dogs in various countries. It was first isolated from clinically infected dogs in 1967 in America (Binn *et al.* 1967), and was subsequently frequently associated with canine respiratory disease in the late 60's and 70's (Crandell *et al.* 1968). Despite the introduction of a vaccine in 1976 (Emery *et al.* 1976), CPIV is still frequently identified in both healthy and diseased dogs. In the UK, 19.4% of tracheal and 10.4% of lung samples from dogs affected by respiratory disease were positive for CPIV. CAV-2 and CRCoV were also identified but not as frequently as CPIV (Erles *et al.* 2004). In Canada, a serological study was conducted at an urban humane shelter, where dogs were divided into two populations: urban and rural. There didn't appear to be a significant relationship between the titre of CPIV antibodies (low or high) and the development of ICT, despite 41/101 dogs with low antibody against CPIV developing ICT. There also appeared to

be no difference in the level of antibodies between rural or urban dogs. This indicated that either CPIV was not involved in ICT at this shelter, or those antibodies against CPIV where not protective against CPIV infection and subsequent disease. (Ellis *et al.* 2011). Recently, there was a report of an outbreak of CPIV at a clinic in Canada. Five dogs were affected, and 4/5 of these dogs had been previously vaccinated for CPIV. The four dogs that had been vaccinated showed at least fourfold increase in titres for CPIV between acute and convalescent samples. Most dogs showed relatively mild signs of disease and made full recoveries, and also tested negative CIV PCR and no bacterial pathogens were identified (Weese and Stull 2013). Hence, this study suggests that vaccination with CPIV may not be protective against mild clinical signs of ICT following CPIV infection.

CPIV has also been identified in Asia, for instance, in Japan and Thailand. CPIV nucleic acid was detected with the highest frequency in diseased Japanese dogs (7.4%) (Mochizuki *et al.* 2008). Viruses tested for included CAV-2, CRCoV, and CIV. In Thailand, the prevalence of antibodies in both healthy and diseased dogs was examined. CPIV had one of the lowest prevalence's in healthy dogs (0.98%) but had the highest prevalence in diseased dogs (11.93%) compared to other viruses examined, which included CAV, CIV and canine distemper virus (CDV) (Posuwan and Payungporn 2011).

Transmission of CPIV occurs via the respiratory route. Little is known about the role of fomites in the transmission of CPIV, but it is likely that several host factors (such as stress, malnutrition and the presence of concurrent infections) are important in the susceptibility of a dog to CPIV infection (Ellis and Krakowka 2012).

1.2.8: CPIV in New Zealand

CPIV has not been isolated from dogs in New Zealand, and no seroprevalence data is currently available. However, CPIV has been isolated in Australia, and implicated in several respiratory disease outbreaks (McGavin *et al.* 1989). It is also unclear how CPIV is maintained in the environment in New Zealand. CPIV is known circulate amongst the wild canid population, including foxes and coyotes (Buonavoglia and Martella 2007). However, neither of these species are present in New Zealand. Hence, it is likely that the virus (if present in New Zealand) is maintained within the domestic dog population, although it is possible other species such as rats may also carry CPIV. CPIV-like viruses have been

frequently isolated from simian species and humans, but there is currently no data to suggest that CPIV can be transmitted from humans to dogs or vice versa.

1.3: Canine Adenovirus type 2 (CAV-2)

1.3.1: General Features

Canine adenovirus type 2 is classified in the genus *Mastadenovirus*, within the family *Adenoviridae* (King *et al.* 2013c). Canine adenovirus type 2 is non-enveloped with icosahedral symmetry, and approximately 70-90 mm in diameter. The adenovirus genome is a double stranded DNA, approximately 26-45 kbp in size. CAV-2 has been found to be genetically different to CAV-1 (75% nucleotide identity), although both viruses cross react in serological tests (Buonavoglia and Martella 2007). CAV-2 is relatively stable in the environment, but is easily inactivated by common disinfectants (Appel 1987).

1.3.2: Replication

There have been very few studies focusing on CAV-2 replication. However, several studies have described the replication of human adenoviruses. The capsid of adenoviruses contains hexon proteins and a pentose base (Russell 2009). In the early stages of CAV-2 infection, the fibre knob attaches to the coxsackie adenovirus receptor (CAR). In human adenovirus infections, conserved regions in the penton base then bind to integrins on the host cell, triggering endocytosis of the viral particle. However, unlike human adenoviruses, the CAV-2 capsid does not appear to contain any integrin interacting motif, yet, by a currently unknown mechanism, is still able to be transported to the nucleus for transcription and translation (Schoehn *et al.* 2008).

In the nucleus, transcription of the early regions E1, E2, E3 and E4 is followed by two intermediate units (IX and Iva2) and five late units (L1-L5) (Maclachlan and Dubovi 2010). The E1A region is essential for the induction of cell cycle progression, avoidance of host immune defences, and the synthesis of viral proteins (Maclachlan and Dubovi 2010). The E2 genes encode three proteins that assist in the initiation and progression of DNA replication within the nucleus (Liu *et al.* 2003). The E3 region of the genome is not essential for replication *in vitro*. *In vivo*, it modulates the host response to CAV-2, specifically inhibition of the major histocompatibility complex class 1 (MHC-1) and tumour necrosis factor (TNF)-induced apoptosis (Benkö and Harrach 2003; Maclachlan and Dubovi 2010). Intermediate

genes are also thought to assist in the switch from the early to late phase (Seth 1999). In the late phase, host cell DNA synthesis is inhibited and synthesis of proteins, which are derived from the major late transcription unit (MLTU), occurs instead. These proteins include capsids proteins as well as non-structural proteins involved in assembly of the virion (Seth 1999). Once assembly of the core proteins has occurred in the nucleus, a cis acting domain containing A repeats acts as a binding site for factors that mediate viral DNA packaging (Ostapchuk and Hearing 2003). The virus is then released via cell lysis (Maclachlan and Dubovi 2010).

1.3.3: Pathogenesis

CAV-2 predominately targets the respiratory tract epithelium, specifically the nasal mucosa, pharynx, tonsillar crypts, mucous cells in trachea, bronchi in peribronchial glands and type 2 alveolar epithelial cells (Appel 1987). Adenoviruses typically cause severe damage to the host cell chromatin, hence nuclei appear abnormal and inclusion bodies can be observed in infected cells (Maclachlan and Dubovi 2010). Typically, infected cells are targeted by cytotoxic T lymphocytes (CTL) which recognise foreign antigen presented on class 1 major histocompatibility complexes (MHC). However, in human adenoviruses, the E3 protein 19K is able to inhibit expression of MHC class 1 by sequestering MHC into the endoplasmic reticulum (ER), thereby inhibiting recognition of infected cells by CTL (Mahr and Gooding 1999). The E3 region also encodes 14.7K protein that inhibits tumour necrosis factor (TNF) mediated apoptosis, as well as a heterodimeric complex of proteins 14.5K and 10.4K (RID complex) that protects cells from TNF and Fas mediated apoptosis (Sparer *et al.* 1996). However, the E3 region of CAdV-1 has little homology to that of human adenoviruses (Morrison *et al.* 1997). Furthermore, an extra 500 bp sequence was identified in the E3 region of CAdV-2 when compared to CAdV-1. Hence, although no studies have compared the CAV-2 and human adenovirus E3 regions, it can be hypothesised that the CAdV-2 E3 region may show little homology with that of human adenovirus E3 region. Therefore, the E3 region of CAdV-2 may interact differently with host defences such as MHC and TNF, and hence may not avoid immune detection in the same way as human adenovirus do.

1.3.4: Clinical signs

CAV-2 predominately causes mild or subclinical infections. Typically, in experimental infection, the peak of replication is reached after 3-6 days post infection, after which time

viral load declines, and usually virus cannot be isolated 9 days post infection (Appel 1987). Experimental infection with CAV-2 resulted in asymptomatic infections for the majority of dogs (Ditchfield *et al.* 1962). However, for dogs that received a large dose of CAV-2 via the parental route, clinical signs were similar to those observed in natural infection (Appel 1987). In naturally occurring cases, CAdV-2 infection is complicated by other disease agents (both viral and bacterial), hence typical clinical signs of acute ICT are observed (Decaro *et al.* 2008). Clinical signs include rhinitis, pharyngitis, tonsillitis, and tracheobronchitis. Interstitial lesions have also been observed, as well as pneumonia and necrotising and proliferative bronchitis in more severe cases (Appel *et al.* 1973).

1.3.5: Immune responses

Antibodies to CAV-2 antigens have been demonstrated by various serological assays. Protection against disease appears to correlate with the level of neutralising antibody (Appel *et al.* 1975). Typically, dogs show a strong antibody response to CAV-2 antigens, and this is shown by the long-lived protection against CAV-2 disease after vaccination (Mouzin *et al.* 2004). The role of cell mediated immunity in protection against CAV-2 has not been investigated, however it is possible, despite low homology, that the E3 region of CAdV-2 plays an important role in suppressing cell mediated immunity.

1.3.6: Diagnosis

CAV-2 can be isolated from nasal or throat swabs in canine kidney cells. Primary cells appear to be more susceptible to infection than established cell lines (Buonavoglia and Martella 2007). Cytopathic effect (CPE) can be observed 2-5 days post infection (dpi), and is characterised by rounded, enlarged cells resembling grape like clusters (Ditchfield *et al.* 1962). Virus neutralisation tests (VNT) and Haemaglutination inhibition (HI) are the most common serological methods for detecting CAV-2 antibody, shown by various studies (Appel *et al.* 1973; Tham *et al.* 1998; Erles *et al.* 2004). Traditional PCR has been used to detect CAV-2 (Posuwan and Payungporn 2011). No qPCR has been reported for diagnosis of CAV-2 infection (Segura *et al.* 2010).

1.3.7: Vaccination

Vanguard plus 5, Nobivac KC, Bronchi Shield and Canigen KC have all been shown to induce induce good serological responses, which are likely to be protective against CAV-2 induced

respiratory disease (Mouzin *et al.* 2004). In one study, 99% of dogs responded to Vanguard plus 5 5-7 days after vaccination with greater than four fold increases in CAdV-2 antibody titres compared to the non-vaccinated group. Furthermore, nearly all dogs had a greater than 2 fold level of CAdV-2 antibody levels of antibodies after 48 months compared to the non-vaccinated group. (Mouzin *et al.* 2004). Modified live vaccines such as Bronchi shield have proved to be highly effective in reducing the circulation of CAV-2 within the canine population (Buonavoglia and Martella 2007). However, in one study at a rehoming shelter, no significant difference in clinical signs of acute ICT (coughing, sneezing, nasal discharge and ocular discharge) was observed for up to 30 days post vaccination between the vaccinated group and the control (placebo vaccine) group. This could have been due to the low sample size, or the possibility that pathogens other than CAdV-2 that were causing clinical signs of ICT in those dogs (Edinboro *et al.* 2004).

1.3.8: Epidemiology

Overseas, CAV-2 is commonly isolated from both diseased and healthy dogs. In Thailand, 9.17% of diseased dogs and 2.94% of healthy dogs were positive for CAdV-2 via PCR (Posuwan and Payungporn 2011). In Japan, CAdV-2 was isolated in MDCK cells from 2.9% (2/68) of household dogs with clinical signs of respiratory disease (Mochizuki *et al.* 2008). Vaccination status of the latter dogs was unknown; however CAdV-2 is included in the core vaccine in Japan (Day *et al.* 2015). In contrast to this study, a study carried out in the UK found no evidence of CAdV-2 (Erles *et al.* 2004), but this could be due to differences in geographical region, timing of sampling, or level of vaccination. In a South African based study, CAdV-2 seroprevalence among unvaccinated dogs brought into clinics for reasons other than respiratory disease was 62% (Wright *et al.* 2013). CAdV-2 has been isolated in New Zealand previously (Tham *et al.* 1998). However, there is currently no data on the prevalence of CAV-2 infection in New Zealand.

CAV-2 appears to be able to infect a wide range of mammalian species via the oronasal route, including canids and marine mammals (in Alaska and Canada) (Buonavoglia and Martella 2007). In Australia, the overall seroprevalence of CAdV-2 in European red foxes was 23.2%, but this varied with location and a seasonal pattern was observed (typically higher seroprevalence in winter) (Robinson *et al.* 2005). In New Zealand, only domestic canines are known to carry CAdV-2.

1.4: Canine Herpesvirus (CHV)

1.4.1: General Features

CHV is classified in the subfamily *Alphaherpesviridae*, which is in the family *Herpesviridae* (King *et al.* 2013b). The virions are enveloped with a diameter of 180-200 nm. The genome consists of a linear, double stranded DNA. The CHV genome has not been completely sequenced, although a few genes have been identified (Buonavoglia and Martella 2007). The virus is sensitive to lipid solvents, and is readily inactivated at temperatures above 40 °C (Appel 1987). Infection with CHV is linked not only with ICT in older dogs, but also with reproductive problems (abortions), and ocular disease. The virus causes fatal necrotising and haemorrhagic systemic disease in young puppies less than 2 weeks of age. The virus is genetically related to FHV-1 (Rota and Maes 1990), phocis herpesvirus and equid herpesvirus type 1 and 4 (Decaro *et al.* 2008). All CHV isolates from dogs appear to be antigenically similar (Poste *et al.* 1972).

1.4.2: Pathogenesis, clinical signs, and immunopathology

Pathogenesis of CHV infection depends on the age of the dog at the time of infection (Decaro *et al.* 2008). Following oronasal exposure, CHV replicates locally in the upper respiratory tract in cells of the nasal mucosa, pharynx, and tonsils. *In vitro*, CHV's attachment to canine kidney cells is mediated by heparan sulphate (Buonavoglia and Martella 2007). During the replication cycle, over 70 virus encoded proteins are made. Viral DNA is replicated in the nucleus and newly synthesised DNA is pulled into immature capsids. These capsids then acquire an envelope by budding through the nuclear membrane. Mature virions accumulate within vacuoles in the cytoplasm, and are released by exocytosis or cytolysis (Maclachlan and Dubovi 2010). After both symptomatic and asymptomatic infections, dogs remain latently infected and virus may be excreted via the oronasal or genital route at unpredictable intervals over periods of several months, or years. Reactivation of latent virus may be provoked by stress, for example, introduction to new kennels. Latent virus persists commonly in the trigeminal ganglia, but also in the lumbo-sacral ganglia, tonsils, and parotid salivary gland (Burr *et al.* 1996; Miyoshi *et al.* 1999)

Most dogs aged over 3 weeks that are infected with CHV remain asymptomatic. Dogs that do show clinical signs often have rhinitis and occasional sneezing. Spontaneous coughing

was observed in experimentally infected dogs, and histological findings include intranuclear inclusion bodies in the trachea and bronchial epithelium (Karpas *et al.* 1968).

Canine herpesvirus is poorly immunogenic. For example, in older dogs, neutralising antibody can be detected within 4-5 weeks of primary infection, but antibody levels decline thereafter (Appel 1987). It is unlikely that antibodies raised against CHV antigen after infection can prevent recrudescence of CHV later in life, given the short amount of time neutralising antibodies are present after primary infection. Maternal antibody has been shown to protect from disease but not from infection (Appel 1987).

1.4.3: Diagnosis, Treatment and Vaccination

Canine herpesvirus is host specific and can be isolated only in canine cells. Growth in cell culture is optimal at around 34-35°C. CHV produces CPE in cell culture, which is characterised by clustered cells with syncytial formation (Xuan *et al.* 1992). For serological diagnosis, VNT, HI and ELISA were used by Xuan et al., 1992, and a specialised plaque reduction assay using mink foetal cells was successfully used by Reading & Field in 1999. Virus specific PCR has been used in many studies to identify latent CHV infections (Miyoshi *et al.* 1999; Erles *et al.* 2004). Recently, a qPCR method for detection of CHV has been developed using a taq-man probe, which may be a better alternative to traditional PCR in future studies (Decaro *et al.* 2010).

Treatment for CHV associated disease is supportive and depends on clinical signs present in affected dogs. Since the optimal temperature for CHV replication is below normal body temperature, raising the core body temperature to 38.5-39.5^{0}C could help limit replication of CHV to the respiratory tract (Appel 1987). This is especially important in puppies since they do not have fully developed thermoregulation (Carmichael and Barnes 1969). Hence, CHV can cause systemic (as opposed to respiratory) disease in hypothermic puppies.

An inactivated subunit vaccine against CHV is available in Europe. This vaccine is used to administer to pregnant bitches in the initial stages of pregnancy, and then again in the sixth to seventh week of gestation (Decaro *et al.* 2008). This vaccine aims to prevent severe disease in puppies but may not prevent CHV's involvement in ICT in older puppies/ adult dogs. A temperature resistant mutant of CHV has been suggested as a modified live vaccine, but is not commercially available (Carmichael and Medic 1978).

1.4.4: Epidemiology

Although seroprevalence of CHV is relatively high among various canine populations, its association with ICT is still questionable. Reported CHV seroprevalences ranged from 3.1% among pet dogs and 27.9% for kennelled dogs in Italy, to 93% for kennelled dogs in England (Reading and Field 1999; Decaro *et al.* 2008). CHV has been isolated in New Zealand, but its seroprevalence is unknown (Horner 1977). Furthermore, it has been 35 years since its isolation, and hence it is possible that CHV is no longer present in New Zealand. Overseas, transmission of CHV usually occurs by direct contact with oronasal or genital secretions (Decaro *et al.* 2008). Since CHV is fragile in the environment, outbreaks can be controlled by isolation of infected dogs and disinfection of the surrounding environment.

1.5: Canine Respiratory Coronavirus (CRCoV)

1.5.1: General Features

Canine respiratory coronavirus is a group 2 coronavirus classified in the family *Coronaviridae* (Erles *et al.* 2003). In general, coronaviruses are enveloped and 80-160 nm in diameter, with a linear positive stranded RNA genome. The structural proteins located within the viral envelope are the spike protein, membrane protein, envelope protein, and the haemagglutinin esterase (HE) protein. These proteins are only present in members of group 2 of the coronaviruses (Buonavoglia and Martella 2007). CRCoV is an emerging pathogen that has been isolated from the respiratory tract in dogs with ICT (Erles *et al.* 2003). In contrast, the group 1 canine enteric coronavirus (CCoV) is a common cause of gastroenteritis in dogs, but is rarely isolated from the respiratory tract (Buonavoglia and Martella 2007). Sequence analysis has shown CRCoV to have only 68.53% nucleotide identity with the polymerase gene of CCoV. However, comparison of a cDNA polymerase sequence revealed that CRCoV has 98.8% and 98.4% similarity with bovine coronavirus (BCoV) and human coronavirus (HCV), respectively (Erles *et al.* 2003)

1.5.2: Pathogenesis, Clinical signs and Immunopathology

To date, there have been no studies on the specific pathogenesis of CRCoV. Given the sequence similarity between CRCoV and BoCV, pathogenesis of CRCoV infection in dogs is likely to be similar to that of BoCV in cattle. The latter comprises one of the several pathogens associated with a shipping fever syndrome, which is a respiratory disease that

typically occurs in stressed, overcrowded cattle (Clark 1993). Bovine coronavirus has been detected from the lung, trachea, and nasal turbinates of cattle with mild upper respiratory disease (McNulty *et al.* 1984).

CRCoV can infect columnar epithelial cells in the bronchi (Ellis *et al.* 2005). It is likely the virus infects these cells either by direct fusion of the virus envelope, or by endocytosis of the virus into the cell. CRCoV RNA has also been detected in the spleen, mesenteric lymph nodes and intestines of dogs, but no clinical signs associated with replication in these tissues have been observed (Decaro *et al.* 2007).

CRCoV replication takes place inside the cytoplasm, followed by virus assembly. Whole virions bud from the membranes of the rough endoplasmic reticulum (RER) and Golgi apparatus into the lumen of the cell. Virions are likely to be released by normal cell secretory mechanisms, or by lysis of the cell (Clark 1993). CRCoV is often detected in samples at the early stages of infection (Erles *et al.* 2004), and is rarely associated with moderate or severe clinical signs of disease. Hence, it is possible that replication of CRCoV may damage the respiratory epithelium, thereby facilitating entry of other viral or bacterial agents, which in turn cause more severe clinical signs (Erles *et al.* 2003).

Canine respiratory coronavirus has been detected in dogs with respiratory disease and in apparently healthy dogs (Kaneshima *et al.* 2006). When CRCoV was isolated from diseased dogs, most had the typical cough associated with ICT, and occasionally sneezing and nasal discharge. In a retrospective study of dogs euthanized due to respiratory disease, CRCoV was detected in fixed tissue samples from two out of 126 cases of bronchitis and bronchiolitis (Ellis *et al.* 2005).

The immune response to CRCoV has not been extensively studied. Given the similarity of the spike gene between BoCV and CRCoV, most studies have successfully used BoCV antigen to detect CRCoV antibodies (Decaro *et al.* 2007).

1.5.3: Diagnosis, Treatment, and Vaccination

Canine respiratory coronavirus has thus far shown no growth in cell culture in multiple cell lines (Erles *et al.* 2007). Previous studies (e.g. Erles, et al., 2003) were unsuccessful at isolating CRCoV in canine adult lung fibroblasts and MDCK cells. Although no CPE was

observed, human colorectal adenocarcinoma cell lines (HRT-18) have been successfully used to isolate CRCoV. The growth of the virus was detected using CRCoV-specific reverse trancriptase (RT)-PCR (Priestnall *et al.* 2006).

A virus neutralisation test can be performed by using bovine antigen to determine a titre of antibodies to CRCoV (Kaneshima *et al.* 2006). A HI test has been used by others, however, the sensitivity of HI test is low in comparison to ELISA. ELISA has been used on many occasions to detect CRCoV antibody, again often using BoCV spike protein (Priestnall *et al.* 2006). A virus-specific conventional (Erles *et al.* 2003) and real-time (Mitchell *et al.* 2009) RT-PCR assays have also been developed.

There is currently no vaccine for CRCoV. Although there is a vaccine available for CCoV, CRCoV antibodies cannot be detected using CCoV antigen (Decaro *et al.* 2007), which is not surprising given their dissimilarity in the spike protein. Hence, the CCoV vaccine is very unlikely to offer protection against CRCoV (Ellis *et al.* 2005).

1.5.4: Epidemiology

In New Zealand, the seroprevalence of CRCoV among 251 healthy and diseased dogs was 29% (Knesl *et al.* 2009). Of the 251 dogs, 210 had a medical history, and of the 210, nine had a history of respiratory disease, two of which (22%) were positive for CRCoV antibodies. In comparison, 67/210 (33%) dogs seropositive for CRCoV had no history of respiratory disease. Prevalence of CRCoV antibodies was very low (7%) in young dogs, but tended to increase with age, up to 31% positive in the 11- to 18-year-old age group. This trend was also reported by others (Priestnall *et al.* 2006). Overseas, CRCoV has been detected in canine sera from several countries. The seroprevalence was found to be 54.5% in the United States, 59.1% in Canada, and 30.3% in Ireland (Priestnall *et al.* 2006). However, within individual regions of the United States, CRCoV seroprevance (for $n \geq 10$ dogs) ranged between 31.8 and 87.5%. In addition, canine blood samples were submitted by various veterinary clinics for unrelated illness; hence this seroprevalence cannot be extrapolated to the prevalence of CRCoV amongst dogs with ICT. The CRCoV seroprevalence was reported to be 17.8% among 898 canine serum samples submitted from various regions in Japan (Kaneshima *et al.* 2006). In Italy, 38.33% and 26.76% of canine serum samples submitted in

2005 and 2006, respectively, were positive for CRCoV antibody (Decaro *et al.* 2007). None of the samples collected before 2005 were positive for CRCoV antibodies in the same study.

1.6: Canine Influenza (CIV)

1.6.1: General Features

The term "canine influenza" refers to Influenza type A viruses, which belong to the family *Orthomyxoviridae* (Beeler 2009). Influenza A viruses are enveloped, 80-120 nm in diameter, with a helical nucleocapsid. The genome consists of linear, single stranded RNA, divided into six to eight segments. The first known case of CIV infection occurred in a greyhound in Florida in 2004 (Payungporn, et al., 2008). Although the most common influenza virus detected in dogs was of H3N8 subtype, viruses from other subtypes, including H3N2, H1N1, H3N1, H5N1 and H5N2 have also been detected, possibly reflecting different sources of trans-species infections (Beeler 2009).

1.6.2: Pathogenesis, Clinical signs, and Immunopathology

Influenza viruses contain haemagglutinin (H) and neuraminidase (N) spikes on the surface of the envelope. Haemagglutinin allows the virus to attach to sialic acid receptors on cell surfaces. The virus enters via receptor mediated endocytosis, and transcription of mRNA takes place within the nucleus (Maclachlan and Dubovi 2010). This mRNA is then translated into viral proteins in the cytoplasm. In early infection, there is enhanced synthesis of the nucleoprotein NS1, whereas later in infection, production of HA, NA and M proteins increase (Lamb and Choppin 1983). NS1 is able to inhibit antiviral response to initial viral replication, hence the enhanced synthesis in early infection (Maclachlan and Dubovi 2010). Virions are released by budding, in which neuraminidase assists the detachment of viruses by cleaving sialic acid receptors that would otherwise bind to the H protein (Beeler 2009).

Clinical signs observed following experimental infection with CIV are similar to those observed in natural acute ICT, and include a low grade fever, lethargy, anorexia, nasal discharge, sneezing, depression, ocular discharge, and cough, which can last up to 3 weeks (Beeler 2009; Dubovi 2010). The onset of disease is usually rapid, with an incubation of 2-3 days. Given that clinical signs of CIV infection cannot be distinguished from clinical signs of other pathogens contributing to acute ICT, it is difficult to access mortality for CIV infection

alone. However, a mortality rate up to 23% has been observed in some populations (Jeoung *et al.* 2013).

The immune response to CIV infections is rapid, with antibodies detected in experimentally infected dogs 10 days post infection (Jirjis *et al.* 2010). The role of cell mediated immunity has not specifically been studied in CIV, however for influenza viruses in general, it is believed that cytotoxic T cells are involved in clearance of the virus (McMichael *et al.* 1983).

1.6.3: Diagnosis, treatment and vaccination

Isolation of CIV has been achieved in MDCK cells or embryonated chicken eggs (Payungporn *et al.* 2008). Canine influenza-specific RT-PCR has been used in many studies (Giese *et al.* 2008; Payungporn *et al.* 2008). Although development of a CIV -specific qPCR has not been reported, a qPCR with primers directed to a conserved region of the matrix protein of all influenza A viruses has been developed (Stone *et al.* 2004). This test can be used for detection of CIV in clinical samples. Serological diagnosis of CIV can also be very useful, especially in countries where CIV is not yet endemic, like New Zealand. Serological testing for previous exposure can be done using HI, however due to the many different subtypes of influenza circulating in dogs this can be problematic (Dubovi 2010). Therefore, ELISA tests that detect anti-nucleoprotein antibodies have been used in some studies to detect exposure to any of the influenza A viruses (Lee *et al.* 2009).

As for all viruses involved in ICT, there is no specific treatment for CIV disease. Although anti-influenza viral drugs have been used in the early stages of infection with human influenza virus, there is no data available on how effective these drugs would be against CIV (Dubovi 2010).

Several vaccines for both H3N8 and H3N2 CIV subtypes have been developed. The first H3N8 vaccine was a killed adjuvant vaccine, in which one group (n=21) of beagle pups aged 6-8 weeks were given 2 doses of the vaccine 3 weeks apart. Antibody titres increased by an average of five fold after the 2nd vaccination. The control group (n=15) were given an adjuvanted placebo vaccine, and no rise in antibody titres against CIV H3N8 were observed. When challenged with CIV H3N8 via aerosolization 13 days after the 2nd vaccination, the mean clinical scores and viral shedding of the vaccinated group were significantly lower than that of the control group. Furthermore, an approximately 14 fold increase in CIV H3N8

antibodies (tested by HI) was observed 11 days post challenge in the vaccinated group (Deshpande *et al.* 2009). This vaccine was licensed for use in the US in 2009 (Dubovi 2010). There was also an experimental H3N8 canarypox – vectored vaccine developed in 2007, however to date it has not been licenced for commercial use (Karaca *et al.* 2007).

In 2010, an inactivated vaccine for subtype H3N2 was developed, and like the H3N8 vaccine, also provided protection against CIV induced disease. (Lee *et al.* 2010). In 2013, virus like particle vaccine for H3N2 was developed and also showed protection against CIV induced disease (Lee *et al.* 2013). After the outbreak of H3N2 is the US (see section 1.6.4), both Zoetis (Anonymous. 2017) and Merck (Anonymous. 2015) licenced vaccines against H3N2 in November 2015. In 2016, a study outlining the efficacy of an inactivated H3N2 vaccine was published (Cureton *et al.* 2016). Neither the 2010 study or the 2016 study of inactivated H3N2 vaccines are listed as references for either the Zoetis or Merck vaccines, and hence it is unclear as to whether the efficacy of the Zoetis or Merck vaccines have been evaluated.

Recently, a bivalent live attenuated vaccine for H3N8 and H3N2 was developed, and like previous vaccines, was shown to be protective against CIV disease (Rodriguez *et al.* 2017). Merck also licenced a bivalent H3N8/H3N2 vaccine in 2016 (Madison 2016), however like the other commercial H3N2 vaccines, there is currently no published studies to confirm the efficacy of the vaccine.

1.6.4: Epidemiology

The first CIV to be identified was a H3N8 virus isolated from a greyhound in Florida in 2004 (Payungporn *et al.* 2008). However, based on serological evidence, H3N8 was circulating among dogs as early as 1999 (Anderson *et al.* 2012). Another study showed that the CIV seroprevalence in the USA decreased from 44% in 2005 to 15% in 2009, with the majority of seropositive dogs in Colorado, Florida and New York (Anderson *et al.* 2013). In contrast, none of the Alaskan sled dogs were positive for antibodies to H3N8 (Pecoraro *et al.* 2012). This is surprising, as 39/399 of the sampled dogs were recorded as vaccinated for CIV, according to the owner's recall. If owner recall was in fact correct, this could suggest that immunity to CIV may be short lived, or other factors such as breed may have affected development of detectable levels of CIV antibody in these dogs.

H3N2 was first reported in dogs with severe respiratory disease in Korea in 2007, and it is thought that the isolate was of avian origin (Song *et al.* 2008). Results of a follow up study in Korea showed that 18.84% of farm dogs had antibodies against H3N2 influenza virus, compared with only 0.48% seropositive pet dogs (Lee *et al.* 2009). In northern China, H3N2 influenza virus was isolated from 12 dogs of various breeds and ages with mild to moderate signs of ICT (Sun *et al.* 2013). An outbreak of H3N2 occurred in the US in March 2015. As at August 2017, approximately 1975 dogs have tested positive to CIV (Network 2017).

Canine influenza virus H3N2 has shown to be experimentally transmitted between species. In one study, droplet transmission between dogs and cats was observed but not between dogs and ferrets. However, both cats and ferrets experimentally infected with CIV via the intranasal route showed influenza-like clinical signs (Kim *et al.* 2013). In addition, an outbreak of influenza-like disease in both dogs and cats occurred in a multi-species shelter, with a 100% morbidity and 40% mortality rate in cats. Influenza virus H3N2 was isolated from the lung of one of the deceased cats, hence H3N2 virus was presumed to be responsible for the outbreak (Song *et al.* 2011). Hence, future surveillance of this virus is important, especially considering the zoonotic potential of influenza viruses.

Highly pathogenic avian influenza virus (H5N1) was identified in a dog in Thailand in 2004 after ingesting an infected duck. However, infections with H5N1 are likely to be dead-end infections, therefore have little relevance in outbreaks of disease (Giese *et al.* 2008). There have also been a few reports of pandemic H1N1 isolated from diseased dogs. In one study, a dog from New York tested positive for H1N1, and a week later the owner also tested positive for H1N1. However, there have been no reports of H1N1 circulating in populations of dogs, so similarly to influenza virus H5N1, it is unlikely to cause outbreaks in kennelled dogs (Dubovi 2010).

Two more influenza subtypes detected in dogs include H5N2 and H3N1. In 2009, a H5N2 subtype was isolated from a dog in China, and found to be closely related to the H5N1 virus described by Giese et al, (2008) (Song *et al.* 2013). In that study, transmission of H5N2 between infected and susceptible dogs was demonstrated. Another influenza virus subtype, H3N1, isolated from a dog with respiratory disease, was found to be a recombinant of H3N2 and H1N1 influenza viruses. The H3N2 isolate was then used to experimentally infect dogs.

The inoculated dogs shed the virus in nasal secretions for up to 4 days without any overt signs of clinical disease (Song *et al.* 2012).

Canine influenza virus has not been reported in New Zealand, although there is no on-going surveillance for the virus, and the only New Zealand-based study that included testing for CIV antibody was published 8 years ago (Knesl *et al.* 2009). Hence, while unlikely, it cannot be excluded that the virus is present in New Zealand but has not yet been detected.

1.7: Mammalian Reovirus (MRV)

1.7.1: General Features

Mammalian Reoviruses are included in the genus *Orthoreovirus*, within the family *Reoviridae* (King *et al.* 2013a). They are non-enveloped, 50-80 nm in diameter and have double stranded RNA genome in 10 segments. They have a broad host range as well as a wide geographical distribution. Three serotypes of MRV have been identified: MRV-1, 2 and 3, with MRV-2 and 3 being the only types isolated from dogs with upper respiratory disease (Buonavoglia and Martella 2007).

1.7.2: Pathogenesis, Clinical signs and Immunopathology

Mammalian reoviruses attach to host cells via the protein σ1 and enter via receptor mediated endocytosis. After viral entry, the host cell protein synthesis decreases rapidly, possibly since translation of the host cell mRNA is less efficient than the translation viral mRNA. Replication takes place in the cytoplasm with virion-associated RNA polymerase in localised areas of the cytoplasm; hence large intracellular inclusion bodies may be formed in infected cells. Virions tend to remain cell associated, but are eventually released by cell lysis (Maclachlan and Dubovi 2010). The virus has been found to persist in the lymphatic tissues of dogs. Infection with MRV has been commonly associated with other pathogens that cause ICT, hence it is possible that MRV replication would cause immunosuppression and aggravate infection with other pathogens (Appel 1987).

The role of MRV in ICT is still questionable. Experimental infection with MRV-1 did not result in an overt disease however MRV-1 has been isolated from dogs with pneumonia (Appel 1987). MRV-2 has been isolated from dogs with upper respiratory disease (Binn *et al.* 1977), and MRV-3 was isolated from a dog with diarrhoea in 1993 (Decaro *et al.* 2005) Hence,

although MRV types 1 and 2 have been isolated from dogs with respiratory disease, previous experimental inoculation would suggest that MRV was not the main causative agent in the latter cases.

The three serotypes of MRV can be distinguished based on antibodies against the σ1 protein (Rosen 1960). Antibodies raised against one serotype are not be cross protective for another serotype. Given MRV's apparent lack of pathogenicity in dogs, it has not been determined whether antibodies raised against MRV would be protective against MRV infection. In addition, there are no studies outlining the role of cell mediated immunity in MRV infection.

1.7.3: Diagnosis, treatment, and vaccination

Mammalian reovirus has been isolated previously in feline kidney cells, but no CPE was observed. The virus heamagglutinates pig and human RBC. Bovine RBC have also been used in HA tests but with a weak result (Decaro *et al.* 2005). Reverse transcriptase-PCR methods have been developed for MRV, and have been used successfully to identify MRV and predict the serotype (Buonavoglia and Martella 2007). No specific treatment or vaccine is available for MRV currently.

1.7.4: Epidemiology

Mammalian reoviruses have been isolated from a wide range of species, including humans (Tyler 2001). Based on serological evidence, dogs can become infected with all three serotypes of MRV (Appel 1987). In one study, MRV RNA was identified in 26% (50/192) of faecal samples from dogs with diarrhoea, 9/12 ocular swabs and 10/19 nasal swabs from dogs with ocular or nasal discharge, via PCR. These results suggest that MRV may be common in the respiratory and/ or enteric tract of dogs, although more research is required to support this (Elia *et al.* 2006).

1.8: *Bordetella bronchiseptica:*

1.8.1: General Features

Bordetella bronchiseptica is a gram negative motile coccobacillus that has been recognised as a canine respiratory pathogen since 1910 (Goodnow 1980). *Bordetella bronchiseptica* is closely related to the human pathogen *B. pertussis,* which commonly causes whopping cough (Goodnow 1980), however *B. bronchiseptica* is antigenically distinct from *B pertussis*

(Keil and Fenwick 1998). *Bordetella bronchiseptica* is strictly aerobic and grows optimally at 35 to 37 °C. They also possess three types of agglutinins that bind and agglutinate erythrocytes from both mammals and fowl (Goodnow 1980).

1.8.2: Pathogenesis, clinical signs, and immunopathology

Bordetella bronchiseptica adheres to ciliated surfaces of epithelial cells in the respiratory tract via fimbriae and agglutinins. This adherence allows colonisation of the respiratory tract (Keil and Fenwick 1998). Although *B. bronchiseptica* is often considered an extracellular pathogen, it has the ability to invade host cells, which offers both protection against host defences and access to cell nutrients (Keil and Fenwick 1998). Furthermore, *B. bronchiseptica* was shown to produce an exotoxin called adenylate cyclase (Gueirard and Guiso 1993). This enzyme was shown to bind to integrin receptors on the surface of the host leukocytes and macrophages (Guermonprez *et al.* 2001), which in turn inhibits phagocytic functions of the host cell. In one study, adenylate cyclase was inoculated into a mouse model and caused general dysfunction of spleen, indicating that this toxin could potentially interfere with both humoral and cell mediated immune responses directed towards *B. bronchiseptica* (Horiguchi *et al.* 1992).

Bordetella bronchiseptica typically has an incubation period of 1-8 days (Wagener et al., 1984). Clinical signs observed in naturally infected dogs are typical of the ICT complex, including a dry hacking cough and occasional nasal discharge (Keil and Fenwick 1998). It is nearly impossible to distinguish *B. bronchiseptica* infection from other causes of ICT since the incubation period overlaps with that of many viral agents, and the clinical signs observed are similar for all pathogens implicated in ICT.

1.8.3: Diagnosis, treatment, and vaccination

Bordetella bronchiseptica can be isolated from samples from the pharynx or trachea on selective media containing penicillin, streptomycin and nystatin (Goodnow 1980). Plates are incubated at 35-37 °C for 40 to 72 hrs. Colonies grown on selective media will appear coccoid. Under EM, flagella can be observed (Richter and Kress 1967)). Suspected *B. bronchiseptica* colonies can then be gram stained and confirmed as *B. bronchiseptica* via biochemical reactions (Goodnow 1980). *Bordetella bronchiseptica* nucleic acid can be detected via PCR, and antibodies can be detected by ELISA (Englund *et al.* 2003; Bhardwaj *et*

al. 2013). However, the results of one study carried out using ELISA, which detected anti-LPS antibodies, suggested that levels of anti-LPS antibodies did not correlate with the history of respiratory disease (Chalker *et al.* 2003b). Hence, detection of antibodies to *B. bronchiseptica* may not be indicative of previous respiratory disease.

Antibiotics are most effective against *B. bronchiseptica* when binding and attachment of the bacterium to host cells is inhibited (Goodnow 1980). The most commonly used antibiotic for treatment of *B. bronchiseptica* infection belongs to the tetracycline family of antibiotics (for example, doxycycline). Erythomycin, clarithomycin and azithromycin have also been used as alternatives to tetracycline (Keil and Fenwick 1998). Antibiotics such as ampicillin and amoxicillin have little efficacy against *B. bronchiseptica* infection, since they have a relatively low concentration in the trachea and bronchi (Keil and Fenwick 1998).

In New Zealand, *B. bronchiseptica* infection is commonly controlled by vaccination with a live attenuated intranasal vaccine that also contains CPIV (Nobivac KC). This type of vaccine has proved to be effective even in the presence of maternal antibodies, hence is more suitable for young puppies than other vaccines. This is because maternal antibodies can interfere with the systemic immune response to vaccines administered via the parenteral route (such as whole cell bacterins) (Keil and Fenwick 1998). The protection provided by the live attenuated vaccine is primarily due to the activation of mucosal immunoglobulin A rather than serum antibodies (Jacobs, et al., 2005). Furthermore, trials of the latter vaccine showed protection against disease for up to 54 weeks, and a considerable reduction in shedding of *B. bronchiseptica* compared to a non- vaccinated control group (Jacobs *et al.* 2005).

1.8.4: Epidemiology

Bordetella bronchiseptica can survive for long periods in the environment; hence environmental contamination is a possible source of infection. *Bordetella bronchiseptica* is sometimes considered as part of the normal flora of the pharynx since it can be commonly isolated from the pharynx of apparently healthy dogs. However, several authors reported that the rates of detection of this bacteria in samples from diseased dogs are higher than in samples from healthy dogs. For example*, B.bronchiseptica* was isolated from lung washings obtained post-mortem from 37% of apparently healthy dogs, compared to 52% of dogs with

clinical signs of respiratory disease (Chalker *et al.* 2003a). Futhermore, *B. bronchiseptica* was more commonly isolated from dogs displaying moderate clinical signs at the time of death (66%) than from dogs with mild or severe disease. In contrast, *B. bronchseptica* was detected in only 1/78 healthy dogs and 2/69 with respiratory disease in another study (Weese and Stull 2013). It should be noted that dogs in that study were not tested for the presence of any other respiratory pathogens. Hence, it is possible that *B. bronchiseptica* was not the cause of respiratory disease among the sampled population. However, other authors reported similar findings. In Canada, most dogs sampled (*n*=358, including healthy and diseased) had moderate to high antibody titres to *B. bronchiseptica*, however no statistically significant correlation between antibody titres and respiratory disease was found (Ellis *et al.* 2011). In Sweden, the seroprevalence of *B. bronchiseptica* was 22% but again no correlation between antibody titre and respiratory disease was found (Englund *et al.* 2003). The results of the latter study suggested that *B.bronchiseptica* and CPIV circulated separately in the population of 287 dogs. Despite these studies indicating that *B. bronchiseptica* may not be as important in ICT as generally presumed, experimental infection of *B. bronchiseptica* in puppies resulted in similar clinical signs to that observed in ICT: paroxysmal coughing, signs of depression, anorexia and fever. In contrast, vaccinated puppies showed reduced or no coughing and no other clinical signs after the same challenge (Ellis *et al.* 2001). Hence, although *B. bronchiseptica* is a likely contributor to ICT, it's involvement in more recent outbreaks may be less prominent, possibly due to widespread vaccination against *B. bronchiseptica*.

1.9: *Streptococcus equi subsp. zooepidemicus*

1.9.1: General features

Streptococcus zooepidemicus is classified as a β- haemolytic, Lancefield group C streptococcus, and was first isolated from a dog with systemic streptococcal disease in 1978 (Wyand and Sherman 1978). Since then, it has been isolated from several different species including horses, llamas, pigs, poultry, lambs, guinea pigs, and humans; hence it is considered a zoonotic pathogen (Chalker *et al.* 2003b). *Streptococcus zooepidemicus* is associated with disease in horses, llamas, and lambs. More recently, it has been involved in several outbreaks of fatal haemorrhagic pneumonia in dogs at various rehoming kennels in different geographical locations (Pesavento *et al.* 2008; Byun *et al.* 2009).

1.9.2: Pathogenesis, clinical signs and immunopathology

Streptococci contain adhesion ligands that can bind to the extracellular matrix molecules on host cells. These adhesion ligands include fibronectin and fibrinogen binding proteins (Harrington *et al.* 2002). In addition to this, hyaluronic acid capsular material of *S. zooepidemicus* has been shown to adhere to HeLa cells *in vitro*, and to offer some protection against phagocytosis by macrophages (Wibawan *et al.* 1999). Furthermore, it has been shown that *S. zooepidemicus* produces an M- like protein termed SzP (Timoney *et al.* 1995). M proteins are cell wall associated proteins that bind to fibrinogen on the host cell and show antiphagocytic properties (Harrington et al., 2002). Recent studies have suggested that some isolates of *S. zooepidemicus* encode up to 3 superantigen proteins, which can cause activation of a large proportion of T lymphocytes. This leads to the activation of cytokines and release of vasoactive factors (Priestnall *et al.* 2010). In addition, elevation of proinflammatory cytokines TNFα, IL-8 and IL-6 was reported in dogs infected with *S. zooepidemicus* (Priestnall and Erles 2011).

