Canine Parvovirus in New Zealand

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ABSTRACT

Since the initial global emergence of canine parvovirus type 2 (CPV-2) in the early 1980s the virus has continued to evolve in its new host. As a result, the original CVP-2 was replaced by newly emerged subtypes designated CPV-2a and CPV-2b. Recently, a third antigenic subtype CPV-2c has emerged in several countries. In New Zealand the evolution of CVP-2 has not been monitored since its emergence in the early 1980s, largely because of the high efficacy of the vaccines available on the market. This lack of monitoring of CPV-2 has left a dearth of knowledge regarding the epidemiological features of CPV-2 in New Zealand. Hence, the aim of this study was to determine what subtypes of CPV-2 circulate in New Zealand and to investigate the phylogenetic relationships between CPV-2 from New Zealand and from other parts of the world.

As part of this project, a virological survey was conducted across New Zealand. A total of 79 faecal samples were collected from dogs suspected to be infected with CPV-2, as judged by submitting veterinarians. Of those, 70 tested positive for CPV-2 DNA. All but one of the CPV-2 sequences were subtyped as CPV-2a. The remaining sequence was subtyped as CPV-2, and most likely represented a vaccine strain of the virus. The majority (74.3%) of CPV-2 positive samples originated from dogs six months of age and younger, with 70% of samples collected from dogs considered not fully vaccinated (unvaccinated dogs or those with only single vaccination), a further 17% of samples originated from dogs with an unknown vaccination history.

Two separate phylogenetic analyses were performed. Seventy one CPV-2 positive sequences originated from New Zealand (61 survey samples, six historic samples, two vaccine sequences and one parvovirus sequence obtained from a cat) and the reference sequence were trimmed to produce contiguous sequences of equal length. These 72 sequences were used to investigate the genetic structure of CPV-2 within New Zealand. Haplotype network analyses revealed that Cook-straight is not an effective geographical barrier to CVP-2 gene flow with an equal distribution of genotypes in the North and South Islands. Translocation of the virus between the islands is likely occurring by transportation of sub-clinically infected animals and fomites.

Additional CPV-2 VP2 sequences (n=95) originating from various countries were obtained from the National Centre for Biotechnology Information (NCBI) database. The selection of 27 samples originating from New Zealand for which a full length contiguous sequence of VP-2

gene was available were aligned with sequences obtained from the NCBI database. The resulting dataset of 123 CPV-2 sequences was used to assess the New Zealand CPV-2 sequences in the context of the worldwide radiation of CPV-2. Phylogenetic analyses of this dataset revealed that New Zealand has a closed monophyletic population of CPV-2 sequences. This suggests that CPV-2 is not being continuously introduced to New Zealand from overseas, but has evolved following a limited number of introductions in the past. Phylogenetic analysis also revealed that CPV-2 subtypes from around the world have emerged independently of one another.

This work has contributed to our understanding of molecular epidemiology of CPV-2 in New Zealand. The knowledge of predominant CPV-2 subtypes circulating in this country is important for evidence driven recommendations with regard to CPV-2 vaccination. Understanding of the genetic structure of the current CPV-2 circulating in New Zealand is also crucial for timely recognition, detection and management of any novel antigenic subtypes that may emerge in the future.

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ACRONYMS USED IN THIS THESIS

Ala	Alanine
Asp	Aspartate
bp	base pair
CPV-1	Canine parvovirus type 1
CPV-2	Canine parvovirus type 2
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FPLV	Feline panleukopenia virus
Glu	Glutamate
Gly	Glycine
HI	Haemagglutination inhibition
Ile	Isoleucine
Leu	Leucine
MAF	Ministry of Agriculture and Fisheries (AKA: MPI – Ministry of Primary Industries)
Met	Methionine
MVC	Minute virus of canines
NCBI	National Centre for Biotechnology Information
NLFK	Northern Line Feline Kidney (cells)
NS1	Non-structural protein 1
NS2	Non-structural protein 2
NZ	New Zealand
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Ser	Serine

ssDNA	Single stranded deoxyribonucleic acid
S.T.A.R	Stool Transport and Recovery (system)
TfR	Transferrin receptor
Thr	Threonine
Tyr	Tyrosine
Val	Valine
VP	Viral protein

1. LITERATURE REVIEW

1.1. CANINE PARVOVIRAL DISEASE AND PATHOGENESIS

Canine parvovirus type 2 infects susceptible dogs via the faecal-oral route. The virus requires rapidly dividing cells in the S phase of the cell cycle (Parker and Parrish 2000). Primary viral replication occurs in the retropharyngeal and mesenteric lymph nodes (Potgieter *et al.* 1981; Meunier *et al.* 1985a). Four to five days post infection the virus can be isolated in the serum (Meunier *et al.* 1985b) and has disseminated from the lymph nodes throughout the body, via the blood stream (Prittie 2004). The highest viral titers are found in the tonsils and intestinal tissues (Meunier *et al.* 1985a). Viral effects can also be seen in the bone marrow (Potgieter *et al.* 1981) and cause the depletion of granulocytes (Potgieter *et al.* 1981), leading to a depletion in the circulating neutrophils (leukopenia).

Clinical signs typically begin to show 5-7 days post exposure (Potgieter *et al.* 1981; Meunier *et al.* 1985a) and include anorexia, vomiting, diarrhoea, dehydration, fever and leukopenia (Appel *et al.* 1979a; Azetaka *et al.* 1981), and in some cases death. Necropsy shows a loss of mucosal epithelium in the small intestine, villous atrophy and dilation of crypts which have no epithelial cells (Azetaka *et al.* 1981). The damage caused in the intestinal tract demands a large neutrophil response which cannot be met due to the viral effects in the bone marrow. Infected dogs shed the virus in the faeces intermittently for a variable period of time, ranging from 4 - 14 days post infection (Azetaka *et al.* 1981; Potgieter *et al.* 1981; Meunier *et al.* 1985b).

Factors which may affect the likelihood and severity of disease include the dose of the infectious virus, the level of virus-specific immunity. Other host related risk factors such as poor body condition, stress and pre-existing bacterial infection can affect occurrence and severity of disease (Carman and Povey 1982; Prittie 2004; Dossin *et al.* 2011; Schoeman *et al.* 2013). In addition, depletion in the numbers of circulating neutrophils leads the dog to be more susceptible to secondary bacterial infections (Azetaka *et al.* 1981; Horner 1983; Prittie 2004). There appear to be some breed predisposition to CPV-2 induced disease, as Doberman Pinschers and Rottweilers have been identified as being at increased risk of CPV-2 enteritis (Glickman *et al.* 1985; Houston *et al.* 1996). Some investigators have also found an element of seasonality to the risk of CPV-2 infection, as dogs were found to be three times more likely to be admitted with CPV-2 enteritis during summer months than in other seasons(Houston *et al.* 1996).

As CPV-2 requires rapidly dividing cells for replication, the pathogenesis and clinical disease in neonatal dogs infected with the virus can be quite different than that seen in dogs over the age of 5 weeks (Shackelton *et al.* 2005). Canine parvovirus was found to cause myocarditis in neonatal pups (Hayes *et al.* 1979). However CPV-2 associated myocarditis is rarely seen today. It can be hypothesised that the rarity of CPV-2 associated myocarditis is due to the fact that the majority of bitches are exposed to the CPV-2 antigens either via vaccination or through environmental contamination. The exposure to CPV-2 antigens results in strong and long lasting immunity, which can prevent the infection of the pups *in utero* and allows for the production of CPV-2 antibodies in colostrum. The maternal antibodies protect new born pups against CPV-2 infection during the first few weeks of life, so during the time when CPV-2 infection can result in development of myocarditis. Myocardial disease among puppies almost disappeared in New Zealand after February of 1981, which coincided with a massive outbreak of CPV-2 in New Zealand (Horner 1983). Although in 2006 a case was reported in a 5-week-old pig dog puppy (Gibson 2006), the affected puppy was born to an unvaccinated bitch who was moved late in her pregnancy from an isolated farm to a different property (Gibson 2006). It is therefore possible that this bitch had no previous exposure to CPV-2 antigens and did not transfer any CPV-2 specific maternal antibody to her pup.

1.2. Spread and Subtype Evolution

Canine parvovirus type 2 (CPV) emerged as a disease of dogs in the late 1970's (Eugster and Nairn 1977; Thomson and Gagnon 1978; Appel *et al.* 1979a; Hayes *et al.* 1979). The first antibody positive sera were found in Greece and were dated to 1974 (Koptopoulos *et al.* 1986). Molecular clock estimates indicate CPV-2 may have been present in the canine population for up to 10 years before it was first described (Shackelton *et al.* 2005).

Within approximately 2 years of the first defined cases of CPV-2 enteritis, suspected and confirmed cases of CPV-2 infection were reported worldwide, including Australia (Johnson 1979), New Zealand (Gumbrell 1979; Horner *et al.* 1979), Great Britain (McCandlish *et al.* 1979), USA (Appel *et al.* 1979a), Canada (Thomson and Gagnon 1978), Belgium (Burtonboy *et al.* 1979) and the Netherlands (Osterhaus *et al.* 1980). During this initial spread of CPV-2, dogs of all ages were affected. The fast worldwide spread of CPV-2 was likely facilitated by the ability of the virus to stay infective in the environment for long periods of time (Gordon and Angrick 1986). Suggested methods of spread for this virus from one country to another include carriage on fomites and possibly the movement of sub-clinically infected dogs

(Hoelzer *et al.* 2008). In addition, the movement of infected wildlife such as raccoons may have played a role in the spread of the virus between adjacent countries (Allison *et al.* 2012).

Since the initial emergence of CPV-2 the virus has been continuously evolving. Within a few years of the first emergence of CPV-2, a new subtype, designated CPV-2a evolved (Parrish *et al.* 1988b). It was characterised by changes in the antibody binding profile of viral proteins, and restriction enzyme digestion profiles of viral DNA, compared to earlier isolates. All viruses collected after 1981 belonged to this new subtype(Parrish *et al.* 1985). CPV-2a spread rapidly around the world replacing the original CPV-2 between 1979 and 1983 (Parrish *et al.* 1988b). Fortunately, dogs that were immune to CPV-2 were also immune to CPV-2a (Parrish *et al.* 1985). Following the emergence CPV-2a, another subtype (CPV-2b) was noted in the USA in 1984, and subsequently became dominant in the United States by 1986 (Parrish *et al.* 1991). After the emergence of CPV-2a and CPV-2b variants, the virus appeared to stabilize. No novel antigenic changes were noted until 2000, when another CPV-2 subtype emerged in Italy. This newest subtype was referred to as CPV-2c (Buonavoglia *et al.* 2001). Thus, the predominant variants of CPV-2 are now classified into these four main subtypes, with the original CPV-2 believed to be no longer circulating among dogs, although it is still incorporated in several vaccine formulations (Nandi and Kumar 2010).

Subtypes CPV-2a and CPV-2b have variable relative distribution from country to country (Pereira *et al.* 2000; Chinchkar *et al.* 2006; Zhang *et al.* 2010). The most recently emerged subtype CPV-2c has now been isolated in many countries around the world (Decaro *et al.* 2006; Hong *et al.* 2007; Calderon *et al.* 2009; Nandi *et al.* 2010); although it appears to be circulating at relatively low levels.

It has been proposed that the initial emergence of CPV-2 among dogs was due to a mutation in the genome of feline panleukopenia virus (FPV) (Parrish *et al.* 1988a). Phylogenetic analysis of various CPV-2 genomes showed that these viruses have evolved from a single common ancestor (Truyen and Parrish 1992; Truyen *et al.* 1995; Horiuchi *et al.* 1998), which suggests that the move into the canine host occurred only once. The subsequent evolution of the virus within the canine host was believed to be driven by natural selection and the high mutation rate observed in CPV-2 (Shackelton *et al.* 2005). Previously, it was believed that recombination did not play a role in the evolution of the virus. However, recombination between CPV-2 and CPV-2a, as well as CPV-2 and CPV-2b has been found to occur (Mochizuki *et al.* 2008). Hence, it is also possible that recombination between FPV and some CPV-2 viruses might have occurred within feline hosts concurrently infected with both

viruses, which might have contributed to the evolution of CPV-2 (Ohshima and Mochizuki 2009).

The original CPV-2 subtype was not able to replicate in cats (Truyen *et al.* 1996). Thus, cats were unlikely to have played a role in the early spread of CPV-2. However, newer subtypes CPV-2a and CPV-2b were isolated from cats with clinical signs of feline panleukopenia (Mochizuki *et al.* 1996; Decaro *et al.* 2010). In addition, cats were shown to develop disease following experimental challenge with CPV-2a, CPV-2b and CPV-2c (Nakamura *et al.* 2001; Gamoh *et al.* 2003). It is therefore possible that cats have also played a role in the evolution, and possibly the spread, of the newer CPV-2 subtypes following the initial adaptation of the virus to the canine host. It has also been found that the CPV-2 sequences of parvoviruses collected from wild raccoons occupy intermediate positions between CPV-2 and CPV-2a (Allison *et al.* 2012), indicating that raccoons may have played a role in the evolution of CPV-2 to CPV-2a. Other wild animals may have also played a role in the evolution and spread of CPV-2. Wild canids such as wolves, foxes, jackals and coyotes have been found to be susceptible to CPV-2 infection and disease (Steinel *et al.* 2001), and experimental infection of mink supported low levels of CPV-2 replication (Parrish *et al.* 1985).

The virus gains entry to the host cell via binding to the canine transferrin receptor (TfR). Results of recent studies investigating the canine TfR suggested that CPV-2 may in fact not be a novel pathogen of dogs. The evolution of the TfR prevented efficient binding of ancient parvoviruses, thereby blocking entry of the virus into target cells. It has been proposed that the variant which emerged in the 1970s overcame this host adaptation to once again allow for efficient entry of the virus to target cells (Kaelber *et al.* 2012).

1.3. VACCINATION AND THE IMMUNE RESPONSE

In dogs experimentally infected with CPV-2 the haemagglutination inhibition (HI) antibody titres increased from 40 at pre-inoculation to 320 six days after inoculation. This spike in antibody level coincided with a decrease in the levels of virus found in tissues (Meunier *et al.* 1985a). Dogs with (HI) titers of 80 or more are protected from CPV-2 infection via oronasal challenge (Kramer *et al.* 1980; Pollock and Carmichael 1982a). Virus-specific immunity post exposure is long lived (Burr *et al.* 1988). In addition, the ability of the virus to remain infective in the environment allows for the natural re-exposure of dogs which are already immune, which can prime the dog's immune system in a similar way to the booster vaccination (Day *et al.* 2010). As a result, CPV-2 induced disease is now considered a disease

of young dogs, with dogs 6 months and under being at highest risk of developing parvovirus enteritis (Horner 1983; Studdert *et al.* 1983).

Many of the commonly available canine vaccines worldwide still contain the original CPV-2 subtype as the antigen. Some vaccines include the newer CPV-2a or CPV-2b subtypes, but none incorporate the CPV-2c subtype. Hence, one of the important questions among the veterinary and scientific communities is: Is the use of the currently available vaccines, particularly those containing the original CPV-2 subtype, still acceptable considering the evidence that the original CPV-2 subtype no longer circulates naturally in countries where characterisation studies have been conducted, and that the virus continues to evolve over time as evidence by recent emergence of CPV-2c?

The concerns related to the efficacy of currently available vaccines against this newest CPV-2c subtype were fuelled by several cases in which CPV-2c was isolated from vaccinated adult dogs with severe haemorrhagic enteritis (Decaro *et al.* 2008a). This lead to several studies which investigated the suitability of the use of CPV-2 as the antigen in canine vaccines. Although results of such trials suggested that the currently used vaccines afford effective and long term protection against all subtypes of CPV-2 in appropriately vaccinated canines (Larson and Schultz 2008; Spibey *et al.* 2008), confirmed clinical cases of CPV-2 enteritis in fully vaccinated dogs are reported occasionally (Decaro *et al.* 2008a). However, the rate of CPV-2c related disease in vaccinated dogs is no greater than the rate of CPV-2 related disease caused by other CPV-2 subtypes (Hong *et al.* 2007).

1.4. Taxonomic Classification and Basic Properties

The *Parvoviridae* family includes two subfamilies, *Parvovirinae* and *Densovirinae*. The subfamily *Parvovirinae* is further divided in to five genera: *Parvovirus*, *Erythrovirus*, *Dependovirus*, *Amdovirus* and *Bocavirus*. Canine parvovirus type 2 belongs to the genus *Parvovirus* of the subfamily *Parvovirinae* in the family *Parvoviridae* (Figure 1). CPV-2 was merged as one species with feline panleukopenia virus in 2002 (Anonymus 2013a).

After its initial emergence, the virus spread rapidly throughout the world (see section 1.2). This newly emerged virus was designated CPV-2 to distinguish it from the antigenically and genetically distinct CPV-1 or minute virus of canines (MVC) (Ohshima *et al.* 2004). Digestions with restriction enzyme of the DNA from CPV-1 and CPV-2 failed to show evidence of genomic relationship between the two viruses (Macartney *et al.* 1988). Taxonomically, CPV-1 belongs to the *Bocavirus* genus.

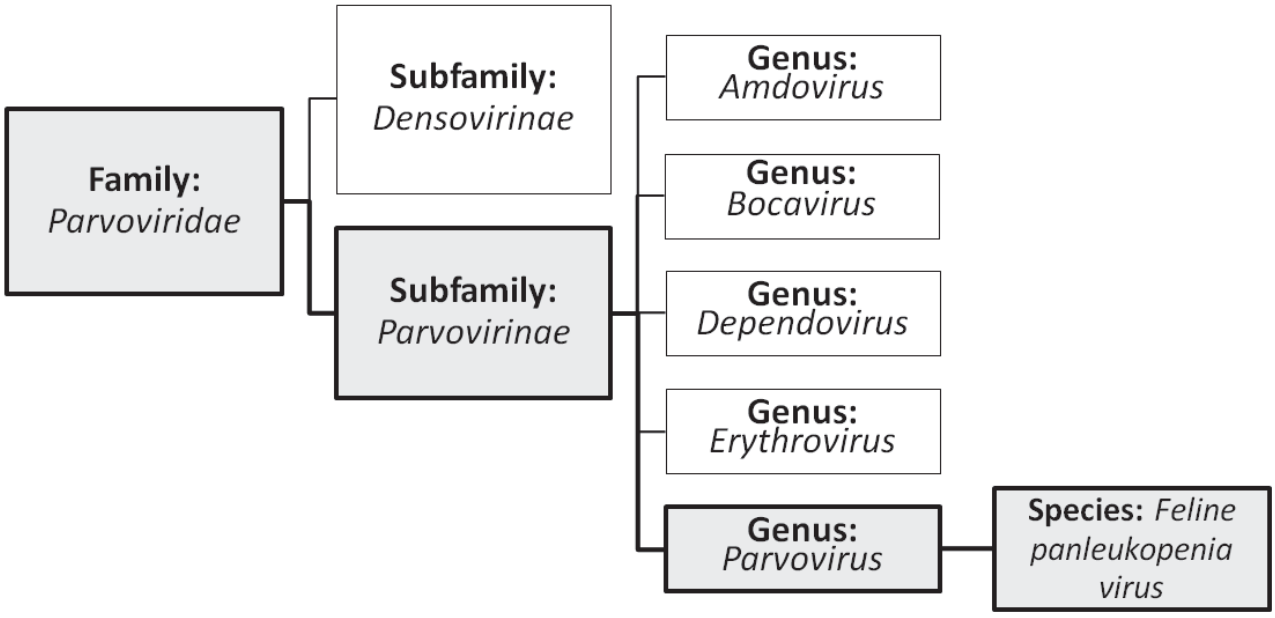

Figure 1 Taxonomic tree of the family *Parvoviridae*.

CPV-2 is a non-enveloped virus which is approximately 26 nm in size. The three dimensional structure of CPV-2 was elucidated by x-ray crystallography (Tsao *et al.* 1990). The virus displays on its surface 60 copies of two size variants of capsid viral proteins (VP), consisting predominantly VP2 and some VP1. Canine parvovirus like many other non-enveloped viruses is an extremely stable virus. It is resistant to inactivation by heat treatment at 50°C for 30 mins, ether treatment and treatment with acid of pH 3 (Azetaka *et al.* 1981). These physiochemical properties help CPV-2 to remain infectious in the environment for long periods of time after being shed in the faeces of an infected dog.

The virus possesses a single stranded DNA (ssDNA) linear genome which is 5,232 nucleotides in length (Reed *et al.* 1988) and contains two major open reading frames (ORFs). The 3' half of the genome encodes non-structural proteins (NS1 and NS2) and the 5' half encodes structural proteins (VP1, VP2 and VP3) (Reed *et al.* 1988). The NS1 protein is involved in the control of viral DNA replication and packing of viral DNA into capsids (Nuesch *et al.* 1998; Daeffler *et al.* 2003; Cotmore and Tattersall 2007). The NS1 protein also has the ability to recognise viral DNA, to nick DNA during replication, and can act as a helicase (Nuesch *et al.* 1998; Christensen and Tattersall 2002). In addition, NS1 also controls cellular apoptosis and binds some cellular proteins (Nuesch *et al.* 1998; Cotmore and Tattersall 2003; Daeffler *et al.* 2003). The role of the NS2 protein is less well understood. Mutation of NS2 had little effect on the replication levels of the CPV-2 in certain tissues (Wang *et al.* 1998) suggesting the NS2 may not play an important role in the CPV-2 life cycle. As mentioned previously, the VP1 and VP2 proteins make up the capsid of the viral particle. The coding regions for the VP1 and VP2 proteins overlap, and the final mRNA is produced by

alternative splicing. The VP2 protein can be cleaved by host proteases to produce the VP3 protein. The VP2 protein has an eight stranded anti-parallel beta barrel confirmation (Tsao *et al.* 1990). The sheets of the beta barrel are joined by loop protrusions which surround the 3-fold axis of symmetry, this is referred to as the 3-fold spike (see Table 1)(Tsao *et al.* 1990).

1.4.1. IMPORTANT TYPING SITES

Canine parvovirus 2a differs from the original CPV-2 by five amino acids at sites 87, 101, 300, 305 and 555 and CPV-2b differs from CPV-2a by only two amino acids (Figure 2) (Parrish *et al.* 1991). CPV-2b also shows a reversion to the original CPV-2 subtype in position 555 (Parrish *et al.* 1991). During the initial emergence of CPV-2c , the virus obtained from one of the presenting cases of an adult dog with signs of severe haemorrhagic gastroenteritis was initially classified as CPV-2b based on reactivity with monoclonal antibodies and CPV-2b specific primers (Buonavoglia *et al.* 2001). However, further investigation revealed a change in the amino acid at position 426 from *Asp* (typical for CPV-2b) to *Glu*, which warranted the new designation of a new subtype CPV-2c (Buonavoglia *et al.* 2001). At around the same time researchers identified yet another new variant in Vietnamese leopard cats (Ikeda *et al.* 2000), which was initially also referred to as CPV-2c and involved a change at amino acid 300 from *Gly* (CPV-2b) to *Asp*. The CPV-2c which possesses the amino acid change at site 426 and was originally isolated for dogs in Italy has now been officially accepted as CPV-2c subtype, while the virus with a Gly300Asp change is considered a variant of CPV-2b. The variable sites which have differentiated the CPV-2 subtypes are all located on the VP-2 protein of the viral capsid (Table 1).

AA Position	Description
87	Residue is surface exposed on the side of the 3-fold-spike of the VP2 protein. This residue is in close proximity to AA residues 300 and 305 of adjacent VP2 unit when the capsid is fully assembled. Due to this close proximity changes here may affect sites 300 and 305 of the adjacent VP2 unit (C. R. Parrish et al. 1991).
101	Is not exposed on the surface of the capsid(C. R. Parrish et al. 1991). This residue changed between CPV-2 and CPV-2a, this change has been conserved in all subsequent subtypes.
300	Residue is surface exposed on the side of the 3-fold-spike of the VP2 protein. Change from *Ala-Asp* results in loss of canine host range.(C. R. Parrish et al. 1991)
305	Is found on the side of the 3-fold-spike of the VP2 protein and is surface exposed(C. R. Parrish et al. 1991).
426	Located at the top of the 3-fold spike and is surface exposed(C. R. Parrish et al. 1991). This residue is used to characterise CPV-2c which possess a unique change to *Glu* at this site.
555	Located in the dimple of the 3-fold-spike and is surface exposed.(C. R. Parrish et al. 1991)

Table 1 A description of sites in the VP2 protein which can affect the antigenicity of CPV-2 and are used for subtyping CPV-2.

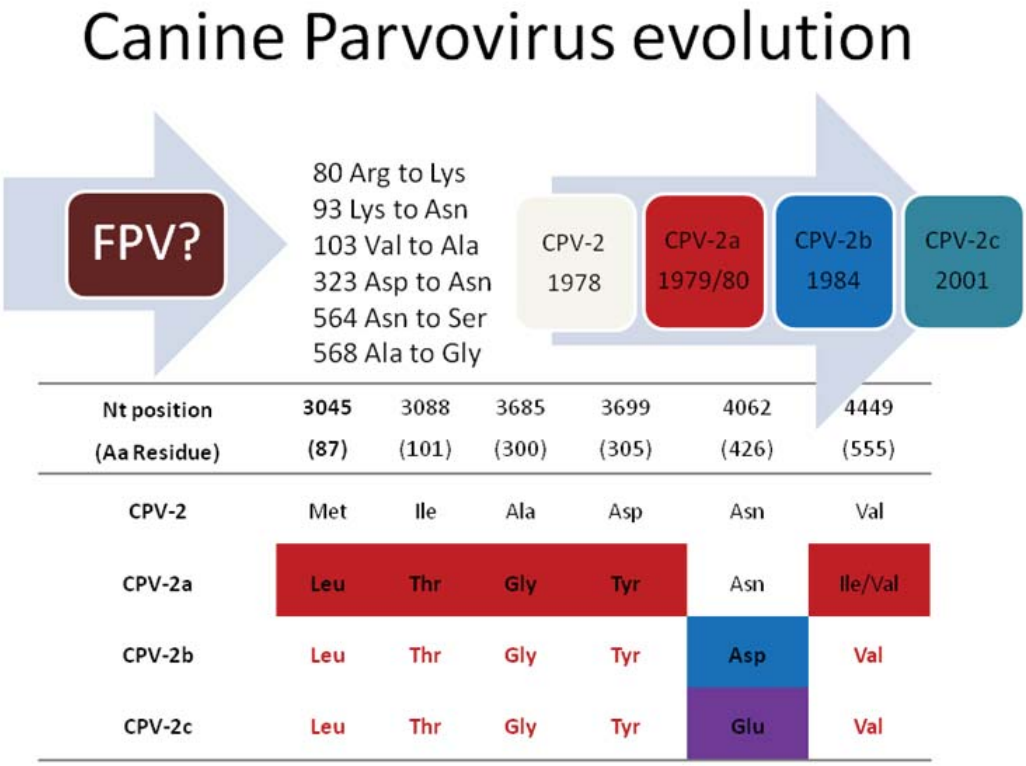

Figure 2 A basic overview of the evolution of the canine parvovirus subtypes. Nucleotide positions based on reference sequence accession number: M38245.1.