Given *S. zooepidemicus*'s pathology above, onset of disease is usually rapid and clinical signs are severe. Despite treatment, the majority of clinical cases often die within 24 – 48 hours of first showing clinical signs (Priestnall and Erles 2011). Clinical signs that are commonly observed include necrotising rhinitis, sinusitis, coughing, depression, lethargy, and haemorrhagic pneumonia (Pesavento *et al.* 2008; Byun *et al.* 2009).

1.9.3: Diagnosis, treatment and vaccination

Streptococcus zooepidemicus has been isolated from nasal, throat and trans-tracheal lavage samples (Priestnall *et al.* 2010). The identity of *S. zooepidemicus* is usually confirmed via biochemical tests, PCR, or a commercially available API20 streptococcus kit (Priestnall *et al.* 2010). Pathological findings in dogs infected by *S. zooepidemicus* are fairly consistent between studies. Gross pathology for *S. zooepidemicus* include rubbery, mottled dark to bright red lungs, infiltration of the lungs with edema fluids. Histopathology typically identifies acute suppurative or necrotising pneumonia with alveolar spaces containing macrophages and neutrophils (Chalker *et al.* 2003b). Chains or clusters of gram positive cocci can be observed in the cytoplasm of macrophages as well as in the extracellular space of the lung (Pesavento *et al.* 2008).

S. zooepidemicus is usually susceptible to penicillin, ampicillin, amoxicillin, and enrofloxacin (Byun *et al.* 2009). However, even early intervention with antibiotics may not prevent the inflammatory cascade, and fatal outcome has been reported even for dogs that received appropriate supportive treatment (Byun *et al.* 2009). Some isolates from the later study were resistant to tetracycline and doxycycline, hence these two antibiotics are not recommended for treatment of infection with *S. zooepidemicus*.

Currently there is no vaccine available for prevention of *S. zooepidemicus*. However, the M-like protein SzP has been used for vaccination of horses against uterine infection with *S. zooepidemicus*, hence it is possible that a modified version of this vaccine could also be used for dogs (Priestnall *et al.* 2010).

1.9.4: Epidemiology

Streptococcus zooepidemicus has been associated with fatal outbreaks of respiratory disease among dogs in the UK (Chalker *et al.* 2003b), the USA (Pesavento *et al.* 2008), and Korea (Byun *et al.* 2009). Although *S. zooepidemicus* had been isolated from the respiratory tract before, an association between *S. zooepidemicus* and ICT was only established in 2003 (Chalker *et al.* 2003b). In that study, *S.* zooepidemicus was isolated from 92% of 209 kennelled dogs, compared to only 1 of 71 pet dogs. A higher proportion of positive isolates were observed for dogs that had severe clinical signs compared to those with mild clinical signs. In a large rehoming centre in the US, in one year over 1,000 dogs suffered from severe haemorrhagic pneumonia out of an approximate intake of 50,000 dogs/ year. Six lung tissue samples from euthanized dogs were submitted for culture, and *S. zooepidemicus* was identified in all six samples (Pesavento *et al.* 2008). Fatal necrotising pneumonia with a morbidity rate of 70-90%, and 50% mortality rate among infected dogs was described among dogs in a Korean study, despite intervention with antibiotics. Histology was performed on three of the deceased dogs, and gram positive cocci were identified in the lung, and culture confirmed the presence of *S. zooepidemicus* in all three dogs. Furthermore, other pathogens including CAdV-1, CAdV-2, CPIV or CDV) were not identified via PCR (Byun *et al.* 2009). It was also noted that the mortality rate rapidly dropped after improvement of hygiene, suggesting that the environmental contamination was a likely source of infection for dogs. Given the large numbers of dogs at each of the outbreak

described above, reduction in the number of kennelled dogs in a given location should be considered if feasible in order to reduce the morbidity of infection.

1.10: *Mycoplasma cynos*

1.10.1: General features

Mycoplasmas are bacteria that lack a cell wall. They are typically 0.3 to 0.8 µM in size, and have a genome size of approximately 1000 Kb (Walker *et al.* 2013). Currently, 15 different species of mycoplasma have been isolated from dogs. Of these, *Mycoplasma cynos* has been most commonly isolated from dogs with respiratory disease (Chalker *et al.* 2004). Other mycoplasmas have also been isolated from the trachea of dogs but have not been fully characterised yet.

1.10.2: Pathogenesis, clinical signs and immunopathology

Little is known about the virulence of *M. cynos in vivo*, and *M. cynos* pathogenesis is poorly understood. It is thought that *M. cynos* attaches to bronchial epithelial cells since deciliated and degenerating bronchial epithelial cells were observed under EM (Rosendal 1982). In the same study, mycoplasma could be observed on the bronchial surface of lung tissue – but were not characterised at the species level. Histological findings of lung tissue from a dog euthanized 15 days after experimental infection with *M. cynos* (originally isolated from a dog with distemper) revealed lung consolidation, proliferation and desquamation of the bronchial epithelium, as well as the presence of neutrophils (Rosendal 1978). However, no overt clinical signs of disease were observed in the latter dog.

The immune response to *M. cynos* is currently not well understood. In one study, a two fold increase in antibodies against a 45 kDa *M. cynos* antigen was demonstrated between acute and convalescent samples in 12/26 (46%) dogs with respiratory disease (Rycroft *et al.* 2007). However, the study does not mention whether an increase in antibodies against *M. cynos* correlated with protection against M. cynos infection.

1.10.3: Diagnosis, treatment and vaccination

Mycoplasma can be isolated on commercially available mycoplasma medium under aerobic conditions within 1-2 days on primary cultivation (Chalker 2005). *Mycoplasma cynos* cannot be identified solely on colony formation since mixed infections with other mycoplasma

species are very common, and colonies are very difficult to see with the naked eye. When observed, mycoplasma colonies form “fried egg” like colonies with a granular appearance (Chalker 2005).

Mycoplasmas are resistant to β-lactam antibiotics, and prolonged treatment with these may exacerbate infection (Chalker 2005). Tetracycline or doxycycline (non- β-lactam antibiotics) are both effective against hemotrophic mycoplasma (Messick 2003), but their effectiveness specifically against *M. cynos* has not been evaluated.

Currently, no vaccine is available against any canine mycoplasmas, although a proposed patent has been put forward in the US for a vaccine containing *M. cynos* (Brownlie *et al.* 2010). In the patent, a suggestion of a live attenuated strain of *M. cynos* (similar to that of *B. bronchiseptica*) has been put forward. More research would be required to determine the efficacy of such a vaccine.

1.10.4: Epidemiology

After *M. cynos* was first isolated in 1972, there have been several studies that aimed to determine the prevalence of *M. cynos* in dogs. Randolph at el (1993) reported isolation of *M. cynos* from all pharyngeal swabs from 26 healthy dogs, and from 84% (*n*=38) swabs from dogs with pulmonary disease. Despite these high rates of *M. cynos* isolation from oropharynx, isolation of *M.cynos* from tracheobronchial lavage was associated with the presence of pulmonary disease, but only for young dogs up to 1-year-old, For dogs equal or greater than 1 year, 21% of dogs with pulmonary disease were positive for M. cynos, whereas M.cynos was isolated from 25% of healthy dogs from this age group. For dogs of all ages with pulmonary disease, M. cynos in tracheobronchial lavage was commonly detected concurrently with other pathogens including *Bordetella* and *Streptococcus* species (Randolph *et al.* 1993). The authors concluded from these data that *M. cynos* was likely to be a part of the normal flora in the upper respiratory tract of older dogs, but may contribute to pulmonary disease in younger dogs, or lead to secondary infections that cause pulmonary disease. In another study (Chalker *et al.* 2004), tracheal tissue samples and bronchial lavage samples from two populations of dogs were tested for the presence of Mycoplasma. The first population (population A) comprised 210 dogs in a rehoming shelter where ICT was endemic. Of this group, both sample types from 9.7% of healthy dogs were positive for *M.*

cynos, whereas for diseased dogs, 23.9% and 21.7% of tracheal and bronchial samples, respectively, were positive for *M. cynos* (Chalker *et al.* 2004). Of the ICT-affected dogs in this population, *M. cynos* was more commonly isolated from dogs with moderate to severe clinical signs. In addition, population A was also examined for the presence of CRCoV, *B. bronchiseptica* and *S.zooepidemicus*, and it was found that these pathogens were associated with mild, moderate, and severe clinical disease, respectively (Chalker *et al.* 2004). The second population (population B) comprised healthy dogs entering a training centre, where only sporadic outbreaks of ICT where observed. Only one healthy dog (0.9%) was positive for *M. cynos*, and none of the 43 dogs that developed ICT were positive for *M. cynos* (Chalker *et al.* 2004). Another interesting finding from this study was that *M. cynos* was isolated from the air, suggesting of the possibility of airborne transmission of *M. cynos* between dogs. In a more recent study, a two fold increase in antibodies to *M. cynos* over a 3 week period was demonstrated for 67% of dogs at a rehoming kennel, and of these dogs, 80% were affected by respiratory disease (Rycroft *et al.* 2007). Altogether, these studies suggest that *M. cynos* may colonise the lower respiratory tract when a dog has been immunocompromised or infected with another agent, hence exacerbating clinical signs of ICT.

1.11: Conclusion

There are several important pathogens involved in ICT, and it is likely that outbreaks of ICT involve several of these pathogens. Most of the canine respiratory pathogens cause mild or subclinical disease on their own, but concurrent infections, under some circumstances, can result in more severe disease. Emerging pathogens such as CRCoV and CIV appear to be involved in ICT outbreaks in various countries, although more research is required to determine whether these pathogens are consistently associated with ICT. Furthermore, few studies have looked at host and environmental risk factors that may be important in the ICT complex.

1.12: Aims of the study

The aims of this study was to identify respiratory pathogens that currently circulate among selected populations of dogs in New Zealand and to determine which of those are most likely to be involved in ICT. The work presented in this thesis focused on viral agents, with

only limited investigation of bacterial pathogens. It is hypothesised that viral agents other than those currently vaccinated for will be identified in diseased dogs. HYPOTHESIS?

Chapter 2: Optimization and development of in-house real-time PCR assays

2.1: Introduction

Real-time quantitative (q) PCR is a fluorescent based assay that has many advantages over conventional PCR. These include potential for increased sensitivity and specificity due to amplification of a relatively short product, as well as time efficiency due to built-in confirmation of the identity of the amplified product (Maclachlan and Dubovi 2010). There is also a lower risk of contamination due to the real-time detection of target nucleic acids, which eliminates the need to open PCR tubes in order to transfer PCR reactions to an agarose gel.

Several factors need to be considered in the development of a reliable and repeatable qPCR assay (Bustin *et al.* 2009). Firstly, the method of nucleic acid extraction should ideally produce nucleic acids that are free of contaminants, with a high purity and optimal yield. For the assay itself, primers that are both sensitive and specific for the target sequence are required. To achieve this, primers should ideally be between 18-30 base pairs long, have no long repeat sequences, have a GC content between 45-60%, and formation of secondary structures within the primer sequence should be minimal (Abd-Elsalam 2003). In addition, the T_m of the forward and reverse primer should not be more than 1°C apart, and hence should have similar %GC content. This is because T_m is determined by the GC content, the higher the GC content, the higher the T_m of the primer (Thornton and Basu 2011).

As well as a verified positive control template, a non-template control (NTC) needs to be included to detect contamination and potential primer dimer formation. It is also advantageous to include a separate negative control, consisting of genomic DNA, expected to be present in the sample (i.e. DNA from the host species when looking for viral sequences) in order to control for the possibility of non-specific amplification. An assay should be optimised for linearity, typically by construction of a standard curve using serial dilutions of the target DNA. Ideally, a qPCR assay should show between 90 and 110% efficiency, and a correlation coefficient (R^2) above 0.99. In order to optimise the assay, the best annealing temperature, based on the T_m of the primer sequence, as well as cycling conditions, should be determined. In some cases, several different protocols (for example two versus three step protocols) may need to be examined to determine conditions for the

optimal performance of the assay (Bustin *et al.* 2009). In addition, the analytical sensitivity of an assay can be determined by the limit of detection (LoD), defined as the lowest concentration of target nucleic acid likely to be reliably distinguished from the NTC (Armbruster and Pry 2008).

At the initiation of this project, only one published qPCR was available for each of the following canine respiratory viruses: CAdV-2 (Segura *et al.* 2010), CHV (Decaro *et al.* 2010), CRCoV (Mitchell *et al.* 2009), and CIV (Payungporn *et al.* 2008). There was no published qPCR protocol for CPIV; hence, CPIV qPCR had to be designed for the current study. For CAdV-2, the published protocol was optimised for detection of CAdV-2 vectors, from which the E1 region had been deleted (Segura *et al.* 2010). Primers targeted the gene for the pentose base protein of CAdV-2 which had not been altered. Hence, the primers reported by Segura et al. were also expected to be suitable for detection of CAdV-2 field viruses. The optimum annealing temperature for the CAdV-2 primers was reported to be 60°C. The qPCR LoD was approximately $1x10^2$ copies/ reaction using 40 cycles. The efficiency of the assay was consistently between 92 and 93% over a range of dilutions between 10^2 and 10^7 copies/reaction. Furthermore, the assay proved to be repeatable and reproducible, with a CAdV-2 load of $8.9\times10^{11} \pm 0.7\times10^{11}$ copies/mL for intra-assay variation (based on a single run of 5 aliquots of the purified CAdV-2 stock solution) and a CAdV-2 load of $8.8\times10^{11} \pm 1.3\times10^{11}$ copies/mL for inter-assay variation (based on 5 independent qPCR runs of the purified CADV-2 stock dilution) (Segura *et al.* 2010).

The LoD of the published CHV qPCR was estimated to be $1x10^1$ copies per 10 µL reaction over 40 cycles (Decaro *et al.* 2010). The slope of the standard curve was -3.165 over a range of 10^1 to 10^9 copies of target DNA, which equates to a qPCR efficiency of 107%. The repeatability/reproducibility of the assay was calculated as the coefficient of variation (CV). The intra- and inter-assay CV was 8.4% for 10^8 copies of target DNA, and 15.9% for 10^6 copies of target DNA, respectively.

The diagnostic sensitivity and specificity was not determined for the CAdV-2 or CHV assays mentioned above. The performance of published CAdV-2 and CHV qPCR was acceptable based on the LoD and intra- and inter- repeatability, hence these primers were selected for use in the current study. The objective of the work presented in this chapter was to adapt

the published CAdV-2 and CHV primers for use with alternative PCR buffers and instruments available in our laboratory, and to develop qPCR for detection of CPIV.

2.2: Canine adenovirus type 2 (CAdV-2)

2.2: CAdV-2

2.2.1: Methodology

2.2.1.1: Cell culture

Madin Darby canine kidney (MDCK) cells (ATCC, Lot 05/2009) were grown in Advanced Dulbecco's Modified Eagle Medium (Adv. DMEM, Invitrogen), supplemented with 2% fetal bovine serum (FBS, Life Technologies) 1% v/v glutaMAX^{TM} (Gibco^{R}), and 1% v/v penicillin/streptomycin antibiotics (Gibco^{R}) (containing penicillin at 10,000 units/mL and streptomycin at 10,000 µg/mL).

MDCK cells were maintained in 75cm^2 tissue culture flasks (Nunk) and subcultured every 3-4 days, when the monolayer was observed to be about 90-95% confluent, according to standard protocols (Freshney 2011). Briefly, cells were washed three times in phosphate buffered saline pH 7.0 (PBS), at room temperature. A small volume (1 mL) of TrypLE^{TM} (Life technologies) was added to the flask and gently swirled across the monolayer of cells, followed by incubation in a 37 °C, 5% CO_2 for up to 10 minutes. Once detachment of the cells from the flask was apparent, any remaining clumps of cells were further separated by banging the side of the closed flask with a hand and then pipetting up and down using a 9 mL volume of fresh growth medium. In some cases, the monolayer did not fully detach from the surface of the flask, and a cell scraper was used to detach the remaining cells. Cells were routinely split at a ratio of 1:10.

2.2.1.2: Preparation of positive control

A New Zealand field isolate (1990) of CAdV-2 with unknown passage history was used as a positive control. MDCK cells were seeded at a density of 2.5x10^5 cells/mL, in three 25cm^2 cell culture flasks (Nunk). The virus was thawed at room temperature, and 200 µL of the stock virus was added to MDCK cells at the time of seeding. One flask was retained as an un-inoculated control. The flasks were observed daily for the presence of viral CPE. Once CPE was evident, the flasks were frozen at -80 ˚C. The virus was passaged a further three times.

Each time, the flasks were thawed at room temperature, and 200 µL of the cell culture lysate was inoculated into fresh MDCK cells at the time of seeding, as described above.

Nucleic acid was extracted from the last passage using the High Pure viral nucleic acid extraction kit (Roche), according to the manufacturer's instructions. The extracted nucleic acid was then subjected to conventional PCR. Each 20 µL reaction consisted of 0.8 µM of each CAdV-2 long-range primer (Table 1) and 2 µL of template DNA in 1 x HotFirePol buffer (Solis BioDyne). The PCR conditions were as follows: 15 minutes at 95 ˚C polymerase activation, followed by 35 cycles at 95 ˚C for 10 seconds, 50 ˚C for 10 seconds and 72 ˚C for 1min. Then a final step of and 72 ˚C for 1min. The primers were designed using Primer3 within Geneious™ software (http://www.geneious.com, Kearse *et al.* 2013). The expected PCR product was further analysed for the absence of predicted secondary structures within primer binding sites using mfold software (http://unafold.rna.albany.edu/?q=mfold). The CAdV-2 PCR long range product was subjected to gel electrophoresis through 0.5% TBE, 2% agarose gel for 45 mins at 100V/cm.

The band of the expected size (811 bp) was then excised from the agarose gel, and used for DNA extraction using DNA gel extraction spin columns (Quantum prep™). Briefly, the extraction spin column containing the PCR product was exposed to liquid nitrogen for 3-5 seconds. The sample was then centrifuged at 13,000 x g for 3 minutes at room temperature. The agarose debris retained in the filter cup were discarded, and the collection tube containing the purified PCR product was placed in the fridge until required.

The quality and quantity of nucleic acid was then assessed spectrophotometrically using Nanodrop (Thermo Fisher Scientific) and the identity of the band was confirmed by sequencing at the Massey Genome Centre. The obtained sequence was identified based on identity scores obtained using Basic Local Alignment Search Tool (BLAST®) (Altschul *et al.* 1990).

2.2.1.3: Preparation of the negative control

Canine DNA extracted from the buffy coat of canine blood was used as a negative control. Approximately 5 mL of uncoagulated canine blood was centrifuged at 2,000 x g for 10 minutes. The buffy coat was removed with a sterile pipette and used for nucleic acid

extraction, using the PCR template preparation kit (Roche), according to the manufacturer's instructions.

Table 1: CAdV-2 long range primer properties

Primer	Primer Sequence	Product size	Target region (GenBank AAB38720)	Position
CAdV-2_L-Forward	5' –CCC ACG GGC GCC AGC ATA AT-3'	811bp	13133-13943	Penton base protein
CAdV-2_L-Reverse	5'- CGC TGC ACC CCG GGA ATG TT-3'			

2.2.1.4: Optimisation of the primer concentration

CAdV-2 primers (Segura et al, 2010) (Table 2) were tested over concentrations ranging from 0.1 µM to 0.5 µM. Each 10 µL qPCR reaction contained the following: 0.5 µL of each primer, 5 µL of PerfeCTa SYBR Green Fast mix® (Quanta Biosciences), 2 µL of distilled water and 2 µL of template DNA, which comprised CAdV-2 long PCR product (positive control) or water (NTC). Each reaction was made in duplicate for each combination of primer concentrations. The following cycling protocol was used: 5 minutes at 95 ˚C polymerase activation, followed by 40 cycles of 1 second at 95 ˚C and 30 seconds at 60 ˚C. The combination of primers that produced an amplification curve with the lowest Cq value without any adverse effects to the assay (such as non-specific amplification) was selected.

Table 2: CAdV-2 real time PCR primers

Primer	Primer Sequence	Product size	Target region	Position (Genbank: AAB38720)	Reference
CAdV-2-Forward	5' -TCATGCCGGGCTTCTACA CTAACG-3'	142bp	Penton base protein	13,228-13,370	(Segura *et al.* 2010)
CAdV-2-Reverse	5'- ATCACAAATCCCTCCTGGTATGGT-3'				

2.2.1.5: Generation of a standard curve

A standard curve was used to determine the performance of CAdV-2 primers under various amplification conditions. The standard curve was prepared using 10 fold serial dilutions of the CAdV-2 long PCR product. Copy numbers for each serial dilution were calculated based on the Nanodrop nucleic acid quantification of the gel-purified band (section 2.2.1.2). The dilutions covered six orders of magnitude, ranging from 10^9 to 10^4 copies of CAdV-2 long PCR product/µL. Serial dilutions were prepared as follows: 90 µL of sterile water was added to each of the 5 sterile microtubes. An aliquot (10 µL) of a 10^9 dilution of CAdV-2 template was added to the first tube. The tube was vortexed for 3-5 seconds, followed by spinning a pop spin micro- centrifuge for 3-5 seconds. An aliquot (10 µL) of the first dilution was then transferred to the second tube. The steps were repeated for the remaining dilutions. Once the dilutions were prepared, they were stored at 4 °C for up to 2 hours until required.

2.2.1.6: Optimisation of the annealing temperature

Serial dilutions (10^9 to 10^4) of the CAdV-2 long PCR product (positive control) in 100 µL of sterile water were made on the day of testing. Several qPCR assays were then run over a range of annealing temperatures between 60 and 64 °C using the same dilution series as templates. Protocol for this assay is outlined below in Table 3. All samples were run in duplicate. A NTC (water) was also included in duplicate in each assay. The annealing temperature that gave the best assay efficiency and the lowest Cq was selected.

Table 3: Real time PCR protocol for annealing temperature optimisation

Temperature	Time	
95 °C	5 minutes	
95 °C	1 seconds	X45 cycles
various	30 seconds	
95 °C	15 seconds	Melt curve
55 °C	15 seconds	
95 °C	15 seconds	

2.2.1.7: Performance of the optimised assay

Once the assay conditions were optimised, the performance of the optimised assay was assessed by determination of its linearity, precision, analytical sensitivity, and reproducibility. Linearity was determined by the assay efficiency and the R^2 value obtained from the analysis of the standard curve. Analytical sensitivity was determined by the LoD of the assay. Precision and reproducibility was determined by calculating intra- and inter-assay variability, respectively. To determine the LoD and intra-assay variability, the standard curve was run with triplicates of serial dilutions of target DNA (long PCR product) covering 10 orders of magnitude, ranging from 10^8 to 10^1 copies per µL. For inter-assay variability, the assay was repeated on three separate occasions using three pre-selected serial dilutions (10^7, 10^6, 10^5) of the template. The intra- and inter-assay variability was expressed as % coefficient of variation (CV), which was calculated as a ratio of the standard deviation over the mean Cq value. In addition, two serial dilutions (1:10 and 1:100) of CAdV-2 virus (NZ isolate 1990, P3) were included in the assay, in order to quantify the amount of CAdV-2 nucleic acid present in the infected cell lysates.

2.2.2: Results

2.2.2.1: Optimisation of primer concentration

The optimal primer concentration for the qPCR CAdV-2 assay was determined to be 0.5 µM, for both forward and reverse primers, as this combination of primers resulted in the lowest Cq with the same template (Figure 1). Furthermore, only one clear melting peak at 80.8 ˚C was observed, which indicated the absence of primer dimers or non-specific amplification. As expected, there was no amplification in negative and non-template controls (Figure 2).

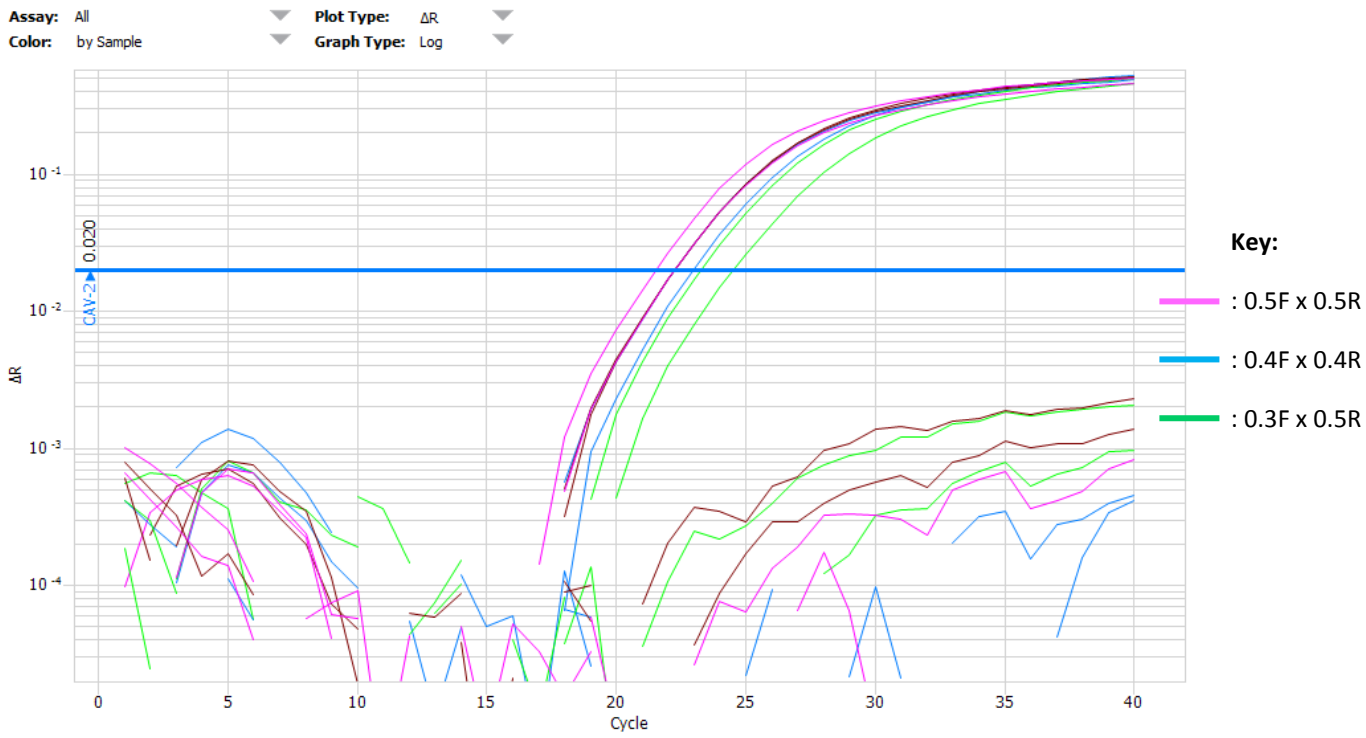

Figure 1: Comparison of three different primer concentrations of primers for optimisation of CaAdV-2 assay. Numbers in the legend indicate the final µM concentration of forward (F) and reverse (R) primers.

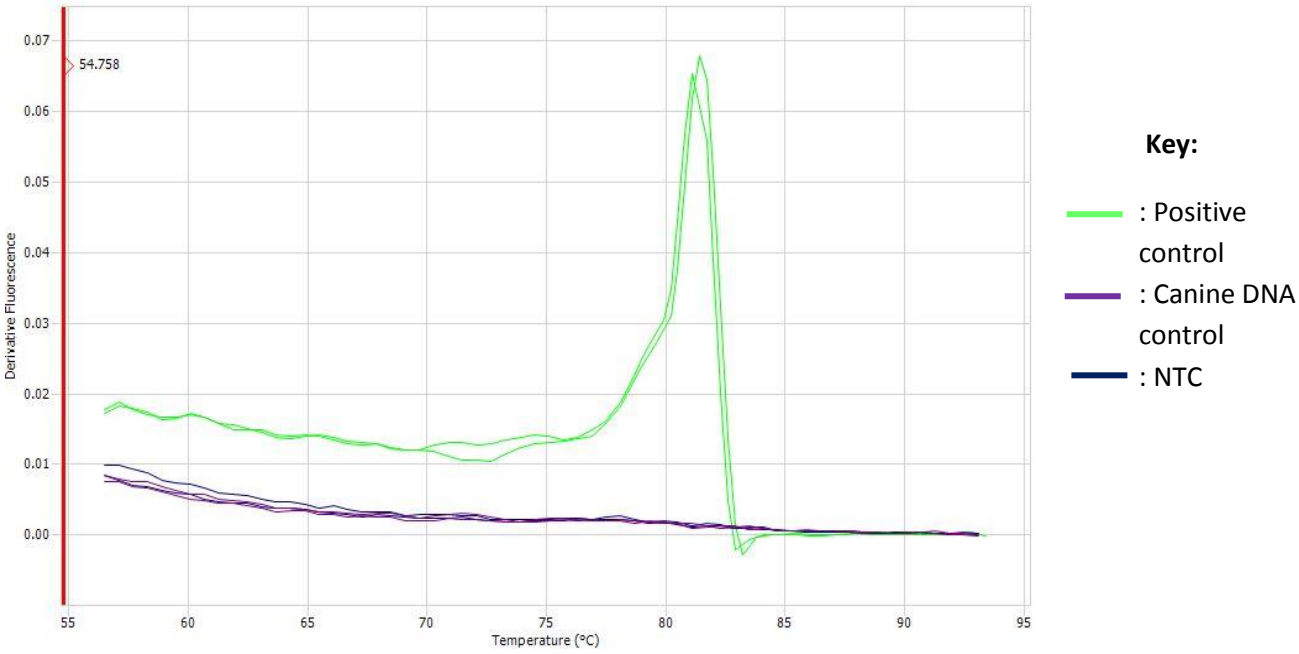

Figure 2: Meting curve for a positive control sample (CAdV-2 long PCR product) with a melting peak at 80.8°C. Negative (canine DNA) and non-template (water) controls were negative.

2.2.2.2: Optimisation of cycling conditions

Of the annealing temperatures tested, 62 ˚C gave the best assay efficiency of 92.41% (slope = -3.518) and an R^2 value of 0.992. Other temperatures tested (60˚C and 63˚C) both gave efficiency of less than 80% (see Appendix 3.1.1: CAdV-2 annealing temperature optimisation).

2.2.2.3: Performance of the assay

The LoD, as determined by the standard curve, was 10^2 copies of DNA/µL of template, with a Cq at approximately 35.5 cycles (Figure 3). The efficiency of the assay was between 90.04% and 92.41%, which was within the acceptable range (90-110%), and the R^2 value was above 0.99 for the assay. The intra- and inter-assay variability were also acceptable (Table 4).

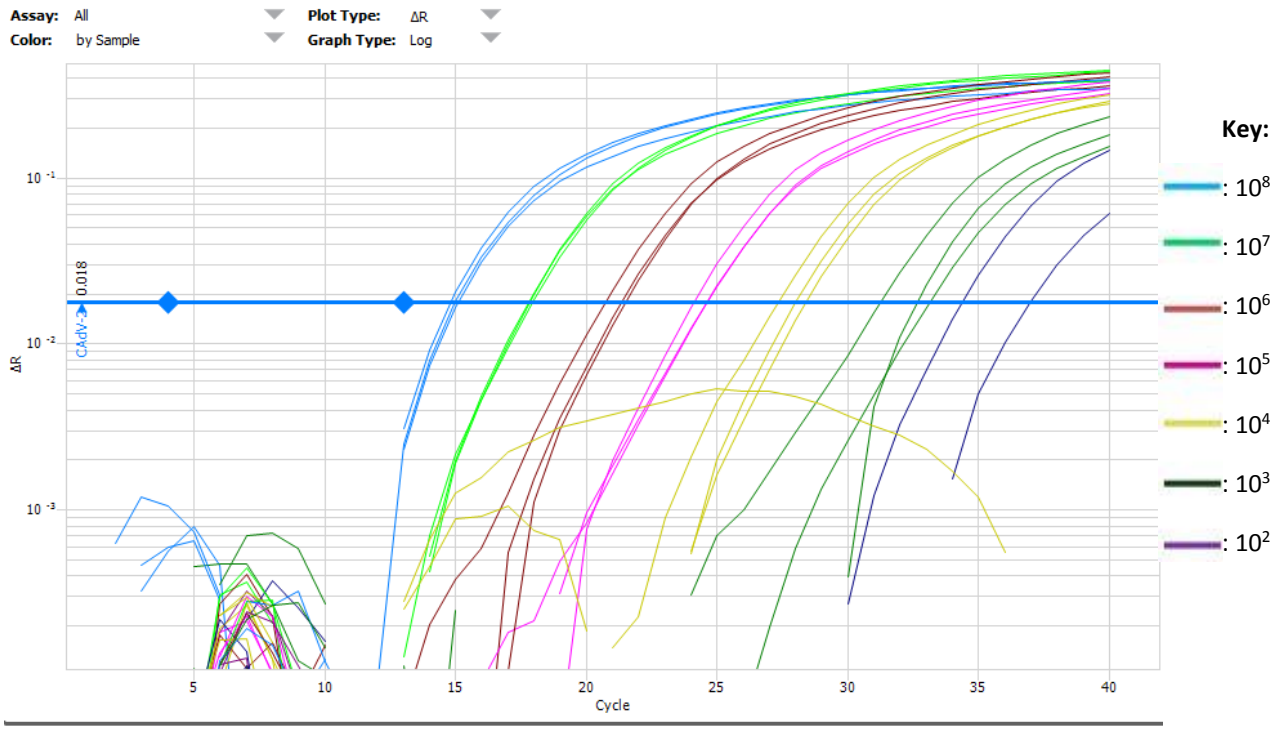

Figure 3: CAdV-2 limit of detection assay, showing the limit of detection at 10^2 copies of CAdV-2 DNA per µL of template.

Table 4: Intra- and inter- assay variation calculations for CAdV-2 qPCR

Variation	Dilution (copies DNA/reaction)	Mean	Standard deviation	CV (%)
Intra-assay	10^8	14.34	0.16	1.08
	10^7	17.50	0.09	0.50
	10^6	20.86	0.45	2.15
	10^5	24.09	0.27	1.14
	10^4	27.55	0.55	2.00
	10^3	31.91	0.96	3.02
	10^2	35.49	2.03	5.73

Table 4: Intra- and inter- assay variation calculations for CAdV-2 qPCR (cont.)

Variation	Dilution (copies DNA/reaction)	Mean	Standard Deviation	CV (%)
Inter-assay	10^7	18.16	0.64	3.52
	10^6	21.82	1.07	4.90
	10^5	25.44	1.32	5.18

The mean intra-assay coefficient of variation (CV) was 2.23 ± 1.75%, and the mean inter-assay CV was 4.53 ± 0.89%. A 1:10 dilution of the virus (containing approximately 4 HA units) was determined to correspond to approximately 7.87^6 copies of viral DNA/µL of template.

2.3: Canine Herpesvirus (CHV)

2.3.1 : Methodology

2.3.1.1: Preparation of the positive control

CHV virus (NZ isolate, 1997 of unknown passage) was thawed at room temperature and inoculated into MDCK cells, which were grown as described in paragraph 2.2.1.1. The virus was then passaged a total of four times. Nucleic acids were extracted from the 4th passage of the virus using High Pure Viral Nucleic Acid Extraction Kit (Roche) and used as a template in conventional PCR. Each 20 µL reaction consisted of 0.8 µM of each primer (Table 5) and 2 µL of template DNA in 1 x HotFirePol buffer (Solis BioDyne).

Table 5: CHV real time PCR primer properties

Primer	Primer Sequence	Product size	Target region (Gen Bank: KJ946357)	Reference
CHV-Forward	5'-ACAGAGTTGATTGATAGAAGAGGTATG-3'	136bp	Glycoprotein B	(Decaro *et al.* 2010)
CHV-Reverse	5'-CTGGTGTATTAAACTTTGAAGGCTTTA-3'			

The amplified product was subjected to electrophoresis through a 2% ethidium bromide-stained agarose gel, and the band of interest was purified using DNA gel extraction spin columns (Quantum prep™) as described in paragraph 2.2.1.2. The gel-purified PCR product was then cloned using the TOPO TA®cloning kit with TOP10 chemically competent cells (Life technologies), according to the manufacturer's instructions. Recombinant *E. coli* colonies were screened for the presence of CHV DNA by PCR. Plasmid containing CHV insert was purified from overnight *E. coli* cultures of selected colonies using High Pure Plasmid purification Kit (Roche) according to the manufacturer's instructions, and submitted for sequencing to Massey Genome centre. The presence of CHV insert was confirmed by BLAST analysis of the obtained sequence.

2.3.1.2: Optimisation of the qPCR conditions

2.3.1.2.1: Optimisation of the primer conditions

The CHV primers (Table 6) were tested over concentrations ranging from 0.1 µM to 0.5 µM, using the following reaction mixture: 0.5 µL of each primer, 5 µL of PerfeCTa SYBR Green Fast mix® (Quanta Biosciences), 2 µL of distilled water and 2 µL of template DNA. The initial qPCR protocol used is outlined in Table 3. The concentration of primers used in combination that resulted in the earliest detection (lowest Cq) of the specific target without any adverse effects to the assay (such as non-specific amplification) was selected for further experiments.

2.3.1.2.2: Optimisation of the cycling conditions

Serial dilutions of the target (gel-purified CHV PCR product) were tested over two annealing temperatures of 60 and 62˚C, using the 2-step protocol (Table 3). In addition, 3-step protocol was carried out, which consisted of the following: 5 minutes at 95˚C polymerase activation, followed by 40 cycles of 1 second at 95°C, 5 seconds at 60°C and 15 seconds at 72 degrees. A NTC (water) was included in duplicate in each run. The best cycling conditions were determined as those that gave the optimal assay efficiency and R^2 value.

2.3.1.3: Generation of the standard curve

The standard curve was prepared using 10-fold serial dilutions of the CHV plasmid (2.3.1.1) ranging from 10^9 to 10^4 copies per reaction. The dilutions were prepared based on DNA copy number, which was calculated based on the Nanodrop nucleic acid quantification of the stock CHV plasmid, using an on-line DNA copy number calculator (ThermoFisher 2016).

Serial dilutions were freshly made for each run and stored at 4°C for up to 2 hours until required.

2.3.1.4: Performance of the optimised assay

The performance of the assay was assessed by its linearity, which was determined by the assay efficiency, as well the R^2 values obtained from the analysis of the standard curve.

2.3.2: Results

2.3.2.1: Preparation of the positive control

Figure 4 below shows the presence of CHV DNA in recombinant E.coli colonies, shown by a band at the expected product size of 136bp, which was observed in all 10 colonies subjected to PCR.

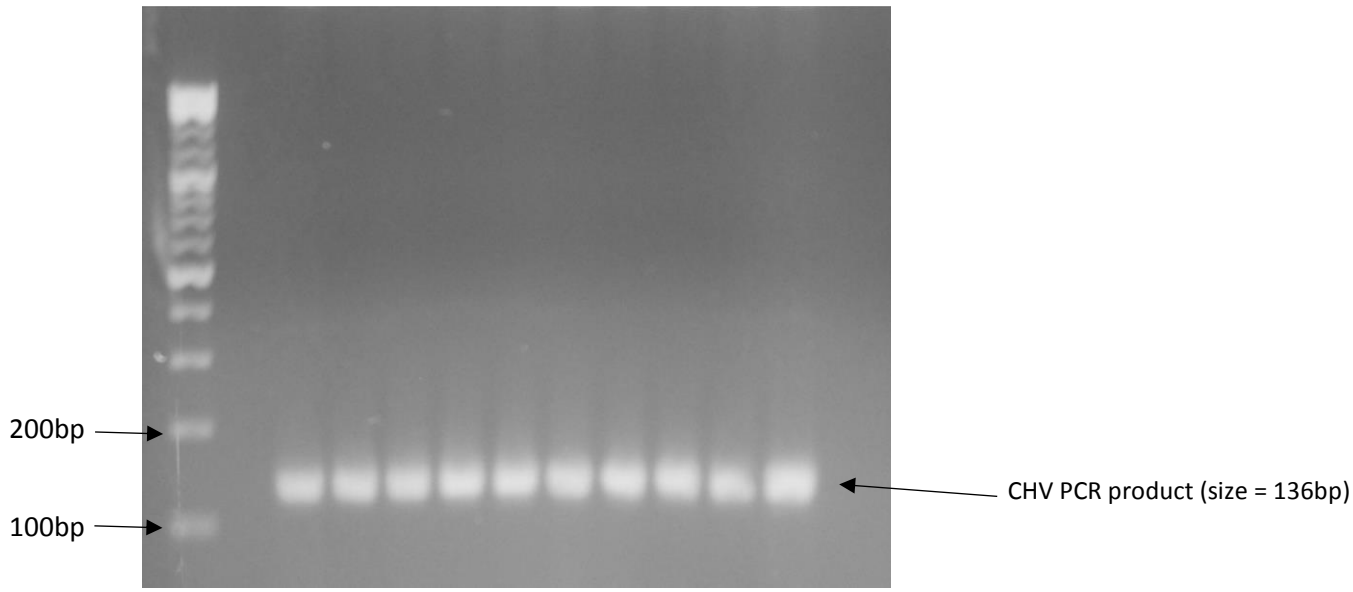

Figure 4: Canine herpesvirus PCR product was cloned into TOP10 chemically competent cells. Ten colonies were screened for the presence of the insert of interest by PCR with (CHV primers). The products were subjected to electrophoresis through a 2% ethidium bromide stained agarose gel. The expected 136 bp product was observed in all 10 colonies.

2.3.2.2: Optimisation of primer concentration

The optimum primer concentration was determined as 0.5 µM for both the forward and reverse primers, as at this concentration, the same template was detected at the lowest Cq value (Figure 5).

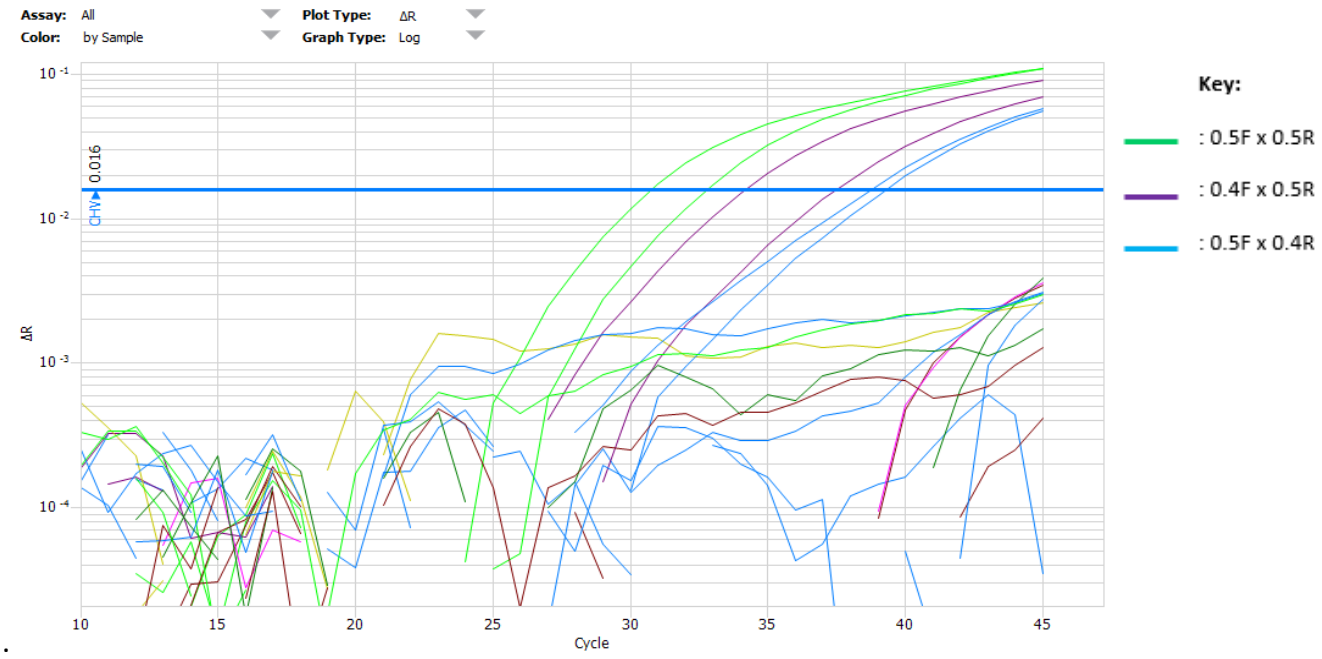

Figure 5: CHV primers tested at various concentrations (0.5, 0.4 and 0.3µM). A forward and reverse primer at 0.5µM concentrations were observed to have the lowest Cq value.