At the nucleotide level, the mutation rate of CPV-2 is closer to the mutation rates of RNA viruses than to those typical for double stranded DNA viruses (Parrish *et al.* 1991; Truyen *et al.* 1995; Shackelton *et al.* 2005). Possible explanations include the single stranded nature of the viral genome and the formation of secondary structures in the nucleic acids during replication, both of which may cause more errors to be introduced (and not repaired) during the replication of the viral genome by cellular polymerases (Lindahl and Nyberg 1974; Shackelton *et al.* 2005); In addition, deamination of cytosine residues is more readily observed in single stranded DNA than in double stranded DNA, leading to increase in the mutation rate (Lindahl and Nyberg 1974; Shackelton *et al.* 2005). However, the genome of FPV appears to be stable, with much lower mutation rates compared with those described for CPV-2 (Battilani *et al.* 2006; Decaro *et al.* 2008b). As these two viruses are very closely related and share many of the same features including a single stranded DNA genome, other factors must play a role in generation of high mutation rates observed for CPV-2. The ratios of non-synonymous to synonymous mutations in the viral capsid protein 2 (VP2) genes have been found to favour synonymous mutations (Truyen *et al.* 1995; Decaro *et al.* 2008c; Yoon *et al.* 2009). In addition, the amino acid substitutions in the sequence of the VP2 gene have been found to be focused in specific areas exposed on the outer and inner surfaces of the capsid (Truyen *et al.* 1995). These findings suggest that CPV-2 is under selection pressure from the immune response, as would be expected for a newly emerging virus. This theory is further supported by the fact that the mutation rate of the NS1 protein is not significantly different between FPV and CPV-2 (Hoelzer *et al.* 2008). As NS1 protein is not antigenically important (Hoelzer *et al.* 2008) and is therefore not expected to be under the same selection pressures as VP2. Immune adaptation is noted as one of the possible major drivers of the evolution of this virus.

1.5. CANINE PARVOVIRUS IN NEW ZEALAND

The earliest suspected case reported in New Zealand can be dated to January 1979 (Gumbrell 1979) and originated from a dog near Christchurch (South Island NZ). The diagnosis in this case was based on clinical signs and necropsy findings. The author reported similar cases from Canterbury (South Island NZ) and the West Coast (South Island NZ). In October 1979 CPV-2 was successfully isolated for the first time in New Zealand from a 9-week-old Afghan hound puppy from Taupo (North Island, NZ) which presented with diarrhea, vomiting and pyrexia and dehydration in June 1979. The virus was isolated in a feline kidney cell-line

(NLFK) (Horner *et al.* 1979). There have been no published investigations of CPV-2 in New Zealand since the early 1980's.

1.6. AIMS OF THE STUDY

The aim of this study was to determine which CPV-2 subtypes are currently circulating among the New Zealand canine populations. In addition, the aims of the phylogenetic analyses were to investigate the evolutionary patterns of CPV-2 within New Zealand and to compare New Zealand CPV-2 sequences to those from other countries.

2. A SURVEY OF CANINE PARVOVIRUS IN NEW ZEALAND

2.1. INTRODUCTION

After the initial emergence of CPV-2 in the early to mid-1970s the virus spread rapidly among the dog population of the world (Carmichael 2005). CPV-2 was first isolated in New Zealand in June of 1979 (Horner *et al.* 1979). Reports of dogs displaying clinical signs consistent with CPV-2 infection were documented as early as January 1979 (Gumbrell 1979). These initial cases included two 16-week-old greyhound puppies displaying clinical signs of parvoviral gastroenteritis three days after introduction to the kennel. Another three days later, a litter of ten 3-month-old puppies also became clinically ill. Three weeks later a one 1-week-old poodle puppy at the same kennel died suddenly (Gumbrell 1979). Attempts to isolate the virus from these early cases were not mentioned in the case reports. Dogs were diagnosed with CPV-2 infection based on the necropsy and histopathology findings.

Soon after the emergence of CPV-2 in New Zealand research articles detailing necropsy findings more thoroughly were published in the New Zealand Veterinary Journal. Colin Parrish, a New Zealander and graduate of Massey University, was the first to publish such a report in 1980 (Parrish *et al.* 1980). It detailed five incidences of CPV-2 enteritis and one case of CPV-2 myocarditis. In addition, 48 blood samples were obtained from local dogs for serological investigation. Twenty seven percent of these samples had HI CPV-2 titers of 2,560 or greater; the remaining 73% had titers of less than 40. These results suggested that several of the tested dogs had been previously exposed to the CPV-2 antigens. It is unclear how the dogs were selected for inclusion in the study.

Given the timing of the publication in relation to the emergence of CPV-2, and that the best method of vaccination for dogs was still under debate at this time, it seems unlikely that these dogs became positive for CPV-2 antibody due to vaccination. During the early stages of the emergence of CPV-2, vaccination of dogs with the FPV vaccines was common place (Appel *et al.* 1979b; Pollock and Carmichael 1982b). The use of vaccines designed to protect cats from FPV disease afforded some protection against canine parvovirus gastroenteritis, although the protection was short-lived even when dogs were vaccinated twice at 3 week intervals (Appel *et al.* 1978; Appel *et al.* 1979b; Mann *et al.* 1980; Pollock and Carmichael 1982b; Povey *et al.* 1983). Approximately 18 months after the first reported CPV-2 cases in

New Zealand, in November 1980, a CPV-2 vaccine licensed for use in dogs became available (Jones *et al.* 1982).

A longitudinal serological survey of CPV-2 antibody levels in dogs presenting for their first vaccination was launched in December of 1980 (Jones *et al.* 1982). As part of this study, 106 samples were collected between the 1st of December 1980 and the 1st of March 1981 from healthy dogs of mixed age, sex, and breed. In addition, 55 historical serum samples collected between June 1974 and October 1980 were also included, nine of which were from dogs displaying clinical signs of CPV-2 disease. The results of this survey showed that all samples collected prior to 1978 were negative for the presence of HI antibodies against CPV-2. A positive sample was defined as a sample with antibody titre of 320 or greater. Of the nine samples collected from dogs with signs suggestive of CPV-2 infection, six were considered seropositive for CPV-2. Of the remaining 106 dogs presenting for their first vaccination, 23% were positive for CPV-2 HI antibody, indicating possible previous CPV-2 infection.

The major antigenic determinants of the CPV-2 are located within viral capsid protein VP2 (Parrish 1991). Antibodies produced by the infected host specifically target residues on the surface of the VP2 protein (Chang *et al.* 1992). Thus, changes to the external appearance of this protein are likely to influence the ability of the antibodies to bind to the virus. For these reasons CPV-2 viruses are typically subtyped based on the amino acid changes at specific positions, corresponding to immune-dominant epitopes on the surface of VP2 (Battilani *et al.* 2002; Wang *et al.* 2005; Chinchkar *et al.* 2006; Ohshima *et al.* 2008; Zhang *et al.* 2010). The main amino acids used for subtyping are described in Table 1.

The VP2 protein is directly involved in the binding of the virus to the host TfR, which facilitates the entry of the virus into the host cell (Harbison *et al.* 2009). Several amino acids in the VP2 protein directly affect the binding efficiency of the virus to the canine TfR and thus, affect the epidemiologically important properties of the virus such as the host range and virulence (Chang *et al.* 1992; Ikeda *et al.* 2002; Hueffer *et al.* 2003; Harbison *et al.* 2009). For example, changes in VP2 residues 93 from Lys to Asn and in residue 323 from Asp to Asn were enough to extend the *in vitro* host range of FPV from feline to canine cells (Horiuchi *et al.* 1992). Another mutation in VP2 residue 300 from Ala to Asp following *in vitro* passage of CPV-2 in feline cells reduced the ability of the viral isolate to infect canine cells (Llamas-Saiz *et al.* 1996).

Studies have been carried out overseas to investigate CPV-2 subtypes circulating in various countries. Although several CPV-2 subtypes may co-circulate in one country, one particular subtype is often predominant in any geographical area. For example, in 2006 and 2010 in India CPV-2a was reported to be the predominant CPV-2 subtype (Chinchkar *et al.* 2006; Raj *et al.* 2010), with other subtypes found at the lower prevalence. However, results of another study suggested that CPV-2b, rather than CPV-2a, was the predominant subtype in most geographical areas in India in 2009 (Nandi *et al.* 2009). This suggested that the composition of CPV-2 viruses in any given area is dynamic and may change over time. The results of a UK-based study showed that 43% of 150 CPV-2 positive samples contained CPV-2a, and 57% CPV-2b (Clegg *et al.* 2011). This indicates the two subtypes were circulating at approximately equal proportions at the time of sample collection. Results of a study in Argentina showed that 33.4% of viruses tested were classified as CPV-2a, 14.8% as CPV-2b and 51.8% as CPV-2c (Calderon *et al.* 2009). To date, there has been no study of the CPV-2 subtypes circulating in New Zealand.

Analysis of CPV-2 genomes showed that CPV-2 from some countries had unique mutations found at sites that are not currently used for the purposes of CPV-2 subtyping (Raj *et al.* 2010; Han *et al.* 2011). The significance of such changes for the epidemiology and biology of locally circulating CPV-2 viruses was not determined.

Since the initial emergence of CPV-2, the interest in the monitoring of this virus in New Zealand seems to have waned, possibly because of the high efficacy of the vaccines available on the market today. The lack of monitoring CPV-2 circulating in New Zealand has left a substantial gap in the collective knowledge of the epidemiological features of CPV-2 in this country; a primary motivation for this study. Although it appears the vaccines currently available around the world do protect dogs against all the currently known subtypes of CPV-2, we are unable to predict how this virus will evolve in the future. As such, close monitoring of viruses circulating locally would facilitate management of any potential outbreaks of CPV-2 disease should a novel CPV-2 variant emerge. This would be particularly important if such a variant was sufficiently different from the vaccine strains as to escape the cross-protection currently provided by the available vaccines.

This survey was initiated in part by the interest of local veterinarians who perceived a recent increase in numbers of CPV-2 associated gastroenteritis in vaccinated, or partially vaccinated, dogs (personal communication, Drs Nick Cave and Magda Dunowska). The question

commonly posted by the field veterinary practitioners was the likelihood of the disease being caused by a new strain of CPV-2, to which the current vaccines may offer little cross-protection. Hence, the aim of the current study was to determine which CPV-2 subtypes circulate among New Zealand dogs, in order to ascertain whether or not the perceived increase in clinical cases is associated with emergence of a novel antigenic variant of CPV-2. In addition, there was interest to determine the basic epidemiological features of CPV-2 in New Zealand, including the vaccination status, age and breed of dogs diagnosed with CPV-2 enteritis. There have been no studies of this nature carried out in New Zealand to date.

2.2. Materials and Methods

2.2.1. Sample Collection and Storage

Faecal samples were collected from November 2009 to December 2010. "Parvo packs" consisting of two sample pots, a submission form (Appendix 6.1.2), a letter (Appendix 6.1.1) explaining the purpose of the study and an addressed prepaid courier envelope were distributed among veterinarians throughout New Zealand by the Intervet representatives during routine clinic visits. Similar packs were sent to SPCA shelters. In addition, samples submitted to Intervet Scherring-Plough of investigation of potential vaccine failure were also included. One sample from a domestic cat (CPV067) was also submitted by the referring veterinarians. This sample was tested for CPV-2 by PCR, but was not included in the analysis presented in this chapter.

The participating veterinarians were asked to collect faecal samples from any dog or puppy that tested positive on a rapid CPV antigen detection kit, or was strongly suspected of CPV-2 infection based on clinical presentation. Veterinarians were asked to store the samples at 4 °C and to courier them to the laboratory as soon as feasible after collection. Upon arrival at Massey University the samples were stored at 4°C until processed. This normally occurred within 12 hours of receipt with the exception of a small number of samples (n=2) which were misplaced on delivery.

Faecal samples were divided into two equal portions for DNA extraction and storage. A small amount of the faecal material (50 to 200 μL) was mixed at a 1:3 ratio with the Stool Transport and Recovery (S.T.A.R) Buffer (Roche Diagnostics GmbH, Roche Applied Science,

Mannheim, Germany). The remaining sample was placed in a cryovial, labelled appropriately and stored at -80°C for future virus isolation.

In addition, 12 other samples were included in the study. These comprised three vaccines (Nobivac [Merck Animal Health, Millsboro, USA], Vangard Plus 5 [Pfizer, Inc., New York, USA] and Protech C3 [Boehringer Ingelheim, Rhineland-Palatinate, Germany]) and 9 historic CPV-2 isolates. These originated from 1979 – 2006 (MAF.WV.1, MAF.WV.2, MAF.WV.3, MAF.WV.4 kindly supplied by Dr Wlodek Stanislawek, and CPV014, CPV015, CPV016, CPV017, CPV018 that represented archival isolates in possession of Massey University) (Appendix 6.1.3).

2.2.2. DNA EXTRACTION

Total DNA was extracted from faecal samples stored in S.T.A.R buffer. During the optimisation of this procedure two kits were trailed: Isolate Faecal DNA Kit (Bioline Pty Ltd, NSW, Australia) and High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Both protocols were carried out as per manufacturer's instructions. The High Pure PCR Template Preparation Kit was used following a protocol for the isolation of nucleic acids from mammalian whole blood, buffy coat or cultured cells, with a small modification. Samples containing substantial amounts of undigested or particulate matter were subjected to an additional centrifugation step (1 minute at 8000 g) after Proteinase K digestion, but before commencing further steps of DNA extraction protocol, in order to pellet any sediment to prevent clogging of the filter. The supernatant was transferred to the High Filter Tube for DNA extraction. DNA was eluted in 200 µL of elution buffer (10 mM Tris HCl, pH 8.5) and stored at 4°C until PCR was performed.

2.2.3. PCR OF THE CPV VP2 GENE

The PCR reaction using primers CPV.VP2.JS1.F and CPV.VP2.JS2.R (as described by Meers et al., 2007) (Table 2) was performed to amplify 1975 bp fragment of VP1/VP2 gene.

Primer	Sequence 5' to 3'	Position	Used For
CPV.VP2.JS1.F	AGCTACAGGATCTGGGAACG	2843-2862	Amplification and Sequencing
CPV.VP2.JS2.R	CCACCCACACCATAACAACA	4799-4818	Amplification and Sequencing
CPV.VP2.JS3.F	GCGCAAACAGATGAAAATCA	3876-3895	Sequencing
CPV.VP2.JS4.R	CCAACCTCAGCTGGTCTCAT	3775-3796	Sequencing

Table 2 Primers used to amplify and sequence 1975 bp fragment of CPV-2 VP1/VP2 gene (from Meers et al, 2007).

Each reaction was performed in a total volume of 20 µL and consisted of 0.4 µM of each primer, 10 µL of 2x FastStart Master Mix (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) and 1µL DNA template. The cycling conditions were as follows: 95 °C 10 min, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 52°C for 10 seconds, and extension at 72°C for 2 min, with the final extension step at 72°C for 7 min, followed by 4°C hold. Positive (a CPV-2 positive sample confirmed by sequencing, obtained before the start of the project and later included as CPV058) and negative (water) controls were included with every PCR run. PCR products (entire 20 µL) were subjected to electrophoresis through 1% agarose (Axygen) gel containing 0.5 mg/mL ethidium bromide in Tris-Acetate-EDTA (TAE) buffer for 90 minutes at 90 V in a mini gel tank (Bio-Rad, Hercules, CA, USA). The PCR bands were visualised using GelDoc reader (Bio-Rad, Hercules, CA, USA). The test was considered valid if positive and negative controls produced expected results. Any bands corresponding to the expected size of the product were extracted from the gel and purified using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin columns (Bio-Rad, Hercules, CA, USA). Briefly, the gel slice was placed in the supplied column, snap frozen in liquid nitrogen and the column was centrifuged at 13,000 g for five minutes at room temperature. The gel remnants were discarded and the DNA-containing elute was stored at 4°C for sequencing. The sample was considered positive for CPV-2 DNA if a band of the expected size was observed and its identity confirmed by sequencing (section 2.2.4).

2.2.4. Sequencing and Sequence Analysis

Purified PCR products were submitted for Sanger sequencing using BigDye Terminator v3.1 (Life Technologies, Carlsbad, CA, USA) at the Massey Genome Centre. The sequencing was performed using four separate primes as described by Meers et al. (Meers *et al.* 2007) (Table 2). The sequences were analysed using bioinformatics software Geneious 5.6.5 (Drummond *et al.* 2012). The low quality/resolution reads were manually removed. Sequences were then

aligned to a pre-selected reference sequence (Accession: M38245.1) and subtyped based on the presence of specific amino acids at selected antigenic typing sites in the predicted amino acid sequence (Table 3).

Sub-type	Nucleotide Position (Amino Acid Residue)					
	3045 (87)	3088 (101)	3685 (300)	3699 (305)	4062 (426)	4449 (555)
CPV-2	A (Met)	T (Ile)	C (Ala)	G (Asp)	A (Asn)	G (Val)
CPV-2a	T (Leu)	C (Thr)	G (Gly)	T (Tyr)	A (Asn)	G (Ile/Val)
CPV-2b	T (Leu)	C (Thr)	G (Gly)	T (Tyr)	G (Asp)	G (Val)
CPV-2c	T (Leu)	C (Thr)	G (Gly)	T (Tyr)	G (Glu)	G (Val)
FPV	A (Met)	T (Ile)	C (Ala)	G (Asp)	A (Asn)	G (Val)

Table 3 Designation of CPV-2 subtypes and FPV based on major antigenic sites, also see Figure 2. Nucleotide positions based on reference sequence Accession number: M38245.1.

2.3. Results

2.3.1. Comparison of Two Commercial DNA Extraction Kits

For the optimisation of the procedures to be used throughout this project two DNA extraction kits were initially tested. The quality of the DNA obtained using the Bioline kit was slightly lower than the quality of DNA obtained with the Roche kit, as determined by calculation of the 260/280 optical density (OD) ratios using Nanodrop (Thermo Fisher Scientific, Maine, USA) (Table 4). Only five out of six samples extracted with the Bioline kit were positive for CPV-2, and non-specific bands were amplified preferentially to the bands of the expected size in a further two samples. By comparison, all six samples tested positive for CPV-2 using DNA extracted with the Roche kit (Figure 3). As the High Pure PCR Template Preparation Kit produced higher quality and more consistent results this was selected for further use throughout the project.

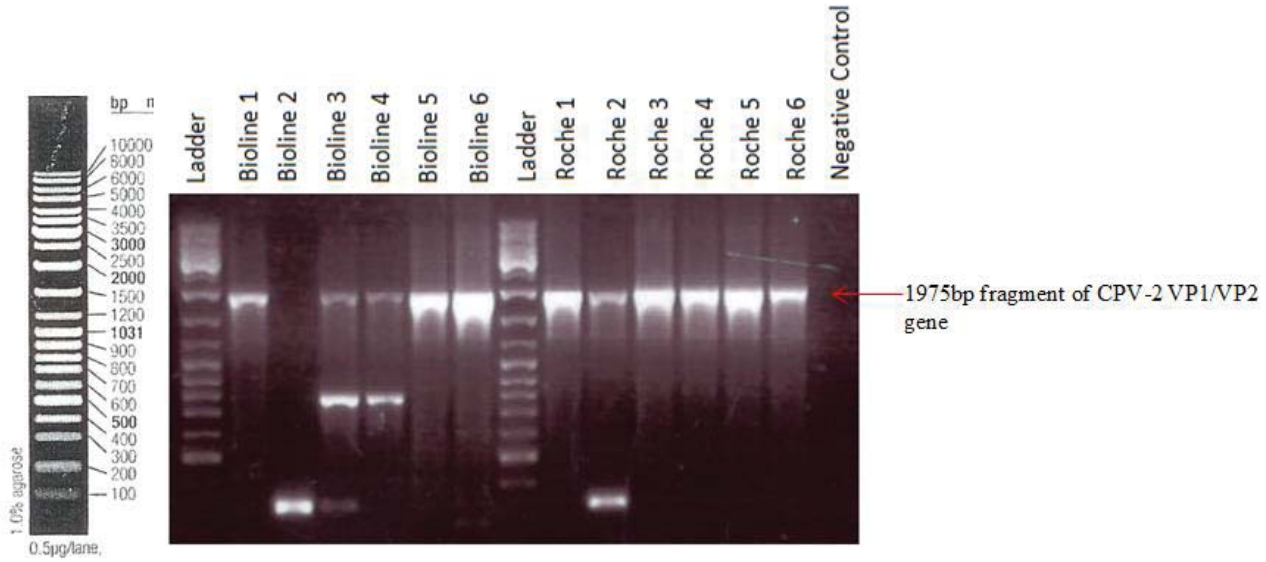

Figure 3 Electrophoresis gel showing a comparison of the same set of samples processed with two different kits: Isolate Faecal DNA Kit (Bioline Pty Ltd, NSW, Australia) and High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). The expected 1975 bp fragment of CPV-2 VP1/VP2 gene can be seen as indicated. O'GeneRuler™ DNA Ladder Mix, Ready-to-use, 100- 10000bp (Thermo Fisher Scientific, Maine, USA) was used as a molecular size marker.

	High Pure PCR Template Preparation Kit (Roche)			Isolate Faecal DNA Kit (Bioline)		
Sample Number	ng/µl	Ratio 260/280	Abs	ng/µl	Ratio 260/280	Abs
CPV001	8.3	1.66	0.166	13.6	1.418	0.271
CPV002	10.5	1.78	0.211	22.6	1.203	0.452
CPV003	53.1	1.749	1.062	25.8	1.407	0.517
CPV004	11.5	1.465	0.229	17.8	1.410	0.355
CPV005	5	1.595	0.101	16.1	1.016	0.323
CPV006	23.1	1.531	0.462	21.8	1.129	0.436

Table 4 The purity and quantity of DNA extracted from six faecal samples using two different kits: Isolate Faecal DNA Kit (Bioline Pty Ltd, NSW, Australia) and High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany), assessed using nanodrop (Thermo Fisher Scientific, Maine, USA) readings.

2.3.2. ORIGIN OF FAECAL SAMPLES SUBMITTED FOR THE SURVEY

During this study a total of 79 faecal samples were collected. The submitting clinics/shelters were distributed throughout New Zealand, including nine regions in the North Island and five regions in the South Island (Figure 4). Majority (n=61) of samples were obtained from privately owned dogs via participating veterinary clinics, which were recruited to the study by Intervet/Schering-Plough Animal Health's representatives during routine visits. The remaining 18 samples were obtained either from one of the SPCA shelters (n=9) or via Dr. Doug Passmore (Intervet/Schering-Plough Animal Health) as part of investigations into potential vaccine failure (n=9) (Appendix 6.1.3).

Based on the information provided in the submission form, 70.9% (56/79) of submitted samples were positive on rapid CPV antigen detection kit, 13.9% (11/79) were negative and 15.2% (12/79) were either not tested or the section pertaining to rapid CPV antigen detection kit on the questionnaire was left blank.

2.3.3. CPV-2 PCR RESULTS

Of the 79 samples received as part of the survey, 70 (88.6%) tested positive for the presence of CPV-2 DNA (Appendix 6.1.4). One sample (CPV044) produced only a weak band, which was not investigated further as the amount of product was insufficient for subtyping. Because the weak PCR band was not confirmed as CPV-2, this sample was regarded as negative for the purpose of the analysis. Of the 9 historic samples 7 tested positive for presence of CPV-2 DNA (Appendix 6.1.4).

Of the 11 samples that were negative on CPV antigen detection kit, 10 (90.9%) were positive for CPV-2 DNA by PCR. In addition, six of the 56 samples (10.7%) which were positive on rapid CPV antigen detection kit were negative for CPV-2 DNA by PCR.

2.3.4. CPV-2 POSITIVE SURVEY SAMPLES

The highest proportion (34/70) of the CPV-2 PCR positive samples were collected from cross breed dogs (48.6%) (Figure 5). Among the samples received from pure breed dogs, the highest numbers came from Huntaways (4/37) and Rottweilers (4/37) (Figure 6). Heading dogs and Pig dogs were also over represented. These types of dogs are generally not considered to be any specific breed type, but often include Staffordshire terrier and Border

collie mixes. The samples which originated from dogs that were six months of age and younger made up 74.3% (52/70) of the total number of samples which tested positive for the presence of CPV-2 DNA by PCR. The distribution of the ages of dogs is presented in Figure 7.

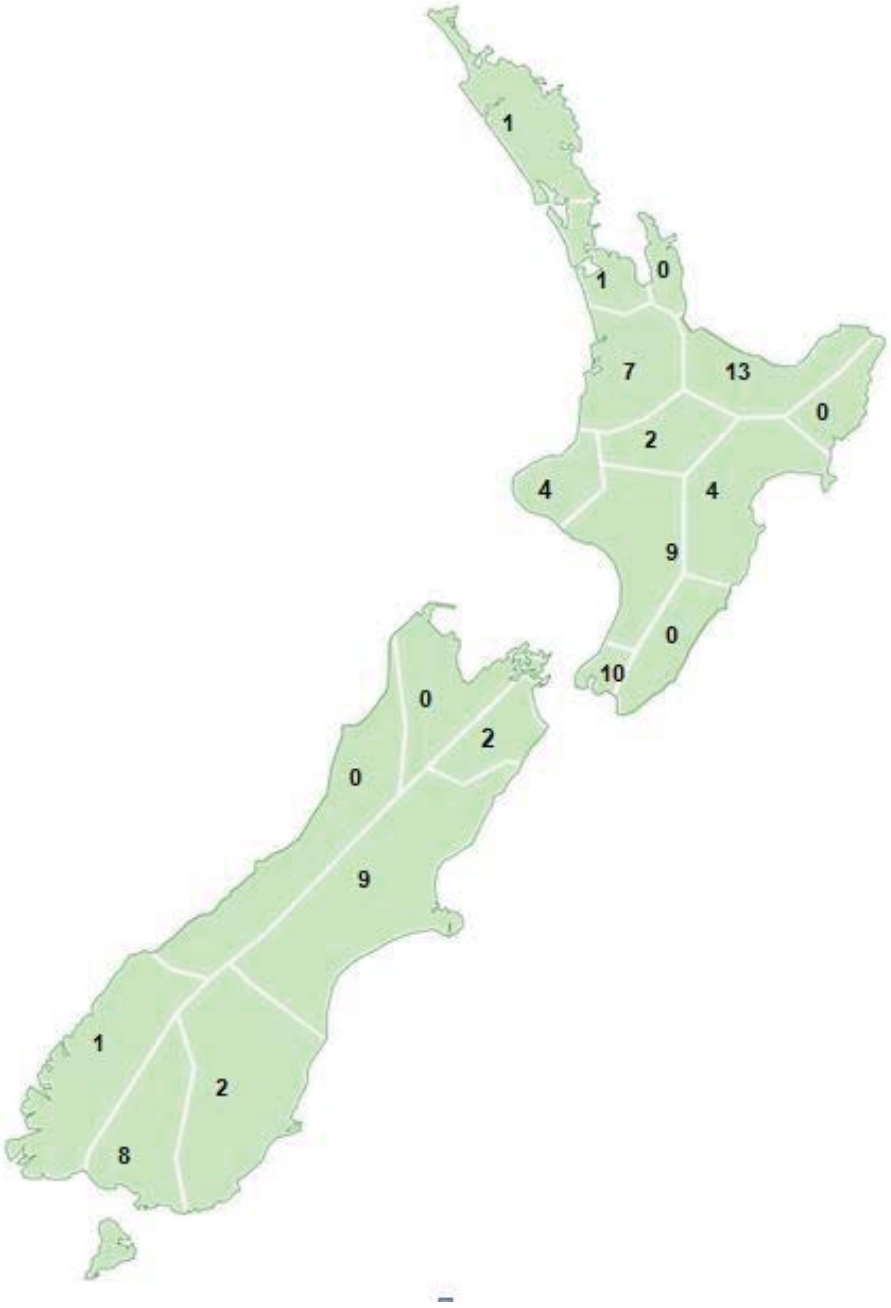

Figure 4 Geographic distribution of locations at which faecal samples (n=79) were collected as part of the survey. The submissions received came from all over New Zealand, including 51 samples from the North Island and 22 samples from the South Island. For 6 samples the location of origin was unknown.