2.3.2.3: Optimisation of cycling conditions

The annealing temperature that gave the best efficiency and R^2 value was 62°C (Figure 6), using a two-step protocol.

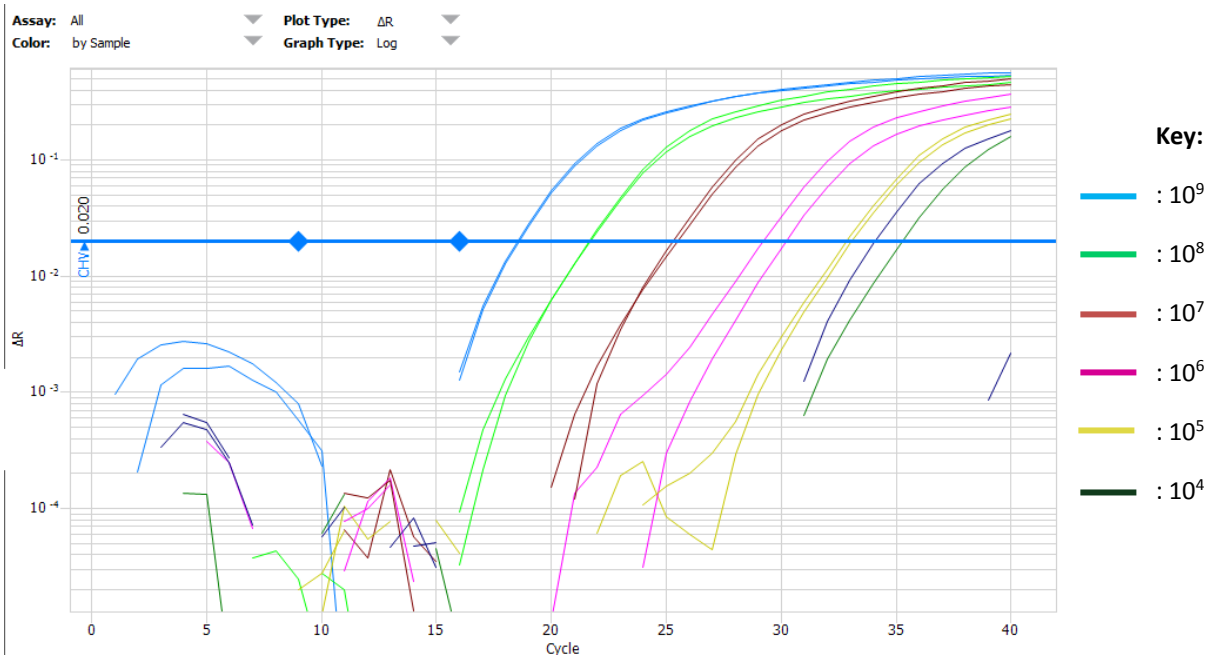

Figure 6: CHV standard curve run over 6 serial dilutions (target DNA copies/µL of template), with an annealing temperature of 62°C.

2.3.2.4: Performance of the assay

The efficiency of the assay outlined in Figure 8 was 90.14% (slope -3.583), and the R^2 value was 0.993. One NTC control was negative; but the other NTC control had a CT value of 33. However, the amplified NTC had a double melting peak, hence it was likely to be the result of non-specific amplification, rather than cross contamination (Figure 7).

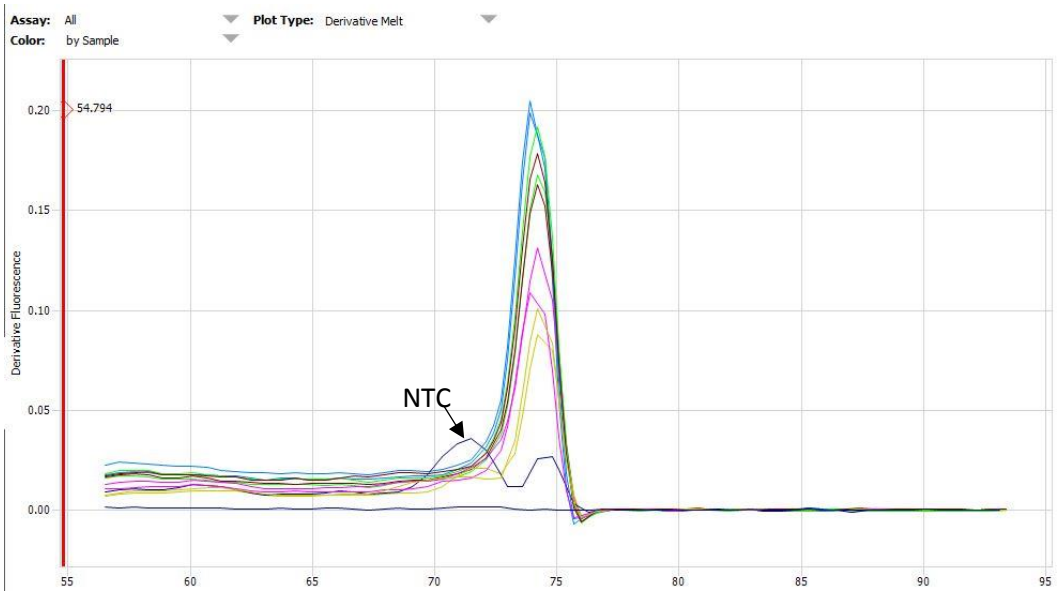

Figure 7: Derivative melt for CHV standard curve, showing a single melt peak at 73.9 for different dilutions of the positive control, and a double melt peak for the NTC.

2.4: Canine Parainfluenza (CPIV)

2.4.1: Methodology

2.4.1.1: Preparation of a positive control

Nobivac®KC intranasal vaccine, containing live, attenuated, freeze-dried *B. bronchiseptica* and canine parainfluenza virus was reconstituted according to the manufacturer's instruction and stored at -80 °C. The reconstituted vaccine was thawed at room temperature,filtered through a 45 µM syringe filter, and inoculated into MDCK cells, which were grown as described in paragraph 2.2.1.1. The virus was blindly passaged 4 times as described in section 2.2.1.2 for CAdV-2. There was no CPE observed in any of the passages, consistent with growth characteristics of CPI- strains. Nucleic acid was extracted from the 4th passage cell lysates using the High Pure Viral Nucleic Acid Extraction Kit (Roche). Complementary DNA was then prepared by adding 2 µL of qSCRIPT^{TM} cDNA SuperMix (Quanta Biosciences) to 8 µL of extracted nucleic acid and incubating the mixture using the thermal profile in Table 6. The 884 bp product was amplified using CPIV cDNA as a template in conventional PCR with long range primers (Table 7).

The primers were designed using Primer 3 within the Geneious™ software (Kearse *et al.* 2013). The expected PCR product was further analysed for the absence of predicted secondary structures within primer binding sites using mfold software (http://unafold.rna.albany.edu/?q=mfold). The band of interest was gel-purified as described for CAdV-2 (section 2.2.1.2). The identity of the purified nucleic acid was confirmed by sequencing and BLAST analysis.

Table 6: Incubation protocol for preparation of CPIV cDNA

Temperature	Time
25°C	5 mins
42°C	30 mins
85C	5 mins
4^0C	Hold

Table 7: CPIV long range primer properties

Primer	Primer Sequence	Product size	Target region (Genbank: EF543648)	Position
CPIV_L-Forward	5' –CTGCAACCGGAGGCTCGACG-3'	884bp	Nucleocapsid protein	782-1665
CPIV_L-Reverse	5'- CCCAGTGCGGCATTCAGGTCA-3'			

2.4.1.2: Optimisation of the qPCR conditions

2.4.1.2.1: Optimisation of the primer concentration

The CPIV primers (Table 8) were tested over concentrations ranging from 0.1 µM to 0.5 µM using the reaction mixture outlined in Table 1 of section 2.2.1.2 and the initial qPCR protocol outlined in Table 3. The primer combination that resulted in earliest detection (lowest Cq) of target nucleic acid without any adverse effects to the assay (such as non-specific amplification) was selected for further experiments.

Table 8: CPIV primer properties

Primer	Primer sequence	Product size	Target region (GenBank: EF543648)	Position
CPIV-Forward:	5'-AGGGTGCAGTTGACATGAGG-3'	136bp	Nucleocapsid	1269-1404
CPIV-Reverse	5'- GAGAACGGGTTGACTCCTCC-3'			

2.4.1.2.2: Optimisation of the annealing condition

An annealing temperature gradient using conventional PCR was carried out using protocol outlined in Table 9, with annealing temperatures ranging from 55 to 69 degrees. The annealing temperature gradient was run with a low concentration (0.1 µM), and a high concentration (0.4 µM) of primers. The best annealing temperature was determined as one that resulted in detection of CPIV cDNA with minimal or no non-specific amplification.

Table 9: Protocol for CPIV annealing temperature gradient, run using a conventional PCR machine (PX2 Thermal Cycler (Thermo Electron Corporation)).

Temperature	Time	
95°C	5min	
95°C	1 sec	} X40 cycles
55-69°C	30sec	

2.4.1.2: Generation of the standard curve

The standard curve was prepared using 10-fold serial dilutions of the 884 bp CPIV PCR product (section 2.4.1.1). The DNA copy number in the undiluted PCR product was calculated based on the Nanodrop nucleic acid quantification using an on-line calculator (ThermoFisher 2016).The dilutions covered a range of three orders of magnitude, ranging from 10^9 to 10^4 copies of CPIV PCR product per µL. Once the dilutions were prepared, they were stored at 4 °C for up to 2 hours until required.

2.4.1.4: Performance of the assay

The performance of the optimised assay was assessed by determination of its linearity and precision. Linearity and precision were determined by the assay efficiency, as well the R^2 values obtained from the analysis of the standard curve.

2.4.2: Results

2.4.2.1: Optimisation of the primer concentration

During the initial optimisation of the primer concentration non-specific amplification products were present in all reactions including positive and non-template controls. Hence, an annealing temperature gradient (section 2.4.1.2.2) was run with a low (0.1 µM) and high primer concentrations (0.4 µM) using a CPIV-positive template in a conventional PCR. CPIV template was not detected in reactions with 0.1 µM of each primer at high annealing temperatures (≥65 °C). At lower annealing temperatures specific 136 bp CPIV product was present, but so were primer dimers (Figure 8). Conversely, CPIV template was detected in a reaction with 0.4 µM of each primer at temperatures above 65 °C with minimal primer dimer formation, and with no non-specific amplification products (data not shown). Hence, a 0.4 µM primer concentration was used in subsequent qPCR assays.

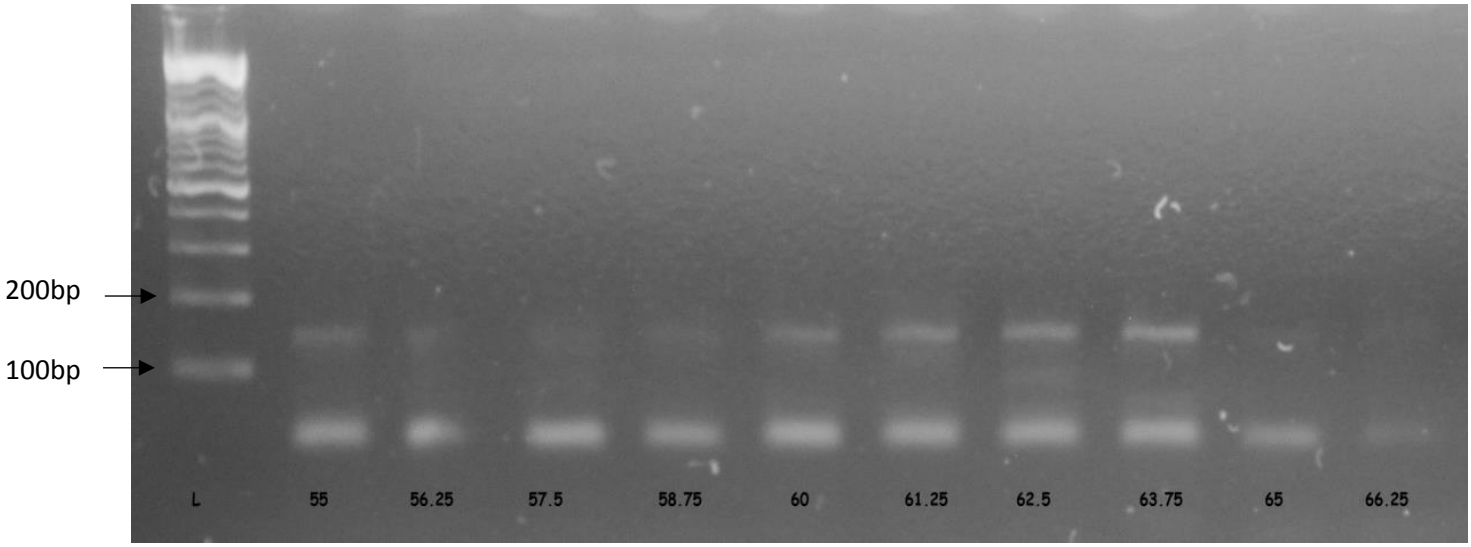

Figure 8: 2% Agarose gel showing the CPIV PCR products amplified with 0.1 µM primers using various annealing temperatures, as indicated. L: Molecular weight ladder (O'GeneRuler™ DNA Ladder Mix, Ready-to-use, 100-10,000bp, Thermo Fisher Scientific). Expected band size was 136 bp. The cleanest and strongest specific band was observed at 63.75 °C, although a second band of lower molecular weight most likely representing primer dimers was also present.

2.4.2.2: Optimisation of the CPIV qPCR cycling conditions

The 67 °C was determined as the optimum annealing temperature. At this temperature, target CPIV cDNA was detected with minimal primer dimer formation or non-specific

amplification. The following protocol was used for the generation of the standard curve: 5 minutes at 95 °C polymerase activation, followed by 40 cycles of 1 second at 95 °C and 30 seconds at 67 °C, followed by the melt step from 55 °C to 95 °C.

2.4.2.2: Performance of the assay

Given the non-specific amplification observed above, efficiency of the assay was always outside of the accepted range (90-110%), as was the R^2 value. Hence, the assay was deemed unsuitable for diagnostic use in its current format.

2.5: Discussion

2.5.1: CAdV-2 qPCR IH assay

2.5.1.1: Problems encountered during optimisation

Overall, the CAdV-2 assay worked well, and few problems were encountered during optimisation. Ideally, the duplicates of the various primer concentrations would have been closer together (Figure 1). However, other factors (such as cycling conditions) had not been optimised at that point, which might have affected the performance of the assay. None-the-less, CAdV-2 DNA was detected in both duplicates of the reaction with 0.5 µM primers earlier than in reactions with primers at lower concentrations. Hence, it was reasonable to assume that 0.5 µM was the optimal primer concentration. The optimum annealing temperature for this assay was concluded to be 62˚C, since other temperatures did not give as good efficiency or linearity. It is possible that the reduction in efficiency and linearity observed at other annealing temperatures was due to inaccuracies in pipetting. However, at least 2-3 assays were run at each annealing temperature, and the inter-assay results were fairly consistent (see appendix 3.1.1: CAdV-2 annealing temperature optimisation). Hence, it is unlikely that the reduced efficiency / linearity of the assay at other annealing temperatures were due to pipetting errors.

2.5.1.2: Usefulness for research and diagnostic purposes

The focus for developing this assay was to determine its analytical sensitivity and specificity. Analytical sensitivity in this context refers to the lowest quantity of the target nucleic acid that can be detected by the assay, whereas specificity refers to the ability of the assay to detect only the target nucleic acid, in this case, CAdV-2 DNA. In comparison, diagnostic performance of the assay refers to the application of the assay to a population, and its ability to differentiate between diseased and healthy animals. For this assessment, another

well-established test (the gold standard) needs to be available for comparison of the results. High analytical sensitivity does not always correlate to high diagnostic sensitivity, since the way a sample is collected, handled, and processed can affect the presence or absence of target nucleic acid in the sample, and hence a test with high analytical sensitivity should not be automatically assumed to have similarly high diagnostic sensitivity (Saah and Hoover 1997).

For CAdV qPCR assay, diagnostic sensitivity and specificity were not calculated due to lack of access to a suitable number of known-positive and known-negative samples, as well as financial constraints of the study. The results obtained could have been confirmed by a number of tests such as virus isolation, haemagglutination, or another validated PCR-based test.

The analytical sensitivity of this assay was determined to be at least 10^2 copies of CAdV-2 nucleic acid per reaction, which was consistent with what has been reported previously (Segura *et al.* 2010). Furthermore, the efficiency (between 90.04% and 92.41%) and R^2 value ($\geq$0.99) of the standard curve indicated that the assay performed well and was linear over the range of target concentrations tested. The fact that no amplification was observed in the negative and non-template controls indicated that the assay would not cross-react with canine DNA. Both the intra- and inter-assay variation was below 5%, indicating that the assay was both reliable and reproducible.

2.5.2: CHV IH assay

2.5.2.1: Problems encountered

Like the CAdV-2 IH assay, there was variation between the duplicates of the various primer concentrations. However, it was clear that CHV DNA was detected earlier in the assay with 0.5 µM primers than in assays with other combination of primer concentrations. The main problem encountered during CHV qPCR optimisation was the presence of a double melting peak in one of the non-template controls. The peak at the lower temperature was likely to be the result of non-specific amplification, most likely primer dimerization. The second peak was close to the melting temperature of the CAdV-2 product, hence could be the result of very low-level cross contamination of the water template in that particular run. However,

the other non-template control showed no amplification and the efficiency and the R^2 value of the assay were both within an acceptable range.

2.5.2.2: Usefulness for research purposes

The purpose of the development of the CHV qPCR assay was to be able to distinguish between samples positive and negative for CHV. Both the efficiency and linearity of this assay were within an acceptable range, and the CHV positive control showed no non-specific amplification nor primer dimer formation. The initial experiments to optimize the assay were performed with limited number of dilutions of the known-positive template, which did not span below 10^4 copies/µL, hence the LoD for this assay was not established as part of this study. The experiments could not be repeated as the qPCR instrument used developed a fault. The standard curve was subsequently re-run using a different model of qPCR instrument (MIC, Bio Molecular Systems) and the assay showed LoD of 10 copies of target DNA per uL of template (data not shown).

2.5.3: CPIV qPCR IH assay

2.5.2.1: Problems encountered

Several problems were encountered during optimisation of this assay. Initially, the aim was to find the optimal primer concentration, then optimise the cycling conditions. However, it was clear from the primer optimisation assay that non–specific amplification was occurring in both the positive and NTCs, regardless of the primer concentration. Hence, a temperature gradient was run to try and identify a temperature at which non-specific amplification would be eliminated or at least minimised. Even at an annealing temperature at which non-specific products weren't visible on an agarose gel, primer-dimers still seemed to be produced as judged by the melting profiles of qPCR products. Non-specific amplification products were easily distinguished from the specific product based on the differences in their melting temperatures. Given the problems encountered with this assay, no further optimisation was carried out.

Another set of primers may have performed better. The primers used in this study were designed using Geneious software and met the criteria for well-designed primers as outlined in section 2.1. Minimal secondary structures were detected within the primer binding sites of the predicted PCR product by the Mfold software. However, other factors that affect primer performance, such as runs of repeated nucleotides, and 3' and 5' stability. For

example, there is a run of 3 repeated nucleotides in each of the forward and reverse primers, and repeated runs should be avoided since they can cause mis-priming (Thornton and Basu 2011). In addition, stability of the last 5 nucleotides of the 3' and 5' end are important since the 3' stability determines the specificity of binding to the template, and the 5' stability determines how strongly the primer sequence will be able to bind to the template (Thornton and Basu 2011). Hence, if the primers were to be re-designed, perhaps more consideration could be given to repeats and stability of the 3'and 5' ends, as these factors could have influenced the performance of the CPIV primers designed for this study.

2.6: Conclusion

Three qPCR assays have been developed for further use in the study. Two of the assays (CAdV-2 and CHV) seemed to perform well enough to be used diagnostically in the survey described in Chapter 3. The remaining one (CPIV) was deemed unsuitable for diagnostic use due to poor performance and therefore was not used in the further part of this study.

Chapter 3: Virological survey of viruses associated with infectious canine tracheobronchitis

3.1: Introduction

Acute ICT, commonly referred to as kennel cough by veterinarians and dog owners, affects a wide range of dogs, but especially those in boarding kennels, animal shelters, and those that normally live in a group environment, such as working farm dogs and greyhounds (Erles, 2004). A number of pathogens has been associated with ICT overseas, including several respiratory viruses such as CPIV, CAdV-2, CHV, CRCoV, and CIV. Canine influenza is exotic to New Zealand (Knesl *et al.* 2009), and currently, little is known regarding the prevalence of the remaining viruses in New Zealand.

Canine adenovirus type 2 was first isolated in New Zealand in 1998 from a greyhound, which presented with signs of tracheitis /tonsillitis (Tham *et al.* 1998). There have been no further reports of detection of CAdV-2 in New Zealand dogs. Canine adenovirus type 2 forms part of the core vaccination for puppies in many developed countries, including New Zealand (Day *et al.* 2010). The vaccine has shown to offer effective protection against CAdV-2 induced respiratory disease for up to 48 months (Mouzin *et al.* 2004). Hence, it is presumed that the majority of younger dogs would have some level of immunity against CAdV-2 induced respiratory disease.

Canine herpesvirus was first isolated in New Zealand in 1977, from a puppy with fatal necrotising and haemorrhagic disease (Horner 1977). Like CAdV-2, there have been no further reports of CHV in New Zealand dogs. It is therefore unknown whether or not this virus is associated with ICT in New Zealand. There is currently an inactivated subunit vaccine against CHV available in Europe, but this vaccine is not available in New Zealand (Decaro *et al.* 2008). The vaccine is labelled for use in pregnant bitches, with the aim of prevention of fatal necrotising and hemorrhagic disease due to CHV infection in their puppies, and not for the prevention of ICT.

A study examining the seroprevalence of CRCoV and CIV in New Zealand was carried out in 2009 (Knesl *et al.* 2009). In total, 29% of 251 dogs of unknown health status were positive for CRCoV antibodies, and none was positive for antibodies against CIV. However, there are

no reports of detection of CRCoV in New Zealand. There is currently no vaccine available for CRCoV. A killed adjuvanted vaccine is available against CIV in the US (Dubovi 2010), but is not licensed in New Zealand.

There are no reports of CPIV in New Zealand dogs, although the virus has been isolated in Australia from dogs as part of investigations of outbreaks of ICT (McGavin *et al.* 1989). There is currently an intranasal vaccine available in New Zealand against CPIV, but it does not form part of the core vaccination, hence protection against CPIV infection may not be as widespread as protection against CAdV-2 infection among New Zealand dogs.

Given the lack of information regarding the prevalence of pathogens involved in ICT in New Zealand, the aim of the current study was to determine the frequency of detection of six canine respiratory viruses from selected populations of healthy and ICT-affected dogs. The rationale of this research was to gain a better understanding of which pathogens are commonly involved in ICT in New Zealand, so that appropriate prevention measures could be recommended.

3.2: Materials and methods

3.2.1: Animals

Samples were collected from three groups of dogs: racing greyhounds, pet dogs and working farm dogs. A total of 96 dogs were sampled, including 47 healthy and 49 diseased dogs. Two types of samples were collected: Oropharyngeal swab samples for PCR and blood samples for serological testing for CRCoV antibody. Oropharyngeal samples were collected for all dogs except two diseased dogs – RG36 and RG37, for which only blood could be collected. Hence, for both IDEXX and IH PCR, 47 diseased and 47 healthy dogs (total of 94) were tested. Blood samples were not collected for one healthy dog – P12, and two diseased dogs – P57 and 59. Hence, 47 diseased and 46 healthy dogs (total of 93) were tested for CRCoV antibody.

Healthy dogs were defined as those with no clinical signs of ICT at the time of sampling, as assessed by either the attending veterinarian or their owners /trainers/handlers. Diseased dogs were defined as those which showed at least one of the following clinical signs commonly associated with ICT: repeated coughing or sneezing for longer than 24 hours, nasal or ocular discharge. Other clinical signs, such as fever, were also recorded, but were

only considered to be associated with ICT if one of the former clinical signs (coughing, sneezing, nasal and/or ocular discharge) was also observed.

For racing greyhounds, a total of 41 samples (all from diseased dogs) were collected by veterinarians who were presented with the diseased dogs at their respective clinics. Racing greyhounds were defined as those that were in race training around the time of sampling and were intended to be raced following the resolution of ICT. Re-homed or retired racing greyhounds were not included in the study population. Samples were collected from various regions across New Zealand, including Whanganui (*n*=30), Hautapu (*n*=5) and Christchurch (*n*=6).

For pet dogs, 6 samples from diseased dogs and 20 samples from healthy dogs were collected by veterinarians at various clinics across the North Island who had previously agreed to take part in the study. Healthy dogs were selected at random and included those that were brought into the clinic for routine preventative procedures, such as dental work or neutering. Samples from diseased pet dogs were collected opportunistically. Most of the healthy dogs sampled (*n*= 17) were from the Palmerton North area, and the remaining healthy pet dogs were sampled in Auckland (*n*= 3). The diseased dogs were from Auckland (*n*= 1) and Cambridge (*n*= 5).

Sampling of the working farm dogs was performed in conjunction with a separate, unrelated, study. As part of that study, farmers were called from a database of 469 farmers from around the Manawatu region (within 1.5 hours driving distance of Palmerston North). In total, 38 farms were selected for sampling. Any farms that only had one working farm dog, or dogs that were not currently working, were excluded from the study population. In addition, farms that did not feed raw meat (homekill) were also excluded from the sample population in accordance with the selection criteria for the other study. Two dogs were selected at random for sampling from each farm. Out of the total of 76 samples collected, 27 samples were randomly chosen for processing and inclusion in the current survey. All farm dogs were considered “healthy” for this study.

The study was approved by the Massey University Animal Ethics Committee (protocol number 12/35).

3.2.2: Sampling

3.2.2.1: Collection of samples

The dogs were sampled from July 2012 to August 2013. The following samples were collected from each dog: a coagulated blood sample for serology and an oropharyngeal swab for virology. For diseased dogs, in addition to an acute blood sample, a convalescent blood sample was also collected 3-4 weeks later.

Whole blood was collected into 10 mL BD vacutainer® tubes by jugular venipunture. Orophangeal swabs were collected by rubbing the orophangeal area with a mini flock swab 80 mm in length (Copan®) for a few seconds. The swab was replaced into 1 mL of Copan® universal transport medium (UTM), which supports the viability of both viruses and mycoplasma, but not bacteria.

Questionnaires were completed for racing greyhounds (Appendix 1.1), pet dogs (Appendix 1.2) and working farm dogs (Appendix 1.3) irrespective of whether the dogs were healthy or diseased. If the dog was showing clinical signs associated with ICT, an additional questionnaire (Appendix 1.4) was completed by the attending veterinarian, which focused on clinical presentation.

For racing greyhounds and pet dogs, the questionnaire included questions about the signalment, reason for presenting to the clinic, vaccination status, diet, housing type, animals the dog was in contact with, time spent in kennels or rescue shelter recently, whether the dog had attended any shows recently (pet dogs only), and any relevant history of respiratory illness.

For farm dogs, the investigator completed a questionnaire which enquired about the age, sex, number of dogs at property, history of respiratory illness, contact with other animals, and diet (such as whether the dog was fed unprocessed meat).

In addition, all questionnaires outlined above enquired about the vaccination status of the dog. Dogs were considered to be vaccinated for the “core” vaccination if they were vaccinated with Vanguard 5 / Vanguard plus 5 (which includes CAV-2 and CPIV antigens) and considered to be vaccinated for “kennel cough” if they also received Nobivac KC (which

includes CPIV and *B. bronchiseptica* antigens) or equivalent, since this meant the dogs were vaccinated for *B. bronchiseptica*, CPIV and CAdV-2 as opposed to just CPIV and CAdV-2.

3.2.3: Processing of samples

Samples from working farm dogs (both blood and virological swabs) were transported on ice packs to the laboratory within 5 hours of collection. Samples collected from veterinary clinics (pet dogs and greyhounds) were couriered overnight on ice packs.

On arrival, samples were kept at 2-8 °C and processed within 5 hours. Blood was centrifuged at 1,500 x g for 10 minutes to separate the serum and packed red blood cells (RBC). The serum was then transferred into 5 mL sterile universal containers and stored at -20 °C until required for serological tests.

Virological swabs in UTM were vortexed for at least 1 minute to dislodge any virus attached to the swab. Transport medium was then transferred into two tubes: a 200 µL aliquot was placed into a sterile microtube to be sent to IDEXX laboratories for qPCR testing, and the remaining volume (approximately 700 µL) was transferred into a cryotube. Both samples were stored at -80 °C until required.

3.2.4: Virus Isolation

3.2.4.1: Cell culture:

Madin Darby canine kidney cells were maintained as described in Chapter 2 (paragraph 2.2.1.1).

3.2.4.2: Virus isolation procedure

Swab samples (processed as described in 3.2.3) were thawed at room temperature. Each sample was then transferred to a sterile microtube and centrifuged at 10,000 g for 10 minutes. The supernatant was then filtered through a 0.45 µm syringe filter (Corning®) into another sterile 1.5 mL microtube. Samples were kept on ice during the procedure.

Virus isolation was performed in 24-well tissue culture plates (Nunc). An aliquot (100 µL) of the filtered swab sample was added to a pre-assigned well containing 1 mL ($2x10^5$ cells) of MDCK cells at the time of seeding. The remaining filtered sample was placed back in a -80°C freezer for storage.

The last well of each plate was left as a negative control and contained uninfected MDCK cells. A control plate with four negative and four positive control wells were also set up at the time of inoculation. Canine adenovirus type 2 (CAdV-2) (New Zealand field isolate, 1990, unknown passage) was used as a positive control.

Plates were then placed in a humidified 37 °C incubator at 5% CO_2. Each plate was checked daily for the presence of CPE up until day 6 post inoculation, at which point the plate was frozen at -80 °C.

3.2.4.3: Passage

After the initial inoculation (P1), each sample was passaged another two times (P2 and P3). Each time, the plates were frozen at -80 °C then thawed at room temperature. The cell lysate from each well was then transferred to a microtube, centrifuged, filtered and re-inoculated into MDCK cells as described above in section 3.2.4.3.

3.2.4.4: Interpretation of results

Samples were considered negative for CPE- producing viruses (CAdV-2, some CPIV isolates, CHV, and CIV) if no CPE were observed after three passages. Samples were considered positive if viral CPE was observed during any of the passages. In addition, cell lysates from the third passage were checked for haemaglutinating activity to detect the growth of non-CPE producing CPIV (see section 3.2.5).

3.2.4.5: Validation of results

All negative controls were expected to show healthy confluent monolayers with no CPE. Positive controls were expected to show extensive CPE (including rounded cells, stranding of cytoplasm, and some clumping of cells) within 2-3 days post inoculation.

3.2.5: Haemagglutination assay (HA) for CPIV

3.2.5.1: Preparation of pig RBC

The blood was collected in 10 mL vaccutainer tubes containing 5 mL of Alsevier's solution. Three tubes of porcine whole blood were processed for each preparation. Red blood cells were washed in PBS before use. Blood in Alsevier's solution was divided between two 50 mL centrifuge tubes (Falcon). The tubes were then filled with PBS and centrifuged for 10 minutes at 1,000 x g. The supernatant was carefully discarded, and fresh PBS added to the cell pellet. The cells were gently mixed and pelleted again as above. The wash process was

repeated three to four times, or until the supernatant was clear. To prepare the 1% solution, 2 mL of packed RBC was added to 198 mL of PBS.

3.2.5.2: Preparation of HA plate

Cell lysates from the third passage were thawed at room temperature. Initially, the samples were screened at three dilutions for the presence of haemagglutinating viruses. Cell lysates (60 µL) were added in duplicate to columns 1, 4, 7, and 10 of one 96- well V-shaped plate (Nunc). Ninety µL of 0.9% saline (pH 7.0) was then added to columns 2, 5, 8 and 11, and 50 µL of saline to columns 3, 6, 9, and 12. Two dilutions were made for each sample (Table 10), by transferring 10 µL of the undiluted (neat) cell lysate to the next column containing 90 µL of saline (1:10 dilution), mixing the content, then transferring 50 µL to the next column (1:20 dilution), with the last 50 µL discarded.

Table 10: Plate layout for HA test plate

	1	2	3	4	5	6	7	8	9	10	11	12
	Neat	1:10	1:20	Neat	1:10	1:20	Neat	1:10	1:20	Neat	1:10	1:20
A	S1			S2			S3			S4		
B	S1			S2			S3			S4		
C	S5			S6			S7			S8		
D	S5			S6			S7			S8		
E	S9			S10			S11			S12		
F	S9			S10			S11			S12		
G	S13			S14			S15			S16		
H	S13			S14			S15			S16		

Control plate included a titration (in triplicate) of a cell culture isolate of CPIV (ex Nobivac vaccine, P3, with an expected HA titre of 160) and 3 rows of negative RBC control. Control virus (100 µL) was added to the top 3 wells in column 1, and 50 µL of saline was added to all

other wells in rows A through C. Serial two-fold dilutions of CPIV were prepared by transferring 50 µL volumes across the plate from column 1 through to column 12. The final 50 µL of the last dilution was discarded. For negative RBC control, 50 µL of saline was added to each well in rows D – F.

Red blood cells (50 µL of a 1% solution) were then added to each well on the test and control plates. The content of each well was mixed briefly using a plate shaker, and the plates were incubated at room temperature until controls showed the expected results (clear button of RBC for negative wells and haemagglutination in the first four dilutions of the control CPIV), typically for 1-2 hours.

3.2.5.3: Validation of results

Results were valid if control CPIV showed a titre within 1 dilution of the expected titre, and there was no haemagglutination in RBC control wells.

3.2.5.4: Interpretation of results

A sample that showed no haemagglutination of RBC was considered negative for haemagglutinating viruses. A sample that showed haemagglutination of RBC at any dilution in the screening test was considered positive for the presence of haemagglutinating viruses. All positive samples were confirmed by re-testing as described above, with the exception that the samples were serially diluted (doubling dilutions from neat to 1:2048) across the entire plate.

3.2.6: Real time (q) PCR

3.2.6.1: IDEXX panel

Aliquots (200 µL) of each swab sample (section 3.2.3) were packaged on ice packs and sent to IDEXX diagnostic laboratory in Queensland, Australia for the IDEXX RealPCR™ Canine Respiratory Disease (CRD) Panel. Nucleic acids were extracted from the samples by IDEXX laboratories and each sample was tested for CPIV, CAdV-2, CHV, CRCoV, CIV, CDV, *Bordetella bronchiseptica*, *Streptococcus equi* subsp. *zooepidemicus,* and *Mycoplasma cynos.*

3.2.6.2: In house qPCR

3.2.6.2.1: Nucleic acid extraction

Nucleic acids were extracted from virological swab samples using the High Pure Viral Nucleic acid extraction kit (Roche) according to the manufacturer's instructions. The extraction

procedure required 200 µL of sample, but in some cases less than 200 µL of swab sample was available. For these samples, the deficit was made up by adding more UTM to the sample. Nucleic acids were eluted with 50 µL of elution buffer supplied with the kit. A 20 µL aliquot was used for cDNA preparation and qPCR for DNA viruses (CAdV-2 and CHV), and the remaining volume (~28 µL) of eluted nucleic acid was placed in a -80 °C freezer.

3.2.6.2.2 cDNA synthesis

Synthesis of cDNA was performed by adding 2 µL of qSCRIPT™ cDNA SuperMix to 8 µL of extracted nucleic acids from each sample. Each reaction was performed using the protocol recommended by the manufacturer (Table 11).

Table 11: Incubation protocol for preparation of cDNA using qSCRIPT cDNA SuperMix.

Temperature	Time
25°C	5 minutes
42°C	30 minutes
85°C	5 minutes
4°C	Hold

Complementary DNA was not used in the current study, but stored at -20 °C for future testing.

3.2.6.2.3: qPCR set up

Reactions were set up in the same way for both assays (CAdV and CHV). The master mix was prepared for 48 reactions (Table 12).

Table 12: Reagents used for qPCR master mix

Reagent	1x Mixture	49x (Master Mix)
PerfeCTa SYBR Green Fast mix	5 µL	245 µL
Forward primer (20 µM)	0.5 µL (0.5 µM)	24.5 µL
Reverse primer (20 µM)	0.5 µL (0.5 µM)	24.5 µL
H_2O	2 µL	98 µL

An aliquot (8 µL) of reaction mix was pipetted into each well of a 48-well PCR plate (Illumina™). Template nucleic acid (DNA or cDNA) was vortexed and pop-spinned for 3-5

seconds, then 2 µL was added to each well in duplicate. The standard curve was included in the first run of each assay (see Chapter 2 for details). In subsequent runs, only 3 selected dilutions of the standard for each virus (in duplicate) were included.

Cycling protocols for CAdV-2 and CHV are outlined in Table 13 below.

Table 13: CAdV-2, CHV and CPIV cycling protocol.

Temperature	Time	
95 °C	5 minutes	
95 °C	1 seconds	X45 cycles
62 °C	30 seconds	
95 °C	15 seconds	Melt cycle
55 °C	15 seconds	
95 °C	15 seconds	

3.2.6.2.4: Interpretation of the results

For both CAdV-2 and CHV qPCR, the results were valid if the following conditions were met: The standard curve fell within 90-110% efficiency, had a ≥0.99 R^2 value, and all NTC showed no specific amplification. Samples were considered positive if amplification occurred and the product showed a melting peak within 1 degree of the melting peak of the positive controls.

3.2.7: Enzyme linked immunosorbent assay (ELISA) for canine respiratory coronavirus antibody

For this assay, a competitive ELISA kit (Bio X diagnostics) for detection of bovine coronavirus (BoCV) antibodies was used, since antibodies raised against CRCoV are cross reactive with bovine coronavirus antigen (Erles and Brownlie 2008). The kit included a 96 well plate that had been sensitised by a monoclonal antibody specific for BCoV, followed by the addition of a BCoV culture. Since the ELISA had a competitive format, if CRCoV antibodies were present in the serum, these would bind to the BoCV attached to the well. Conjugate was then added, that comprised a specific monoclonal antibody against BCoV coupled to horseradish peroxidise. If cross reactive CRCoV antibodies had already bound to the BCoV antigen, then the conjugate would not bind. Hence, an absence of colour (after the addition of chromogen) indicated a positive result, whereas a colour change indicated that the sample was negative for CRCoV antibodies. For each sample, an initial optical density (OD) value

was read at 450 nm, which was then converted to a percentage of inhibition (POI) according to the following formula: POI = [(OD neg – OD sample)/OD neg]*100. The POI was a measure of relative levels of CRCoV antibodies bound to the BCoV antigen, which in turn inhibited the binding of the conjugate. Any sample that had a POI levels greater than 20% was considered positive for CRCoV antibody. The degree of postivity was defined according to the manufacturer's recommendations as follows: 1(+): (20-40%), 2 (++): (40-60%), 3(+++): (60-80%) and 4(++++) (>80%).

3.2.8: Statistical analyses

A database was set up in Microsoft Excel. For each dog, the age, sex, vaccination status and the results of each test were recorded (appendix 4.3 and 4.4). Data were analysed using Minitab software (version 17) and GraphPad Prism version 5.04 (San Diego California USA, www.graphpad.com). Data was assessed for its normal distribution by plotting a normal probability plot. Measurement variables for healthy and diseased dogs were compared using a two–sided t-test. Either Mann-Whitney U test or Kruskal Wallis test was applied to non-normal data. Nominal variables were compared using Fisher's exact test. Correlation between two measurement variables was calculated using Pearson correlation test, and correlation between disease status and nominal variables was expressed as relative risk (RR) with 95% CI. Significance was set at $p<0.05$.

Agreement between the IDEXX and IH qPCR results was assessed using the Cohen's Kappa coefficient.

3.3: Results

3.3.1: Data distribution

3.3.1.1: Age

Overall, the mean age of all sampled dogs was 3.99 years (range <0.5-17 years). The puppies for which the recorded age was <0.5 years were considered to be 0.3 years of age for the purpose of the analysis. The data was found to be not normally distributed (Figure 9 and 10), hence the p value was determined using the Mann-Whitney U test. The mean age of diseased dogs (2.68 years, range <0.5 to 8.5) was significantly ($p=0.01778$, $\alpha=0.05$) lower than the mean age of healthy dogs (5.31 years, range <0.5 to 17). This was due to the fact that diseased dogs sampled consisted predominately (41/47) of racing greyhounds. Hence,

the range of ages for the diseased dogs was narrower, compared to that of healthy dogs (which comprised pet and working farm dogs).

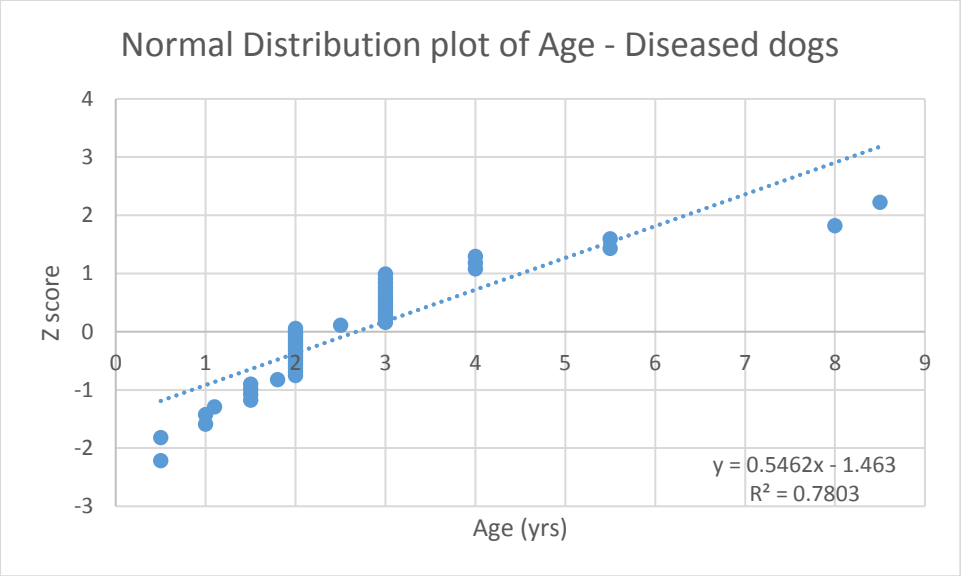

Figure 9: Normal distribution plot for the ages of diseased dogs. The plot shows that the data does not follow a normal distribution.

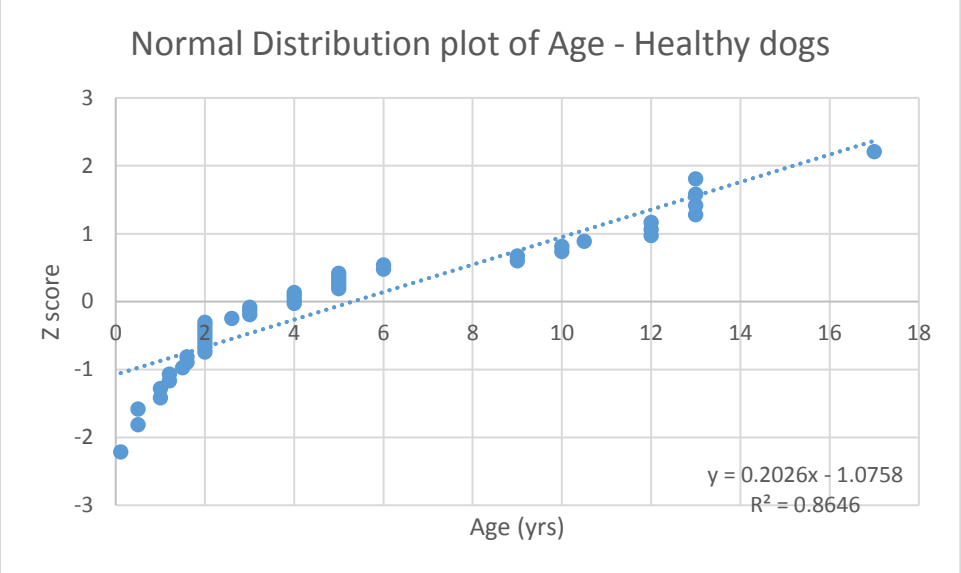

Figure 10: Normal distribution plot for the ages of healthy dogs. The plot shows that the data does not follow a normal distribution.

3.3.1.2: Sex

The sex distribution was similar for healthy (23/46, 50.0% females, with one dog of unrecorded sex) and diseased (22/49, 44.9% females) dogs.