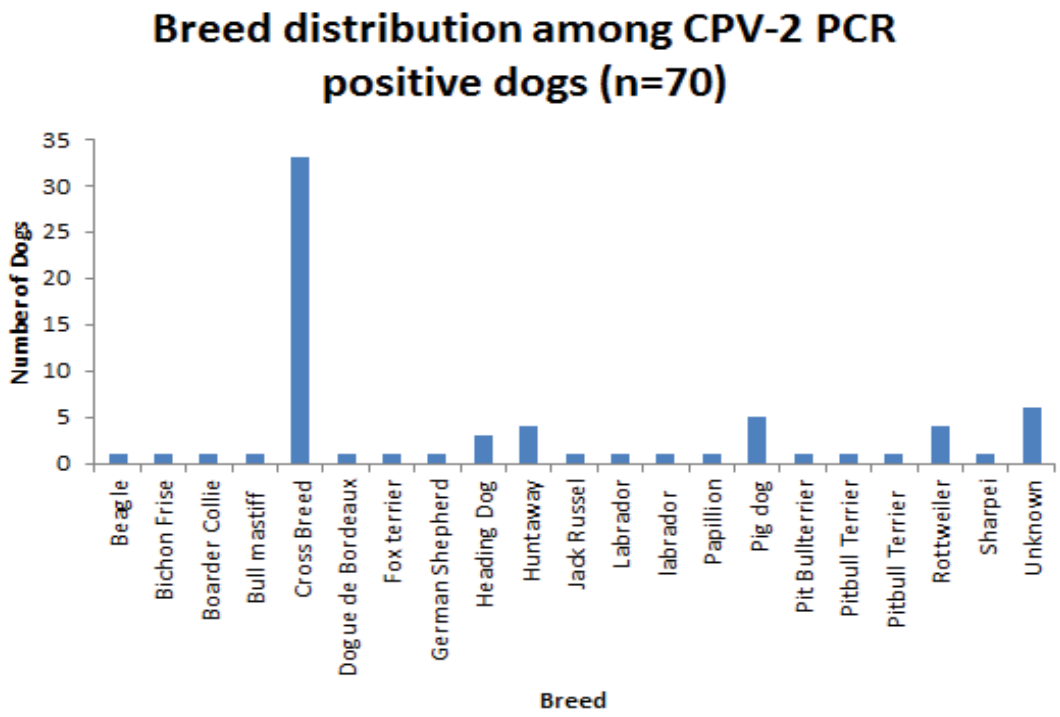

Figure 5 Distribution of breeds among CPV-2 positive dogs.

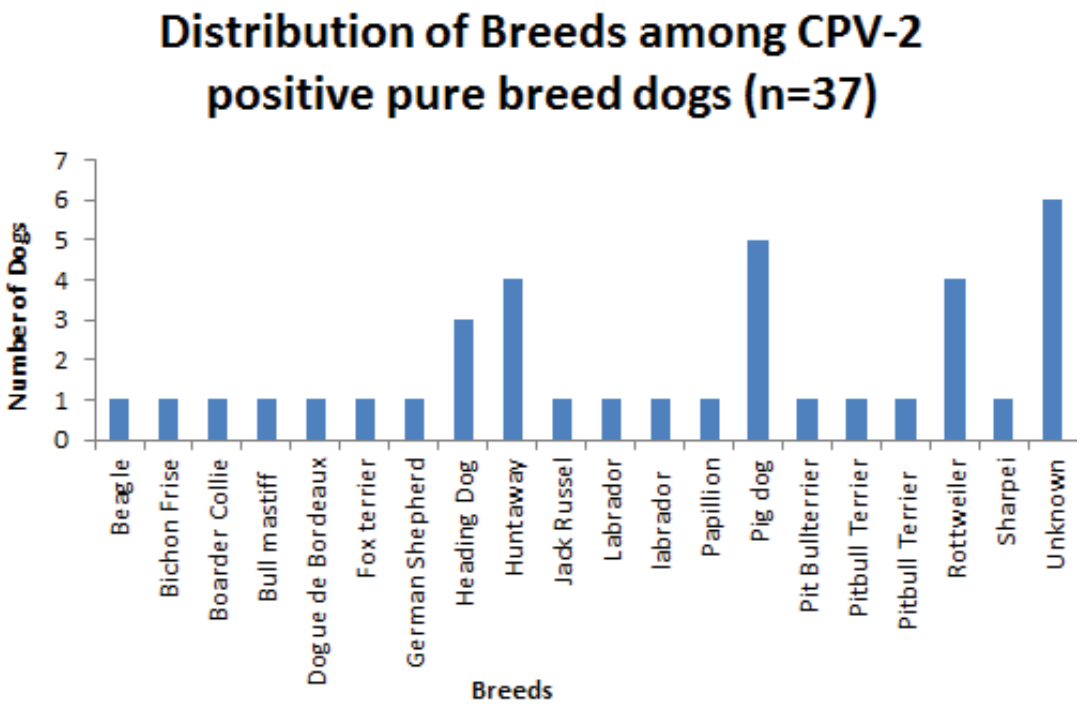

Figure 6 Distribution of breeds among CPV-2 positive purebred dogs. Heading dogs and Pit bull dogs are included as dogs bred for specific traits, although they are not breeds officially recognised by the New Zealand Kennel Club.

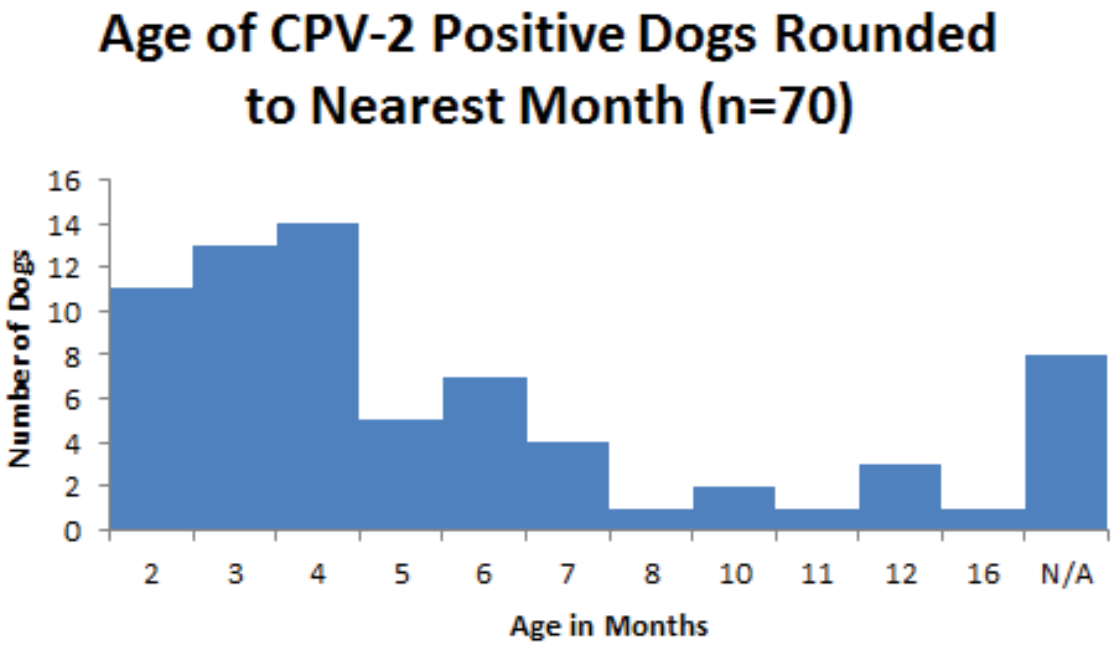

Figure 7 Age of CPV-2 positive dogs (n=70) in months. This graph shows a left skew indicating that the majority of samples in this survey originated from dogs under the age of 6 months old. N/A indicates samples for which age was unknown (n=8).

2.3.5. SUBTYPING OF CPV-2

Canine parvovirus DNA was detected in 70 (88.6 %) of the 79 faecal samples. Of the CPV-2 positive survey samples that were submitted between 2009 and 2010, 69 (98.6%) were subtyped as CPV-2a. A single sample was subtyped as CPV-2 (Table 6). Of the three vaccines which were sequenced were subtyped one was CPV-2b (Protech C3) and the remaining two were CPV-2 (Nobivac and Vangard Plus 5). Among the historic samples four were subtyped as CPV-2 (CPV015 [2006], CPV016 [2006], MAF.WV.3 [1986], MAF.WV.4 [1980]) and 3 were subtyped as CPV-2a (CPV017 [2008], MAF.WV.1 [2009], MAF.WV.2 [1990]).

Subtype	Survey (2009-2010)	Historic	Vaccine	TOTALS
CPV-2	1	4	2	7
CPV-2a	69	3	0	72
CPV-2b	0	0	1	1
CPV-2c	0	0	0	0
TOTALS	70	7	3	80

Table 5 CPV-2 subtypes based on sequencing results of the CPV-2 PCR products. The samples included those collected from various New Zealand regions as part of a prospective survey conducted between November 2009 and December 2010, historic New Zealand CPV-2 isolates, and commercial vaccines.

2.3.6. VACCINATION HISTORY

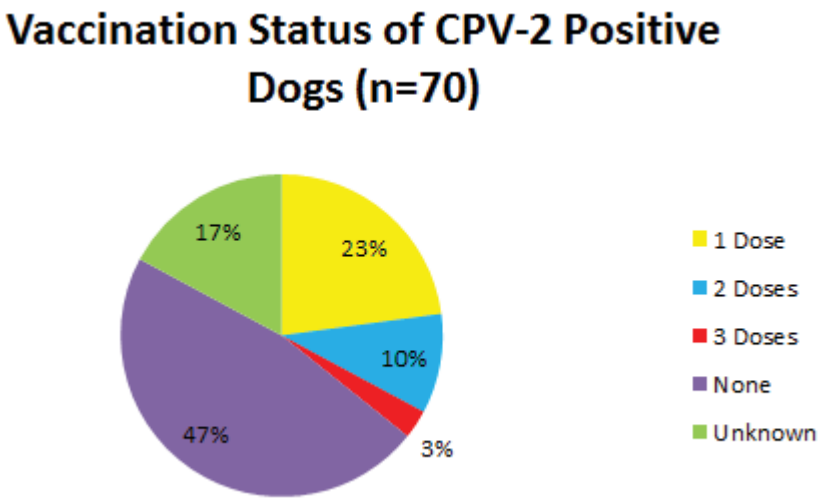

Figure 8 Vaccination status' of dogs positive for CPV-2 DNA, as reported by the submitting veterinarian.

Of the 70 samples that were positive for CPV-2 by PCR, 33 (47.1%) came from dogs with no vaccination history, 25 (35.7%) came from dogs that had been vaccinated at least once, and the remaining 12 (17.1%) samples came from dogs with an unknown vaccination history (Figure 8 and Appendix 6.1.4). The records provided by veterinarians showed that only two of

the 25 dogs with vaccination history had three doses administered; seven dogs were reported to have had two vaccination doses, the remaining 16 dogs had one vaccination dose.

Of the 16 dogs which had one dose of vaccination, five were over 10 weeks old at the time of vaccination. One of these 16 dogs (CPV044) presented with clinical signs of CPV-2 enteritis one week after vaccination. The PCR results from this dog were considered negative for the presence of CPV-2 DNA as the band observed in the electrophoresis gel was weak and did not yield sufficient DNA for sequencing purposes. Another dog of an unknown age (CPV037) had been vaccinated one day before it presented to the clinic with clinical signs of gastroenteritis; this sample was negative for the presence of CPV-2 DNA by PCR, although it was reported to be positive when tested by the rapid CPV antigen detection kit. The second dose of vaccination for puppies that were vaccinated twice was given at the age of 10 to 12 weeks.

2.4. DISCUSSION

2.4.1. SUBTYPES AND DISTRIBUTION

This survey was the first of its kind to be carried out in New Zealand. Samples were submitted from all over New Zealand with only five regions failing to submit any samples (Figure 4).The majority (64.6%) of samples collected in this survey originated from the North Island. This is most likely due to the higher density of people (and therefore, presumably pet-dogs) found in the North Island (Anonymus 2013b), as opposed to any potential higher risk of CPV-2 infection for dogs in the North Island compared with the South Island. However, warmer average annual temperatures and more annual sunlight hours (NIWA 2001) may allow for more favourable conditions for exercising dogs in public places, thereby possibly increasing the risk of exposure to infectious CPV-2, leading to possible disease. Other authors have also described higher admission rates for CPV-2 enteritis in warmer months (Houston *et al.* 1996).