3.3.1.3: Vaccination

The questionnaire included two questions regarding vaccination: whether the dog had ever been vaccinated with the core vaccination (Vanguard 5), and whether the dog had been vaccinated for kennel cough (with either Nobivac KC, Bronchi-shield, or Canigen KC). Overall, 11/47 (27.7%) and 1/47 (2.1%) of diseased and healthy dog owners, respectively, did not respond to this question. Of those who did respond (*n* = 82), all indicated that their dogs were vaccinated with the standard vaccine (Vanguard 5) containing the core antigens. Nearly half 37/82 (45.1%) of sampled dogs were specifically vaccinated for kennel cough, with similar proportions of vaccinated dogs within healthy and diseased groups (Table 14). In addition, the mean number of days since a dog was last vaccinated before been sampled was 642 days, with a range of 11-3149 days, and a standard deviation of 525 days.

Table 14: Number of dogs vaccinated for kennel cough among healthy and diseased dogs.

	Vaccinated for Kennel cough
Diseased	15/36 (41.7%)
Healthy	22/46 (47.8%)
Total	37/82 (45.1%)

3.3.1.4: Clinical signs of ICT

Clinical signs that were recorded included sneezing, nasal discharge, coughing (including type and frequency), rectal temperature, and on rare occasions, pneumonia and altered respiration. In some cases, specific clinical signs were not recorded in the questionnaire, and these were categorised as “not recorded” in the data set.

All diseased dogs for which clinical signs were recorded (*n*=35), had a cough with some showing additional signs of respiratory disease (Table 15). The overall average duration of clinical signs was 5.5 days, with a range between 1 and 14 days. Only 19/35 (54.3%) dogs had a temperature recorded. Of these, five (14.3%) had fever (temperature higher than

39°C). In addition, one dog (RG36) was reported to have pneumonia and altered respiration in addition to other typical clinical signs of ICT.

Table 15: Frequency of selected clinical signs that were reported for diseased dogs at the time of sampling.

Clinical sign	No. of dogs (%)
Coughing	35/35 (100%)
Sneezing	5/35 (14.2%)
Nasal discharge	12/35 (34.3%)
Fever	5/19 (26.3%)

3.3.1.5: Previous respiratory disease

Unfortunately, limited data were available on previous disease history, as this question was often left unanswered in questionnaires. Overall, 10/35 (28.6%) of diseased dogs had a history of previous respiratory disease in the past 12 months. None of the healthy dogs had a history of previous respiratory disease within the same time frame, although it was noted that one dog (F34) previously displayed sneezing related to allergies and another dog (F39) had respiratory difficulties, most likely related to non-infectious causes (old age).

3.3.1.6: Housing

Housing was categorised into “mainly indoors” (MI), “indoors/outdoors” (IO), or ‘mainly outdoors’ (MO). Overall, 20/32 (62.5%) and 12/32 (37.5%) of diseased dogs were categorised as MI and IO respectively. For healthy dogs, 4/19 (21.1%) and 15/19 (78.9%) of pet dogs were MI and IO, respectively, and all farm dogs were kept MO.

3.3.1.7: Diet

All greyhounds were fed a commercial diet. Seven pet dogs were fed raw meat, and the remaining 12 were fed a commercial diet. Of those that were fed raw meat, it was often fed occasionally in conjunction with a commercial diet. All farm dogs were fed raw meat on a regular basis (more than 3 times per week).

3.3.2: Detection of live virus

No CPE was observed in MDCK cells infected with orophangeal swab samples from healthy or diseased dogs after three passages. None of the cell lysates from the third passage haemagglutinated porcine RBC, indicating that CPIV was not isolated on MDCK cells. The positive and negative controls showed the expected results (Figure 11).

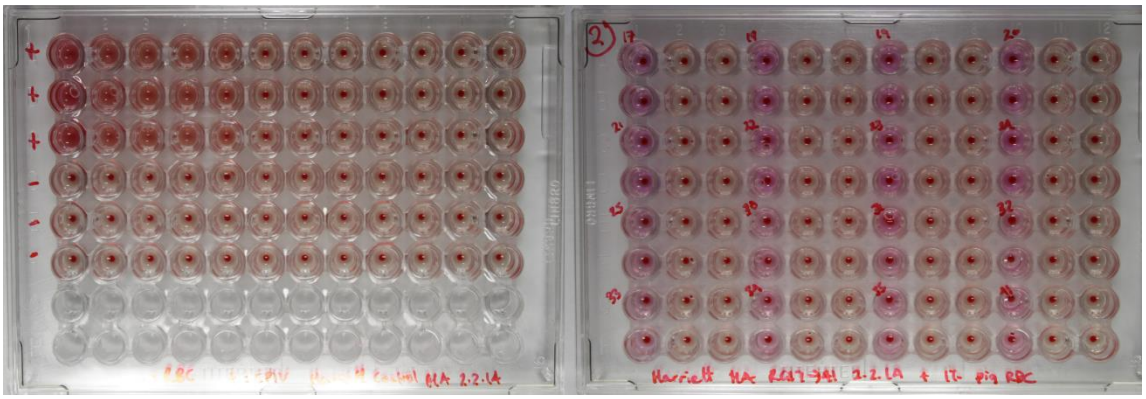

Figure 11: Typical HA plate. Left: Control plate. Right: test plate with samples. Samples were tested in duplicate and diluted 3 times.

3.3.3: IDEXX qPCR canine respiratory disease panel

In total, 20/94 (21.3%) of dogs were positive for at least one agent tested. Of these, CPIV (7.3%), *Bordetella bronchiseptica* (7.3%) and *Mycoplasma cynos* (17.0%) were the most common amongst diseased dogs, whereas CAdV-2 (10.6%) was the most common amongst healthy dogs (Table 16). Although *M. cynos* appeared to be more common in diseased dogs, this association was not statistically significant ($p= 0.091$; $\alpha = 0.05$). None of the samples tested were positive for CRCoV or CIV. The bacteria *M. cynos* and *B. bronchiseptica* were detected from both healthy and diseased dogs, whereas the viruses CPIV and CHV were only present in samples from diseased dogs.

Canine adenovirus type 2 was the most commonly detected pathogen in healthy dogs. However, it was only identified in farm dogs. The presence of CAdV-2 nucleic acid was statistically significantly associated with healthy dogs in comparison to diseased dogs ($p=0.02$, $\alpha=0.05$).

Table 16: Number of dogs (%) positive for respiratory pathogens as detected by IDEXX canine respiratory panel, stratified by the health status. Note that there were no "diseased" farm dogs sampled in this study, so only racing greyhounds and pet dogs fell under this category.

Disease agent	Healthy (*n*=47)			Diseased (*n*=47)		
	Pet	Farm	Total	RG	Pet	Total
CPIV	0/20 (0%)	0/27 (0%)	0/47 (0%)	3/41 (7.3%)	0/6 (0%)	3/47 (6.4%)
CAdV-2	0/20 (0%)	6/27 (22.2%)	6/47 (12.8%)	0/41 (0%)	0/6 (0%)	0/47 (0%)
CHV	0/20 (0%)	0/27 (0%)	0/47 (0%)	1/41 (2.4%)	0/6 (0%)	1/47 (2.4%)
Bordetella Bronchiseptica	0/20 (0%)	1/27 (2.1%)	1/47 (2.1%)	7/41 (17.1%)	0/6 (0%)	3/47 (7.3%)
Mycoplasma cynos	1/20 (5%)	1/27 (3.7%)	2/47 (4.3%)	7/41 (17.1%)	1/6 (16.7%)	8/47 (17.0%)

3.3.3.1: Multiple agents

In total, three dogs were positive for multiple agents, which included two diseased and one healthy dog. The two diseased dogs were positive for CHV and *M. cynos* (RG35), and CPIV and *M. cynos* (RG1). No distinguishing clinical signs (compared to dogs positive for one agent) or other variables (such as age) were seen with either diseased dog. The healthy dog was positive for CAdV-2, *B. bronchiseptica* and *M. cynos*. This dog was a 5-year-old female that was not vaccinated for kennel cough but did have the routine vaccination course, and had no previous history of respiratory disease (according to the owner).

3.3.3.2: Association between age and presence of respiratory pathogens based on the results of the IDEXX respiratory panel

To investigate whether age was a factor for the presence of viral or bacterial nucleic acids, diseased and healthy dogs were grouped into two groups: those who were positive for at least one agent ("positive"), and those who were negative for all pathogens tested ("negative") (Table 17). Age, in association with each specific agent, was not examined due to the low numbers of positive results for healthy and diseased dogs. There was no statistically significant association between age and presence of respiratory pathogens for either healthy (p value = 0.8355, at CI95) or diseased dogs (p value = 0.3294, α=0.05).

Table 17: 2x2 table showing the association of age with the presence of at least one respiratory pathogen (positive dogs), as determined by results from IDEXX respiratory panel qPCR. The numbers indicate average age of each group of dogs in years.

	All dogs	Positive dogs (*n=21*)	Negative dogs (*n=73*)
Diseased (*n=47*)	2.68	2.31	2.82
Healthy (*n=47*)	5.31	5.64	5.25

3.3.3.3: Association between sex and presence of respiratory pathogens based on the results of the IDEXX respiratory panel qPCR

For positive diseased dogs, 7/15 (46.7%) were females and 8/15(53.3%) were males. For positive healthy dogs, 5/9 (55.6%) and 4/9 (45.4%) were females and males, respectively. Hence, despite low numbers of positives, there appeared to be a relatively even number of males and females amongst positive dogs. Therefore, sex was unlikely to be a factor in positivity for viral or bacterial nucleic acid.

3.3.3.4: Association between ICT vaccination and presence of respiratory pathogens based on the results of the IDEXX respiratory panel

Based on the results of the IDEXX panel qPCR, 5 different pathogens circulated amongst both healthy and diseased dogs sampled. Of these five agents, only CAdV-2, CPIV and *B. bronchiseptica* are included in canine vaccines offered in New Zealand. Thus, only results for CAdV-2, CPIV and *B*. *bronchiseptica* were used to investigate whether the proportion of dogs that were positive for any of these three pathogens differed between vaccinated and unvaccinated dogs. All six healthy dogs that were positive for CAdV-2 were vaccinated with Vangaurd 5 (containing CAdV-2). For diseased dogs, there were 6/15 (40%) dogs that were positive for CPIV or *B. bronchispetica*. Of these, one (16.7%) was vaccinated against CPIV and *B*. *bronchiseptica*. For healthy dogs, 7/9 (77.8%) dogs were positive for CPIV or *B. bronchiseptica.* Of these, three were vaccinated for CPIV and *B. bronchiseptica* (Table 18).

Table 18: Number of vaccinated and unvaccinated dogs (%) that tested positive for CAdV-2, CPIV or *B. bronchispetica* by IDEXX respiratory panel qPCR.

	Vaccinated for ICT	Not vaccinated for ICT
Diseased	1/6 (16.7%)	5/6 (83.3%)
Healthy	3/7 (42.9%)	4/7 (57.1%)
Total	4/13 (30.8%)	9/13 (69.2%)

3.3.3.5: Association between other factors and presence of respiratory pathogens based on the results of the IDEXX respiratory panel qPCR.

Due to low numbers of positive diseased dogs (*n*=13), it was difficult to assess if there was any significant association between other factors and disease, especially since, in some cases, data were not recorded in the questionnaire. Of the dogs whose clinical signs were recorded (*n*=11), 6/8 (75%) diseased dogs that were positive for *M. cynos* had nasal discharge, which was significantly higher (p=0.0077, α=0.05) than the number of diseased dogs negative for *M. cynos* that had nasal discharge (6/23, 20.7%). No other significant association between a specific clinical sign and disease agent was found.

Table 19: List of dogs positive for at least one pathogen tested for which clinical signs were recorded. Correlation between dogs that were positive (by IDEXX respiratory panel qPCR) for a specific disease agent, and the clinical signs that were recorded for those dogs (*n=11*). Key: a: M= Moist/ Productive, H= Harsh, S= soft, U = unknown. b: T= only with tracheal palpitation, O = Occasionally, F = Frequent, P= Occurring in paroxysms, U = unknown. N/A: not recorded

Dog ID	Positive for	Nasal Discharge (Yes/No)	Sneezing (Yes/No)	Cough[a] Type	Cough[b] Freq	Temp (°C)	Duration (days)
P60	*M. cynos*	Y	N	M	N/A	38.8	1
RG1	CPIV + Bord	N	N	S	O	N/A	1
RG3	CPIV	N	Y	H	F	N/A	1
RG8	*M. cynos*	Y	N	H	F	N/A	1
RG15	M.cynos	N	N	H	P	N/A	14
RG21	CPIV	N	N	M	O	38.1	1
RG30	M.cynos	Y	N	M	P	39.3	N/A
RG31	M.cynos	Y	N	M	P	38.7	14
RG33	M.cynos	Y	N	S	F	38.9	N/A
RG34	M.cynos	Y	N	S	T	38.7	N/A
RG35	CHV + *M. cynos*	N	N	H	F	39.5	4

The proportion of positive diseased dogs with previous respiratory disease (2/10, 20%) was very similar to that of the total number of negative diseased dogs with previous respiratory disease (8/47, 17%), hence it is unlikely that previous respiratory disease was associated with the presence of nucleic acid detected by IDEXX qPCR. However, it should be noted that 5/15 (33.3%) dogs had no data recorded for previous respiratory disease.

Housing type (MI versus IO) was only assessed for diseased dogs since 7/8 of the positive healthy dogs were farm dogs which were all housed outdoors. Of the positive diseased dogs (*n*=15), 3/20 (15%) and 4/20 (20%) were housed MI and IO, respectively. The remaining 6 positive dogs had no recorded housing response.

3.3.4: In-house qPCR

3.3.4.1: CAdV-2

In total, 71/94 (75.5%) of dogs were positive for CAdV-2 DNA, including 34/47 (72%) diseased dogs and 37/47 (78.6%) healthy dogs. A typical run of an assay is shown in Figure 12. All positive dogs were considered “low level” positives, as Cq for all samples was >30. The positive and negative controls showed the expected results.

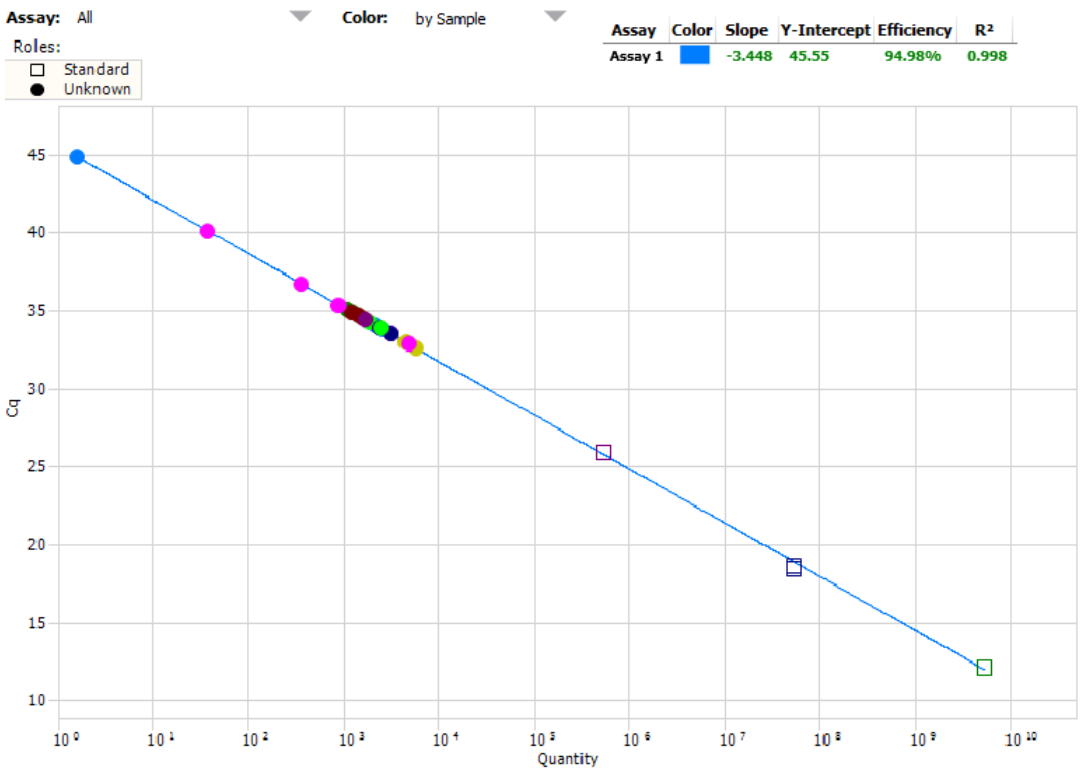

Figure 12: Typical CAdV-2 qPCR sample run, showing 3 standard curve points for assessment of linearity and efficiency of the assay.

Because of the unexpectedly high level of samples positive for CAdV-2 DNA, amplification products of a subset of eight randomly selected samples that were positive for CAdV-2 on qPCR were run on an agarose gel. All positive samples (RG1, RG3, RG42, F5, F8, F76, P13 and P60), including the positive control, showed a band at the expected molecular weight (142 bp), whereas no band was observed for the NTC (Figure 13). The melting temperatures of these amplicons ranged from 79.9 degrees to 80.5 °C. Thus, they were all within 1 °C of the

melting peak of the positive control (80.8 °C). In addition, the identity of the eight randomly selected PCR products (RG1, RG3, RG42, F5, F8, F76, P13, P60) was confirmed by sequencing.

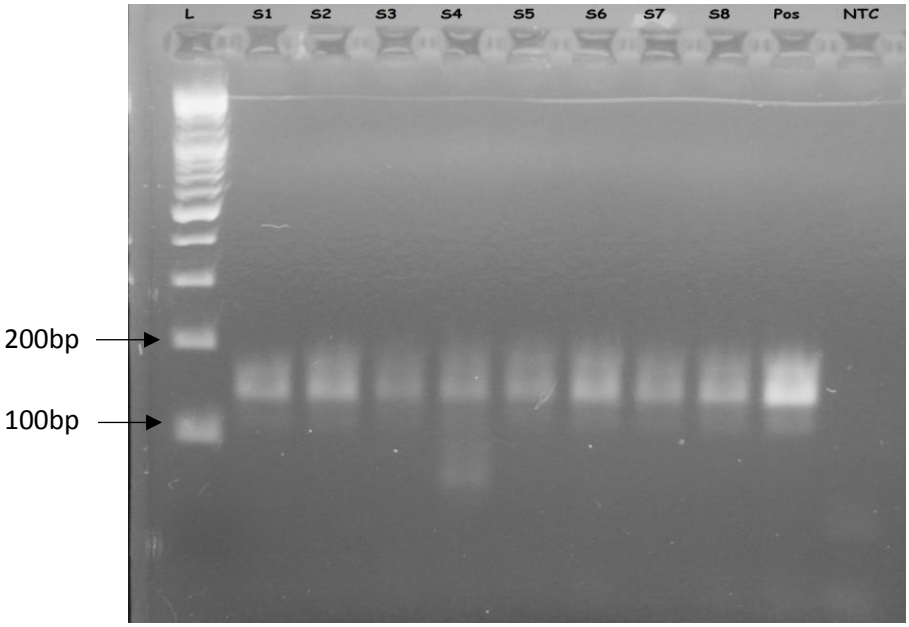

Figure 13: Agarose gel of positive samples identified by IH qPCR. Lane 1: Molecular weight ladder (O'GeneRuler™ DNA Ladder Mix, Ready-to-use, 100-10,000bp (Thermo Fisher Scientific)) (L), Lanes2-9: positive qPCR samples: RG1, RG3, RG42, F5, F8, F76, P13, P60. Lane 10: Positive control (pos), Lane 11: non template control (NTC).

Figure 14 shows that the Cq values for CAdV-2 positive samples were not normally distributed ($p>0.005$). Hence, to determine if there was a significant difference between Cq values for samples collected from healthy versus diseased dogs, a Kruskal-Wallis test was performed. There was no significant difference between the Cq values of these two groups of dogs ($p=0.375$, $\alpha=0.05$, Figure 15).

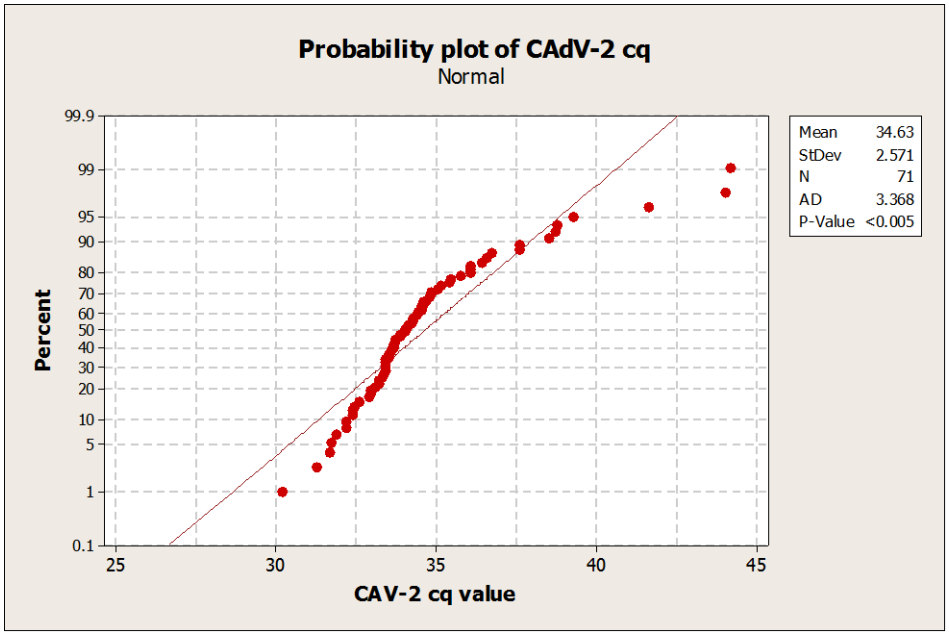

Figure 14: Probability plot of CAdV-2 Cq values for positive healthy and diseased dogs, showing plot deviation from the expected (normal) plot.

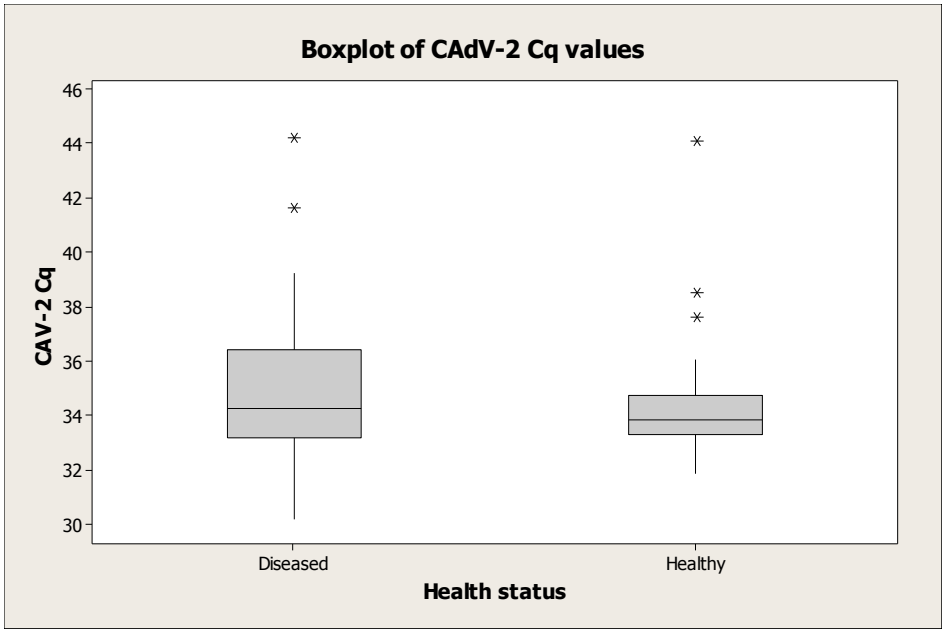

Figure 15: Boxplot of CAdV-2 Cq values for healthy and diseased dogs, showing no significant difference in mean/ range between the two groups (p value = 0.375, CI95). Middle line = median. Bars either side of the box = range of values in lower / upper quartile respectively. * = Outlier values from data.

3.3.4.2: Association between variables and the presence of CAdV-2

Overall, none of the variables that were able to be tested (age and sex,) were significantly associated with the presence of CAdV-2 DNA (Table 20). However, there were a few points of interest. Dogs positive for CAdV-2 DNA within both the healthy and diseased groups

appeared to be slightly younger compared to those who were negative. There appeared to be a greater proportion of males amongst diseased, CAdV-2 positive dogs (61.7%) compared to diseased CAdV-2 negative dogs (45.5%). Healthy dogs appeared to have a reasonably even spread of males and females across CAdV-2 positive and negative dogs (Table 20). Given that the core vaccination includes CAdV-2, the core vaccination status was considered as a variable. All dogs were vaccinated according to owner/handler response to the vaccination question in the questionnaire, hence time since vaccination was examined as a possible predictor of CAdV-2 Cq value.

Table 20: Correlation between CAdV-2 nucleic acid and variables

Variable	**Diseased**				**Healthy**			
	Positive (*n= 34*)	**Negative (*n=13*)**	**p value (at CI95)**	**Overall**	**Positive (*n=37*)**	**Negative (*n=10*)**	**p Value (at CI95)**	**Overall**
Average age (years)	2.3	2.5	0.9329	**2.4**	5.2	5.6	0.7791	**5.3**
Sex								
Male	21	5	0.5959	**26**	17	6	0.4747	**23**
Female	13	6		**19**	19	4		**23**

There was no association between days since vaccination and CAdV-2 Cq value for either healthy or diseased dogs (p value =0.942 at CI95; R^2 =0.0% (no correlation) (Figure 16).

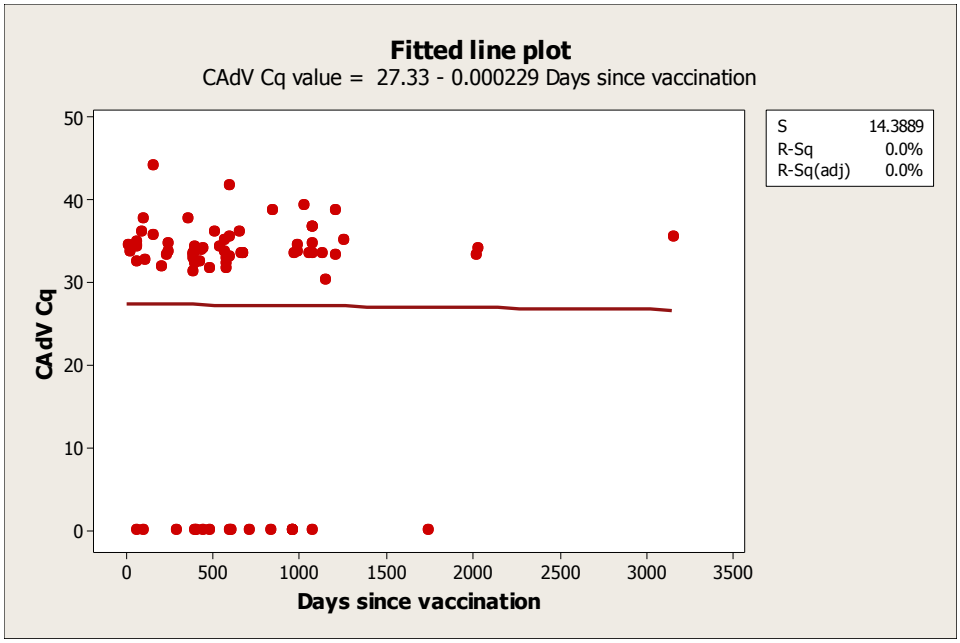

Figure 16: Correlation between days since vaccination and the CAdV-2 Cq value of the corresponding dog. "O" denotes samples negative for CAdV-2 DNA.

3.3.4.3 CHV

In total, 9/94 (9.6%) of dogs tested positive for CHV, two of which were diseased. A further 13 (13.8%) dogs were classified as suspect CHV positives including seven healthy and six diseased ones (see below for explanation). A typical run of the test is shown in Figure 17.

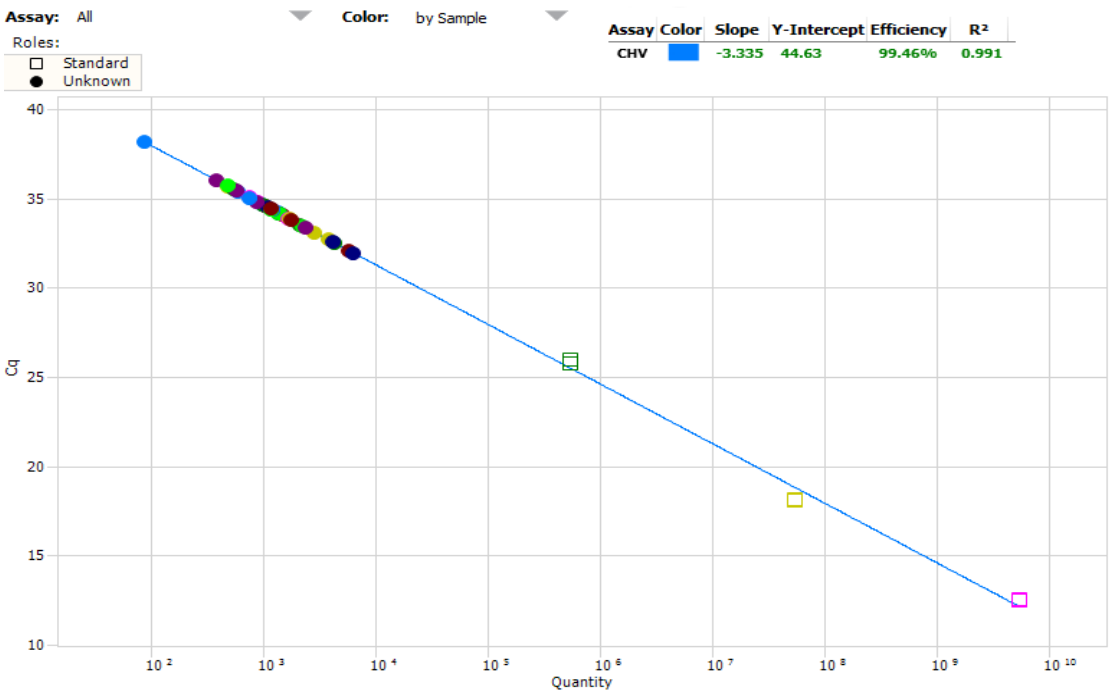

Figure 17: Typical run of samples for CHV qPCR, where square boxes represent standard and circles represent samples. The standards consisted of 106, 104, and 102. Note: not all of the samples are true positives.

According to the criteria set up for interpretation of CHV qPCR (section 3.2.6.2.4), any sample that crossed the threshold and had a T_m value between 72.9 and 74.9°C was considered positive for CHV DNA. Based on these criteria, 20 dogs (F2, F31, F33, F39, F41, F42, F43, F44, F76, P1, P17, P37, P38, P58, P59, P61, RG22, RG47, RG49, RG50) were identified as positive for CHV. Since a comparatively large number of samples showed amplification with T_m outside of that expected for the specific product (73.9 °C ± 1 °C), a selection of 12 products with various T_ms, ranging from 69.7 to 73.9 °C, were subjected to gel electrophoresis through a 2% ethidium bromide stained agarose gel (Figure 18).

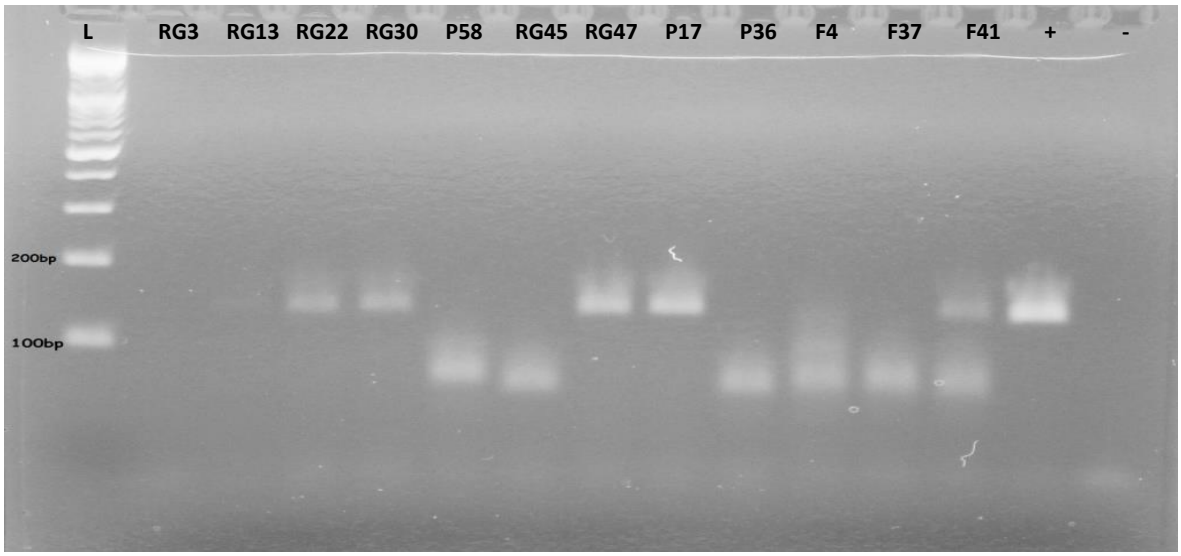

Figure 18: CHV samples from qPCR run on a 2% agarose gel. L: Molecular weight ladder (O'GeneRuler™ DNA Ladder Mix, Ready-to-use, 100-10,000bp (Thermo Fisher Scientific). Tm's of samples as follows: <70°C = RG45; >70≤73°C =RG3, RG13, RG30, P58, P36, F4 and F37; >73≤74°C= RG22, RG47, P17 and F41. + = Positive CHV template from standard curve. - = NTC

All four samples that generated products with a T_m within 1 °C of the expected T_m (RG22, RG47, P17 and F41) produced bands at the expected size (146 bp), with sample F41 also showing a second band >100 bp in size. This corresponded well with two melting peaks observed for this sample, one at 73.9 °C (within the expected T_m range) and the other at 71.2 °C. In addition, sample RG30 also produced a band of the expected size, even though its T_m was 72.1 °C. All other samples for which T_ms were < 72 °C (RG3, RG13, P58, RG45, P36, F4 and F37) produced bands <100bp in size, which were likely due to primer dimers (Figure 18). Based on these data, the criteria used for classification of a sample as positive for CHV DNA were revised to include a broader Tm range spanning from 72.1 °C to 74.9 °C. This resulted in classification of two additional samples (RG30 and P7) as positive for CHV DNA. However, taking into consideration a high proportion of samples with non-specific melting

peaks (Figure 19), the decision was made to consider only nine samples (F39, F41, F43, F76, P1, P17, P38, P59, P61) for which both duplicates produced positive results as true positives. The remaining 13 samples that fulfilled the criteria for being classified as CHV-positive only in one duplicate of the run were classified as suspect CHV positives.

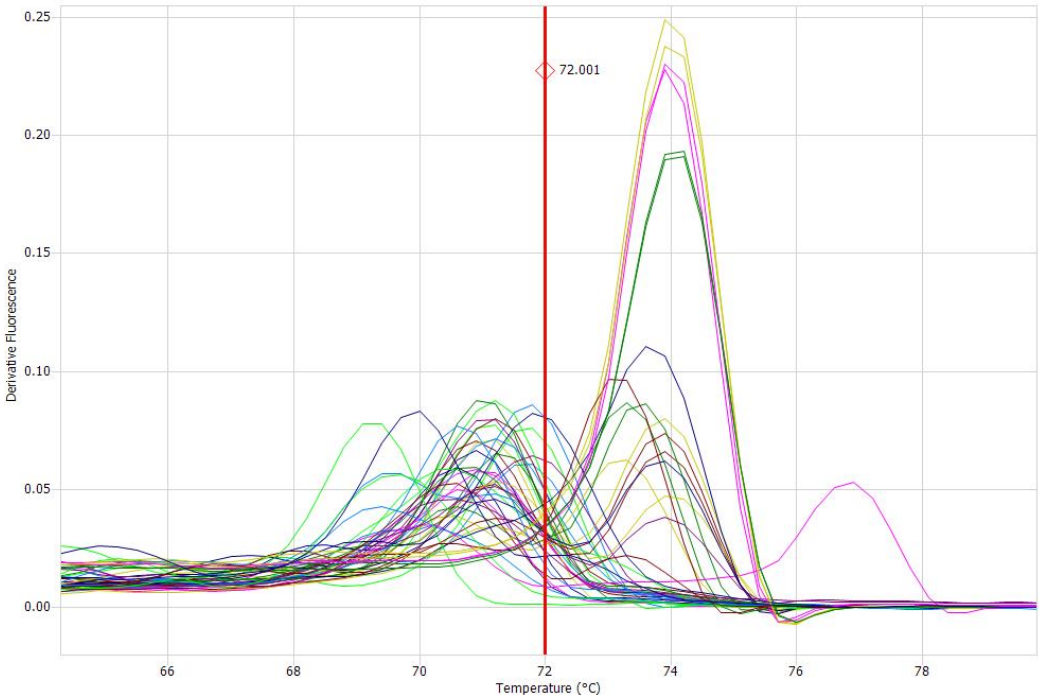

Figure 19: Example of CHV qPCR run illustrating a large number of samples with non-specific melting peaks (Tm < 72°C)

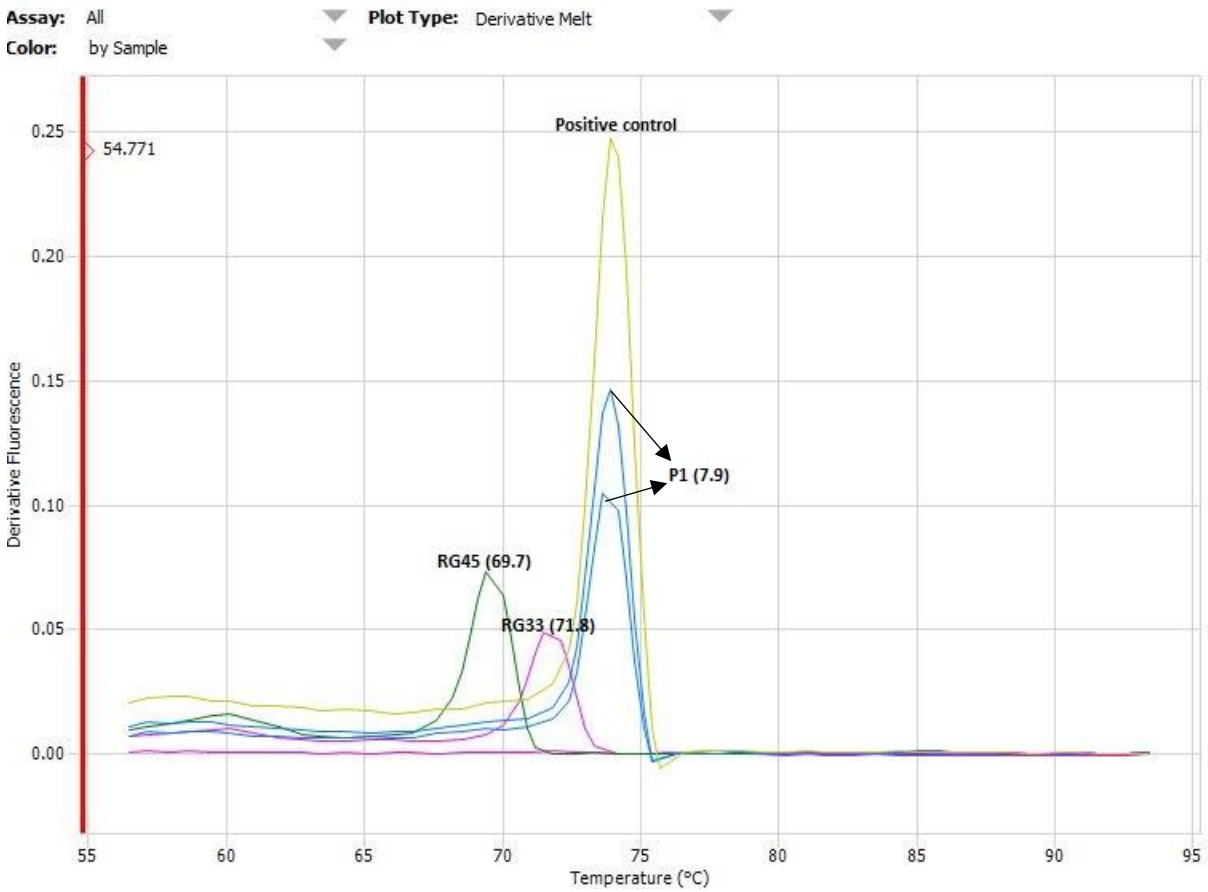

Figure 20: Example of melting peaks for three samples of various TM's, and CHV positive control. P1 was considered to be a true positive, RG33 suspect positive and RG45 negative for CHV DNA.

3.3.4.4: Significance of variables for CHV results

The median age of the seven healthy dogs positive for CHV was 7.2 years. Of these seven dogs, five were ≥ 5yrs, one dog was under 1yr, and the other dog's age was not recorded. Thus, on average, CHV positive healthy dogs were older than CHV negative dogs (average age 5.0 years), but the mean age of CHV positive dogs was not significantly higher than that of CHV negative dogs ($p=0.2520$, $\alpha=0.05$). The 2 CHV positive diseased dogs (P59 and P61) were aged 8 and 0.5 years, respectively. Since such a small proportion of diseased dogs were positive for CHV, statistical analysis was of little value.

3.3.4.5: Multiple agents

In total, seven dogs were positive for CAdV-2 and CHV DNA, including six healthy and one diseased dog (Table 21). Given the small number of dogs that were positive for these two infectious agents, statistical analysis was not carried out.

Table 21: Variables for dogs positive for CAdV-2 and CHV DNA

	Dog I.D.	Age	Sex	Clinical signs (Diseased only)
Healthy	P1	10.5	F	
	P17	N/A	N/A	
	P38	0.5	M	
	F39	10	M	
	F43	12	F	
	F76	5	F	
Diseased	P61	0.5	M	Nasal discharge, sneezing, mucoid cough.

3.3.5: Comparison of IDEXX qPCR and inhouse qPCR results

All samples that were positives for CAdV-2 DNA by IDEXX were also positive by IH CAdV-2 qPCR (Table 22). However, an additional 64 samples (healthy and diseased) were positive for CAdV-2 by IH qPCR. The level of agreement between the two tests was considered low (kappa value of -0.155).

The one dog (RG35) that tested positive for CHV DNA by IDEXX qPCR was also positive for CHV by IH qPCR. In addition, 8 and 13 other samples were classified as true and suspect positives for CHV DNA, respectively, by IH CHV qPCR.

Table 22: IDEXX positive qPCR results compared with IH qPCR positive results. Only samples classified as "true positives" for CHV were included in the comparison.

	CAdV-2 positives		CHV positives		CPIV positives	
	Diseased	Healthy	Diseased	Healthy	Diseased	Healthy
IDEXX	0/47 (0.0%)	6/47 (12.7%)	1/47 (2.1%)	0/47 (0.0%)	3/47 (6.4%)	0/46 (0.0%)
In House	34/47 (72%)	36/47 (76.6%)[a]	4/47 (8.5%)[b]	5/47 (10.6%)	0/47 (0.0%)	0/47 (0.0%)
Kappa level	-0.155		0.200		N/A	

a: All 6 positives identified by IDEXX qPCR were also positive IH qPCR

b: IDEXX qPCR positive dog was not identified as positive in the IH qPCR assay

3.3.7: Coronavirus ELISA results

In total, 48/93 dogs (51.6%) were seropositive for CRCoV on at least one sampling occasion by BCoV competitive ELISA. Of the 48 seropositive dogs, 16 were healthy and 32 were diseased (Table 23). For diseased dogs, a total of 47 acute samples were collected, for which there were 30 corresponding convalescent samples.

Table 23: Proportion of dogs positive for canine respiratory coronavirus (CRCoV) ELISA antibody stratified by health status and use.

		Positive	Negative	Total
Healthy	*Pet dogs*	6 (30%)	13 (70%)	**19**
	Farm dogs	10 (37.0%)	17 (63%)	**27**
	Total	**16 (34.0%)**	**31 (66.0%)**	**46**
Diseased	*Greyhounds*	29 (67.0%)	14 (33%)	**43**
	Pet dogs	3 (75.0%)	1 (25%)	**4**
	Total	**32 (68.0%)**	**15** (32%)	**47**
Total		**48 (51.5%)**	**46 (48.5%)**	**93**

The following groups were then examined for statistical significance: sera from diseased dogs (acute vs convalescent), sera from diseased dogs (acute samples) vs healthy dogs, and sera from diseased dogs (convalescent samples) vs healthy dogs. The POI values of acute serum samples from diseased dogs was normally distributed, as shown in Figure 21.

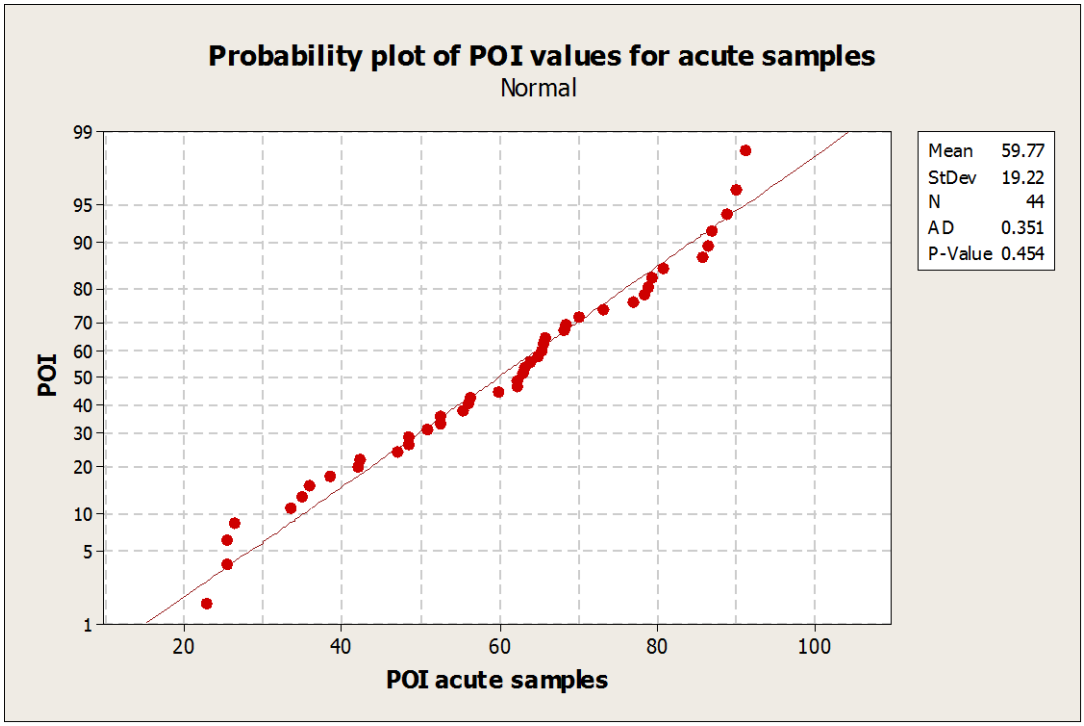

Figure 21: Probability plot showing that canine respiratory coronavirus antibody levels, expressed as percent of inhibition (POI) values in bovine coronavirus blocking ELISA, for acute samples from diseased dogs were normally distributed.

The CRCoV POI for convalescent serum samples were, however, not normally distributed, and followed more of a bi-modal distribution (Figure 22). Hence, a Kruskal-Wallis test was used for comparison of convalescent samples with acute samples and healthy samples, respectively.

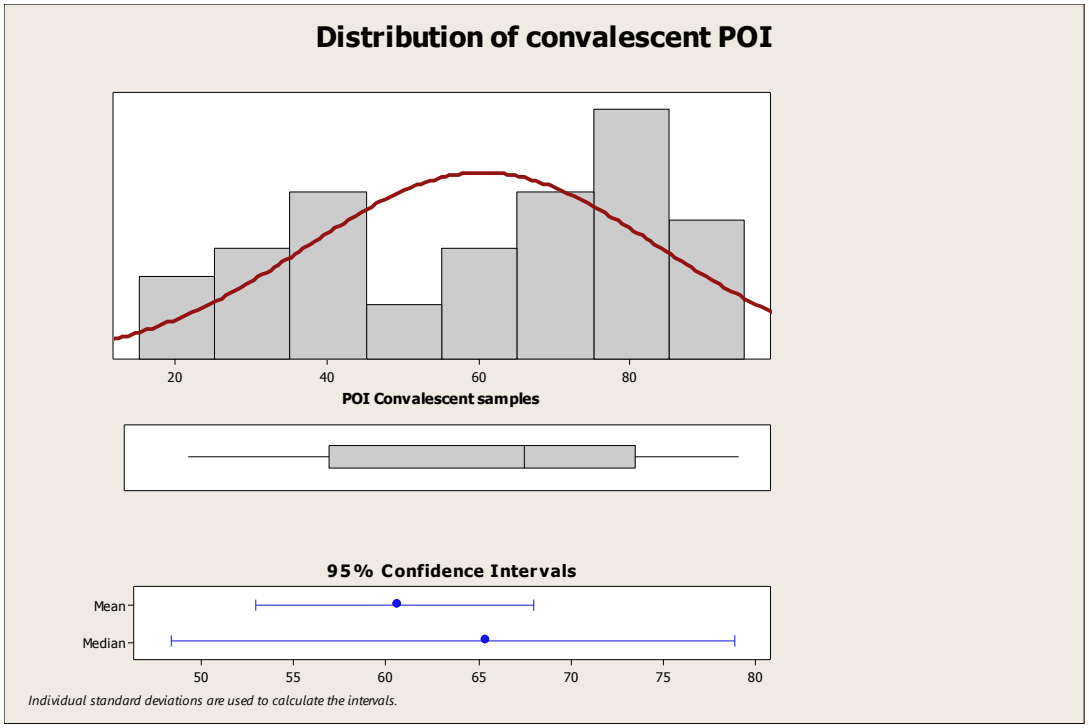

Figure 22: Canine respiratory coronavirus antibody levels, expressed as percent of (POI) in bovine coronavirus blocking ELISA for convalescent serum samples from diseased dogs. The values were not normally distributed but followed a bimodal distribution.

Figure 23 shows that convalescent serum samples did not have significantly higher coronavirus POI values compared to acute serum samples from diseased dogs (p=0.20408, α=0.05).

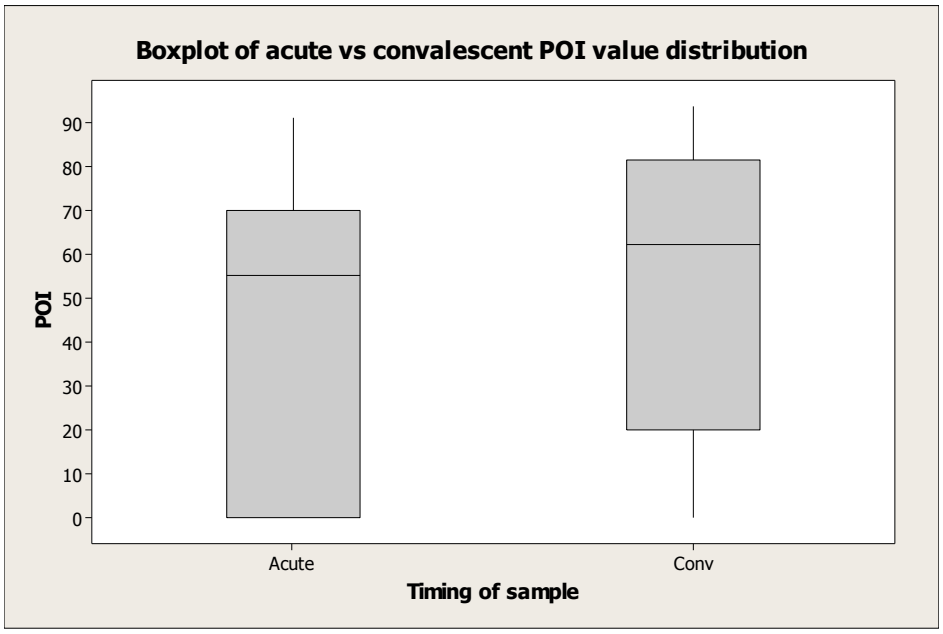

Figure 23: Box plot showing the distribution of canine respiratory coronavirus antibody levels, expressed as percent of inhibition (POI) value in bovine coronavirus blocking ELISA for acute (*n*=47) and convalescent (conv) serum samples from diseased dogs (*n*=30). The middle line of the box represents the median, whereas the bottom line of the box and top line of the box represent the lower and upper quartile values respectively.

However, convalescent sera from diseased dogs showed a significantly higher CRCoV POI compared to sera from healthy dogs (p=0.023, α=0.05; Figure 24).

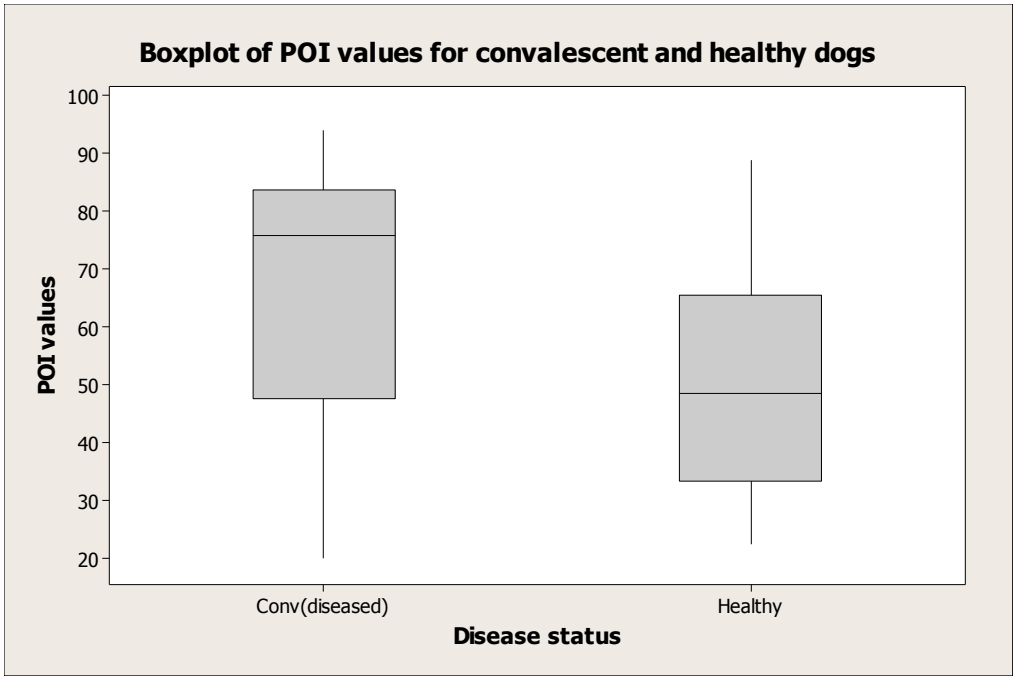

Figure 24: Box plot showing the distribution of canine respiratory coronavirus antibody levels, expressed as percent of inhibition (POI) values in bovine coronavirus blocking ELISA for convalescent (conv) serum samples from diseased dogs (*n*=30), and all samples from healthy dogs (*n*=47). The middle line of the box represents the median, whereas the bottom line of the box and top line of the box represent the lower and upper quartile values respectively.

Acute samples from diseased dogs, like convalescent samples, also had a significantly higher POI than serum samples from healthy dogs (p=0.013, α=0.05; Figure 25). In addition, acute serum samples from diseased dogs were significantly more likely to be positive for CRCoV blocking antibodies compared to sera from healthy dogs (RR 5.22, CI95 1.972, 14.115, p=0.0003).

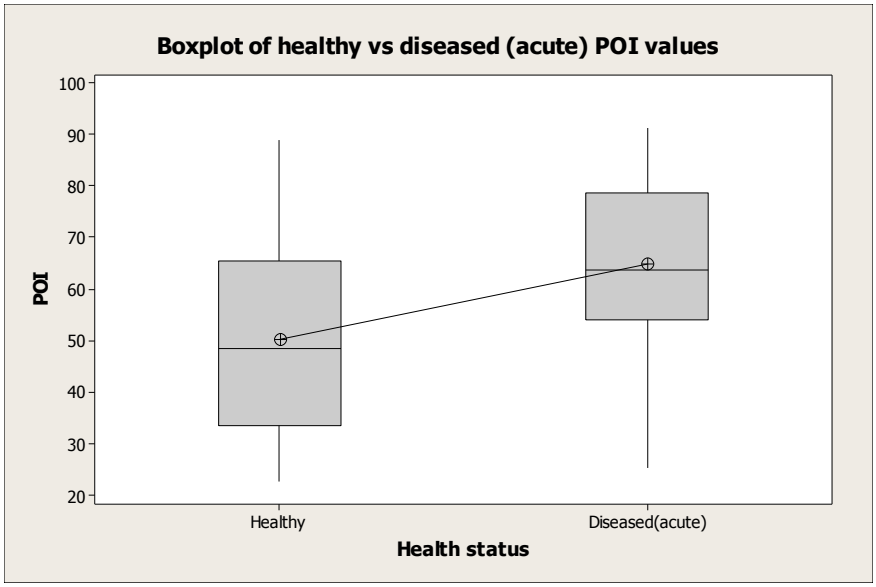

Figure 25: Box plot showing the distribution of canine respiratory coronavirus antibody levels, expressed as percent of inhibition (POI) values in bovine coronavirus blocking ELISA for all serum samples from healthy dogs (*n*=47), compared to acute serum samples from diseased dogs (*n*=47). The middle line of the box represents the median, whereas the bottom line of the box and top line of the box represent the lower and upper quartile values respectively. The line from healthy to the diseased (acute) box represents a significant increase between the medians of each box.

All together, these results are in accordance with the finding that diseased dogs were more likely to be positive for CRCoV antibodies compared to healthy dogs.

3.3.7.1: Correlation between age and CRCoV blocking antibody levels.

Overall, the median age for CRCoV seronegative and seropositive dogs was 3.8 and 3.9 years respectively (Table 24).

Table 24: Average age (years) for canine respiratory coronavirus seronegative and seropositive dogs, with the age range in brackets.

	Diseased	Healthy	Overall
Seronegative	1.4 (0.5-3)	4.69 (0.5-17)	3.8 (0.5-17)
Seropositive	2.8 (0.5-8)	6.14 (1.5-13)	3.9 (0.5-13)

Although there appeared to be a very weak upward trend line for levels of CRCoV blocking antibodies with increasing age for diseased dogs, and conversely a weak downwards trend for the levels of CRCoV blocking antibodies with increasing age for healthy dogs, neither

trend was statistically significant given the R^2 values of 0.0133 and 0.1923 for diseased and healthy dogs, respectively (Figure 26).

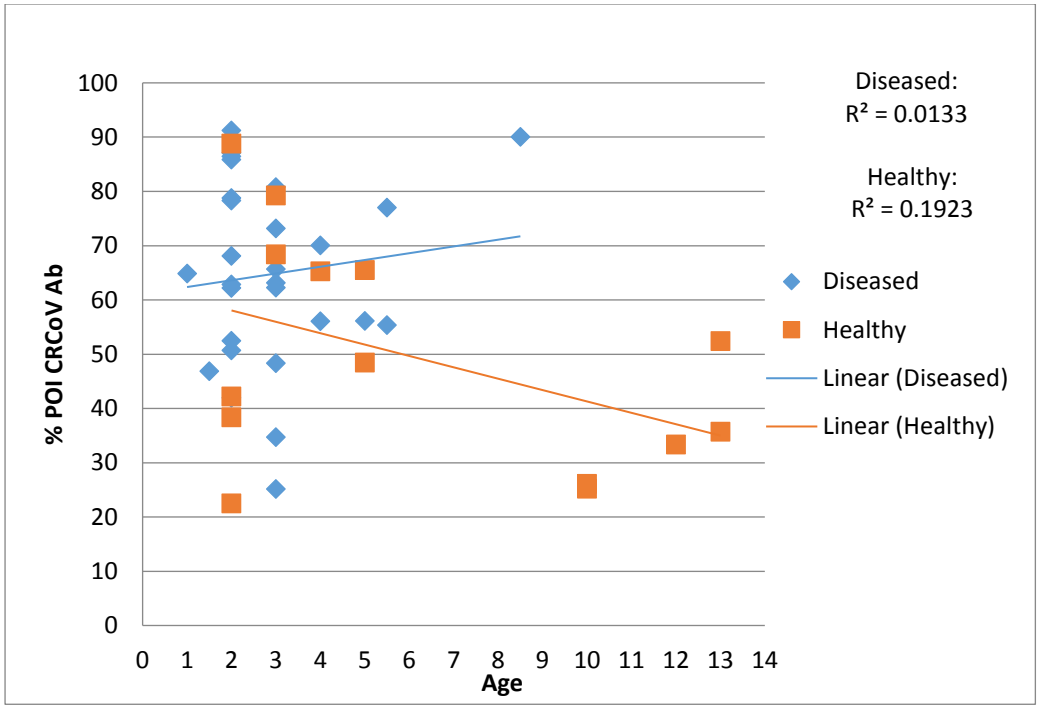

Figure 26: Correlation between dog's age and canine respiratory coronavirus antibody levels expressed as a percent of inhibition (POI) in blocking ELISA using bovine coronavirus antigen, stratified by the health status of the dogs.

3.3.7.2: Correlation between sex and CRCoV antibodies

Overall, the percentage of female versus male dogs was fairly similar in both CRCoV seropositive and seronegative dogs. Both groups had a higher percentage of males compared to females, which is in concordance with the overall study population having more males than females (see section 3.3.1.2) (Table 25).

Table 25: Numbers of canine respiratory coronavirus seropositive dogs, stratified by disease status and sex.

		Female	Male
Diseased	*Negative*	6/15 (40%)	9/15 (60%)
	Positive	14/32 (43.80%)	18/32 (56.20%)
Healthy	*Negative*	14/31 (45.20%)	17/31 (55.80%)
	Positive	8/14 (57%)	6/14 (43%)
Total	*Negative*	20/46 (43.4%)	26/46 (56.6%)
	Positive	22/47 (46.8%)	24/47 (53.2%)

3.3.7.3: Correlation between clinical signs and CRCoV antibody status.

Overall, no clinical signs were significantly associated with the presence of CRCoV blocking antibodies. However, nasal discharge was observed in over half (6/13) of dogs that were highly positive (3/4+) for CRCoV antibody (Table 26).

Table 26: Proportion of clinical signs observed in canine respiratory coronavirus seronegative and seropositive diseased dogs.

Clinical sign	Seronegative	Low positive (1-2+)	High positive (3/4+)
Sneezing	1/13	2/9	2/13
Clinical sign	Seronegative	Low positive (1-2+)	High positive (3/4+)
Nasal discharge	3/13	3/9	6/13
Coughing	13/13	9/9	13/13
Elevated temperature	2/13	1/9	2/13
Other signs	0/13	0/9	1/13[a]
Duration (average - days)	6.3	5.1	5.0

a= Signs included pneumonia and altered respiration (dyspnoea).

In addition, dogs who were described as having a harsh, frequent cough were significantly more likely to have high level of CRCoV antibodies compared with dogs who didn't have high levels of CRCoV antibodies (aka seronegative and low levels (1/2+) of CRCoV antibodies) (p-value =0.009, at α=0.05). Likewise, dogs who were seronegative were significantly more likely to have a moist cough than other types of cough, compared to dogs who were seropositive (p-value =0.009, at α=0.05) (Figure 27)

For cough frequency, dogs who were described as having a "frequent" cough were significantly more likely to have high level of CRCoV antibodies compared with dogs who

didn't have high levels of CRCoV antibodies (aka seronegative and low levels (1/2+) of CRCoV antibodies) (p-value =0.0008, at α=0.05) (Figure 28).

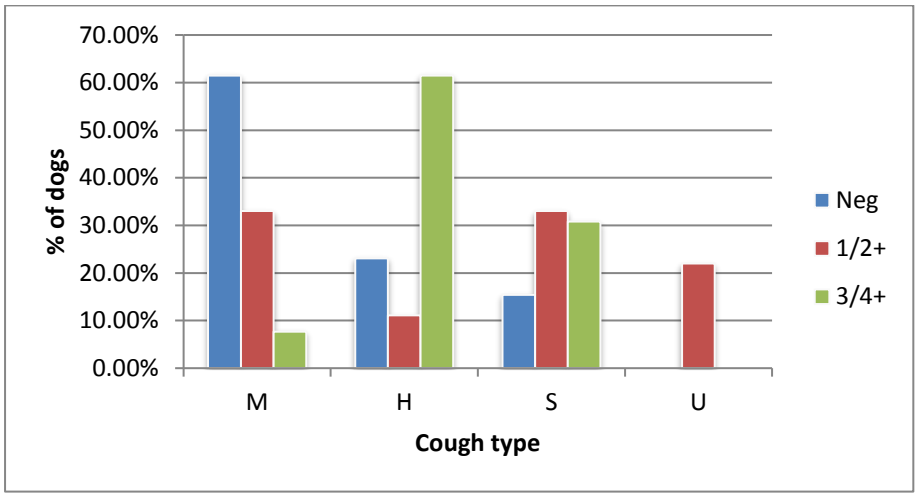

Figure 27: Cough type stratified by the degree of canine respiratory coronavirus seropositivity expressed as percent of inhibition (POI) in bovine coronavirus blocking ELISA, where M= Moist/ Productive, H= Harsh, S= soft, U = unknown (not recorded). The numbers in the legend on the right of the graph correspond to the following ELISA POI values: 1: 20-40%), 2: 40-60%, 3: 60-80% and 4: >80%).

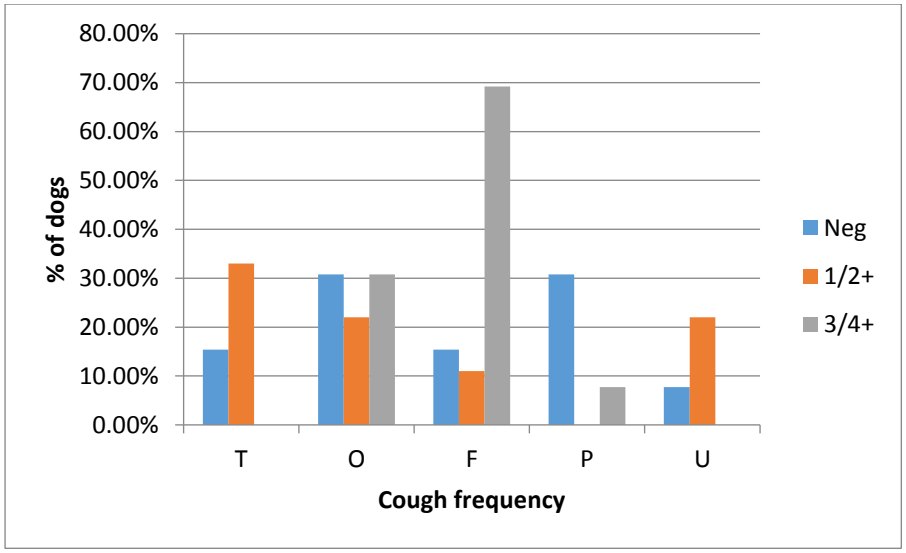

Figure 28: Cough frequency stratified by the degree of canine respiratory coronavirus seropositivity, expressed as percent of inhibition (POI) in bovine coronavirus blocking ELISA, where T= only with tracheal palpitation, O = Occasionally, F = Frequent, P= Occurring in paroxysms, U = unknown. The numbers in the legend on the right of the graph correspond to the following ELISA POI values: 1: 20-40%), 2: 40-60%, 3: 60-80% and 4: >80%).

3.3.7.5: Other variables

Of the 10 diseased dogs that had a history of previous respiratory disease, low levels (1/2+) of CRCoV antibodies was most common (Figure 29). Dogs that had no history of previous

respiratory disease (*n=30*) were typically either seronegative, or had high levels (3/4+) of CRCoV blocking antibodies.

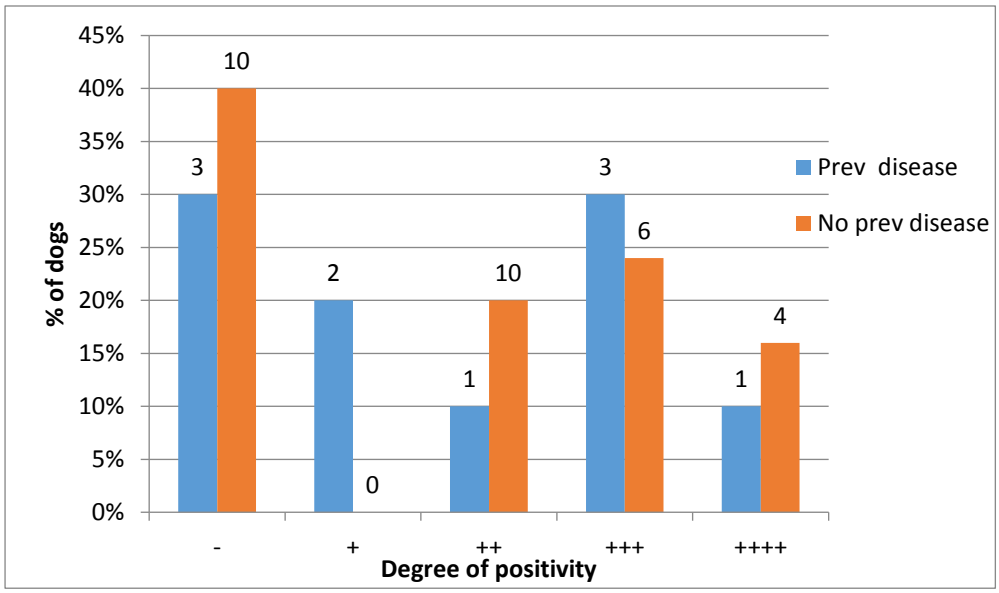

Figure 29: Previous respiratory disease status stratified by the degree of CRCoV seropositivity expressed as percent of inhibition (POI) in bovine coronavirus blocking ELISA. The numbers above each column represent the number of dogs that contributed to that column.

The question regarding housing was not comprehensively answered in questionnaires, particularly those filled for diseased dogs. Since the majority of dogs were recorded as "IO", no trend between degree of CRCoV seropositivity and housing was observed. In addition, all four dogs that were recorded as "MI" were seronegative for CRCoV (Table 27).

Table 27: Number of healthy dogs housed indoors/outdoors (IO) or mainly indoors (MI) stratified by degree of canine respiratory coronavirus seropositivity, expressed as percent of inhibition (POI) in bovine coronavirus blocking ELISA from: – (< 20%), + (20-40%), ++ (40-60%), +++ (60-80%) and ++++ (>80%)

Degree of pos	IO	MI	Total
-	27	4	31
+	6	0	6
++	3	0	3
+++	4	0	4
++++	1	0	1
Total	41	4	45

For diseased dogs, MI dogs did not show any trend in regard to the level of CRCoV antibodies detected. However, IO dogs tended to either be seronegative or have high levels of CRCoV antibodies (Figure 30).

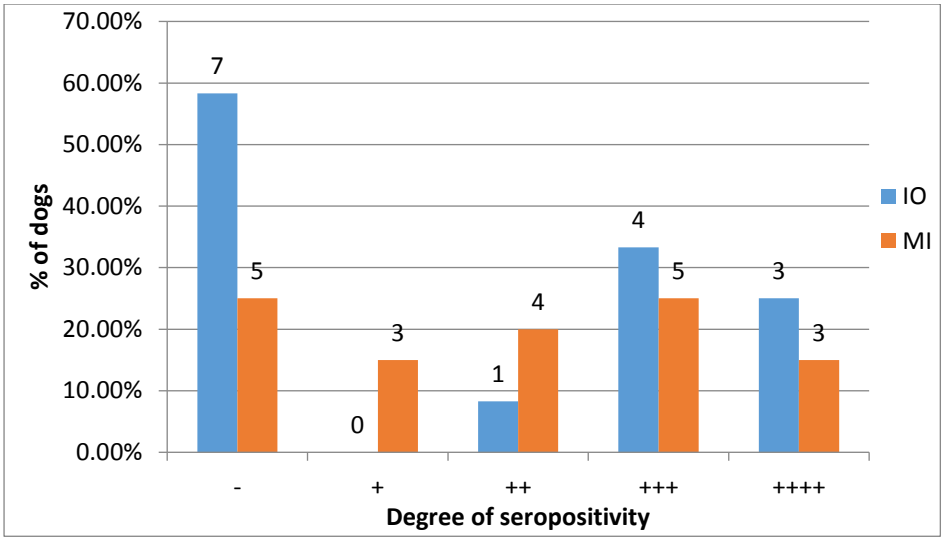

Figure 30: Degree of canine respiratory coronavirus seropositivity expressed as percent of inhibition (POI) in bovine coronavirus blocking ELISA from: – (< 20%), + (20-40%), ++ (40-60%), +++ (60-80%) and ++++ (>80%) for diseased dogs housed mainly indoors (MI) or indoors-outdoors (IO). The numbers above each column represent the number of dogs that contributed to that column.

3.4 Discussion

Infectious canine tracheobronchitis presents a common problem for dog owners and veterinarians, particularly for dogs that are kennelled (Buonavoglia and Martella 2007). It is well recognised that a variety of pathogens are likely to contribute to ICT. Hence, the aim of the current study was to determine which respiratory pathogens, with an emphasis on viruses, circulate among New Zealand dogs, and which ones of those are associated with ICT. The secondary aim was to develop IH qPCR assay for detection of selected canine respiratory viruses in order to facilitate further research and to provide diagnostic support for cases used for teaching at the Massey University Veterinary Teaching Hospital.

As expected, the results of this study have shown that CPIV, CAV-2, CHV, and CRCoV circulate among both healthy and diseased dogs in New Zealand, as well as the bacterium *M. cynos* and *B.bronchiseptica*. However, only 13/47 diseased dogs sampled were positive for at least one of the agents mentioned previously via the IDEXX canine respiratory disease panel. While this was slightly higher than the frequency of detection of respiratory pathogens in samples from healthy dogs (7/47), no respiratory pathogens were identified from the majority of diseased dogs tested by the IDEXX panel.

This could be due to a number of reasons. Firstly, the low numbers of positive samples could reflect the sensitivity of the IDEXX assay, below the level of pathogens' nucleic acids present in the samples. This is discussed further below with relations to CAdV-2 results. Secondly, it is also possible that pathogens other than those tested for were involved in development of CIRD in the sampled dogs. This view is supported by the fact that a number of novel pathogens have been recently described as possibly involved in ICT overseas. These include canine pneumovirus (Mitchell *et al.* 2013), canine bocavirus and canine hepacivirus (Priestnall *et al.* 2014).

3.4.1: Absence of live virus in samples

In this study, no viruses were isolated in cell culture. The cell line used (MDCK) was expected to support the growth of CIV (Payungporn *et al.* 2008), CPIV (Ellis and Krakowka 2012), CHV (Xuan *et al.* 1992), and CAV-2 (Mochizuki *et al.* 2008). Since no viral CPE was apparent after three passages, and the cell lysate from the third passage did not show any HA activity, it was concluded that live CPIV, CHV and CAV-2 were not present in the samples. This result could be interpreted in several ways. Firstly, it is possible that the samples were collected too late in the infection, after the dogs cleared the virus from the upper respiratory tract. However, damage to the epithelium in the upper respiratory tract may have predisposed them to secondary infection with agents such as Mycoplasma cynos. This has been suggested to be the case with CRCoV (Erles *et al.* 2003). If so, CIV, CPIV, CHV and CAV-2 may have still contributed to the development of clinical disease despite lack of detection.

Secondly, the viruses may have been present in the samples, but were not viable due to suboptimal sampling or processing. This possibility cannot be fully excluded considering discrepancies between virus isolation and qPCR data. However, care was taken to maintain the cold chain and to process the samples soon after the arrival at the laboratory. In addition, the samples were collected into commercial viral transport media, which have been shown to support viability of viruses for up to 72 hours at room temperature (Weissfeld 2004).

Canine respiratory coronavirus has proved difficult to isolate in previous studies. Up until the completion of this study, CRCoV has only been isolated in HRT-18 cells and no other cell line, including MDCK cells (Erles *et al.* 2007) was shown to be supportive of CRCoV growth.

The HRT-18 cells were not available. Hence, lack of isolation of CRCoV in the current study is not surprising and does not exclude the possibility that live CRCoV may have been present in the samples collected.

3.4.2: Canine adenovirus infections are common among New Zealand dogs.

Canine adenovirus was the most frequently detected virus, both among healthy and diseased dogs in relatively similar proportions. These results suggest that while CAdV-2 circulates among dogs in New Zealand, it is uncertain as to whether CAdV-2is an important contributor to clinical CIRD in the sampled population. This is in agreement with results of some other investigations, for example in 2004 where no evidence of CAdV-2 was found in a rehoming shelter were ICT was endemic (Erles *et al.* 2004). In addition, CAdV-2 was detected from only 2.9% of 68 diseased dogs, and hence was not linked to outbreaks of ICT in that population. However, in contrast to these studies, In Thailand, 9.17% of diseased dogs and 2.94% of healthy dogs were positive for CAdV-2 via RT-PCR, indicating CAdV-2 may have contributed to ICT in the study population (Posuwan and Payungporn 2011).

The correlation between results of a commercial qPCR (IDEXX panel) and in-house qPCR assays for CAdV-2 was poor. Based on the IH CAdV-2 qPCR results, 73% and 78% of diseased and healthy dogs, respectively, were positive for CAdV-2 DNA. This was in contrast to the IDEXX finding, which showed the presence of CAdV-2 DNA in 12.8% of healthy dogs, and no CAdV-2 DNA in diseased dogs. These discrepancies could be explained in a number of ways. Firstly, they could reflect differences between sensitivity of the IDEXX assay and IH CAdV-2 qPCR. The limit of detection for IH CAdV-2 qPCR was determined to be 10^2 copies of DNA/µL of template, which was consistent with what was reported previously (Segura *et al.* 2010).The exact sensitivity and specificity of the IDEXX qPCR was unknown beyond a generic statement that they were “generally around 90%”. We were unable to source the actual data on which this statement was made and it was not clear what tests were considered as golden standard for the calculations. Similarly, LoDs were not stated for each of the pathogens included in the panel. Hence, it is possible that a much higher proportion of dogs were positive for CAdV-2 DNA in IH qPCR because the LoD of the IH assay was lower than that of the IDEXX assay.

Secondly, the high proportion of positives in IH qPCR could represent false positive results due to non-specific amplification or cross-contamination between samples. Cross

contamination might have occurred at the time of sampling, during DNA extraction of samples, or during the setup of the qPCR assay. Cross contamination at the time of sample collection is unlikely, as similar results would then be expected in the IH CHV assay, as well as in the IDEXX panel. Similarly, if cross-contamination occurred during nucleic acid extraction, similar results with high proportion of positive samples would be expected from the IH CHV assay, which was not the case. Furthermore, samples from each dog were collected into individual sealed tubes, which minimised the risk of cross contamination. For DNA extraction, several precautions were taken to minimise cross contamination, including preparing samples in individual, closed Eppendorf tubes, and using filtered pipette tips. For the setup of the IH qPCR assay, reagents were set up in a separate room to where nucleic acid was handled. Cross-contamination during the set-up of each run is unlikely as amplification was not present in any of the negative controls included in each run. Non-specific amplification is also unlikely to explain our results, as 8 randomly chosen amplicons that were sequenced all contained CAdV-2 sequences.

Altogether, this suggests that the high proportion of dogs positive for CAdV-2 DNA most likely represented a real finding. This would be in contrast not only to the IDEXX panel results, but also to results of virus isolation. Thus, the presence of CAdV-2 DNA may not have represented the presence of infectious virus in the samples. It is also possible that the levels of CAdV-2 in the samples were very low, below the limit of detection of the other two assays, which would fit well with the high Cq recorded in IH CAdV-2 PCR for most of the samples.

The prevalence of CAdV-2 DNA found in our study was higher than that reported by several other authors. For example, there was no evidence of CAdV-2 DNA amongst 211 tracheal and lung samples in a UK-based study (Erles *et al.* 2004). In contrast, and similarly to our results, 30/51 (58.8%) of pet dogs presented for a variety of reasons to the veterinary clinic in Bologne in Italy tested positive for CAdV-2 DNA in faecal swabs or urine samples (Balboni *et al.* 2014)

The source of CAdV-2 amplified in the current study is unclear. It is possible that the dogs were infected with viruses circulating in the field, similarly to the Italian dogs described by Balboni et al (2014). It is also possible that the dogs were shedding the vaccine strain of the

virus. This would be in contrast to older studies on CAdV vaccination, in which no (Bass *et al.* 1980) or limited shedding within one week of vaccination (Cornwell *et al.* 1982)) was described. However, detection of the virus in the two older studies was based on virus isolation, while detection of the virus in the more recent Italian study was based on PCR. Thus, the results of our study are in agreement with all three studies cited above – we have detected no CAdV-2 shedding by virus isolation, but a large numbers of dogs sampled were positive for CAdV-2 by qPCR. The CAV-2 DNA fragments amplified in the current study were most similar to the vaccine strain Toronto A26/61. However, due to the short length of the fragment (142 bp), as well as lack of genetic data on New Zealand field CAdV-2 isolates, no conclusions can be drawn from these findings with regard to the origin of CAdV-2 DNA. Altogether, the source of the virus, as well as clinical implications of our findings, need to be further investigated.

3.4.3 Canine herpesvirus circulates among New Zealand dogs but is unlikely to be important for ICT.

Canine herpesvirus was detected in 4/47 diseased and 5/47 healthy dogs by IH CHV qPCR and in one diseased dog based on the IDEXX panel. As with CAdV-2, the IH CHV qPCR results did not correlate well with the findings from the IDEXX panel, nor with the results of virus isolation. The possible explanations for these discrepancies are discussed above under CAdV-2 heading, although none of these can explain the fact that one sample positive by IDEXX was negative by IH CHV qPCR.

One apparent problem with the IH CHV qPCR was amplification of non-specific products, most likely primer-dimers, in addition to the specific product. This was not apparent during the optimisation of an assay for which a less complex template (long PCR product) was used. In addition, the Tm of the specific product seemed to vary between samples more than originally anticipated. Although it was possible to distinguish between specific and non-specific products based on melting analysis, the cut off was established based on the size of amplified product in a number of reactions that were subjected to post-PCR gel electrophoresis combined with Tm analysis. Ideally, a larger number of amplicons should have been examined, and a selection of these should have also been sequenced. Direct sequencing was attempted but was not successful (data not shown), possibly due to the short length of the product. Cloning of the PCR products would have overcome these

difficulties but was not done due to financial constraints. Overall, the test requires further optimisation before it can be used confidently for diagnostic testing.

Irrespective of these limitations, CHV did not appear to be an important contributor to the ICT observed in the sampled dogs. In other studies, CHV was detected in the trachea of 14.4% of dogs with a history of ICT (Posuwan and Payungporn 2011), whereas in this study, 8.5% of diseased dogs (4/47) were positive for CHV. However, samples for the current study were collected from the oropharynx, not the trachea. In contrast to these findings, CHV DNA was detected in a total of 32/40 samples (nasal swabs and tracheal lavage samples combined) from dogs with clinical signs of ICT (Kaczorek *et al.* 2017). Thus, it may be that the importance of CHV infection varies between different populations of dogs or the site of collection of the sample.

3.4.4 Canine parainfluenza virus circulates among New Zealand dogs, and may contribute to ICT.

According to the IDEXX panel results, CPIV was identified in 6.4% of 47 diseased dogs and in none of the healthy dogs sampled. This indicates that CPIV circulates among New Zealand dogs and is a likely contributor to the ICT syndrome in some of the dogs. This is in agreement with several other studies. For example, in Japan, frequency of CPIV detection among 64 diseased dogs was 7.4% (Mochizuki *et al.* 2008), and in Thailand 11.93% of diseased dogs were positive for CPIV RNA, compared to 0.98% healthy dogs (Posuwan and Payungporn 2011).

More recently, CPIV was detected in 5/40 (12.5%) nasal swabs from diseased dogs in Poland. Canine parainfluenza virus was identified in 27/40 (67.5%) of tracheal lavage samples collected from the same dogs (Kaczorek *et al.* 2017). This suggests that oropharyngeal swabs may not be the best type of sample for detection of CPIV. As the only sample type used in the current study was oropharyngeal swabs, it is possible that a larger proportion of diseased dogs would have tested positive for CPIV RNA if BAL samples had been also collected. The latter procedure is, however, more invasive than collection of oropharyngeal swabs and requires sedation. Hence, owner's willingness to participate would have been lower and it would have been considerably more difficult to enrol dogs into the study outside of the veterinary clinic settings.

There is some confusion in the literature with regard to the identity of CPIV. The virus has been often referred to simply as "canine parainfluenza virus", without a reference to its taxonomic classification. The target virus in the IDEXX CRD panel was defined as CPIV-3. However, the majority CPIV that have been described in the literature were PIV-5 like viruses (Ellis and Krakowka 2012; Oem *et al.* 2015; Liu *et al.* 2017). Our attempts to verify the identity of the target virus in the IDEXX panel were unsuccessful (no reply to several emails asking for clarification of the issue). Parainfluenza type 5 virus is currently classified within species *Mammalian rubulavirus 5* (Anonymous 2017). The name of the species has been recently changed from *Parainfluenza virus 5* to reflect a broad host range for its type virus PIV-5, hence the prefix "canine" in CPIV-5 indicates simply the host species from which PIV-5 was detected/isolated (Rima *et al.* 2014) It is unlikely that CPIV-5 would be detected by the primers specific for CPIV-3, as PIV-5 and PIV-3 belong to different genera (*Rubulavirus* and *Respirovirus*, respectively) (Karron and Collins 2013). Hence, the low level of detection of CPIV RNA in by the IDEXX panel in comparison with some previous studies (Decaro *et al.* 2016) may simply reflect differences in the viral targets. Unfortunately, initial attempts to optimize the IH assay for CPIV-5 failed to produce acceptable results and it was therefore decided not to use the assay for the survey. Hence, we were unable to compare IDEXX results with the results of the IH test.

None-the-less, the current data, as well as results published by others using the same IDEXX panel (Schulz *et al.* 2014) suggest that CPIV-3 may also circulate among dogs (in addition to CPIV-5) and should be considered as a potential contributor to ICT in future studies.

3.4.5: CRCoV infection is common among New Zealand dogs and has likely contributed to ICT observed in the sampled population.

The overall prevalence of CRCoV antibodies was 51% among dogs sampled, including 34% of healthy and 68% of diseased dogs. This was higher than 29% CRCoV seroprevalence reported previously among New Zealand dogs (Knesl *et al.* 2009). This may indicate that CRCoV infection rates have increased in New Zealand over the past 7 years. Alternatively, the differences between the results of these two studies may reflect differences between populations sampled. Knesl and colleagues did not target dogs with any particular clinical presentation, as the serum samples were collected opportunistically from samples

submitted to a veterinary diagnostic laboratory for a variety of reasons. It is therefore possible that the majority of sampled dogs have not been recently affected by ICT, in which case the CRCoV seroprevalence reported in 2009 would be very comparable to the seroprevalence found among healthy dogs in the current study.

It is also possible that the differences between the results of the two studies reflected differences in the sensitivity or specificity of the tests used. The study of Knesl et al utilised an indirect fluorescent antibody test (IFT) using CRCoV isolate. In our study, a commercially available blocking ELISA test with BCoV antigen was used. It has been shown by others that BCoV is antigenically very similar to CRCoV and serological tests based on BCoV antigen have been used by others to detect CRCoV antibody (Erles *et al.* 2003; Priestnall *et al.* 2006) In addition, ELISA tests with BCoV and CRCoV antigens produced very similar results when both tests were compared to IFA with CRCoV used as antigen (Priestnall *et al.* 2006). Thus, cross-reactivity between BCoV and CRCoV is well documented. However, bovine and canine sera may differ in the level of non-specific binding. If so, the cut-offs for bovine and canine sera for the same commercial ELISA test may not be the same. The cut-off applied in the current study was based on the manufacturer's recommendation for bovine sera. Ideally, we should have validated the recommended cut-off with known positive and known negative canine sera. Since such sera were not available, this was not done. Hence, we cannot exclude the possibility that the cut-off for canine sera should have been set up differently, which may have affected the classification of low-positive sera. However, even if we considered all low-positive sera (POI between 20% and 40%) as negative for CRCoV antibodies, the CRCoV seroprevalence among sampled dogs would change from 47/93 (51%) to 38/93 (41%), with a larger difference among healthy dogs (34% to 20%) than among diseased dogs (68% to 62%). Thus, the overall interpretation of these data and conclusions reached would not have changed with a higher cut-off for CRCoV positive samples.