Of the 79 survey samples received, 70 tested positive for the presence of CPV-2 DNA. One sample (CPV044) did produce a weak band, which was not confirmed by sequencing. As such, this sample was regarded as negative based on criteria used in the current study. The sample originated from a 6-week-old puppy which had been vaccinated once at five weeks of age. Considering the timing of sample collection with respect to vaccination, it is possible that the weak band detected on the electrophoresis gel represented the vaccine strain of CPV-2. It is also possible that the puppy was

exposed to the field CPV-2 and was able to control the replication of the virus (and therefore faecal shedding) effectively due to either appropriate levels of maternal antibodies or development of active immunity following recent vaccination. Finally, it is also possible that the band represented non-specific PCR product. As we were unable to confidently interpret CPV-2 PCR result for this puppy, and no other diagnostic test results were available to us, it was not possible to assess the putative CPV-2 contribution to the clinical disease in this puppy.

All samples collected during the survey part of this study were of subtype CPV-2a, except one which was subtyped as CPV-2. This was similar to results described in a recent Australian-based survey (Meers 2007). The one sample which contained CPV-2 originated from an 11.5 week old puppy, which had been vaccinated three weeks prior to the onset of clinical signs of severe gastroenteritis. The sequence of CPV-2 obtained from that puppy was over 97% identical to the sequence of a vaccine strain of CPV-2 over two fragments 454 and 786 nt in length. The only differences between the vaccine strain and the virus obtained from the puppy were at poorly resolved sequence peaks (data not shown). As such, the CPV-2 obtained from this puppy most likely represented a vaccine strain of CPV-2. It remains unresolved whether or not the clinical disease observed in the puppy was induced by the vaccine strain of CPV-2. Reversion to virulence among vaccine strains of CPV-2 is rare (Decaro *et al.* 2007). In addition, the puppy was positive for *Campylobacter*. Hence it is possible that the clinical signs seen in this dog were due to bacterial infection. Alternatively, the vaccine strain of CPV-2 could have contributed to disease in the puppy whose immune responses might have been compromised due to co-infection with other pathogens.

Four historic sequences were subtyped as CPV-2. Two of these originated from faecal samples collected from two puppies from the same litter in 2006, and the remaining two represented archival virus isolates from 1980 and 1986. No other information was available for any of these samples, including the age of dogs or their vaccination status. Thus, it is difficult to hypothesise on the likely origin of these viral sequences. However, considering the timing of sample collection, it is likely that the archival isolate from 1980 represents a field virus circulating in New at the time, while the remaining three sequences are more likely to have been derived from the vaccine strains of the virus than from the viruses circulating in the field.

The dominance of CPV-2a may indicate that only CPV-2a is in active circulation in New Zealand or may indicate that this is the only virulent CPV-2 subtype in New Zealand. This study is not able to exclude the presence of other CPV-2 subtypes because only dogs with clinical signs were sampled; other subtypes may be present in New Zealand dogs sub-clinically. The dominance of

CPV-2a may also be driven by the fact that CPV-2 arrived in New Zealand early in the viruses' history and that new strains have not arrived since (See Chapter 3). However, subtypes of CPV-2 have emerged spontaneously globally. The reasons for the apparent genetic stability of New Zealand CPV-2 remain undetermined. These may include geographical separation of New Zealand and strict quarantine protocols preventing introduction of newer CPV-2 subtypes. Alternatively, the stability of New Zealand parvoviruses may be linked to the absence of wild carnivores in this country. It has been proposed that circulation of CPV-2 or CPV-2 like viruses in wildlife may have contributed to evolution of the virus overseas (Allison *et al.* 2012).

2.4.2. CPV-2 RISK FACTORS

The results of this survey suggested that unvaccinated dogs were more likely to develop CPV-2 associated gastroenteritis, as 47% (33/70) of the CPV-2 PCR positive samples originated from dogs in this category. It is likely that the percentage of unvaccinated dogs was in fact higher, as some of the 17% (12/70) of the CPV-2 positive dogs with an unknown vaccination history may also have not been vaccinated. Dogs which received only one vaccination made up 23% (16/70) of the CPV-2 positive samples. Of the 16 dogs with only a single vaccination, five had their vaccinations administered after the age of 10 weeks. Advances in vaccination technology, and the behavioural need for early socialisation in puppies, has led to some vaccination products being licenced for a course of two vaccinations where the final vaccination is administered at 10 weeks of age. However, vaccination guidelines still recommend when possible a third vaccination be given at between 14-16 weeks of age (Day *et al.* 2010). Only two dogs in the survey received three vaccinations. In both cases the final vaccination was administered at 12 weeks of age, two weeks earlier than what is recommended in the WSAVA vaccination guidelines (Day *et al.* 2010); but within the manufacturers guidelines.

In this survey 74.3% (52/70) of the samples positive for CPV-2 DNA by PCR originated from dogs six months of age and younger. This finding correlates with the findings of other studies carried out around the world which have shown that dogs in this age group are at the highest risk of CPV-2 associated disease (Horner 1983; Studdert *et al.* 1983).

While higher numbers of CPV-2 positive samples were obtained from Rottweiler's and Huntaways than from other breeds, the number of samples collected in this survey would not lend enough power to statistical analysis to draw any conclusions regarding the risk of clinical

disease as a result of CPV-2 infection in these specific breeds. However, results of the previous studies have shown that Rottweilers are at an increased risk of CPV-2 enteritis (Glickman *et al.* 1985; Houston *et al.* 1996)

2.4.3. Rapid CPV Antigen Detection Kits and PCR

While originally it was requested that samples from dogs which tested positive on rapid CPV antigen detection kits be submitted for CPV-2 PCR testing, a number of samples (n=11) which were negative on these in-house tests were also received. Of these, 10 returned a positive result by CPV-2 PCR. Results of other studies have shown that conventional PCR is highly sensitive for the purposes of detecting CPV-2 DNA (Desario *et al.* 2005). In the current study, the identity of the PCR products was confirmed by sequencing thus, it is unlikely that the positive PCR results represented false positives. Instead, it is more likely that the negative results obtained from the rapid CPV-2 antigen detection kit were false negatives. While rapid CPV antigen detection kits have value as a fast diagnostic tool in a clinical setting the sensitivity of these kits has been shown to be poor in comparison to PCR (Schmitz *et al.* 2009). The principle of the test relies on the detection of the CPV-2 antigen in the sample, if the dog has already mounted and immune response to infection the antigen may be coated in antibody, this can cause the test to give a negative result despite the dog being infected with CPV-2.

In addition, six samples positive for CPV-2 using a rapid CPV antigen detection kit were negative by CPV-2 PCR. In five of these cases 'classical' risk factors for CPV-2 related disease were cited in the notes section of the submission form; the sixth case came with very little information (CPV085). Two cases (CPV086 and CPV042) were litters of 5-week-old, recently weaned puppies that were born to unvaccinated bitches. Another case (CPV063) was a 3-month-old, unvaccinated, Rottweiler puppy, which had spent time in the pound on the weekend before the onset of clinical signs of gastroenteritis. It is likely in these cases CPV-2 infection was the cause of disease as these animals were in a high risk category for CPV-2 infection, due to their age (Horner 1983; Studdert *et al.* 1983) and unvaccinated status (Ling *et al.* 2012). The fourth case (CPV011) was a French bulldog which had been vaccinated at 6 and 11 weeks of age. In this case there was a significant delay (three weeks) between the rapid CPV antigen detection kit testing and the arrival of the sample which was submitted to our survey. Thus, it is possible that faecal sample for PCR testing was collected too late in the disease progression, after the puppy has cleared the virus. The final case (CPV037) involved a Rottweiler of unknown age; this dog had its first vaccination one day before the onset of clinical signs. It is unlikely that the positive result of the rapid CPV antigen

detection kit was due to recent vaccination, as research suggests that the levels of CPV-2 antigen present in faeces after vaccination are not sufficient to cause a positive result in these kits (Larson *et al.* 2007; Day *et al.* 2010). It is also unlikely that the clinical signs in this case were caused by the vaccine strain of the virus as the time between vaccination and the onset of clinical signs was shorter than the typical incubation period in CPV-2 infection (Meunier *et al.* 1981; Pollock 1982).

Shedding of viral particles has been found to be variable in CPV-2 infection (Carmichael *et al.* 1980; Azetaka *et al.* 1981; Carman and Povey 1982), therefore it is possible that in all of the mentioned cases samples for CPV-2 PCR were collected during a time when the virus was not being shed, while samples for in-house rapid CPV-2 antigen testing were collected during the times of shedding. It is also possible that rapid CPV-antigen test results represented false-negatives. Finally, the negative PCR result may have been due to the presence of PCR inhibitors, which is a common problem for DNA extraction from faecal samples (Rådström *et al.* 2004). The amplification of the house-keeping gene would have allowed assessment of this possibility, but was not performed as part of the study.

2.5. CONCLUSIONS

The results of this survey have shown that CPV-2 was circulating among New Zealand dogs. All, but one, CPV-2 PCR positive samples were subtyped as CPV-2a. The lack of detection of CPV-2b and CPV-2c in this survey does not rule out the presence of these subtypes in New Zealand, as the survey focused on clinically sick animals. There is a possibility that other CPV-2 subtypes could be circulating at low levels sub-clinically. A further survey focusing on, or including, clinically normal dogs would be useful to estimate the frequency and subtypes of CPV-2 that may be circulating sub-clinically in New Zealand. In agreement with results of other studies (Horner 1983; Studdert *et al.* 1983), unvaccinated dogs aged 6 months and under appeared to be at the highest risk of CPV-2 associated disease.

3. PHYLOGENETIC ANALYSIS OF CANINE PARVOVIRUS TYPE 2 IN NEW ZEALAND AND COMPARISON WITH WORLDWIDE SEQUENCES.

3.1. INTRODUCTION

Canine parvovirus type 2 (CPV-2) is a non-enveloped, single stranded DNA virus approximately 26 nm in size and is a member of the family *Parvoviridae*. Despite being a DNA virus, CPV-2 was shown to have a high mutation rate, which approaches rates more commonly found among RNA viruses (Parrish *et al.* 1991; Truyen *et al.* 1995; Horiuchi *et al.* 1998). The observed high mutation rate may also be due to the virus still being in the process of adapting to its new host. It has been suggested that the single stranded nature of this virus may account for its high mutation rate (Lindahl and Nyberg 1974).

Racoons have been found to have played a key role in the evolution of CPV-2 to CPV-2a (Allison *et al.* 2012). Furthermore, racoons may also have played a role in the evolution of the original CPV-2. Further investigations of other wild carnivore species may reveal more potential intermediate hosts.

For the purposes of this project, sequences of the CPV-2 VP2 genes were analysed. The genome of CPV-2 comprises 5232 nucleotides, of which 1755 code for the VP2 gene (Hirayama *et al.* 2005; Decaro *et al.* 2008c). The protein expressed by the VP2 gene comprises a structural component of the virus particle. It contains all the major antigenic typing sites, and has important roles in host range determination (Allison *et al.* 2012). It is therefore expected to be under a high degree of selection pressure in this relatively newly emerged virus of dogs. The current typing methods rely on changes in the antigenic sites in VP2. Thus, changes in other parts of the gene are typically not considered. All contemporary New Zealand CPV-2 samples examined as part of the current study (Chapter 2) were subtyped as CPV-2a. To further examine evolution of CPV-2 in New Zealand, phylogenetic analysis of VP-2 gene sequence was carried out.

Unvaccinated dogs between the ages of six weeks to six months have been found to be at the highest risk of developing CPV-2 related disease (Horner 1983; Studdert *et al.* 1983; Ling *et al.* 2012). Even puppies vaccinated at the recommended schedule may succumb to CPV-2 disease. This can occur if a high dose of virus is encountered, when the level of CPV-2 specific maternal antibody is no longer protective. Because maternal antibody may interfere with the development of active immunity to the virus even at levels that are no longer protective against CPV-2 infection, it

has been recommended that puppies receive their final vaccination at 14 - 16 weeks of age (Carmichael 1999; Schultz 1999; Schultz 2006). If there are differences in the viruses which infect vaccinated versus unvaccinated dogs, clustering in haplotype networks to reflect this would be expected.

As New Zealand is geographically isolated from the rest of the world, we are able to more easily prevent the entry of many animal borne diseases. A prime example of this is the absence of rabies in New Zealand (Rupprecht and Shlim 2013), which is due to strict quarantine measures that are in place at the boarders. However these quarantine measures failed to prevent the initial introduction of CPV-2 to New Zealand. As a survey of this sort has not previously been carried out in New Zealand, it is unclear if CPV-2 entered New Zealand on a single occasion, or if the virus was, and is being imported regularly. Both scenarios are possible due to the highly contagious and stable nature of this virus. If CPV-2 was introduced on a single occasion, geographical clustering of samples confined to one area of a haplotype network would be expected. However, if CPV-2 was introduced on multiple occasions, geographical scattering throughout a haplotype network, and possibly close relationships between New Zealand CPV-2 sequences and those obtained from cases in other parts of the world would be expected.

New Zealand is an island nation divided into two regions separated by the 24 km wide Cook Strait. One might assume then that the physical barrier imposed by the Cook Strait may impair the movement of CPV-2 between the South and North islands. For CPV-2 to move from one island to the other, a contaminated fomite or infected dog must be shipped or flown between the islands. Subsequently, the virus needs to come into contact with a susceptible individual and successfully establish infection in a new host. The chances of this occurrence appear more remote than the movement of a virus over the same comparable distance across one land mass. Thus, investigation of whether the spread and evolution of CPV-2 in New Zealand occurred independently within the South and North Island dog populations was carried out.