The CRCoV seroprevalence in the current study was similar to that reported from the United States (54.5% of 956 dogs), and Canada (59.1% of 44 dogs). Both of the latter populations of dogs had unknown disease status (Priestnall *et al.* 2006). However, it was higher than what had been reported from some European countries. For example, 26.76 to 38.33% of 216 Italian dogs with unknown disease status (Decaro *et al.* 2007), and 30.3% of 66 Irish dogs

with unknown disease status were seropositive for CRCoV (Priestnall *et al.* 2006) It should be taken into account that CRCoV was first identified in 2003, with the earliest retrospective detection of the virus dating back to 1996 (Ellis *et al.* 2005). Hence, the epidemiology of CRCoV infections may have changed over the past decade or so in various geographical regions, to reflect the adaptation of the virus to its new canine host.

Diseased dogs were 5.22 times more likely to be seropositive for CRCoV compared to healthy dogs in the current study. However, higher levels of CRCoV antibodies were detected in both acute and convalescent sera from diseased dogs compared to healthy dogs. This suggests that either the sampled dogs had not been recently infected with CRCoV, (and therefore the virus was not responsible for the clinical signs of ICT observed) or acute serum samples were potentially collected not at the onset of disease, but after antibody levels had already increased and virus had already begun to be cleared from the upper respiratory tract.

Both scenarios fit in well with the fact that no CRCoV nucleic acid was detected in the oropharyngeal swabs from the dogs. However, the latter is more likely for a couple of reasons. Firstly, the average POI of convalescent serum samples was significantly higher than POI of acute serum samples for those dogs for which two serum samples were available. Secondly, acute samples may not have been collected soon after CRCoV infection if clinical sings of ICT in sampled dogs were due to secondary infections rather than primary CRCoV infection. The virus has been shown to infect ciliated epithelial and goblet cells, as well as having antiviral effects on the innate immune system, in particular, reduction of the pro-inflammatory cytokines TNF-a and IL-6, and the chemokine IL-8 (Priestnall *et al.* 2009). Based on these findings, as well as epidemiological data from a large UK-based kennel where the virus was first discovered (Erles *et al.* 2003), it has been hypothesised that infection with CRCoV may be mild or subclinical, but may predispose to secondary bacterial infection. While CRCoV may be the initiating agent, overt clinical signs typically associated with ICT may be due to these secondary infections.

A proportion of healthy dogs in the current study also showed serological evidence of prior exposure to CRCoV, despite the fact that none of the seropositive dogs had a history of respiratory disease within the 12 months prior to sampling. This positive CRCoV serology

suggests a past exposure to the virus, which may have occurred with or without overt clinical disease. Serological evidence of recent exposure to CRCoV in clinically healthy dogs has been documented by others (Erles and Brownlie 2005). The length of time that CRCoV antibodies persist following infection is not very well established, but likely depends on factors such as the infectious dose of the virus or immunological status of each individual dog. In one study (Erles and Brownlie 2005), the levels of CRCoV ELISA antibody declined over three to eight months for 11 dogs and remained stable for three dogs for which paired serum samples were available.

There was no association between the level of CRCoV antibodies in either healthy or diseased dogs and sex/age of the dogs. This was in contrast to data reported by Priestnall et al (2006), who showed that the frequency of detection of CRCoV antibodies increased in dogs older than 1 year, with the peak seroprevalence recorded in the 7 to 8-year-old group. Although the trend line observed was not significant, diseased highly CRCoV seropositive dogs did tend to be older than diseased seronegative dogs in the current study. This may be because older dogs were more likely to have been exposed to CRCoV in the past. Within the group of seropositive healthy dogs, dogs over the age of 8 appeared to have lower levels of CRCoV antibodies compared to younger healthy dogs, however, the overall association was considered very weak (R^2<0.2).

None of the other variables investigated was significantly associated with CRCoV seropositivity. This may represent a true finding, or may simply reflect a comparatively small sample size, which was further decreased for some analyses by poor response rate to selected questions.

In the present study, coughing was further categorised into cough type and cough frequency. Dogs that had a high CRCoV titre often were described as having a harsh, frequent cough, whereas seronegative dogs tended to have moist/ productive type of cough with no tendency towards a particular cough frequency. This could suggest that a harsh, frequent cough is characteristic of CRCoV infection. However, a dry, harsh cough has often been noted as a clinical sign of the ICT complex (Erles *et al.* 2004), hence a harsh frequent cough is more likely to be a clinical sign of ICT in general, rather than being specific to CRCoV infection. Furthermore, these observations are based on a small sample size (*n*=35), so, as

with other clinical signs, more samples would be required in order to evaluate the true significance of these results. In addition, the description of the cough type/ frequency was evaluated by the attending veterinarians; hence it is possible that different veterinarians may not have been consistent with their definitions of cough types/ frequency.

Altogether, our data suggest that out of the pathogens investigated, CRCoV was most likely to be associated with ICT among the sampled dog population. However, the involvement of other pathogens was judged based on the presence/absence of these pathogens in the samples, rather than on serology. Have we relied on the virological data only, CRCoV would not have been considered a likely aetiological agent, as none of the dogs sampled was positive for CRCoV RNA by IDEXX panel.

3.4.6: The role of other pathogens.

Canine influenza virus was not identified by either PCR or virus isolation, which is consistent with the results of a serological survey among 251 New Zealand dogs of unknown health status (Knesl *et al.* 2009)(Knesl et al 2009).

Although this was not the focus of the current study, it is possible that bacteria, rather than viruses, were responsible for clinical disease observed in the sampled dogs. *Bordetella bronchiseptica* was unlikely to be an important pathogen among the dogs sampled as only three diseased dogs were positive for *B. bronchiseptica* DNA by IDEXX qPCR. In addition, *B. bronchiseptica* was not grown from orophangeal swabs taken at the same time as the orophangeal swabs used for virus isolation (data not shown).

Streptococcus zooepidemicus DNA was not identified in any of the dogs sampled based on IDEXX qPCR, indicating that *S. zooepidemicus* was not aetiologically involved in ICT among dogs in the current study. Furthermore, *S. zooepidemicus* has typically been associated with more severe signs of ICT (Priestnall and Erles 2011) than those observed in the current study. The majority of diseased dogs sampled had clinical signs that would typically be classed as mild (no treatment was required in most cases and no deaths due to ICT were observed.

Mycoplasma cynos, however, was identified by IDEXX qPCR in 17% of diseased dogs, in comparison with only two (4%) of healthy dogs. This bacterium has been described as a commensal of the upper respiratory tract by some authors (Randolph *et al.* 1993). However,

its aetiological role in ICT has been suggested by others. In one study, *M. cynos* was significantly associated with the presence of ICT, with 23.9% and 21.7% of trachea and bronchial samples, respectively, positive for *M. cynos,* compared to 9.7% of samples from healthy dogs (Chalker *et al.* 2004). In contrast, 29.2% of 503 asymptomatic dogs were positive for *M.cynos* in another study. The frequency of detection of *M.cynos* among this group of dogs was the highest out of several other respiratory pathogens tested for (including CAdV-2, CDV, CHV, CIV , CRCoV, CPIV, *S. zooepidemicus* and *B. bronchiseptica*) (Lavan and Knesl 2015). Altogether, the results of above studies suggest that although *M. cynos* can be found in healthy dogs, under some circumstances its presence in the upper respiratory tract may be facilitating secondary infections, and hence exacerbating clinical signs of ICT. Further research wold be required to clarify the role of *M.cynos* in ICT.

Chapter 4: General Discussion and Concluding remarks

Up until this study, very little was known about the prevalence of pathogens involved in ICT in New Zealand. The findings of this study suggest that CRCoV does circulate among New Zealand dogs, and may be one of the viral agents aetiologically involved in ICT in New Zealand. Out of other viruses tested for, CPIV was likely to contribute to ICT in the sampled dogs, while CHV and CAdV-2 were less likely to be aetiologically involved.

This study had several limitations typical for field-based studies utilising client-owned dogs. These included variabilities in timing of sample collection with respect to the onset of clinical signs, and missing responses to some of the survey questions. The former was likely to affect the results, as ability to detect respiratory viruses is typically highest in samples collected early in the disease process. Ideally, serology would have been performed for all viral agents, which would have allowed to detect recent exposure to a given agent based on rising antibody titres. This was performed only for CRCoV due to financial and time constraints.

In addition to CRCoV and CPIV, this study also implicated *M. cynos* as a possible pathogen involved in ICT, either as primary or secondary pathogen. There are currently no vaccines available in New Zealand (and other countries) for CRCoV or *M. cynos*. Hence, aetiological involvement of these agents in ICT in New Zealand may explain why outbreaks of ICT have anecdotally occurred on occasions among dogs that were considered protected against "kennel cough" through vaccination.

Only 8/47 (17.02%) of diseased dogs in this study were negative for all agents tested, if one considers results of IDEXX panel and IH qPCR in parallel. However, if one excluded dogs positive for CAdV-2 (as there was no difference in levels of detection of this virus between healthy and diseased dogs) then the number of diseased dogs negative for any of the remaining pathogen tested would rise to 34/47 (72.4%). Hence, 13/47 (27.6%) of diseased dogs would have been positive for at least one agent. This corresponds well with results of overseas studies. For example, only 29.4% of 64 dogs in Japan tested positive for CAdV-2, CPIV, CCoV, CRCoV, CIV, or *B. bronchispetica* via conventional PCR (Mochizuki *et al.* 2008). Similarly, no significant correlation between the presence of antibodies CPIV, CRCoV or *B.*

bronchiseptica and ICT was found in a rehoming shelter in Canada, indicating that these agents were likely not involved in ICT among that study population (Ellis *et al.* 2011).

Lack of identification of possible aetiologically involved pathogens in some of the dogs from the current study suggest that other pathogens that wern't tested for may have contributed to the disease syndrome. Identification of such pathogens may be challenging as they may not grow readily in cell culture and would not be detected by virus-specific molecular tests used. Advanced molecular tools, such as next generation sequencing, has proven very useful for detection of novel pathogens. For example, a novel canine bocavirus was identified using random PCR and NGS in a dog litter with severe enteritis recently (Bodewes *et al.* 2014). Thus, submission of the samples from the current study for NGS may provide a logical follow-up to the current study.

In addition, availability of CRCoV qPCR in New Zealand would aide further investigation of the role that CRCoV plays in development of ICT among New Zealand dogs. Such test is currently not available due to lack of New Zealand CRCoV isolate to use as a positive control. This can be, however, overcome by the use of plasmid containing target sequence or by the use of synthetic piece of DNA. For example, Integrated DNA Technologies offers production of DNA fragments called gBLOCKS of up to 500 nt in size for a very affordable price of NZ$120.

Better understanding of the frequency of infection, and relative importance in causing ICT, or different pathogens circulating among New Zealand dogs would ultimately lead to better control strategies, which may require development of new vaccines. The current study provides some initial data towards this long-term goal.

Appendices

Appendix 1: Questionnaires

1.1: Questionnaire for dogs with suspected acute tracheobronchitis

Pxx

Questionnaire for dogs with suspected acute tracheobronchitis

Name: ……

Age: ……..

Breed: ……..

(Please circle as appropriate)

Female speyed Male castrated Entire female Entire male

Cough	Yes / No			
If yes, describe the type	Soft	Harsh	Moist / productive	
If yes, describe the frequency	Only with tracheal palpation	Occasional	Frequent	Occurring in paroxysms
Nasal discharge	Yes / No			
If yes, describe the type	Serous	Mucoid	Purulent	Haemorrhagic
Sneezing	Yes / No			
Rectal temperature	……..°C			
Lymphadenopathy	Yes / No			
Altered respiration	No	Tachypnoea	Dyspnoea	

Other signs: ……
……

Duration of clinical signs: ……..

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

Treatment:
……
……
……

(We may contact your clinic in the future to obtain further details on this case)

1.2: Racing greyhound questionnaire

A survey of Kennel Cough in New Zealand Racing Greyhounds

RGxx

Name: …… Tattoo: …….

Gender: ☐ Female ☐ Male

Age: ……..

Number of dogs in your facility/premises: .....

When the dog was last vaccinated? ……..

Was kennel cough included? ☐ No ☐ Yes

If yes, what type was the kennel cough vaccination? ☐ Injection ☐ Intranasal

If known, what was the brand name? .....

Housing: ☐ Mainly indoor ☐ Indoors/Outdoors

My dog is in contact with other animals:

☐ Cats ☐ Livestock ☐ Horses ☐ Poultry

Have you had respiratory disease in any dogs in your kennel in the last 12 months? ☐ No ☐ Yes

Do you feed your dogs with raw horse meat? ☐ No ☐ Yes

This is not a diagnostic service that will allow identification of all the possible organisms in a timely fashion.

By signing this form, you agree for your dog to participate in the study.

I have read and understood the information that is provided for the survey of Kennel Cough in New Zealand Racing Greyhounds and have agreed to take part in the survey.

1.3: Pet dog questionnaire

Pxx

Questionnaire Card
CIRD Project, Massey University

Reason for presenting: ☐Neutering ☐Vaccination/Check-up ☐Dental treatment

When was the last vaccination? Last date**.....**
Vaccine name**.....**

Was kennel cough included? ☐ No ☐ Yes
If yes, last date.....
☐ Injection ☐ Intranasal
Vaccine name.....

Diet: ☐ Raw meat ☐ Commercial

Does the pet have any history of respiratory illness? ☐No ☐Yes

Which treatment was administered? **.....**

What was this discontinued? **.....**

Any other disease in last 6 months? .....

In kennels within last 6 months: ☐ No ☐ Yes

My dog is obtained from rescue shelter within last 6 months: ☐ No ☐ Yes

My dog is mostly: ☐ Indoors ☐ Outdoors ☐ Both

☐ Town ☐ Rural

My dog is in contact with animals:

☐ Dogs ☐ Cats ☐ Livestock ☐ Horses ☐ Poultry

How often: .....

Any illness: .....

My dog has attended in shows in last 6 months: ☐ No ☐ Yes

If yes, which type and how often: .....

Date: ……..

Pet name, owner: ……

Breed: ……..

Age: ……..

Gender: ☐ Male ☐ Female

Neutered: ☐ Yes ☐ No

Address: ……

Phone: ……

Case number/label: ……

I agree to be contacted: ☐ Yes ☐ No

This is not a diagnostic service that will allow identification of all the possible organisms in a timely fashion.

I am the owner of the above pet and give consent for nasal and throat swabs, and a blood sample to be collected.

1.4: Working farm dog questionnaire

Fxx

TO BE COMPLETED BY INVESTIGATOR

Name: ……

Age: ……..

Breed/Type: ……

Gender: ☐ Female ☐ Male

Neutered: ☐Yes ☐ No

Number of dogs in your farm: ……

When was your dog last vaccinated? ……..

Was kennel cough included? ☐ No ☐ Yes

If yes, what type of kennel cough vaccination? ☐ Injection ☐ Intranasal

Housing: ☐ Mainly outdoors ☐ Indoors/Outdoors

Livestock on farm: ☐ Sheep ☐ Beef cattle ☐ Dairy cattle ☐ Deer

Dog(s) is/are in contact with: ☐ Other dogs ☐ Cats ☐ Horses ☐Poultry

How often: ……

Have you had respiratory disease in your dogs in the last 12 months? ☐ No ☐ Yes

Do you feed your dogs with raw meat? ☐ No ☐ Yes

If yes, what type? ☐ Horse meat ☐ Beef ☐ Sheep meat ☐ Chicken

This is not a diagnostic service that will allow identification of all the possible organisms in a timely fashion.

I……..have read and understood the information that is provided for the survey of Kennel Cough in New Zealand and have agreed to take part in the survey.

Signature …….. Date……..

Appendix 2: Database for healthy and diseased dogs

2.1: Diseased dogs: Signalment and medical history

Dog ID	Age (years)	Sex[a]	Date	Vaccination (core-Vangaurd 5)[b]	Vaccination (KC[c])	Housing[d]	Previous Respiratory Disease
RG1	2	M	6/12/2012	Y	N	IO	N
RG2	3	F	7/12/2012	Y	Y	IO	N
RG3	2	F	9/12/2012	Y	Y	IO	N
RG4	2	M	9/12/2012	Y	Y	IO	N
RG5	1.5	F	7/12/2012	Y	Y	IO	N
RG6	1.5	M	7/12/2012	Y	N	IO	N
RG7	3	M	9/12/2012	Y	N	MI	N
RG8	2	M	9/12/2012	Y	N	MI	N/A
RG9	2	F	9/12/2012	Y	N	MI	N/A
RG10	2	F	9/12/2012	Y	N	IO	N
RG11	1.5	F	14/12/2012	Y	Y	MI	N/A
RG12	8.5	F	14/12/2012	Y	Y	MI	N/A
RG13	5.5	F	14/12/2012	Y	Y	IO	N/A
RG14	3	F	14/12/2012	Y	Y	MI	N/A
RG15	2.5	F	14/12/2012	Y	N	IO	N
RG16	4	M	15/12/2012	Y	Y	N/A	N/A
RG17	5.5	M	15/12/2012	Y	Y	MI	N
RG18	4	F	15/12/2012	Y	Y	MI	N
RG19	2	F	15/12/2012	Y	Y	MI	N
RG20	4	F	15/12/2012	Y	Y	IO	N
RG21	1.1	M	11/12/2012	Y	N	IO	N
RG22	1.8	M	11/12/2012	Y	N	IO	N
RG23	2	F	11/12/2012	Y	N	IO	N
RG24	3	F	11/12/2012	Y	N	IO	N
RG25	2	M	11/12/2012	Y	Y	MI	N
RG30	2	F	12/12/2012	N/A	N/A	N/A	N/A
RG31	1.5	M	12/12/2012	N/A	N/A	N/A	N/A
RG32	2	M	12/12/2012	N/A	N/A	N/A	N/A
RG33	2	M	12/12/2012	N/A	N/A	N/A	N/A
RG34	2	M	12/12/2012	N/A	N/A	N/A	N/A
RG35	3	F	12/12/2012	N/A	N/A	N/A	N/A
RG36	2	F	5/12/2012	N/A	N/A	N/A	N/A
RG37	2	M	5/12/2012	N/A	N/A	N/A	N/A
RG41	2	M	30/07/2013	Y	Y	MI	Y
RG42	3	M	30/07/2013	Y	N	MI	Y
RG43	3	M	30/07/2013	Y	N	MI	Y
RG44	3	M	30/07/2013	Y	N	MI	Y
RG45	3	M	30/07/2013	Y	N	MI	Y
RG46	3	F	30/07/2013	Y	N	MI	Y
RG47	3	M	30/07/2013	Y	N	MI	Y
RG48	3	M	30/07/2013	Y	N	MI	Y
RG49	3	M	30/07/2013	Y	N	MI	Y
RG50	3	F	30/07/2013	Y	N	MI	Y

a: M= Male, F= Female. b: Y= Yes. N= No. c= Kennel cough. d: IO = Indoors /outdoors, MI= mainly indoors.

Dog ID	Age (years)	Sex[a]	Date	Vaccination (core-Vangaurd 5)[b]	Vaccination (KC[c])	Housing[d]	Previous Respiratory Disease
P27	1	M	19/3/2013	Y	N	IO	N
P57	<0.5	M	29/8/2013	N/A	N/A	N/A	N/A
P58	5	F	29/8/2013	N/A	N/A	N/A	N/A
P59	8	M	29/8/2013	N/A	N/A	N/A	N/A
P60	1	F	29/8/2013	N/A	N/A	N/A	N/A
P61	0.5	M	29/8/2013	N/A	N/A	N/A	N/A

a: M= Male, F= Female. b: Y= Yes. N= No. c= Kennel cough. d: IO = Indoors /outdoors, MI= mainly indoors.

2.2: Healthy dogs: Signalment and medical history

Dog ID	Age	Sex[a]	Date	Vaccination (core-Vanguard 5)[b]	Vaccination (KC[c])	Housing[d]	Previous disease	Shows /Kennels	Fed raw meat
P1	11	F	1/8/2012	Y	N	I/O	N	N	Y
P2	12	M	9/8/2012	Y	N	MI	N/A	N	N
P3	1	F	7/8/2012	Y	N	I/O	N	N	N
P4	2	F	7/8/2012	Y	Y	I/O	N	N	Y
P5	6	M	7/8/2012	Y	N	MI	N	N	N
P6	4	F	8/8/2012	Y	N	IO	N	N	N
P7	4	M	8/8/2012	Y	N	IO	N	N	N
P8	13	F	8/8/2012	Y	N	IO	N	N	Y
P9	1.5	M	8/8/2012	Y	N	IO	N	N	Y
P10	9	F	9/8/2012	Y	N	IO	N	N	N
P11	10	F	9/8/2012	Y	Y	IO	N	Y	Y
P12	13	F	9/8/2012	Y	N	IO	N	N	N
P13	2.5	F	10/8/2012	Y	N	IO	N	N	N
P14	1	F	11/9/2012	Y	N	IO	N	N	Y
P15	0.5	M	11/8/2012	Y	Y	O	N	N	N
P16	0.5	F	18/9/2012	Y	Y	IO	N	Y	Y
P17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
P36	6	M	16/6/2013	Y	Y	MI	N	N	N
P37	1	M	16/6/2013	Y	N	MI	N	N	N
P38	0.5	M	16/6/2013	Y	Y	IO	N	N	N
F1	3	F	24/7/2012	Y	N	O	N	N	Y
F2	1.5	F	24/7/2012	Y	N	O	N	N	Y
F3	2	M	24/7/2012	Y	Y	O	N	N	Y
F4	5	F	24/7/2012	Y	Y	O	N	N	Y
F5	2	F	24/7/2012	Y	N	O	N	N	Y
F6	2	M	24/7/2012	Y	N	O	N	N	Y
F7	9	M	24/7/2012	Y	Y	O	N	N	Y
F8	1	M	24/7/2012	Y	Y	O	N	N	Y

a: M= Male, F= Female. b: Y= Yes. N= No. c= Kennel cough. d: IO = Indoors /outdoors, MI= mainly indoors, O=outdoors

Dog ID	Age	Sex[a]	Date	Vaccination (core-Vanguard 5)[b]	Vaccination (KC[c])	Housing[d]	Previous disease[b]	Shows /Kennels[b]	Fed raw meat[b]
F9	13	F	24/7/2012	Y	Y	O	N	N	Y
F10	3	M	24/7/2012	Y	Y	O	N	N	Y
F31	4	F	26/3/2013	Y	Y	O	N	N	Y
F32	12	F	26/3/2013	Y	Y	O	N	N	Y
F33	2	M	26/3/2013	Y	Y	O	N	N	Y
F34	17	M	27/3/2013	Y	Y	O	Sneezing	N	Y
F35	5	M	27/3/2013	Y	Y	O	N	N	Y
F36	2	M	27/3/2013	Y	N	O	N	N	Y
F37	3	M	27/3/2013	Y	N	O	N	N	Y
F38	2	F	3/4/2013	Y	N	O	N	N	Y
F39	10	M	3/4/2013	Y	Y	O	Difficulty breathing	N	Y
F40	2	F	3/4/2013	Y	Y	O	N	N	Y
F41	5	M	3/4/2013	Y	Y	O	N	N	Y
F42	13	M	11/4/2013	Y	N	O	N	N	Y
F43	12	F	11/4/2013	Y	N	O	N	N	Y
F44	4	M	11/4/2013	Y	Y	O	N	N	Y
F45	5	M	11/4/2013	Y	Y	O	N	N	Y
F75	1.5	F	2/5/2013	Y	N	O	N	N	Y
F76	5	F	2/5/2013	Y	N	O	N	N	Y

a: M= Male, F= Female. b: Y= Yes. N= No. c= Kennel cough. d: IO = Indoors /outdoors, MI= mainly indoors, O=outdoors

2.3: Diseased dogs: clinical signs reported

Note: RG12 and RG13 did not have clinical signs recorded, but were diseased according to the vet who submitted the samples, and hence are excluded from this table

Dog I.D.	Nasal discharge (S,M,P,H)[e]	Sneeze (Y/N)[b]	Elevated Temp	Duration of signs (days)	Cough[b]	Cough Type (S,H,M)[f]	Cough freq (T,O,F,P)[g]
RG1	N	N	N	1	Y	S	O
RG2	N	N	N	7	Y	H	F
RG3	N	Y	N	1	Y	H	F
RG4	S	Y	N	6	Y	H	F
RG5	N	N	N	7	Y	H	F
RG6	N	N	N	7	Y	S	O
RG7	N	N	N	5	Y	H	F
RG8	P	N	N	1	Y	H	F
RG9	N	N	N	10	Y	H	F
RG10	N	N	N	7	Y	H	F
RG11	N	N	N	N/A	Y	S	T
RG14	N	N	N	N/A	Y	N/A	N/A
RG15	N	N	N	14	Y	H	P
RG16	N	N	N	3	Y	N/A	T
RG17	N	N	N	5	Y	S	T
RG18	N	N	N	6	Y	H	F
RG19	N	N	N	5	Y	S	T
RG20	N	N	N	5	Y	S	O
RG21	N	N	38.1	1	Y	M	O
RG22	N	N	38.6	2	Y	M	O
RG23	N	N	37.8	4	Y	M	O
RG24	N	N	38	3	Y	M	O
RG25	N	Y	38.3	4	Y	M	O
RG30	Y	N	39.3	N/A	Y	M	P
RG31	M	N	38.7	14	Y	M	P
RG32	S	N	38.5	14	Y	S	O
RG33	S	N	38.9	N/A	Y	S	F
RG34	P	N	38.7	N/A	Y	S	T
RG35	N	N	39.5	4	Y	H	F
RG36[1]	S	N	39.8	N/A	Y	M	P
RG37	N	N	39.5	N/A	Y	M	P
P27	S	N	38.7	5	Y	H	F
P57[2]	Y	N/A	38.8	6	Y		
P58[3]	Y	Y	39.1	6	Y	M	N/A
P59	Y	N	38.3	1	Y	N	N
P60	S	N	38.8	1	Y	M	N/A
P61	Y	Y	39.1	6	Y	M	N/A

B: Y=Yes, N=No. e: Y= Yes but type not recorded, S= serous, M= mucoid, P= Purulent, H = haemorrhagic. f: M= Moist/ Productive, H= Harsh, S= soft, U = unknown. g: T= only with tracheal palpitation, O = Occasionally, F = Frequent, P= Occurring in paroxysms, U = unknown.1= Pnuemonia and dyspnoea noted. 2=Tachypnoea noted. 3= Mild tachypnoea noted.

Appendix 3: Real time qPCR3.1 IH qPCR optimisation

3.1.1: CAdV-2 annealing temperature optimisation

3.1.1.1: 60°C annealing

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
Assay 1		-5.252	49.65	55.03%	0.978

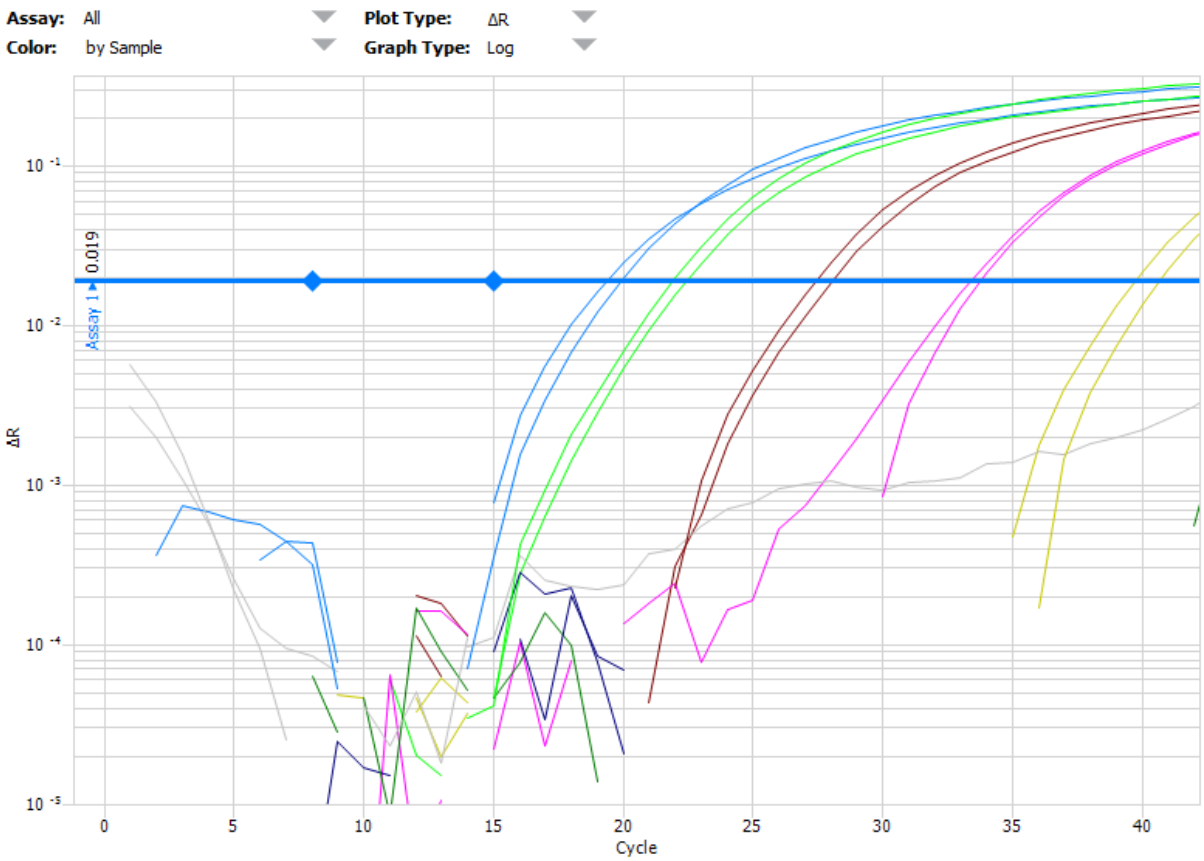

Figure 31: CAdV-2 standard curve (annealing temperature = 60 degrees)

3.1.1.2: 63°C annealing:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
CAV-2		-4.479	40.17	67.21%	0.958

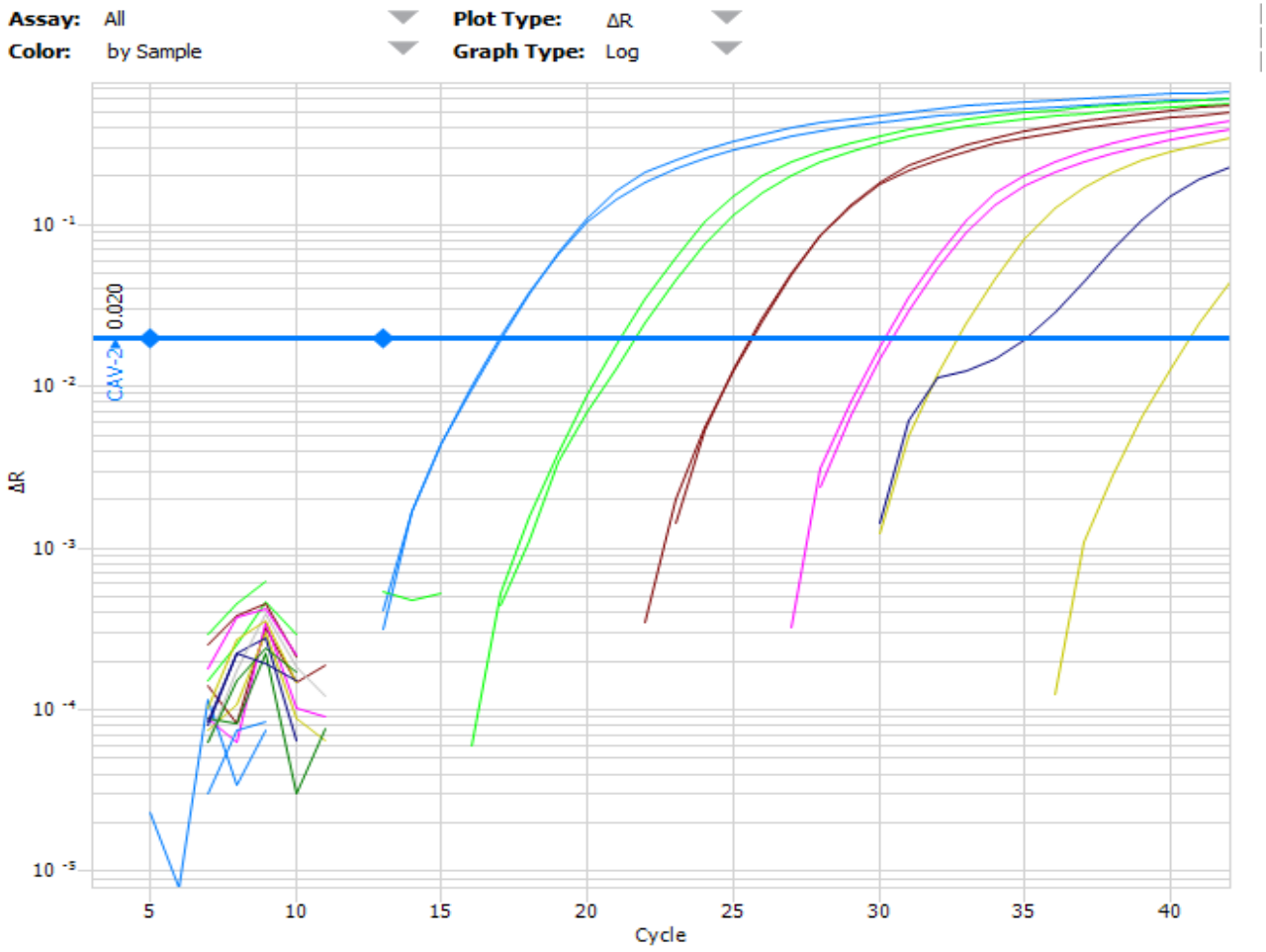

Figure 32: CAdV-2 standard curve (annealing temperature = 63 degrees)

3.1.2: CHV annealing temperature optimisation

3.1.2.1: 60°C annealing:

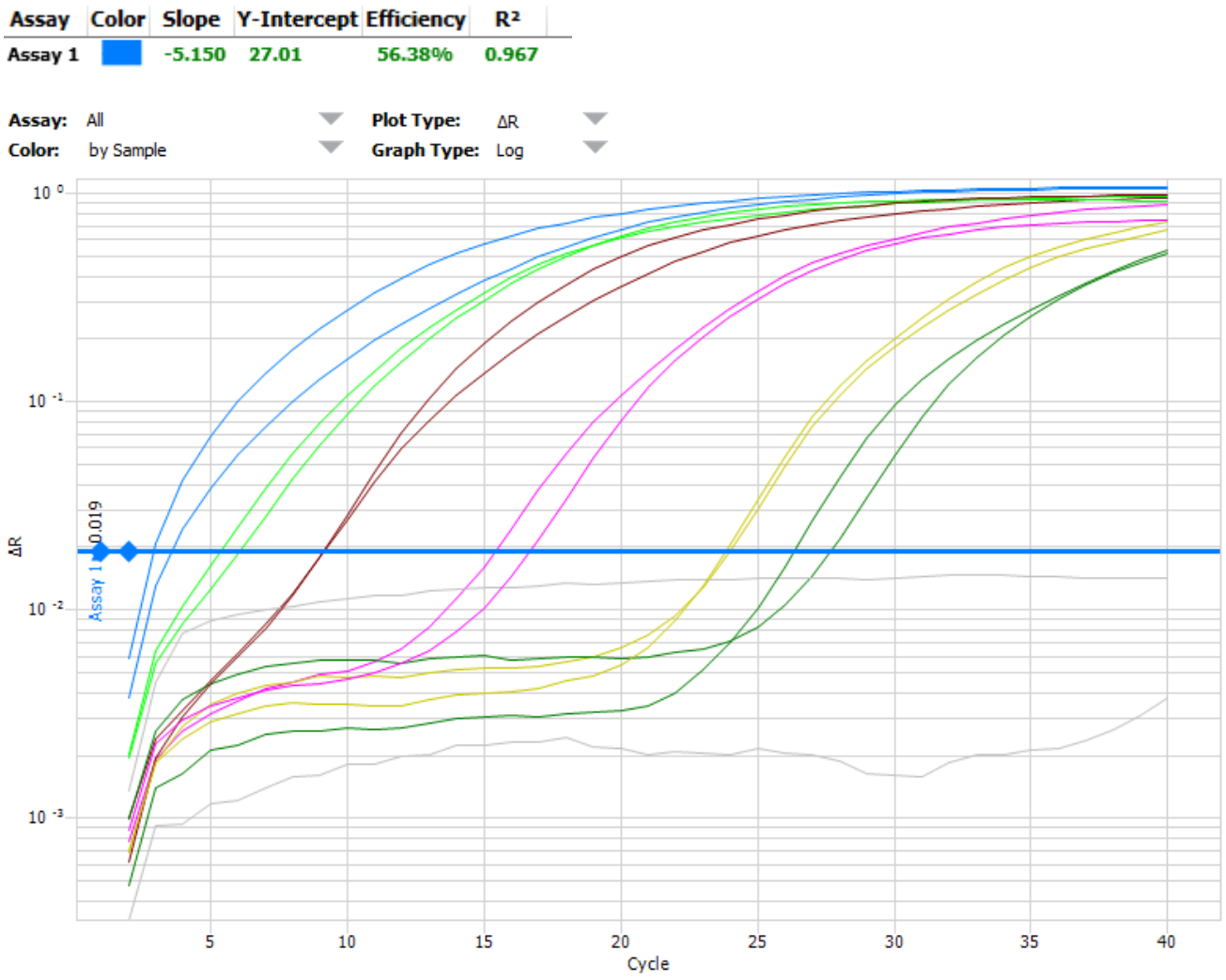

Figure 33: CHV standard curve (annealing temperature = 60 degrees)

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3.1.2.2: 64°C annealing:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
Assay 1		-6.128	59.02	45.61%	0.914

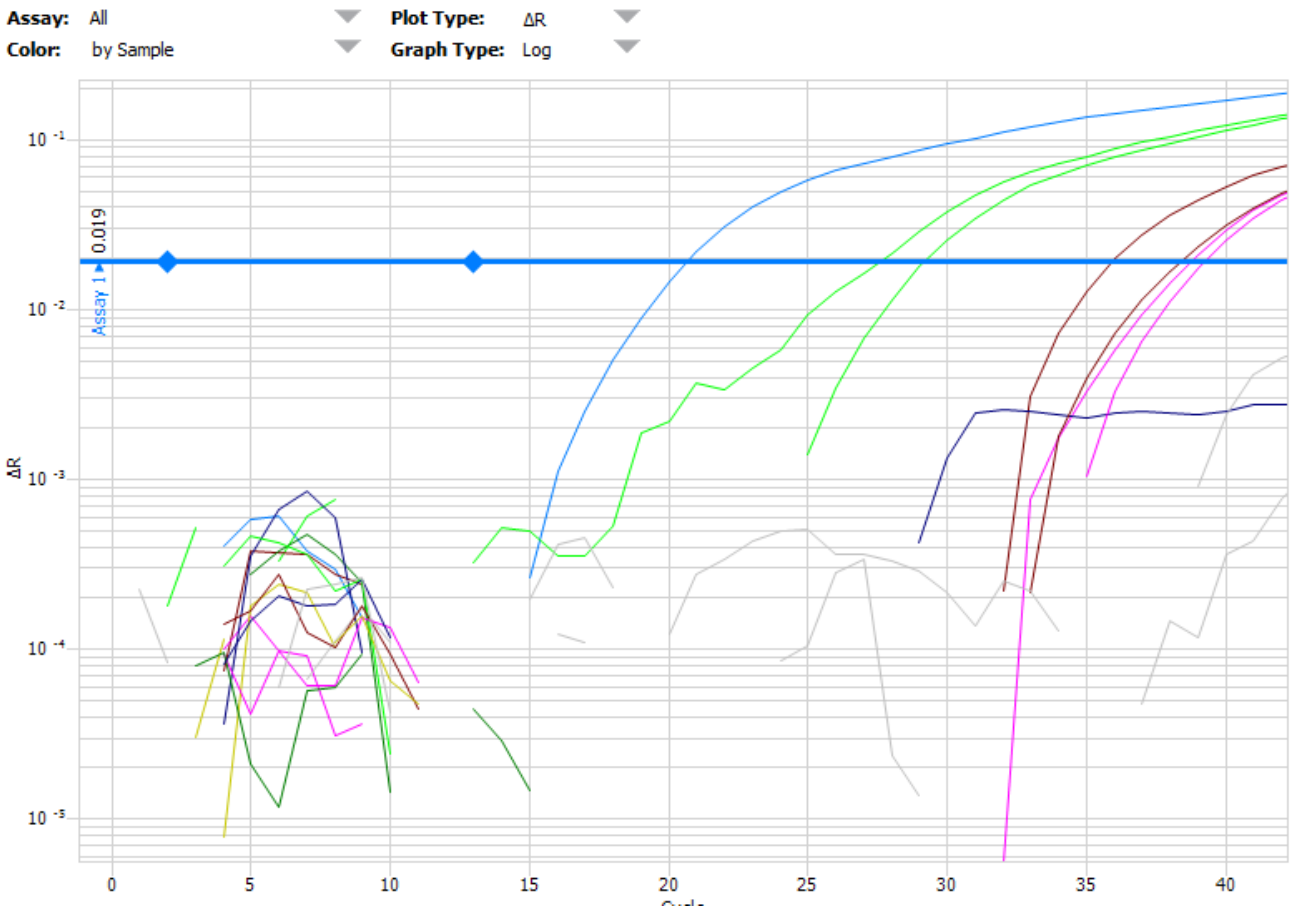

Figure 34: CHV standard curve (annealing temperature = 64 degrees)

3.1.3: CPIV annealing temperature optimisation

3.1.3.1: 60°C annealing:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
CPIV		-1.590	34.27	325.52%	0.932

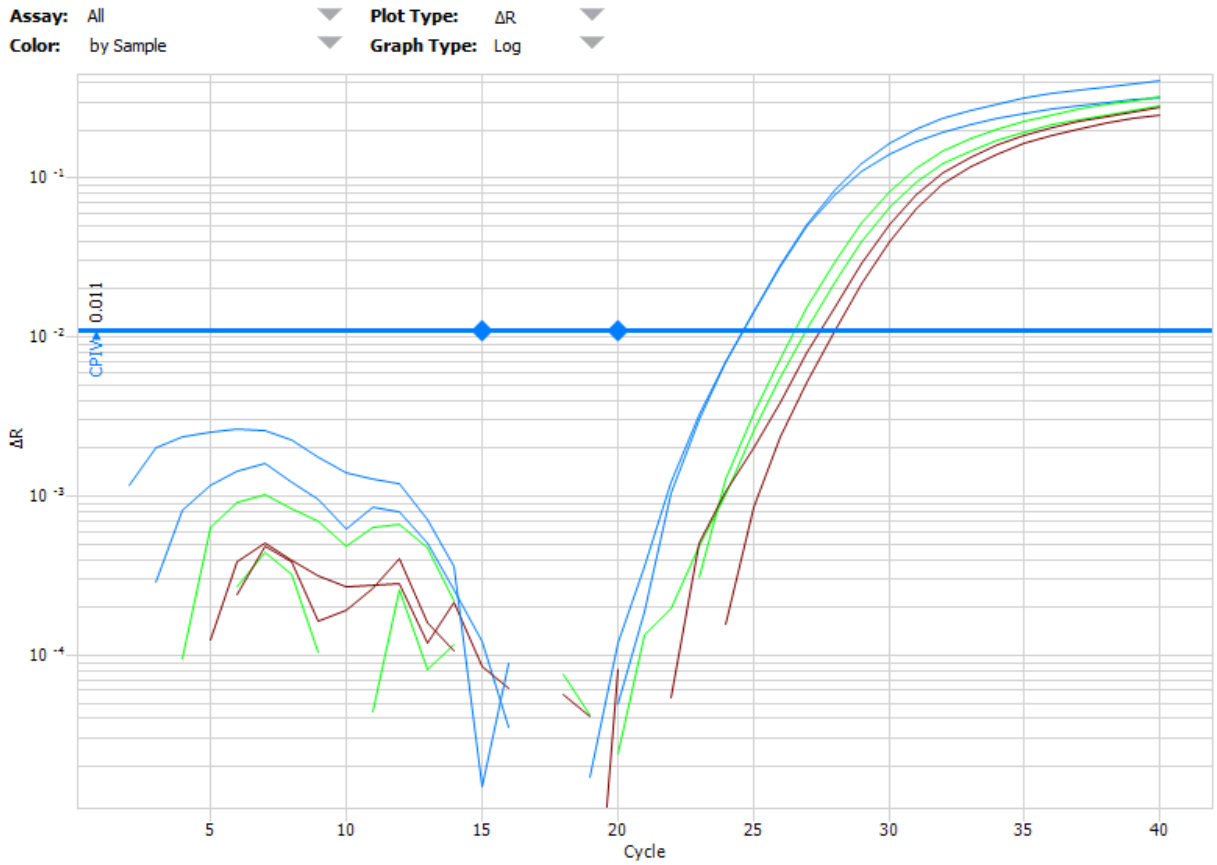

Figure 35: CPIV standard curve (annealing temperature = 60 degrees)

3.2: In-house qPCR results

3.2.1: CAdV-2:

3.2.1.1: RG1-21:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
Assay 1		-3.382	27.94	97.57%	0.984

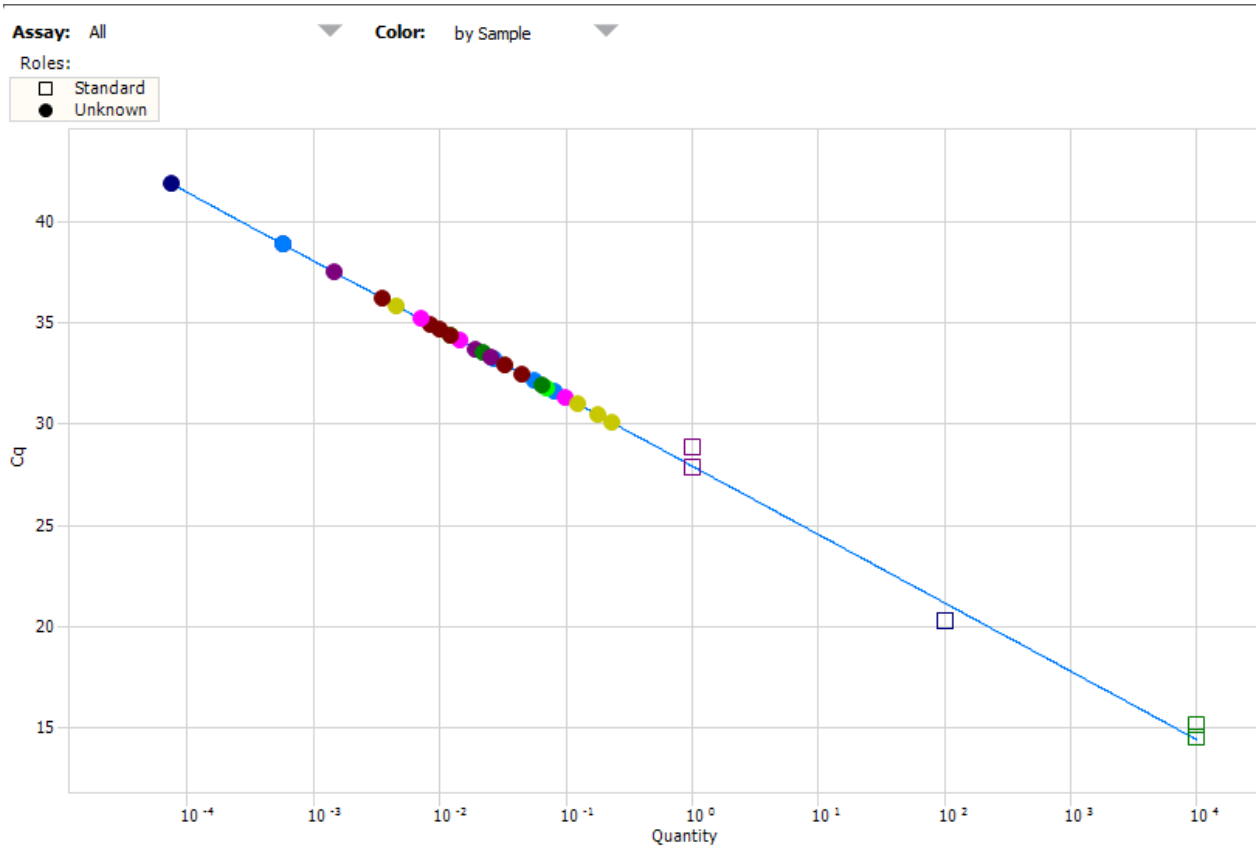

Figure 36: Samples RG1-21 run with CadV-2 primers

3.2.1.2: RG22-47:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
Samples		-3.578	33.28	90.32%	0.992

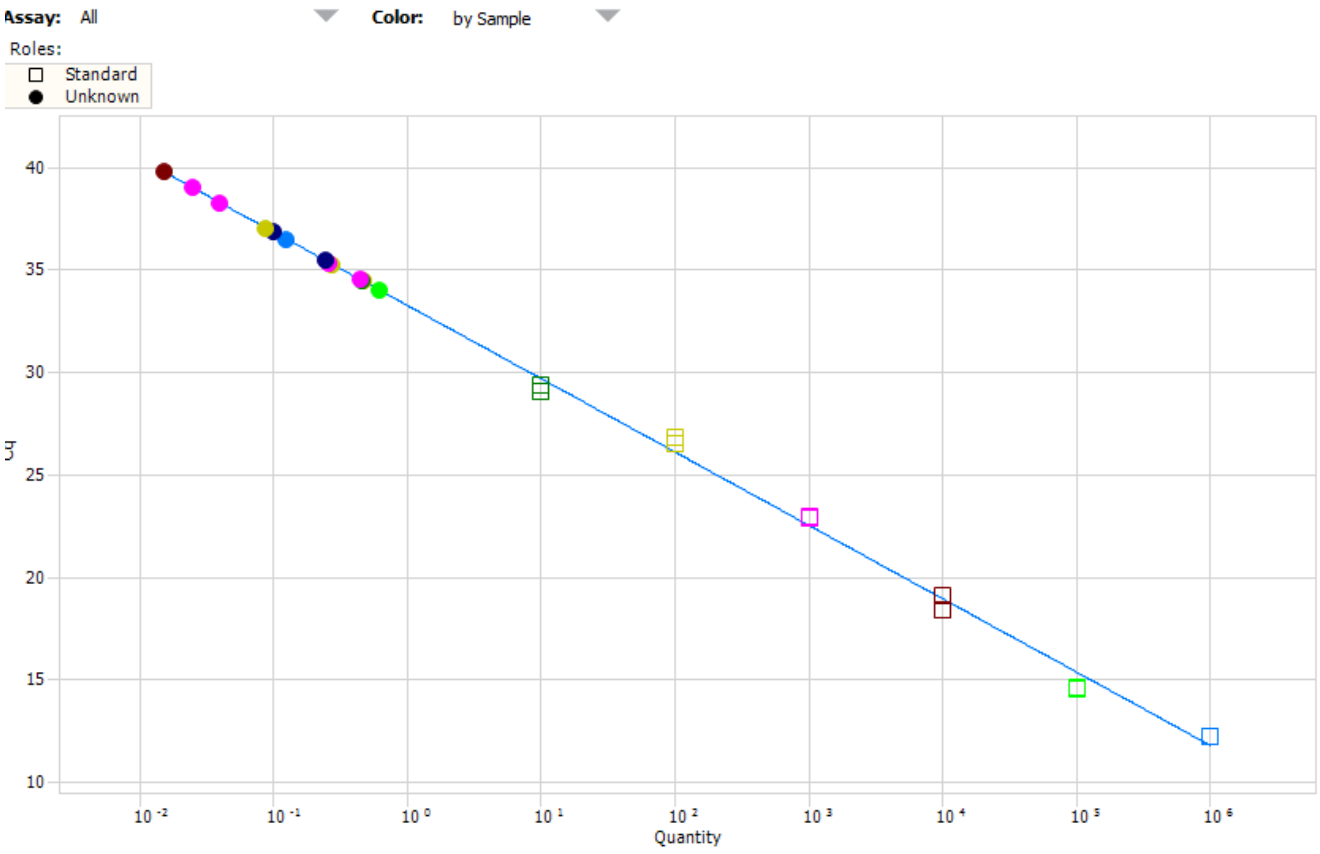

Figure 37: Samples R221-47 run with CadV-2 primers

3.2.1.3: RG50-P10:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
Assay 1		-3.315	28.97	100.30%	0.997

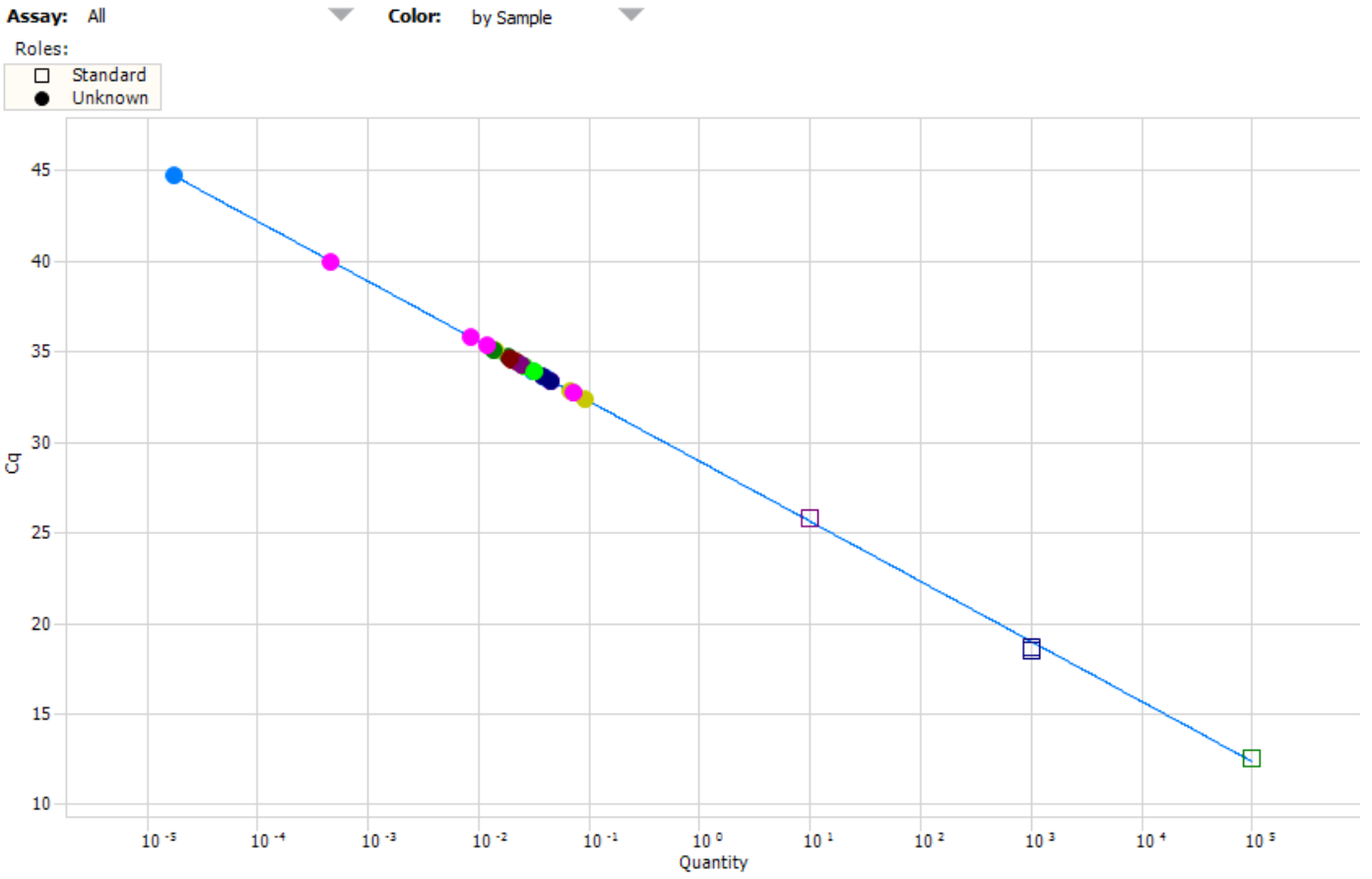

Figure 38: Samples RG50-P10 run with CadV-2 primers

3.2.1.4: P11-F33:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
Assay 1		-3.439	29.46	95.34%	0.999

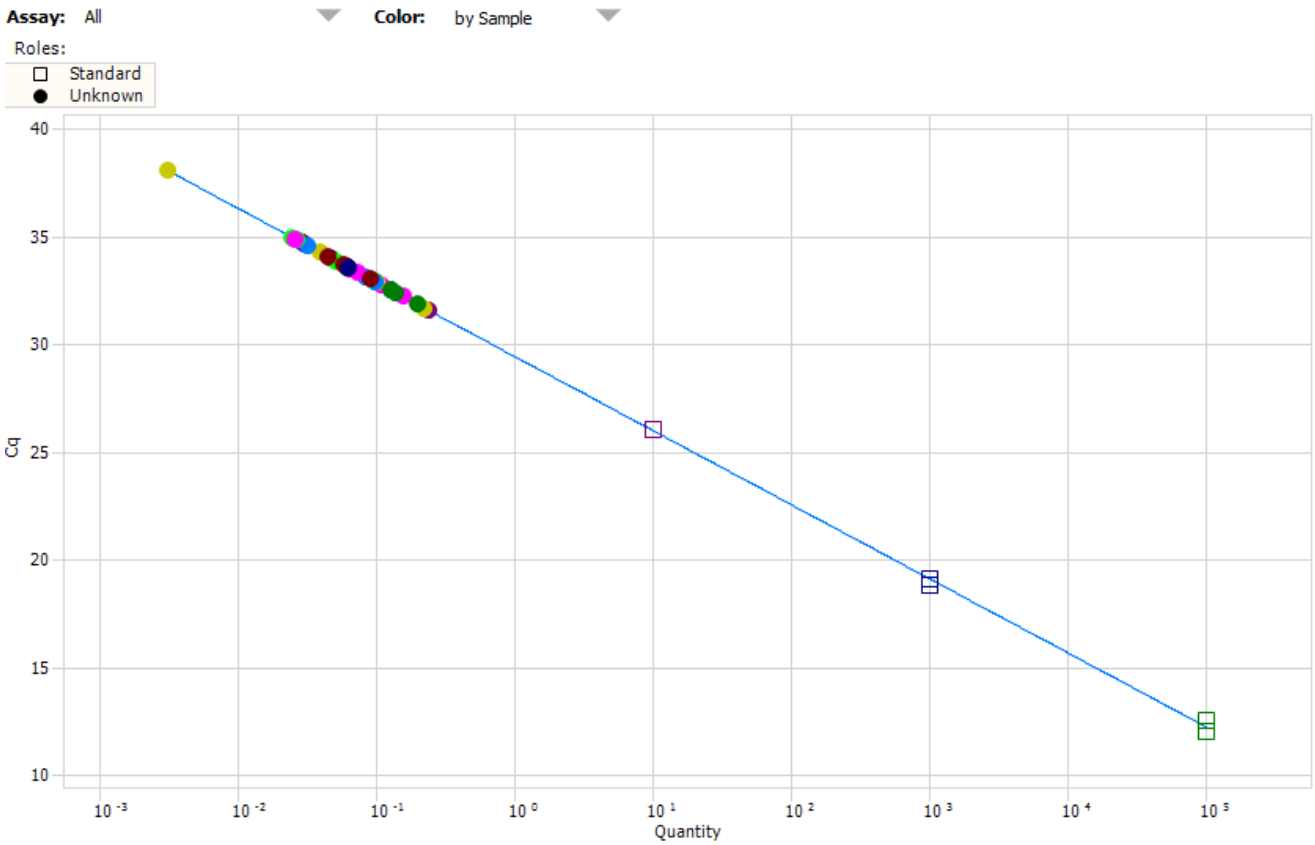

Figure 39: Samples P11-F33 run with CAdV-2 primers

3.2.1.5: F34-F76:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
Assay 1		-3.454	29.70	94.76%	0.980

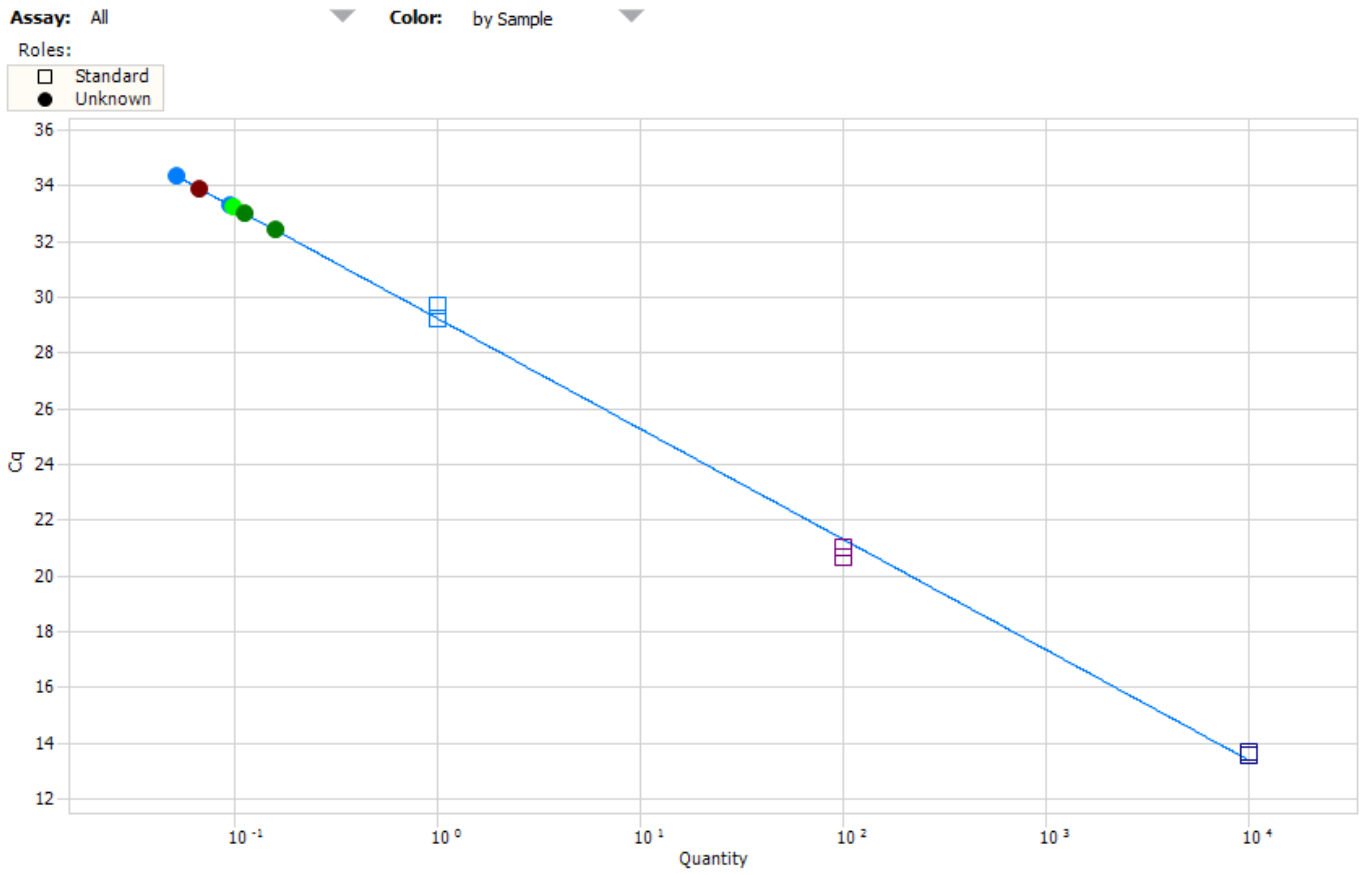

Figure 40: Samples F34-F76 run with CAdV-2 primers

3.2.2: CHV:

3.2.2.1: RG1-17:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
CHV		-4.298	39.30	70.88%	0.978

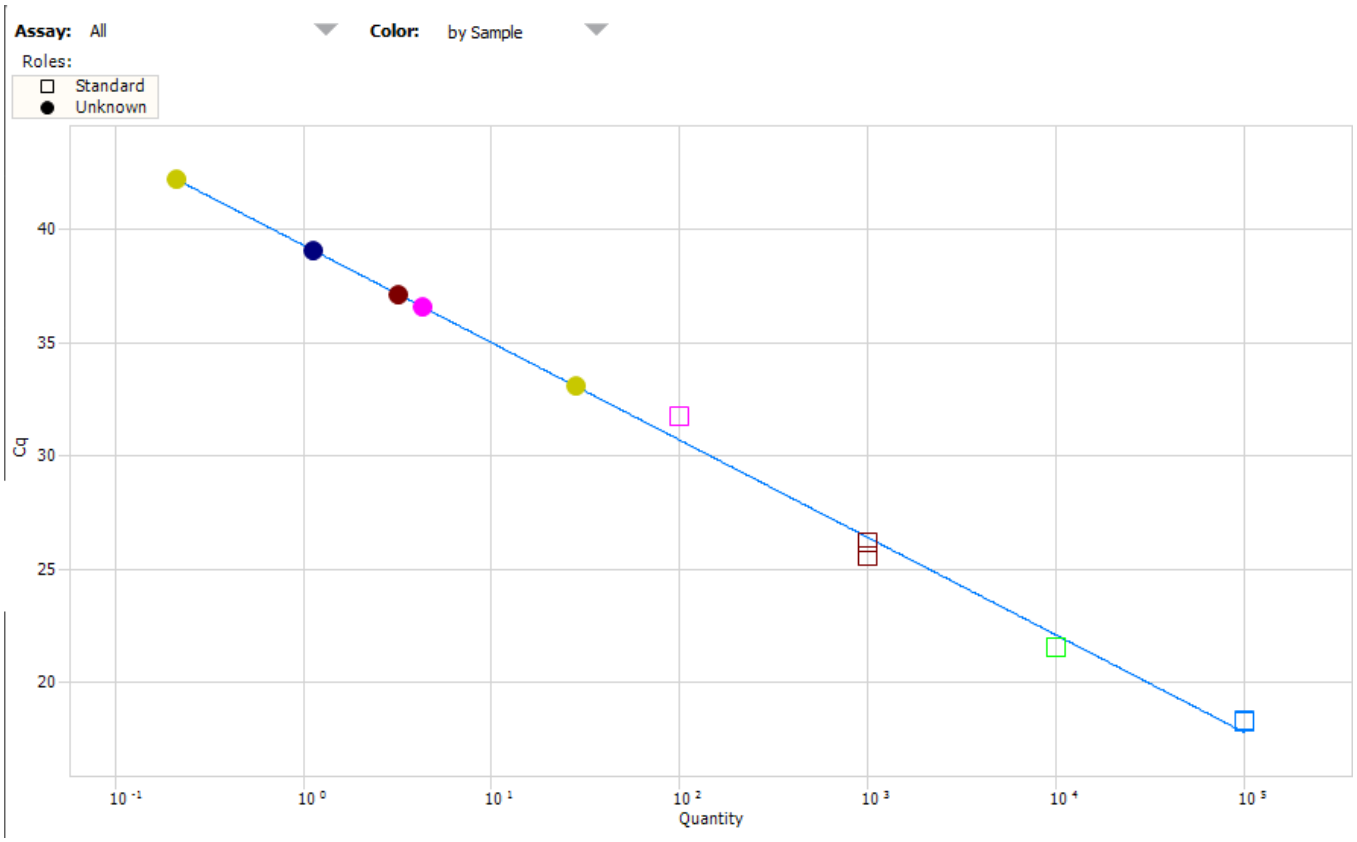

Figure 41: Samples RG1-17 run with CHV primers

3.2.2.2: RG18-41:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
CHV		-3.003	26.19	115.29%	1.000

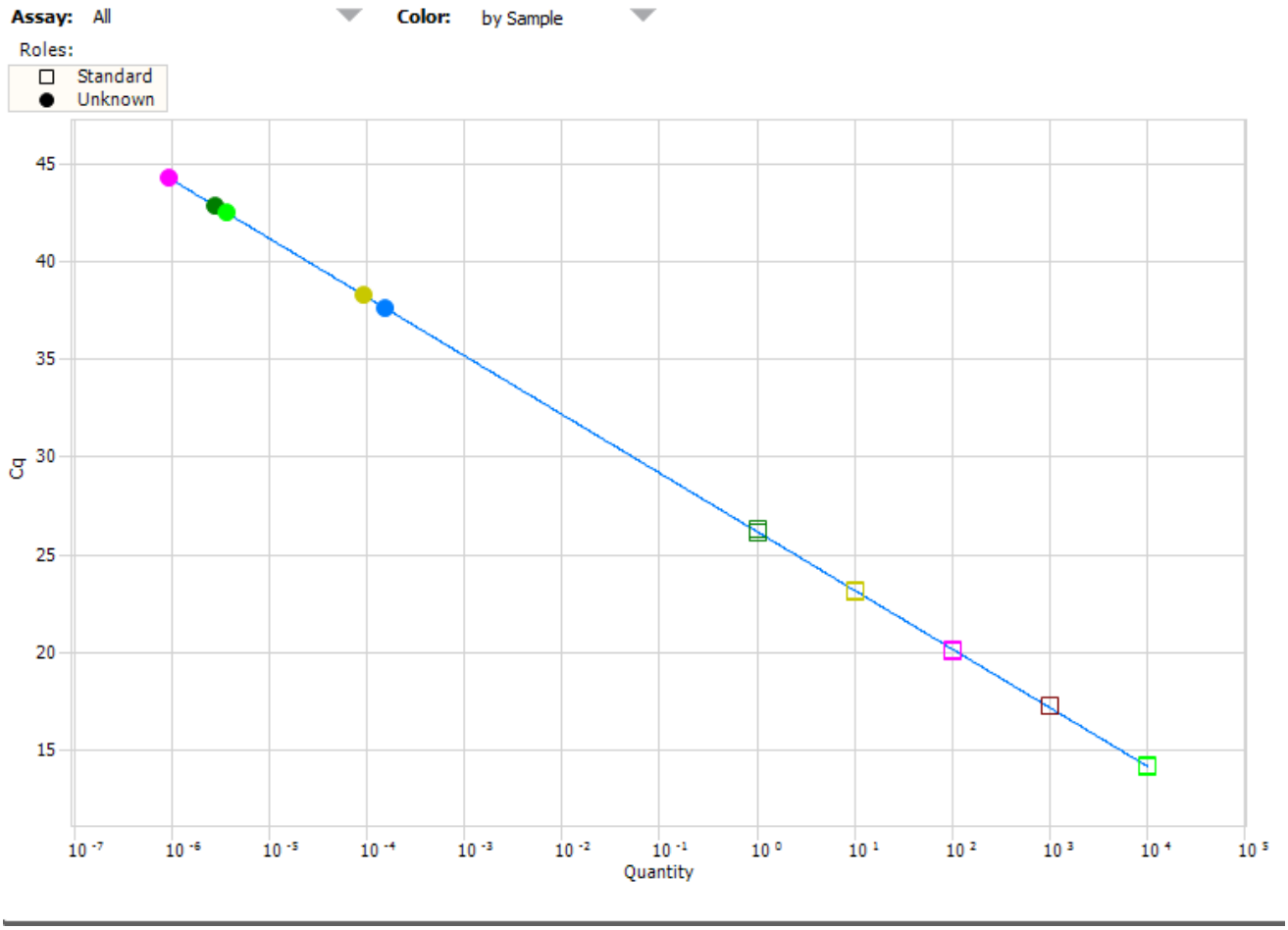

Figure 42: Samples RG18-41 run with CHV primers

3.2.2.3: RG33 (repeat), RG42-50, P1-4; 57-61:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
chv		-3.677	27.48	87.05%	0.991

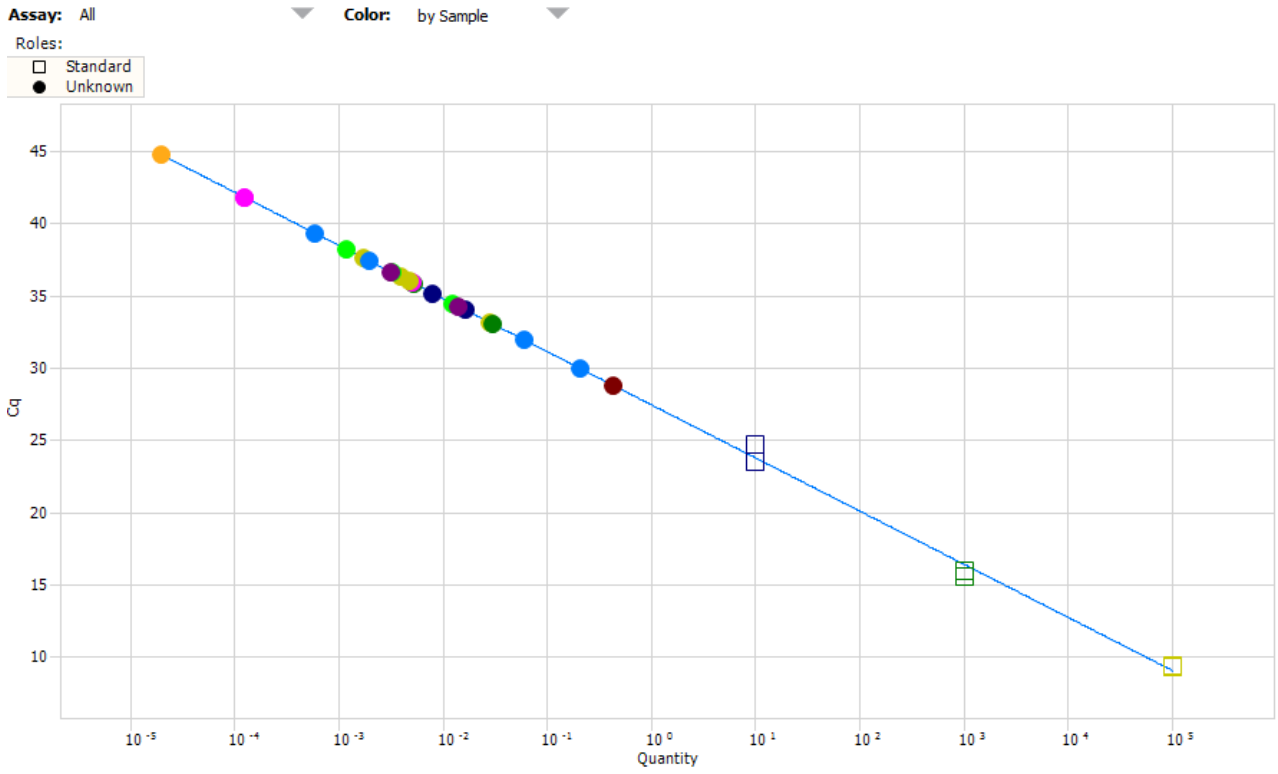

Figure 43: Samples RG33 (repeat), RG42-50, P1-4; 57-61 run with CHV primers

3.2.2.4: P5-F5:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
chv		-3.408	29.32	96.52%	0.999

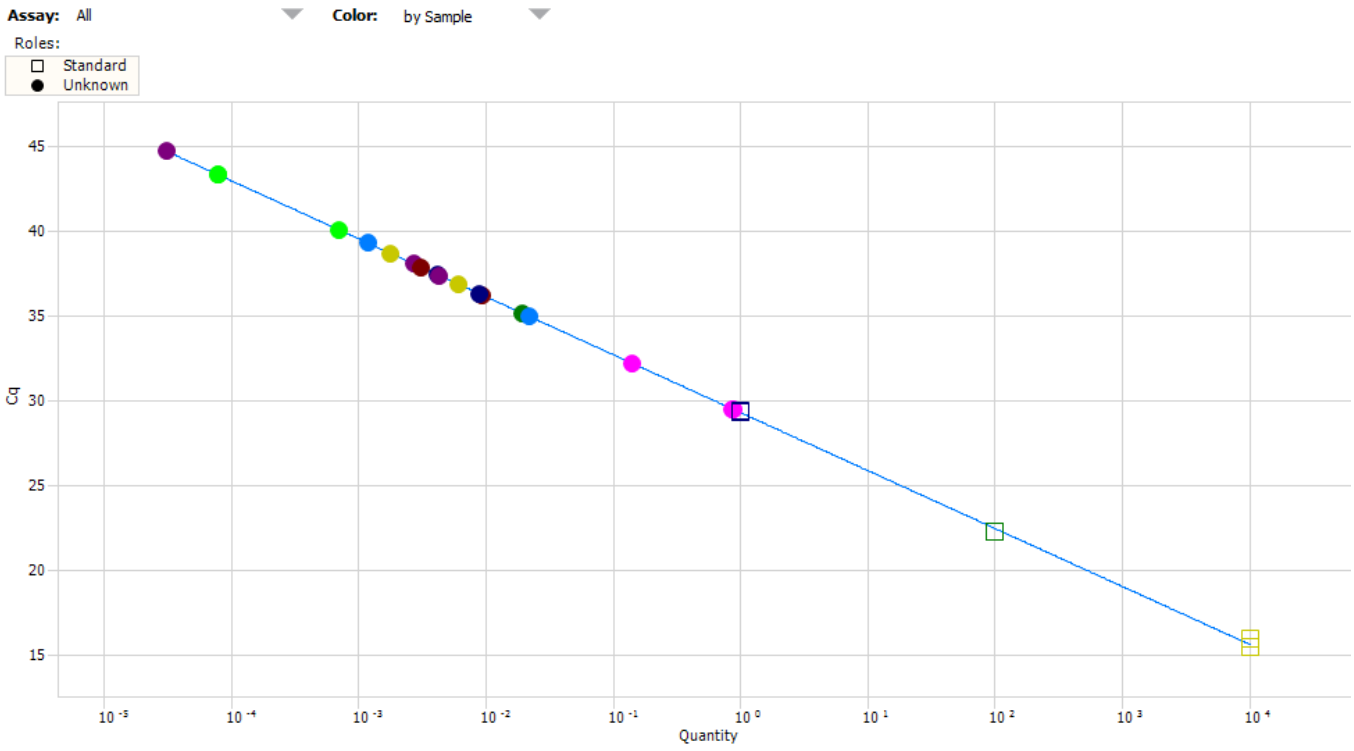

Figure 44: Samples P5-F5 run with CHV primers

3.2.2.5: F6-F76:

Assay	Color	Slope	Y-Intercept	Efficiency	R^2
CHV		-3.304	30.23	100.74%	0.991

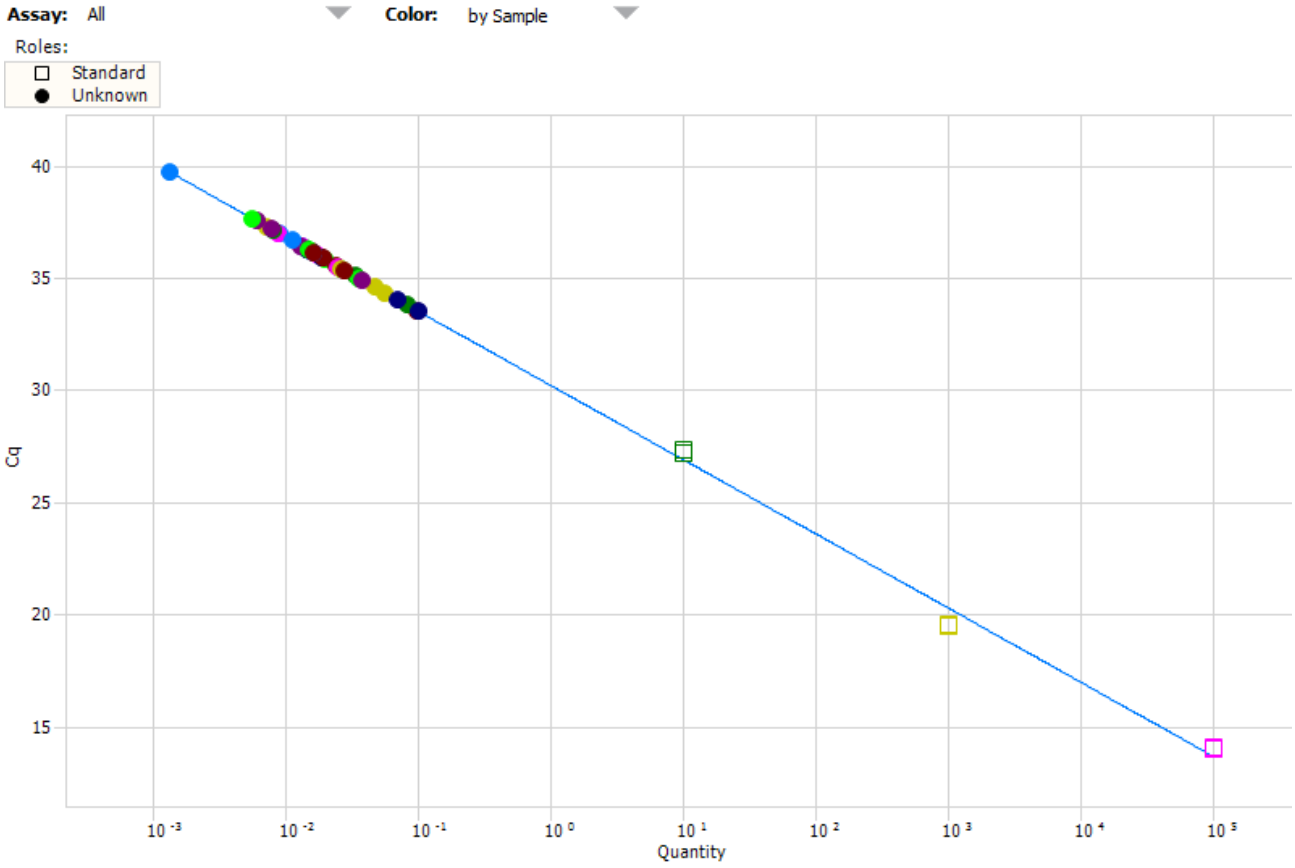

Figure 45: Samples F6-F76 run with CHV primers

3.3: IDEXX and IH qPCR results

3.3.1: Diseased dogs

	qPCR IDEXX (Cq values)							IH qPCR (Cq values)	
Dog ID	**CPIV**	**CAV-2**	**CHV**	**CRCoV**	**CIV**	**M.cynos**	**B.Bord**	**CAV-2**	**CHV**
RG1	36.86	0	0	0	0	0	37.18	31.72	0
RG2	0	0	0	0	0	0	0	32.91	0
RG3	39	0	0	0	0	0	0	33.39	0
RG4	0	0	0	0	0	0	0	31.25	0
RG5	0	0	0	0	0	0	0	33.19	0
RG6	0	0	0	0	0	0	0	31.63	0
RG7	0	0	0	0	0	0	0	41.61	0
RG8	0	0	0	0	0	32.95	0	35.42	0
RG9	0	0	0	0	0	0	0	33.06	0
RG10	0	0	0	0	0	0	0	0	0
RG11	0	0	0	0	0	0	0	0	0
RG12	0	0	0	0	0	0	0	35.4	0
RG13	0	0	0	0	0	0	0	34.01	0
RG14	0	0	0	0	0	0	0	30.15	0
RG15	0	0	0	0	0	0	34.19	33.40	0
RG16	0	0	0	0	0	0	0	38.72	0
RG17	0	0	0	0	0	0	0	33.16	0
RG18	0	0	0	0	0	0	0	38.74	0
RG19	0	0	0	0	0	0	0	0	0
RG20	0	0	0	0	0	0	0	33.54	0
RG21	0	34	0	0	0	0	0	35.11	0
RG22	0	0	0	0	0	0	0	36.03	38.3
RG23	0	0	0	0	0	0	0	33.48	0
RG24	0	0	0	0	0	0	0	39.25	0
RG25	0	0	0	0	0	0	0	37.6	0
RG30	0	0	0	0	0	0	31.42	34.55	0
RG31	0	0	0	0	0	0	28.86	34.04	0
RG32	0	0	0	0	0	0	0	36.40	0
RG33	0	0	0	0	0	0	34	0	0
RG34	0	0	0	0	0	0	34.23	0	0
RG35	0	0	0	40	0	0	33.84	0	0
RG41	0	0	0	0	0	0	0	0	0
RG42	0	0	0	0	0	0	0	34.58	0
RG43	0	0	0	0	0	0	0	36.57	0
RG44	0	0	0	0	0	0	0	0	0
RG45	0	0	0	0	0	0	0	35.04	0
RG46	0	0	0	0	0	0	0	0	0
RG47	0	0	0	0	0	0	0	33.37	0
RG48	0	0	0	0	0	0	0	0	0

	qPCR IDEXX (Cq values)							IH qPCR (Cq values	
Dog ID	CPIV	CAV-2	CHV	CRCoV	CIV	M.cynos	B.Bord	CAV-2	CHV
RG49	0	0	0	0	0	0	0	0	0
RG50	0	0	0	0	0	0	0	36.73	0
P27	0	0	0	0	0	0	0	32.37	0
P57	0	0	0	0	0	0	0	0	0
P58	0	0	0	0	0	0	0	34.49	0
P59	0	0	0	0	0	0	0	0	32.65
P60	0	0	0	0	0	0	35	44.18	0
P61	0	0	0	0	0	0	0	33.86	33.03

3.3.2: Healthy dogs

	qPCR IDEXX (Ct values)							qPCR I.H (Ct values)	
Dog ID	CPIV	CAV-2	CHV	CRCoV	CIV	M.cynos	B.Bord	CAV-2	CHV
P1	0	0	0	0	0	0	0	34.23	0
P2	0	0	0	0	0	0	0	34.53	0
P3	0	0	0	0	0	0	0	33.63	0
P4	0	0	0	0	0	0	0	34.76	0
P5	0	0	0	0	0	0	0	33.30	0
P6	0	0	0	0	0	0	0	34.09	0
P7	0	0	0	0	0	0	0	0	0
P8	0	0	0	0	0	0	0	33.33	0
P9	0	0	0	0	0	0	0	34.40	0
P10	0	0	0	0	0	0	0	33.67	0
P11	0	0	0	0	0	0	0	32.4	0
P12	0	0	0	0	0	0	0	34	0
P13	0	0	0	0	0	0	0	34.83	0
P14	0	0	0	0	0	0	0	0	0
P15	0	0	0	0	0	0	0	32.57	0
P16	0	0	0	0	0	0	0	36.05	0
P17	0	0	0	0	0	0	0	32.89	0
P36	0	0	0	0	0	0	0	33.68	0
P37	0	0	0	0	0	36.51	0	31.87	34.34
P38	0	0	0	0	0	0	0	32.36	0
F1	0	0	0	0	0	0	0	33.39	0
F2	0	0	0	0	0	0	0	33.5	0
F3	0	0	0	0	0	0	0	32.92	0
F4	0	0	0	0	0	0	0	32.16	0
F5	0	0	0	0	0	0	0	32.14	0
F6	0	0	0	0	0	0	0	33.68	0
F7	0	0	0	0	0	0	0	0	0
F8	0	0	0	0	0	0	0	34.25	0

	qPCR IDEXX (Ct values)							qPCR I.H (Ct values)	
Dog ID	**CPIV**	**CAV-2**	**CHV**	**CRCoV**	**CIV**	**M.cynos**	**B.Bord**	**CAV-2**	**CHV**
F9	0	0	0	0	0	0	0	33.59	
F10	0	0	0	0	0	0	0	34.69	
F31	0	0	0	0	0	0	0	33.39	
F32	0	0	0	0	0	0	0	0	
F33	0	0	0	0	0	0	0	34.36	
F34	0	36.59	0	0	0	0	0	34.81	
F35	0	0	0	0	0	0	0	34.27	
F36	0	0	0	0	0	0	0	35.72	
F37	0	37.47	0	0	0	0	0	44.04*	
F38	0	0	0	0	0	0	0	0	
F39	0	35.24	0	0	0	0	0	37.57	33.24
F40	0	36.75	0	0	0	0	0	33.84	
F41	0	0	0	0	0	0	0	0	33.5
F42	0	0	0	0	0	0	0	0	34.04
F43	0	0	0	0	0	0	0	38.48	32.29
F44	0	0	0	0	0	0	0	0	0
F45	0	0	0	0	0	0	0	0	0
F75	0	33.73	0	0	0	0	0	0	0
F76	0	34.3	0	0	0	27.36	29.26	36.04	0

Appendix 4: CRCoV ELISA results

4.1: Calculation of the percentage of inhibition

The percentage of inhibition (POI) for each sample was calculated using the following formula:

POI = [(OD neg – OD sample)/OD neg]*100

The POI of the positive test serum was calculated using the following formula:

POI = [(OD neg – OD pos)/ OD neg]*100

4.2: Interpretation of results

Calculated value	Degree of positivity
POI<20	0
20 <=POI <40	+
40 <=POI <60	++
60 <=POI <80	+++
80 <= POI	++++

4.3: CRCoV serology results: diseased dogs.