3.2. METHODS

3.2.1. SOURCES OF CPV-2 SEQUENCES

A description of samples obtained from New Zealand, including the signalment, vaccination history and geographical location are presented in Chapter 2 and in Appendix 6.1.4. The samples were processed as described in section 2.2. A total of 81 CPV-2 sequences were considered for phylogenetic investigation. These comprised 70 of the sequences generated from samples collected during the survey period (November 2009 – December 2010) as described in Chapter 2, the sequences obtained from the three vaccines (also as described in Chapter 2), the 7 positive historic samples and a single sample originating from a cat (CPV0067).

The four sequencing products described in section 2.2.4 were assembled to reconstruct a single contiguous sequence. Sequence data were manually curated in Geneious v 5.6.5 using chromatograms to resolve ambiguous base calls and base calling errors.

Only 27 of the 81 CPV-2 sequences were assembled into a single contiguous sequence. In each of the remaining 54 CPV-2 sequences, the sequencing products failed to assemble into a single contiguous sequence, with a missing sequence in the region between primers JS4R and JS3F (see primer placement in Figure 9). These fragmented sequences were aligned to a reference sequence (Accession: M38245.1), manually curated and concatenated to form shortened contiguous sequences. Concatenated products (n=10) that were considered to be too short for the purpose of the analysis were discarded. These included seven sequences from the surveyed dogs (CPV002, CPV057, CPV058, CPV072, CPV073, CPV078, CPV086, CPV087, CPV088) and one historic CPV-2 isolate (CPV014).

All remaining 71 sequences and the reference sequence were then trimmed as required to produce a data set containing 72 contiguous sequences of equal length (1218 nucleotides). These 72 shortened sequences were used to investigate the genetic structure of CPV-2 within New Zealand.

Additional CPV-2 VP2 sequences (n=95) originating from various countries (including one New Zealand sequence) were obtained from the National Centre for Biotechnology Information (NCBI) database (Appendix 6.1.5). These sequences were required to be at least 5 Kb in length and submitted with information about the country, year of origin, and author's subtype designation. Sequences from the NCBI database that originated from vaccines, had been reported to have been passaged in cell lines or those originating from felines were excluded.

The selection of 27 samples originating from New Zealand which produced a full length contiguous sequence where aligned with those obtained from the NCBI database, and with the reference sequence. The resulting dataset of 123 CPV-2 sequences (Appendix 6.1.5) was used to assess the New Zealand virus samples in the context of the worldwide radiation of CPV-2. All alignments and editing was carried out in Geneious v 5.6.5., and exported in nexus file format for downstream analysis.

Number of Sequences in International Networks.

Country Of Origin	Number of Samples
New Zealand	26
Argentina	11
Brazil	2
China	14
Germany	3
Greece	2
Italy	15
Nigeria	6
South Korea	6
South Africa	20
Uruguay	1
USA	14
Vaccines	2
Reference Sequence	1
TOTAL	**123**

Table 6 Number of sequences included in the international networks and categorised by country of origin.

3.2.2. HAPLOTYPE NETWORK ANALYSIS

The population structure of CPV-2 was analysed in Network 4.6.1.0 (Bandelt *et al.* 1999) and PopART version 1 (Leigh 2013) using default parameters to produce median joining haplotype networks.

The population analysis carried out in Network focused on comparisons based on the location, age and vaccination status of the CPV-2 infected dogs in New Zealand. The analysis carried out in PopART compared the New Zealand population with the rest of the world. This network focused on comparisons between the subtype of the sample, year of isolation and origin of the sample by continent.

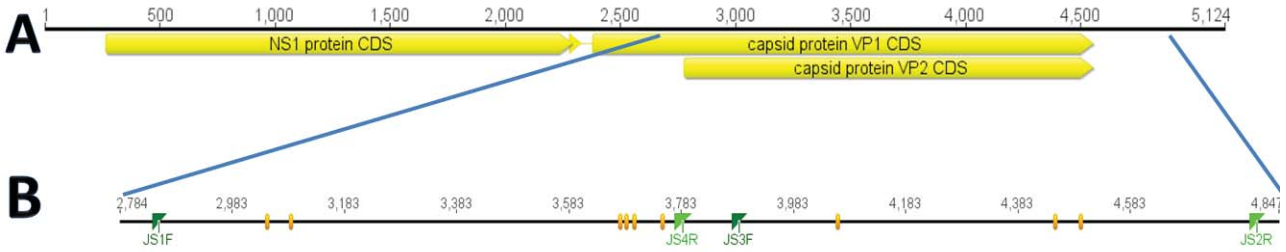

Figure 9 A) CPV-2 genome; yellow bars indicate coding regions for CPV-2 viral proteins (VP), with number at the top indicating nucleotide positions. B) Coding sequence of the VP2 gene and partial coding sequence of the VP1 gene which was investigated in this study. Green triangles indicate primer binding sites JS1F, JS2R, JS3F and JS4R. Light green triangles indicate reverse primers and dark green triangles indicate forward primers, the orange ovals are the specific sites that were used for the sub-typing of the viral sequences in this study. Images produced in Geneious (Drummond *et al.* 2012) with parts manually assembled.

3.3. RESULTS

3.3.1. GENETIC STRUCTURE OF CPV IN NEW ZEALAND

The haplotype networks produced illustrate the genetic structure of CPV-2 population in New Zealand (Figure 10), based on the analysis of samples collected as part of the survey described in Chapter 2. The nodes represent individual haplotypes; these nodes were sized in proportion to the number of individuals that share a particular haplotype. Nodes are separated by branches which are numbered; the numbers indicate the number of mutational steps between two haplotypes. Reticulation in a haplotype network indicates that the data did not contain appropriate signal to resolve the true pathway through a network, indicating there is more than one possible sequence of mutations to reach a particular haplotype.

The network produced when considering CPV-2 sequences from New Zealand formed two major groups, which were separated by 60 mutational steps. Samples collected during the survey period (November 2009 – December 2010) formed a larger cluster containing the samples collected from dogs displaying clinical signs of CPV-2 infection. A small, relatively distantly related cluster of samples was made up of sequences obtained from vaccines, historical sequences and one sequence from a cat, which was further separated from the small cluster. Assessment of the New Zeeland sequences based on location (Figure 10 A), vaccination status (Figure 10 B) and age (Figure 10 C), revealed no population genetic structure.

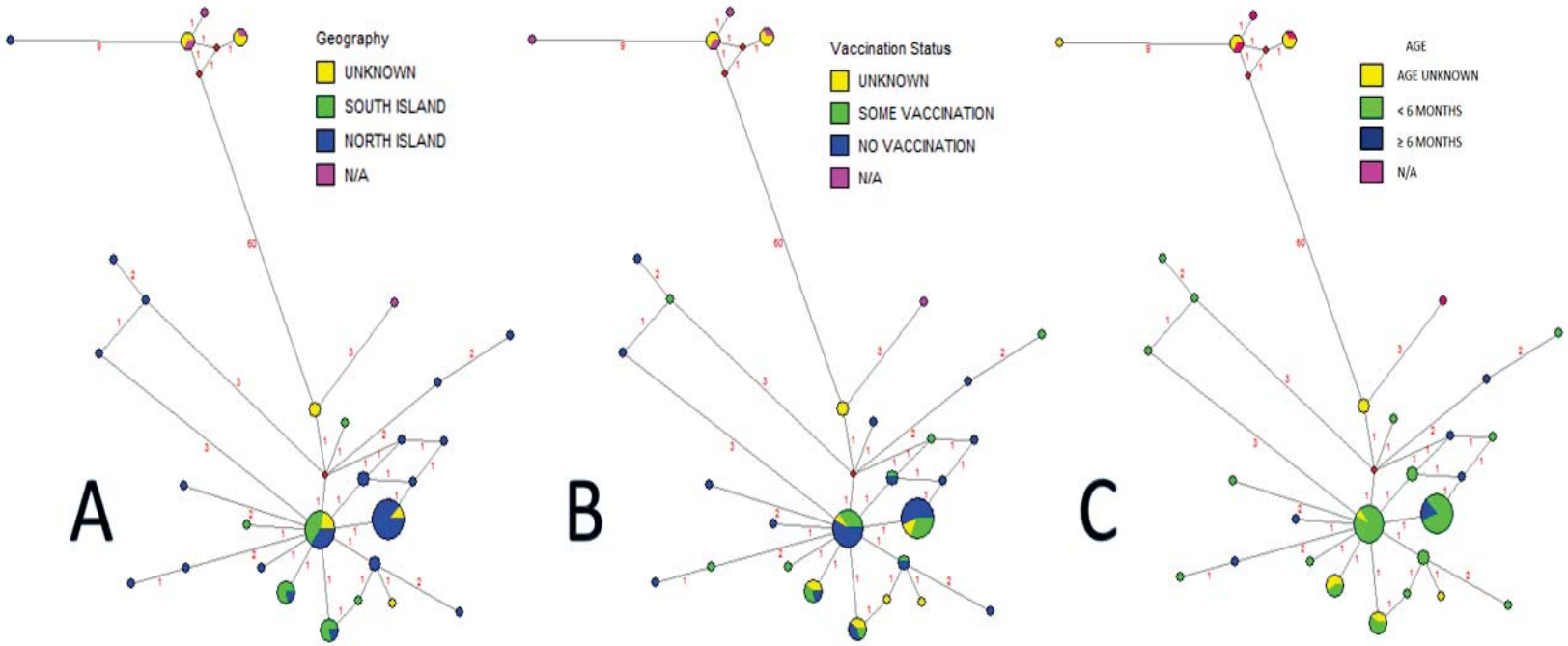

Figure 10 Networks displaying CPV-2 sequences from samples originated in New Zealand (n=71) and a reference sequence (M38245.1). The numbers on the branches indicate the number of mutational steps, the small red squares represent evolutionary intermediate which must have occurred between nodes but which were not represented in the sample. Nodes are sized proportional to the number of samples contained within them. A) Samples are coloured by their origin. Unknown samples are those received from unknown locations B) Samples are coloured by the vaccination status of the dog. Samples categorised as having some vaccination indicates the dog received at least one vaccination at some time. Unknown samples are those with an unknown vaccination status C) Samples are coloured by the age of the dog from which the sample originated. Unknown samples are those originating from dogs of an unknown age.
N/A - For all diagrams samples designated N/A indicates the sequences originating from a vaccine or the Reference sequence.
Figures produced in PopART (Leigh 2013)

3.3.2. WORLDWIDE CONTEXT OF NEW ZEALAND CANINE PARVOVIRUS

The haplotype networks produced illustrate the genetic structure of the CPV-2 population in New Zealand and provide a representative view of the genetic structure of CPV-2 throughout the world. The sequences of CPV-2 from the rest of the world were obtained through the collection of sequences from the NCBI sequence database. The nodes represent individual haplotypes; these nodes are sized in proportion to the number of individuals that share a particular haplotype. Nodes are separated by branches; the branches in the international network have hatch marks to indicate the number of mutational steps between each of the haplotypes. Reticulation in a haplotype network indicates that the data do not contain appropriate signal to resolve the true pathway through a network, meaning there is more than one possible sequence of mutations to reach a particular haplotype.

In the networks of world-wide CPV-2 (Figures 11, 12 and 13), the small black circles represent inferred un-sampled haplotypes. These networks showed little distinct clustering. The network contained sequences from all currently know subtypes of CPV-2 (CPV-2, CPV-2a, CPV-2b and CPV-2c). All the sequences included in the network appeared to be closely related despite the geographical distances separating them. In the alignments produced to create the networks, pairwise identity values were around 99.5% and overall 91.3% of sites were identical.

In the investigation of the evolutionary relationships of the sequences based on their country of origin (Figure 11), it was found that different subtypes were present within several countries. Furthermore, sequences of similar geographic origin were often scattered throughout the network. Often sequences originating from a geographically similar area were more closely related to sequences from a different country than to each other. The exception to this however were the samples obtained from New Zealand. The New Zealand samples clustered almost exclusively together, with the exception of two archival samples (MAF2 *(year 1990)* and MAF3 *(year 1986)*).

Investigation of the structure of the international sequence set revealed that subtypes of the same designation were not necessarily most closely related (Figure 12). This could be seen specifically with relation to CPV-2c sequences, which were not directly connected to each other in the haplotype network. The subtype CPV-2b also formed two distinct clusters which couldn't be directly connected to each other.

When the haplotype network was coloured by the decade from which the sequence was collected (Figure 13), there was little indication that the sequences collected during the same decade were

more closely related to each other than to sequences collected during different decades. There were some well-structured regions (e.g. the NZ samples) which were predominantly comprised of recent samples. A loose grouping of the oldest sequences could be seen in the upper central region of the network. Considerable reticulation was associated with those samples.