Note: Samples from dogs P57 and P59 were not available for testing.

Dog I.D.	Acute POI	Degree of positivity	Conv POI	Degree of Positivity
RG1	87.056	++++	N/A	-
RG2	0	-	7.664	-
RG3	91.224	++++	94.065	++++
RG4	78.767	+++	83.832	++++
RG5	0	-	7.664	-
RG6	46.832	++	72.804	+++
RG7	73.159	+++	83.224	++++
RG8	85.83	++++	71.682	+++
RG9	41.952	++	39.019	+
RG10	68.065	+++	62.43	+++
RG11	0		3.224	-
RG12	90.026	++++	88.645	++++
RG13	77.012	+++	91.542	++++
RG14	19.221	-	20.748	+
RG15	0	-	9.206	-

Dog I.D.	Acute POI	Degree of positivity	Conv POI	Degree of Positivity
RG16	56.036	++	90.047	++++
RG17	55.351	++	79.673	+++
RG18	63.741	+++	78.925	+++
RG19	0	-	-5.981	-
RG20	70.034	+++	81.776	++++
RG21	6.542	-	N/A	N/A
RG22	-3.131	-	N/A	N/A
RG23	-4.579	-	N/A	N/A
RG24	-2.71	-	N/A	N/A
RG25	50.654	++	N/A	N/A
RG30	3.879	-	N/A	N/A
RG31	7.804	-	N/A	N/A
RG32	62.85	+++	N/A	N/A
RG33	86.402	++++	N/A	N/A
RG34	52.43	++	N/A	N/A
RG35	63.131	+++	N/A	N/A
RG36	78.271	+++	N/A	N/A
RG37	14.673	-	N/A	N/A
RG41	62.158	+++	59.813	++
RG42	0	-	-6.308	-
RG43	65.668	+++	71.589	+++
RG44	80.779	++++	84.019	++++
RG45	18.536	-	28.598	+
RG46	25.128	+	55.467	++
RG47	0	-	6.776	-
RG48	34.717	+	42.757	++
RG49	62.243	+++	78.925	+++
RG50	48.33	++	44.907	++
P27	64.86	+++	N/A	N/A
P58	56.079	++	80.327	++++
P60	2.44	-	20.047	+
P61	-3.553	-	N/A	N/A

4.4: CRCoV serology results: healthy dogs.

Note: Sample from dog P12 was not available for testing.

Dog I.D.	POI	Degree of Positivity
P1	26.075	+
P2	-18.037	-
P3	4.533	-
P4	1.729	-
P5	-2.196	-
P6	3.738	-
P7	6.168	-
P8	52.383	++
P9	-4.579	-
P10	3.645	-
P11	25.187	+
P12	N/A	N/A
P13	68.364	+++
P14	11.682	-
P15	4.252	-
P16	-14.346	-
P17	59.86	++
P36	-8.458	-
P37	3.832	-
P38	-16.822	-
F1	79.252	+++
F2	22.477	+
F3	6.075	-
F4	10.28	-
F5	38.364	+
F6	88.785	++++
F7	3.131	-
F8	6.262	-
F9	-5.047	-
F10	12.43	-
F31	-5.047	-
F32	19.673	-
F33	42.196	++
F34	7.477	-
F35	48.411	++
F36	19.159	-
F37	-4.72	-
F38	19.813	-
F39	15.467	-
F40	2.897	-
F41	65.467	+++
F42	35.701	+
Dog I.D.	POI	Degree of Positivity
F43	33.318	+
F44	65.28	+++

F45	15.701	-
F75	17.103	-
F76	13.598	-

4.5: Reagents and buffers used

4.5.1: PBS:

For 1L PBS at pH 7.0:

1. Add 8 g of NaCl.
2. Add 0.2 g of KCl.
3. Add 1.15 g of Na_2HPO_4.
4. Add 0.2 g of KH_2PO_4.
5. Mix and pH to between 7.0-7.2.

4.5.2: Electrophoresis Gel (2% TAE):

Agarose LE (Axygen): 0.8g

TAE 0.5%: 40mL

Ethidium Bromide: 5µL

4.5.3: Electropheresis Gel (2% TBE)

Agarose LE (Axygen): 0.8g

TBE 0.5%: 40mL

Ethidium bromide: 5µL

4.5.4: Tris/Borate/EDTA buffer (TBE Buffer) x5 (1L)

Tris-Base: 54g

Boric acid: 27.5g

0.5M EDTA (pH 8): 20mL

Distilled H_2O: 800mL

4.5.5: Cell culture maintenance media

To 500 mL bottle of Advanced MEM (Invitrogen) add:

10 mL FBS (Life technologies) (Total 2% FBS)

5 mL Penicillin-Streptococcus (Pen-Strep - Gibco^{R}) antibiotic (Total 1% Pen-Strep)

5mL Glutamine (Gibco^{R}) (Total 1% Glutamine).

Reference List

Abd-Elsalam KA. Bioinformatic tools and guideline for PCR primer design. *african Journal of biotechnology* 2, 91-5, 2003

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *Journal of molecular biology* 215, 403-10, 1990

Anderson T, Bromfield C, Crawford P, Dodds W, Gibbs E, Hernandez J. Serological evidence of H3N8 canine influenza-like virus circulation in USA dogs prior to 2004. *The Veterinary Journal* 191, 312-6, 2012

Anderson TC, Crawford PC, Dubovi EJ, Gibbs EPJ, Hernandez JA. Prevalence of and exposure factors for seropositivity to H3N8 canine influenza virus in dogs with influenza-like illness in the United States. *Journal of the American Veterinary Medical Association* 242, 209-16, 2013

Andrejeva J, Young D, Goodbourn S, Randall R. Degradation of STAT1 and STAT2 by the V proteins of simian virus 5 and human parainfluenza virus type 2, respectively: consequences for virus replication in the presence of alpha/beta and gamma interferons. *Journal of Virology* 76, 2159-67, 2002

Anonymous. Virus Taxonomy: The Classification and Nomenclature of Viruses The Online (10th) Report of the ICTV. https://talk.ictvonline.org/ictv-reports/ictv_online_report/, 2017

Anonymous. Merck Animal Health Pioneers H3N2 Canine Influenza Vaccine. http://www.merck-animal-health-usa.com/products/nobivac-canine-flu-h3n8/productdetails_130_121109.aspx (accessed 13.08.2017, 2017). Merck Sharp & Dohme Corp, 2015

Anonymous. CanineInfluenzaVaccineH3N2. https://www.zoetisus.com/products/dogs/CanineInfluenzaVaccineH3N2/ (accessed 13.08.2017, 2017). 2017 Zoetis Services LLC, 2017

Appel M, Bistner SI, Menegus M, Albert DA, Carmichael LE. Pathogenicity of low-virulence strains of two canine adenovirus types. *Am J Vet Res* 34, 543-50, 1973

Appel M, Carmichael L, Robson D. Canine adenovirus type 2-induced immunity to two canine adenoviruses in pups with maternal antibody. *American journal of veterinary research* 36, 1199, 1975

Appel MJ. *Virus infections of Carnivores.* Elsevier Science Publisher, Amsterdam, 1987

Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* 29 Suppl 1, S49-52, 2008

Balboni A, Mollace C, Giunti M, Dondi F, Prosperi S, Battilani M. Investigation of the presence of canine adenovirus (CAdV) in owned dogs in Northern Italy. *Research in Veterinary Science* 97, 631-6, doi:http://dx.doi.org/10.1016/j.rvsc.2014.10.010, 2014

Bass E, Gill M, Beckenhauer W. Evaluation of a canine adenovirus type 2 strain as a replacement for infectious canine hepatitis vaccine. *Journal of the American Veterinary Medical Association* 177, 234-42, 1980

Baumgartner W, Krakowka S, Durchfeld B. In vitro cytopathogenicity and in vivo virulence of two strains of canine parainfluenza virus. *Vet Pathol* 28, 324-31, 1991

Beeler E. Influenza in dogs and cats. *Vet Clin North Am Small Anim Pract* 39, 251-64, doi:S0195-5616(08)00191-5 [pii]

10.1016/j.cvsm.2008.10.011, 2009

Benkö M, Harrach B. Molecular evolution of adenoviruses. In: *Adenoviruses: Model and Vectors in Virus-Host Interactions*. Pp 3-35. Springer, 2003

Bhardwaj M, Singh BR, Kumar S, Pawde AM. Poor Association of Bordetella Bronchiseptica Infection with Kennel Cough in Dogs in Northern India. 2013

Binn L, Marchwicki R, Keenan K, Strano A, Engler W. Recovery of reovirus type 2 from an immature dog with respiratory tract disease. *American journal of veterinary research* 38, 927-9, 1977

Binn LN, Eddy GA, Lazar EC, Helms J, Murnane T. Viruses recovered from laboratory dogs with respiratory disease. *Proc Soc Exp Biol Med* 126, 140-5, 1967

Binn LN, Lazar EC, Rogul M, Shepler VM, Swango LJ, Claypoole T, Hubbard DW, Asbill SG, Alexander AD. Upper respiratory disease in military dogs: bacterial, mycoplasma, and viral studies. *Am J Vet Res* 29, 1809-15, 1968

Bodewes R, Lapp S, Hahn K, Habierski A, Förster C, König M, Wohlsein P, Osterhaus AD, Baumgärtner W. Novel canine bocavirus strain associated with severe enteritis in a dog litter. *Veterinary microbiology* 174, 1-8, 2014

Brownlie J, Chalker VJ, Erles K. VACCINE COMPOSITION FOR VACCINATING DOGS AGAINST CANINE INFECTIOUS RESPIRATORY DISEASE (CIRD). Google Patents, 2010

Buonavoglia C, Martella V. Canine respiratory viruses. *Vet Res* 38, 355-73, doi:10.1051/vetres:2006058

v06186 [pii], 2007

Burr P, Campbell M, Nicolson L, Onions D. Detection of canine herpesvirus 1 in a wide range of tissues using the polymerase chain reaction. *Veterinary microbiology* 53, 227-37, 1996

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55, 611-22, doi:10.1373/clinchem.2008.112797

clinchem.2008.112797 [pii], 2009

Byun M, Verweij MC, Pickup DJ, Wiertz EJ, Hansen TH, Yokoyama WM. Two mechanistically distinct immune evasion proteins of cowpox virus combine to avoid antiviral CD8 T cells. *Cell Host Microbe* 6, 422-32, doi:S1931-3128(09)00350-3 [pii]

10.1016/j.chom.2009.09.012, 2009

Carmichael LE, Barnes FD. Effect of temperature on growth of canine herpesvirus in canine kidney cell and macrophage cultures. *J Infect Dis* 120, 664-8, 1969

Carmichael LE, Medic BL. Small-plaque variant of canine herpesvirus with reduced pathogenicity for newborn pups. *Infect Immun* 20, 108-14, 1978

Chalker J, Toomey C, Opperman S, Brooks HW, Ibuoye MA, Brownlie J, Rycroft AN. Respiratory disease in kennelled dogs: serological responses to Bordetella bronchiseptica

lipopolysaccharide do not correlate with bacterial isolation or clinical respiratory symptoms. *Clinical and diagnostic laboratory immunology* 10, 352-6, 2003a

Chalker V. Canine mycoplasmas. *Research in veterinary science* 79, 1-8, 2005

Chalker VJ, Brooks HW, Brownlie J. The association of Streptococcus equi subsp. zooepidemicus with canine infectious respiratory disease. *Vet Microbiol* 95, 149-56, doi:S037811350300155X [pii], 2003b

Chalker VJ, Owen WM, Paterson C, Barker E, Brooks H, Rycroft AN, Brownlie J. Mycoplasmas associated with canine infectious respiratory disease. *Microbiology* 150, 3491-7, 2004

Chancock RM, Murphy BR, Collins PL. Parainfluenza viruses. In: *Fields Virology*. 4 Edtn. Pp 1341-79. Lippincott Williams & WIlliams, Philadelphia, 2001

Chatziandreou N, Stock N, Young D, Andrejeva J, Hagmaier K, McGeoch DJ, Randall RE. Relationships and host range of human, canine, simian and porcine isolates of simian virus 5 (parainfluenza virus 5). *J Gen Virol* 85, 3007-16, doi:10.1099/vir.0.80200-0

85/10/3007 [pii], 2004

Childs K, Stock N, Ross C, Andrejeva J, Hilton L, Skinner M, Randall R, Goodbourn S. mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. *Virology* 359, 190-200, doi:10.1016/j.virol.2006.09.023, 2007

Clark MA. Bovine coronavirus. *Br Vet J* 149, 51-70, doi:S0007-1935(05)80210-6 [pii]

10.1016/S0007-1935(05)80210-6, 1993

Cornwell H, Koptopoulos G, Thompson H, McCandlish I, Wright N. Immunity to canine adenovirus respiratory disease: a comparison of attenuated CAV-1 and CAV-2 vaccines. *The Veterinary Record* 110, 27-32, 1982

Crandell RA, Brumlow WB, Davison VE. Isolation of a parainfluenza virus from sentry dogs with upper respiratory disease. *Am J Vet Res* 29, 2141-7, 1968

Cureton DK, Scott-Garrard M, Parker DS, House A, Veal E, Aitcheson T, Chiang Y-W. An Inactivated H3N2 Canine Influenza Virus (CIV) Vaccine Aids in the Prevention of Clinical Disease and Virus Shedding in Dogs Challenged with Virulent H3N2 CIV. *International Journal of Applied Research in Veterinary Medicine* 14, 128-34, 2016

Day MJ, Horzinek MC, Schultz RD. WSAVA Guidelines for the Vaccination of Dogs and Cats. *Journal of Small Animal Practice* 51, e1-e32, doi:10.1111/j.1748-5827.2010.00959a.x, 2010

Day MJ, Karkare U, Schultz R, Squires R, Tsujimoto H. Recommendations on vaccination for Asian small animal practitioners: a report of the WSAVA Vaccination Guidelines Group. *Journal of Small Animal Practice* 56, 77-95, 2015

Decaro N, Campolo M, Desario C, Ricci D, Camero M, Lorusso E, Elia G, Lavazza A, Martella V, Buonavoglia C. Virological and molecular characterization of a mammalian orthoreovirus type 3 strain isolated from a dog in Italy. *Vet Microbiol* 109, 19-27, doi:S0378-1135(05)00178-1 [pii]

10.1016/j.vetmic.2005.05.014, 2005

Decaro N, Desario C, Elia G, Mari V, Lucente MS, Cordioli P, Colaianni ML, Martella V, Buonavoglia C. Serological and molecular evidence that canine respiratory coronavirus is circulating in Italy. *Vet Microbiol* 121, 225-30, doi:S0378-1135(06)00501-3 [pii]

10.1016/j.vetmic.2006.12.001, 2007

Decaro N, Martella V, Buonavoglia C. Canine adenoviruses and herpesvirus. *Vet Clin North Am Small Anim Pract* 38, 799-814, viii, doi:S0195-5616(08)00068-5 [pii]

10.1016/j.cvsm.2008.02.006, 2008

Decaro N, Amorisco F, Desario C, Lorusso E, Camero M, Bellacicco AL, Sciarretta R, Lucente MS, Martella V, Buonavoglia C. Development and validation of a real-time PCR assay for specific and sensitive detection of canid herpesvirus 1. *J Virol Methods* 169, 176-80, doi:S0166-0934(10)00263-6 [pii]

10.1016/j.jviromet.2010.07.021, 2010

Decaro N, Mari V, Larocca V, Losurdo M, Lanave G, Lucente MS, Corrente M, Catella C, Bo S, Elia G, Torre G, Grandolfo E, Martella V, Buonavoglia C. Molecular surveillance of traditional and emerging pathogens associated with canine infectious respiratory disease. *Veterinary Microbiology* 192, 21-5, doi:10.1016/j.vetmic.2016.06.009, 2016

Deshpande MS, Jirjis FF, Tubbs A, Jayappa H, Sweeney D, Spencer S, Lakshmanan N, Wasmoen T. Evaluation of the efficacy of a canine influenza virus (H3N8) vaccine in dogs following experimental challenge. *Veterinary Therapeutics* 10, 103, 2009

Ditchfield J, Macpherson LW, Zbitnew A. Association of Canine Adenovirus (Toronto A 26/61) with an Outbreak of Laryngotracheitis ("Kennel Cough"): A Preliminary Report. *Can Vet J* 3, 238-47, 1962

Dubovi EJ. Canine influenza. *Vet Clin North Am Small Anim Pract* 40, 1063-71, doi:S0195-5616(10)00092-6 [pii]

10.1016/j.cvsm.2010.07.005, 2010

Edinboro CH, Ward MP, Glickman LT. A placebo-controlled trial of two intranasal vaccines to prevent tracheobronchitis (kennel cough) in dogs entering a humane shelter. *Preventive veterinary medicine* 62, 89-99, 2004

Elia G, Lucente M, Bellacicco A, Camero M, Cavaliere N, Decaro N, Martella V. Assessment of reovirus epidemiology in dogs. p 1674. 2006

Ellis J, Anseeuw E, Gow S, Bryan H, Salb A, Goji N, Rhodes C, La Coste S, Smits J, Kutz S. Seroepidemiology of respiratory (group 2) canine coronavirus, canine parainfluenza virus, and Bordetella bronchiseptica infections in urban dogs in a humane shelter and in rural dogs in small communities. *Can Vet J* 52, 861-8, 2011

Ellis JA, Haines DM, West KH, Burr JH, Dayton A, Townsend HG, Kanara EW, Konoby C, Crichlow A, Martin K, Headrick G. Effect of vaccination on experimental infection with Bordetella bronchiseptica in dogs. *J Am Vet Med Assoc* 218, 367-75, 2001

Ellis JA, McLean N, Hupaelo R, Haines DM. Detection of coronavirus in cases of tracheobronchitis in dogs: a retrospective study from 1971 to 2003. *Can Vet J* 46, 447-8, 2005

Ellis JA, Krakowka GS. A review of canine parainfluenza virus infection in dogs. *J Am Vet Med Assoc* 240, 273-84, doi:10.2460/javma.240.3.273, 2012

Emery JB, House JA, Bittle JL, Spotts AM. A canine parainfluenza viral vaccine: immunogenicity and safety. *Am J Vet Res* 37, 1323-7, 1976

Englund L, Jacobs AA, Klingeborn B, Chriel M. Seroepidemiological survey of Bordetella bronchiseptica and canine parainfluenza-2 virus in dogs in Sweden. *Vet Rec* 152, 251-4, 2003

Erles K, Toomey C, Brooks HW, Brownlie J. Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease. *Virology* 310, 216-23, doi:S0042682203001600 [pii], 2003

Erles K, Dubovi EJ, Brooks HW, Brownlie J. Longitudinal study of viruses associated with canine infectious respiratory disease. *J Clin Microbiol* 42, 4524-9, doi:42/10/4524 [pii]

10.1128/JCM.42.10.4524-4529.2004, 2004

Erles K, Brownlie J. Investigation into the causes of canine infectious respiratory disease: antibody responses to canine respiratory coronavirus and canine herpesvirus in two kennelled dog populations. *Arch Virol* 150, 1493-504, doi:10.1007/s00705-005-0533-x, 2005

Erles K, Shiu KB, Brownlie J. Isolation and sequence analysis of canine respiratory coronavirus. *Virus Res* 124, 78-87, doi:S0168-1702(06)00300-5 [pii]

10.1016/j.virusres.2006.10.004, 2007

Erles K, Brownlie J. Canine respiratory coronavirus: an emerging pathogen in the canine infectious respiratory disease complex. *Vet Clin North Am Small Anim Pract* 38, 815-25, viii, doi:10.1016/j.cvsm.2008.02.008

S0195-5616(08)00070-3 [pii], 2008

Freshney RI. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications.* Wiley, 2011

Gale M, Jr., Sen GC. Viral evasion of the interferon system. *J Interferon Cytokine Res* 29, 475-6, doi:10.1089/jir.2009.0078, 2009

Giese M, Harder TC, Teifke JP, Klopfleisch R, Breithaupt A, Mettenleiter TC, Vahlenkamp TW. Experimental infection and natural contact exposure of dogs with avian influenza virus (H5N1). *Emerg Infect Dis* 14, 308-10, doi:10.3201/eid1402.070864, 2008

Goodnow RA. Biology of Bordetella bronchiseptica. *Microbiological Reviews* 44, 722, 1980

Gueirard P, Guiso N. Virulence of Bordetella bronchiseptica: role of adenylate cyclase-hemolysin. *Infect Immun* 61, 4072-8, 1993

Guermonprez P, Khelef N, Blouin E, Rieu P, Ricciardi-Castagnoli P, Guiso N, Ladant D, Leclerc C. The adenylate cyclase toxin of Bordetella pertussis binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18). *J Exp Med* 193, 1035-44, 2001

Harrington DJ, Sutcliffe IC, Chanter N. The molecular basis of Streptococcus equi infection and disease. *Microbes Infect* 4, 501-10, doi:S1286457902015654 [pii], 2002

He B, Leser GP, Paterson RG, Lamb RA. The paramyxovirus SV5 small hydrophobic (SH) protein is not essential for virus growth in tissue culture cells. *Virology* 250, 30-40, doi:S0042-6822(98)99354-0 [pii]

10.1006/viro.1998.9354, 1998

Horiguchi Y, Matsuda H, Koyama H, Nakai T, Kume K. Bordetella bronchiseptica dermonecrotizing toxin suppresses in vivo antibody responses in mice. *FEMS Microbiol Lett* 69, 229-34, 1992

Horner GW. Some recent virus isolations and their importance in New Zealand. *N Z Vet J* 25, 335-6, doi:10.1080/00480169.1977.34446, 1977

Jacobs AA, Theelen RP, Jaspers R, Horspool LJ, Sutton D, Bergman JG, Paul G. Protection of dogs for 13 months against Bordetella bronchiseptica and canine parainfluenza virus with a modified live vaccine. *Vet Rec* 157, 19-23, doi:157/1/19 [pii], 2005

Jeoung H-Y, Lim S-I, Shin B-H, Lim J, Song J-Y, Song D-S, Kang B-K, Moon H-J, An D-J. A novel canine influenza H3N2 virus isolated from cats in an animal shelter. *Veterinary microbiology* 165, 281-6, 2013

Jirjis FF, Deshpande MS, Tubbs AL, Jayappa H, Lakshmanan N, Wasmoen TL. Transmission of canine influenza virus (H3N8) among susceptible dogs. *Vet Microbiol* 144, 303-9, doi:S0378-1135(10)00119-7 [pii]

10.1016/j.vetmic.2010.02.029, 2010

Kaczorek E, Schulz P, Małaczewska J, Wójcik R, Siwicki AK, Stopyra A, Lew-Kojrys S, Pomianowski A, Hryniewicka K, Mikulska-Skupień E. Prevalence of respiratory pathogens detected in dogs with kennel cough in Poland. *Acta Veterinaria Brno* 85, 329-36, 2017

Kaneshima T, Hohdatsu T, Satoh K, Takano T, Motokawa K, Koyama H. The prevalence of a group 2 coronavirus in dogs in Japan. *J Vet Med Sci* 68, 21-5, doi:JST.JSTAGE/jvms/68.21 [pii], 2006

Karaca K, Dubovi EJ, Siger L, Robles A, Audonnet J-C, Jiansheng Y, Nordgren R, Minke JM. Evaluation of the ability of canarypox-vectored equine influenza virus vaccines to induce humoral immune responses against canine influenza viruses in dogs. *American journal of veterinary research* 68, 208-12, 2007

Karpas A, Garcia F, Calvo F, Cross R. Experimental production of canine tracheobronchitis (kennel cough) with canine herpesvirus isolated from naturally infected dogs. *American journal of veterinary research* 29, 1251, 1968

Karron R, Collins P. Parainfluenza viruses. In: Knipe D, Howley P (eds). *Fields Virology*. Pp 996-1023. Lippincott, Williams & Wilkins, Philadelphia, 2013

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. 28. http://www.geneious.com Bioinformatics, 2013

Keil DJ, Fenwick B. Role of Bordetella bronchiseptica in infectious tracheobronchitis in dogs. *J Am Vet Med Assoc* 212, 200-7, 1998

Kim H, Song D, Moon H, Yeom M, Park S, Hong M, Na W, Webby RJ, Webster RG, Park B, Kim JK, Kang B. Inter- and intraspecies transmission of canine influenza virus (H3N2) in dogs, cats, and ferrets. *Influenza Other Respir Viruses* 7, 265-70, doi:10.1111/j.1750-2659.2012.00379.x, 2013

King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. Mammalian Reovirus. http://ictvonline.org/taxonomyHistory.asp?taxnode_id=19982435&taxa_name=Mammalian reovirus (accessed 22/05/2014, Elsvier, 2013a

King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. Canid Herpesvirus 1. http://ictvonline.org/taxonomyHistory.asp?taxnode_id=20130269&taxa_name=Canid herpesvirus 1 Elsevier, 2013b

King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. Canine adenovirus http://ictvonline.org/virusTaxonomy.asp?taxnode_id=19910315 (accessed 22/05/2014, 2014). Elsevier Academic Press, 2013c

Knesl O, Allan FJ, Shields S. The seroprevalence of canine respiratory coronavirus and canine influenza virus in dogs in New Zealand. *N Z Vet J* 57, 295-8, doi:10.1080/00480169.2009.58624, 2009

Kraus TA, Garza L, Horvath CM. Enabled interferon signaling evasion in an immune-competent transgenic mouse model of parainfluenza virus 5 infection. *Virology* 371, 196-205, doi:S0042-6822(07)00642-3 [pii]

10.1016/j.virol.2007.10.001, 2008

Lamb RA, Choppin PW. The gene structure and replication of influenza virus. *Annu Rev Biochem* 52, 467-506, doi:10.1146/annurev.bi.52.070183.002343, 1983

Lavan R, Knesl O. Prevalence of canine infectious respiratory pathogens in asymptomatic dogs presented at US animal shelters. *Journal of Small Animal Practice* 56, 572-6, 2015

Lee C, Song D, Kang B, Kang D, Yoo J, Jung K, Na G, Lee K, Park B, Oh J. A serological survey of avian origin canine H3N2 influenza virus in dogs in Korea. *Vet Microbiol* 137, 359-62, doi:S0378-1135(09)00038-8 [pii]

10.1016/j.vetmic.2009.01.019, 2009

Lee C, Jung K, Oh J, Oh T, Han S, Hwang J, Yeom M, Son D, Kim J, Park B. Protective efficacy and immunogenicity of an inactivated avian-origin H3N2 canine influenza vaccine in dogs challenged with the virulent virus. *Veterinary microbiology* 143, 184-8, 2010

Lee D-H, Bae S-W, Park J-K, Kwon J-H, Yuk S-S, Song J-M, Kang S-M, Kwon Y-K, Kim H-Y, Song C-S. Virus-like particle vaccine protects against H3N2 canine influenza virus in dog. *Vaccine* 31, 3268-73, 2013

Liu C, Li X, Zhang J, Yang L, Li F, Deng J, Tan F, Sun M, Liu Y, Tian K. Isolation and genomic characterization of a canine parainfluenza virus type 5 strain in China. *Archives of virology* 162, 2337-44, 2017

Liu H, Naismith J, Hay R. Adenovirus DNA replication. In: *Adenoviruses: Model and Vectors in Virus-Host Interactions*. Pp 131-64. Springer, 2003

Maclachlan NJ, Dubovi EJ. *Fenner's veterinary virology.* Academic Press, 2010

Madison NJ. Merck Animal Health Announces USDA Approval of Innovative Canine Flu Bivalent Vaccine. http://www.merck-animal-health.com/news/2016-10-7.aspx (accessed 13.08.17, 2017). 2016

Mahr JA, Gooding LR. Immune evasion by adenoviruses. *Immunological reviews* 168, 121-30, 1999

McGavin DR, Mellows AJ, Choice L. Evidence for Australia-wide canine parainfluenza infection. *Aust Vet J* 66, 221, 1989

McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *N Engl J Med* 309, 13-7, doi:10.1056/NEJM198307073090103, 1983

McNulty M, Bryson D, Allan G, Logan E. Coronavirus infection of the bovine respiratory tract. *Veterinary microbiology* 9, 425-34, 1984

Messick JB. New perspectives about Hemotrophic mycoplasma (formerly, Haemobartonella and Eperythrozoon species) infections in dogs and cats. *Vet Clin North Am Small Anim Pract* 33, 1453-65, 2003

Mitchell JA, Brooks H, Shiu KB, Brownlie J, Earles K. Development of a quantitative real-time PCR for the detection of canine respiratory coronavirus. *J Virol Methods* 155, 136-42, doi:S0166-0934(08)00374-1 [pii]

10.1016/j.jviromet.2008.10.008, 2009

Mitchell JA, Cardwell JM, Renshaw RW, Dubovi EJ, Brownlie J. Detection of canine pneumovirus in dogs with canine infectious respiratory disease. *Journal of clinical microbiology* 51, 4112-9, 2013

Miyoshi M, Ishii Y, Takiguchi M, Takada A, Yasuda J, Hashimoto A, Okazaki K, Kida H. Detection of canine herpesvirus DNA in the ganglionic neurons and the lymph node lymphocytes of latently infected dogs. *J Vet Med Sci* 61, 375-9, 1999

Mochizuki M, Yachi A, Ohshima T, Ohuchi A, Ishida T. Etiologic study of upper respiratory infections of household dogs. *J Vet Med Sci* 70, 563-9, doi:JST.JSTAGE/jvms/70.563 [pii], 2008

Morrison MD, Onions DE, Nicolson L. Complete DNA sequence of canine adenovirus type 1. *Journal of General Virology* 78, 873-8, 1997

Mouzin DE, Lorenzen MJ, Haworth JD, King VL. Duration of serologic response to five viral antigens in dogs. *Journal of the American Veterinary Medical Association* 224, 55-60, 2004

MSDAnimalHealth. NOBIVAC® KC. http://www.msd-animal-health.co.nz/products/Nobivac__KC/020_Product_Details.aspx (accessed 13th February, 2018). 2017

Network CIS. H3N2 test results from March 2015 to present. https://cornell.carto.com/u/nick-hollingshead/builder/e777506b-8f1c-44b0-a809-57f942b10f2b/embed Cornell University College of Veterinary Medicine, 2017

Oem JK, Kim SH, Kim YH, Lee MH, Lee KK. Molecular characteristics of canine parainfluenza viruses type 5 (CPIV-5) isolated in Korea. *Can J Vet Res* 79, 64-7, 2015

Ostapchuk P, Hearing P. Regulation of adenovirus packaging. In: *Adenoviruses: Model and Vectors in Virus-Host Interactions*. Pp 165-85. Springer, 2003

Paterson R, Lamb R, Moss B, Murphy B. Comparison of the relative roles of the F and HN surface glycoproteins of the paramyxovirus simian virus 5 in inducing protective immunity. *Journal of Virology* 61, 1972-7, 1987

Payungporn S, Crawford PC, Kouo TS, Chen LM, Pompey J, Castleman WL, Dubovi EJ, Katz JM, Donis RO. Influenza A virus (H3N8) in dogs with respiratory disease, Florida. *Emerg Infect Dis* 14, 902-8, doi:10.3201/eid1406.071270, 2008

Pecoraro HL, Lee JS, Achenbach J, Nelson Jr S, Landolt GA. Seroprevalence of canine influenza virus (H3N8) in Iditarod racing sled dogs. *The Canadian Veterinary Journal* 53, 1091, 2012

Pesavento PA, Hurley KF, Bannasch MJ, Artiushin S, Timoney JF. A clonal outbreak of acute fatal hemorrhagic pneumonia in intensively housed (shelter) dogs caused by Streptococcus equi subsp. zooepidemicus. *Vet Pathol* 45, 51-3, doi:10.1354/vp.45-1-51

45/1/51 [pii], 2008

Poste G, Lecatsas G, Apostolov K. Electron microscope study of the morphogenesis of a new canine herpesvirus in dog kidney cells. *Archiv für die gesamte Virusforschung* 39, 317-29, doi:10.1007/bf01241010, 1972

Posuwan N, Payungporn S. Prevalence of respiratory viruses isolated from dogs in Thailand during 2008-2009. *Asian Biomed* 4, 563, 2011

Priestnall S, Erles K. Streptococcus zooepidemicus: an emerging canine pathogen. *Vet J* 188, 142-8, doi:10.1016/j.tvjl.2010.04.028

S1090-0233(10)00149-8 [pii], 2011

Priestnall S, Mitchell J, Walker C, Erles K, Brownlie J. New and emerging pathogens in canine infectious respiratory disease. *Veterinary pathology* 51, 492-504, 2014

Priestnall SL, Brownlie J, Dubovi EJ, Erles K. Serological prevalence of canine respiratory coronavirus. *Vet Microbiol* 115, 43-53, doi:S0378-1135(06)00071-X [pii]

10.1016/j.vetmic.2006.02.008, 2006

Priestnall SL, Mitchell JA, Brooks HW, Brownlie J, Erles K. Quantification of mRNA encoding cytokines and chemokines and assessment of ciliary function in canine tracheal epithelium during infection with canine respiratory coronavirus (CRCoV). *Veterinary immunology and immunopathology* 127, 38-46, 2009

Priestnall SL, Erles K, Brooks HW, Cardwell JM, Waller AS, Paillot R, Robinson C, Darby AC, Holden MT, Schoniger S. Characterization of pneumonia due to Streptococcus equi subsp. zooepidemicus in dogs. *Clin Vaccine Immunol* 17, 1790-6, doi:10.1128/CVI.00188-10

CVI.00188-10 [pii], 2010

Randolph J, Moise N, Scarlett J, Shin S, Blue J, Bookbinder P. Prevalence of mycoplasmal and ureaplasmal recovery from tracheobronchial lavages and prevalence of mycoplasmal recovery from pharyngeal swab specimens in dogs with or without pulmonary disease. *American journal of veterinary research* 54, 387, 1993

Reading MJ, Field HJ. Detection of high levels of canine herpes virus-1 neutralising antibody in kennel dogs using a novel serum neutralisation test. *Res Vet Sci* 66, 273-5, doi:10.1053/rvsc.1998.0222

S0034-5288(98)90222-3 [pii], 1999

Richter GW, Kress Y. Electron microscopy of a strain of Bordetella bronchiseptica. *J Bacteriol* 94, 1216-24, 1967

Rima BK, Gatherer D, Young DF, Norsted H, Randall RE, Davison AJ. Stability of the parainfluenza virus 5 genome revealed by deep sequencing of strains isolated from different hosts and following passage in cell culture. *J Virol* 88, 3826-36, doi:10.1128/JVI.03351-13, 2014

Robinson AJ, Crerar SK, Waight Sharma N, Muller WJ, Bradley MP. Prevalence of serum antibodies to canine adenovirus and canine herpesvirus in the European red fox (Vulpes vulpes) in Australia. *Aust Vet J* 83, 356-61, 2005

Rodriguez L, Nogales A, Murcia PR, Parrish CR, Martínez-Sobrido L. A bivalent live-attenuated influenza vaccine for the control and prevention of H3N8 and H3N2 canine influenza viruses. *Vaccine*, 2017

Rosen L. Serologic groupings of reovirus by hemagglutination inhibition. *American Journal of Epidemiology* 71, 242-9, 1960

Rosenberg FJ, Lief FS, Todd JD, Reif JS. Studies of canine respiratory viruses. I. Experimental infection of dogs with an SV5-like canine parainfluenza agent. *Am J Epidemiol* 94, 147-65, 1971

Rosendal S. Mycoplasmas as a possible cause of enzootic pneumonia in dogs. *Acta Vet Scand* 13, 137-9, 1972

Rosendal S. Canine mycoplasmas: pathogenicity of mycoplasmas associated with distemper pneumonia. *Journal of Infectious Diseases* 138, 203-10, 1978

Rosendal S. Canine mycoplasmas: their ecologic niche and role in disease. *J Am Vet Med Assoc* 180, 1212-4, 1982

Rota PA, Maes RK. Homology between feline herpesvirus-1 and canine herpesvirus. *Arch Virol* 115, 139-45, 1990

Russell WC. Adenoviruses: update on structure and function. *J Gen Virol* 90, 1-20, doi:10.1099/vir.0.003087-0

90/1/1 [pii], 2009

Rycroft AN, Tsounakou E, Chalker V. Serological evidence of Mycoplasma cynos infection in canine infectious respiratory disease. *Vet Microbiol* 120, 358-62, doi:S0378-1135(06)00458-5 [pii]

10.1016/j.vetmic.2006.11.011, 2007

Saah AJ, Hoover DR. "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings. *Annals of internal medicine* 126, 91-4, 1997

Schmitt P, Ray G, Schmitt A. The C-terminal end of parainfluenza virus 5 NP protein is important for virus-like particle production and M-NP protein interaction. *J Virol* 84, 12810-23, doi:10.1128/JVI.01885-10

JVI.01885-10 [pii], 2010

Schoehn G, El Bakkouri M, Fabry CM, Billet O, Estrozi LF, Le L, Curiel DT, Kajava AV, Ruigrok RW, Kremer EJ. Three-dimensional structure of canine adenovirus serotype 2 capsid. *J Virol* 82, 3192-203, doi:10.1128/JVI.02393-07

JVI.02393-07 [pii], 2008

Schulz B, Kurz S, Weber K, Balzer H-J, Hartmann K. Detection of respiratory viruses and Bordetella bronchiseptica in dogs with acute respiratory tract infections. *The Veterinary Journal* 201, 365-9, 2014

Segura MM, Monfar M, Puig M, Mennechet F, Ibanes S, Chillón M. A real-time PCR assay for quantification of canine adenoviral vectors. *Journal of virological methods* 163, 129-36, 2010

Seth P. *Adenoviruses : Basic Biology to Gene Therapy.* R.G. Landes Co, Austin, Tex, 1999

Song D, Kang B, Lee C, Jung K, Ha G, Kang D, Park S, Park B, Oh J. Transmission of avian influenza virus (H3N2) to dogs. *Emerg Infect Dis* 14, 741-6, doi:10.3201/eid1405.071471, 2008

Song D, An D, Moon H, Yeom M, Jeong H, Jeong W, Park S, Kim H, Han S, Oh J. Interspecies transmission of the canine influenza H3N2 virus to domestic cats in South Korea, 2010. *Journal of General Virology* 92, 2350-5, 2011

Song D, Moon HJ, An DJ, Jeoung HY, Kim H, Yeom MJ, Hong M, Nam JH, Park SJ, Park BK, Oh JS, Song M, Webster RG, Kim JK, Kang BK. A novel reassortant canine H3N1 influenza virus between pandemic H1N1 and canine H3N2 influenza viruses in Korea. *J Gen Virol* 93, 551-4, doi:10.1099/vir.0.037739-0

vir.0.037739-0 [pii], 2012

Song QQ, Zhang FX, Liu JJ, Ling ZS, Zhu YL, Jiang SJ, Xie ZJ. Dog to dog transmission of a novel influenza virus (H5N2) isolated from a canine. *Vet Microbiol* 161, 331-3, doi:10.1016/j.vetmic.2012.07.040

S0378-1135(12)00442-7 [pii], 2013

Sparer TE, Tripp RA, Dillehay DL, Hermiston TW, Wold W, Gooding LR. The role of human adenovirus early region 3 proteins (gp19K, 10.4 K, 14.5 K, and 14.7 K) in a murine pneumonia model. *Journal of Virology* 70, 2431-9, 1996

Stone B, Burrows J, Schepetiuk S, Higgins G, Hampson A, Shaw R, Kok T. Rapid detection and simultaneous subtype differentiation of influenza A viruses by real time PCR. *J Virol Methods* 117, 103-12, doi:10.1016/j.jviromet.2003.12.005

S0166093403004038 [pii], 2004

Sun D, Luthra P, Xu P, Yoon H, He B. Identification of a phosphorylation site within the P protein important for mRNA transcription and growth of parainfluenza virus 5. *J Virol* 85, 8376-85, doi:10.1128/JVI.00618-11

JVI.00618-11 [pii], 2011

Sun Y, Sun S, Ma J, Tan Y, Du L, Shen Y, Mu Q, Pu J, Lin D, Liu J. Identification and characterization of avian-origin H3N2 canine influenza viruses in northern China during 2009-2010. *Virology* 435, 301-7, doi:10.1016/j.virol.2012.09.037

S0042-6822(12)00489-8 [pii], 2013

Tham KM, Homer GW, Hunter R. Isolation and identification of canine adenovirus type-2 from the upper respiratory tract of a dog. *New Zealand Veterinary Journal* 46, 102-5, doi:10.1080/00480169.1998.36068, 1998

ThermoFisher. DNA Copy Number and Dilution Calculator. https://www.thermofisher.com/nz/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html 2015). 2016

Thornton B, Basu C. Real‐time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education* 39, 145-54, 2011

Thrusfield MV, Aitken CGG, Muirhead RH. A field investigation of kennel cough: Incubation period and clinical signs. *Journal of Small Animal Practice* 32, 215-20, doi:10.1111/j.1748-5827.1991.tb00550.x, 1991

Timoney JF, Walker J, Zhou M, Ding J. Cloning and sequence analysis of a protective M-like protein gene from Streptococcus equi subsp. zooepidemicus. *Infection and immunity* 63, 1440-5, 1995

Tyler KL. Mammalian Reoviruses. In: Knipe DM, Howley P (eds). *Fields Virology*. 4th Edtn. Lippincott Williams & Wilkins, Philadelphia, 2001

Vetmedica BI. BRONCHI-SHIELD III. http://www.bi-vetmedica.com/species/pet/products/bronchi-shield/Bronchi-Shield-III.html (accessed 13th February, 2018). Boehringer Ingelheim Vetmedica, Inc., 2018

Virbac. Canigen® KC. https://nz.virbac.com/home/cat-dog/products/vaccines/canigen-kc.html#_ednref3 (accessed 13th February

2018). Virbac group, 2017

Wagener JS, Sobonya R, Minnich L, Taussig LM. Role of canine parainfluenza virus and Bordetella bronchiseptica in kennel cough. *Am J Vet Res* 45, 1862-6, 1984

Walker CA, Mannering SA, Shields S, Blake DP, Brownlie J. Complete Genome Sequence of Mycoplasma cynos Strain C142. *Genome Announcements* 1, e00196-12, doi:10.1128/genomeA.00196-12, 2013

Weese JS, Stull J. Respiratory disease outbreak in a veterinary hospital associated with canine parainfluenza virus infection. *Can Vet J* 54, 79-82, 2013

Weissfeld A. DEVELOPMENT OF A QUANTITATIVE CULTURE ASSAY TO ACCESS THE SUITABILITY OF NEW VIRAL TRANSPORT MEDIA. *ASM 104th General Convention.* New Orleans, 2004

Wibawan IW, Pasaribu FH, Utama IH, Abdulmawjood A, Lammler C. The role of hyaluronic acid capsular material of Streptococcus equi subsp. zooepidemicus in mediating adherence to HeLa cells and in resisting phagocytosis. *Res Vet Sci* 67, 131-5, doi:10.1053/rvsc.1998.0287

S0034528898902879 [pii], 1999

Wright N, Jackson FR, Niezgoda M, Ellison JA, Rupprecht CE, Nela LH. High prevalence of antibodies against canine adenovirus (CAV) type 2in domestic dog populations in South Africa precludes the use ofCAV-based recombinant rabies vaccines. *Vaccine* 31, 4177-82, 2013

Wyand D, Sherman B. STREPTOCOCCAL SEPTICEMIA IN RACING GREYHOUNDS. *Journal of the American Animal Hospital Association* 14, 399-401, 1978

Xuan X, Horimoto T, Limcumpao JA, Tohya Y, Takahashi E, Mikami T. Glycoprotein-specific immune response in canine herpesvirus infection. *Arch Virol* 122, 359-65, 1992

Young D, Randall R, Hoyle J, Souberbielle B. Clearance of a persistent paramyxovirus infection is mediated by cellular immune responses but not by serum-neutralizing antibody. *Journal of Virology* 64, 5403-11, 1990