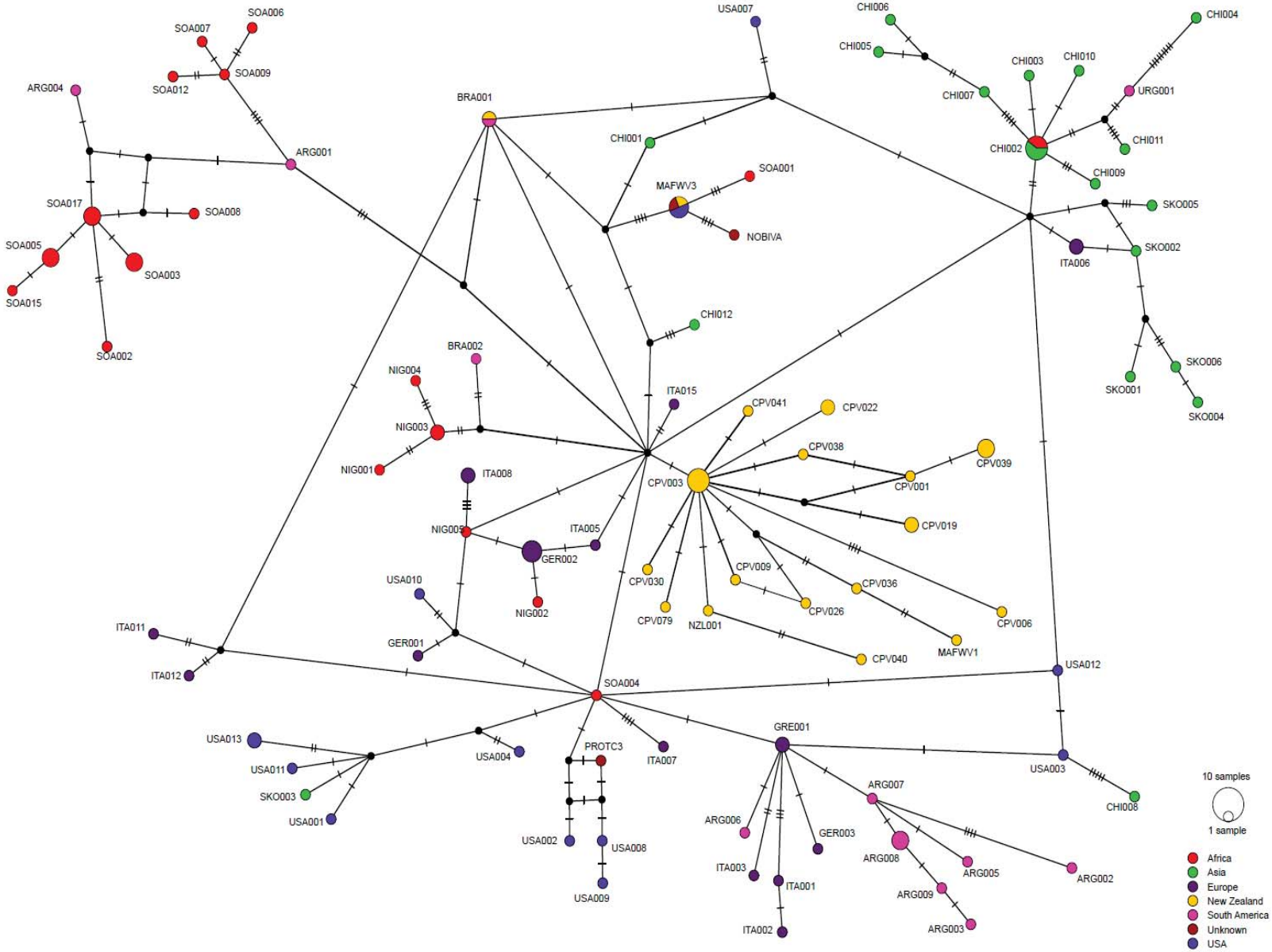

Figure 11 Haplotype Network of CPV-2 sequences obtained from NCBI and those obtained from the CPV-2 survey in New Zealand coloured by continent of origin. Figures produced in PopART(Leigh 2013)

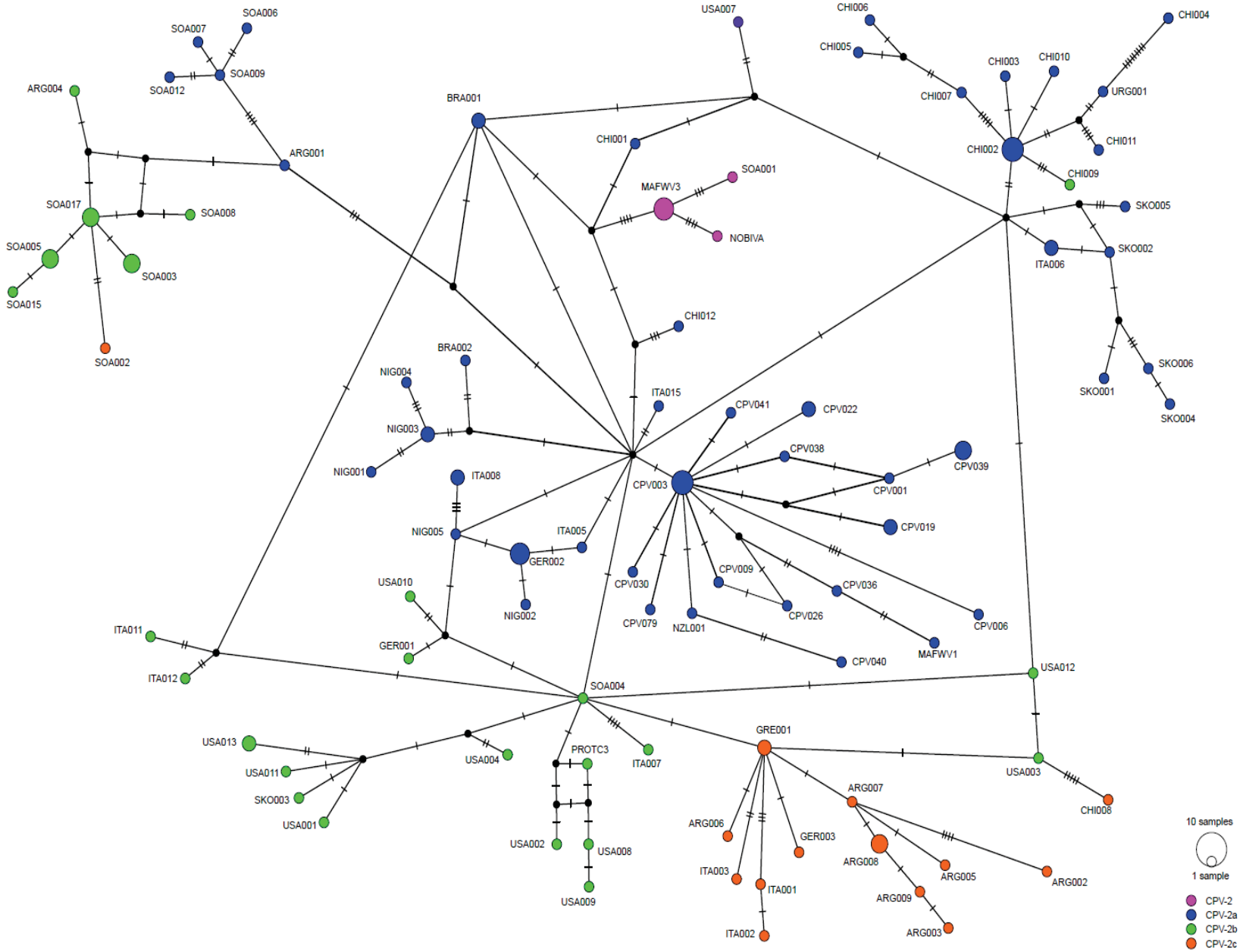

Figure 12 Haplotype Network of CPV-2 sequences obtained from NCBI and those obtained from the CPV-2 survey in New Zealand coloured by subtype. Figures produced in PopART(Leigh 2013)

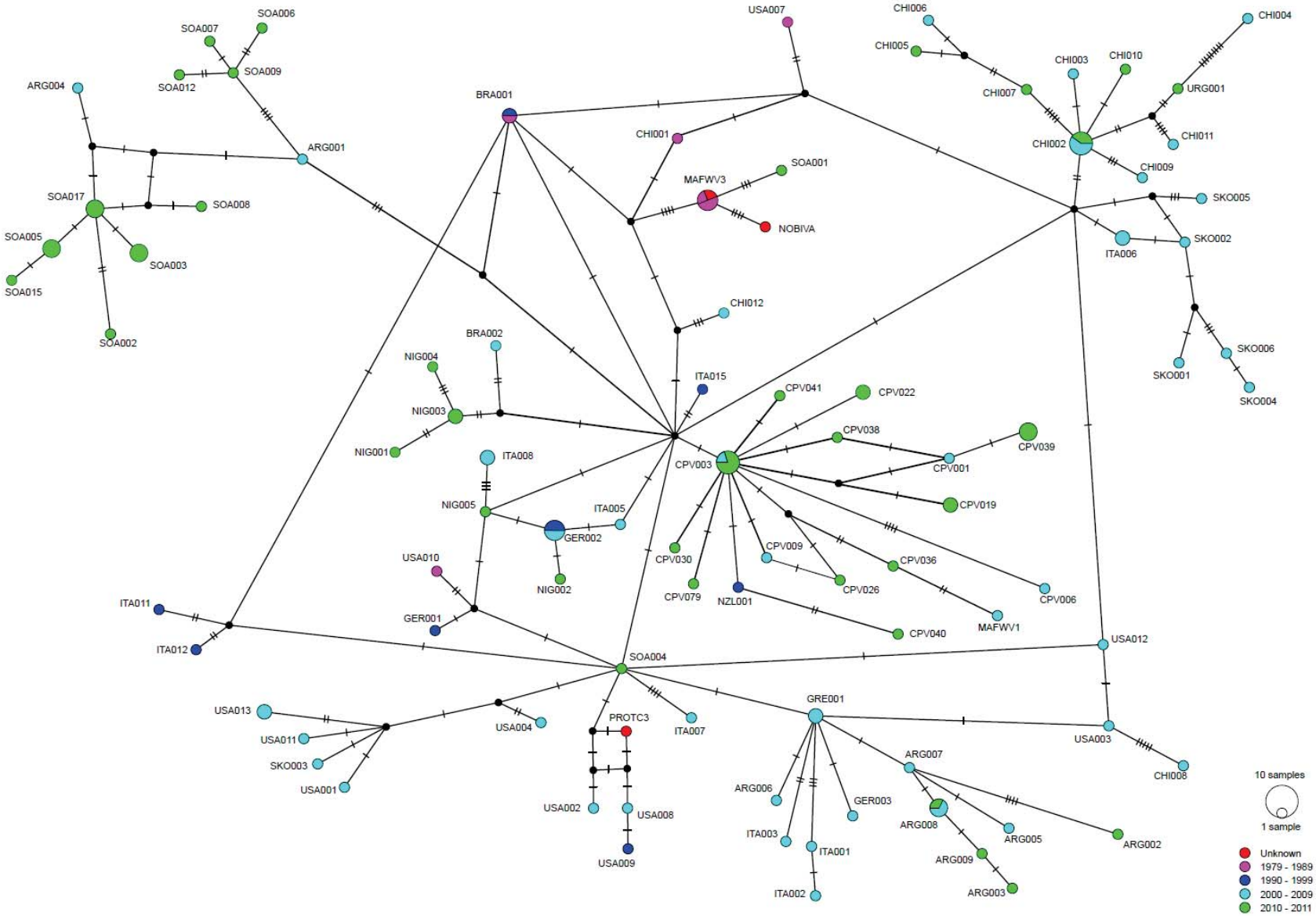

Figure 13 Haplotype Network of CPV-2 sequences obtained from NCBI and those obtained from the CPV-2 survey in New Zealand coloured by decade. Figures produced in PopART (Leigh 2013)

3.4. DISCUSSION

Traditionally neutral markers, which are not under any known selection pressure, are used for phylogenetic analyses. When using a neutral gene which is not under any known selection pressure inferences can be drawn with regard to differences found within a population or between populations. The major focuses of studies investigating the VP2 gene investigate its antigenic properties. The gene selected for this analysis has been found to be under selection pressure (Truyen *et al.* 1995; Decaro *et al.* 2008c; Yoon *et al.* 2009). However, as sampling in this survey was at a population genetics level, evolutionary selection pressure was expected to be approximately the same across all of the samples, therefore the use of the VP2 gene in this instance was acceptable.

A single sample from a cat was included in the haplotype networks which focused on CPV-2 sequences from New Zealand. The original intention was to use this sample to root the network, however results of these attempts indicated that this was not the appropriate action for this data set (data not included).

The placement of the primers for sequencing the PCR products was an issue in this study. More of the samples could have been used in the international networks had primers CPV.VP2.JS3F and CPV.VP2.JS4R been placed differently. In their current configuration there was a small gap in the VP2 gene between the two primers (Figure 9). This gap was only sequenced successfully for the subset of samples which were used in the international networks, where it was desirable to have maximal nucleotide length in each sequence. If the primers had traded places, this section of the DNA would have been successfully sequenced more frequently and more of the samples collected through the survey could have been utilised in the international network.

While much of the available sequencing data have been generated in the last 15 years, the use of smaller bin sizes in the international network, which was coloured by decade, did not aid to further resolve the network (data not shown).

3.4.1. CANINE PARVOVIRUS GENE FLOW IN NZ

In order to investigate if the geographical division of New Zealand had a significant impact on the evolutionary patterns of the CVP-2 in New Zealand, sequences of CPV-2 samples obtained in this survey were compared from the North and South Islands. The absence of genotypic clustering in the network (Figure 10A) indicates the Cook Strait does not act as a barrier to gene flow in the New

Zealand CPV-2 population. Possible reasons for this include the movement of CPV-2 particles on fomites, or the translocation of sub-clinically infected animals. The highly stable nature of CPV-2 would allow for the transport of this virus on fomites. The translocation of young dogs in New Zealand may occur as a result of purchases of puppies from breeders. These animals are often under six months of age; a high risk age group for infection and disease caused by CPV-2 (Horner 1983; Studdert *et al.* 1983). It is possible that these young animals are facilitating the spread of CPV-2 between the North and South islands. As New Zealand does not have any wild animals that have been identified as potential intermediate hosts of CPV-2 it is unlikely the virus is being spread through the movement of other animals which may be carrying the virus.

There was also lack of structure in the network to suggest a relationship between the vaccination status of the host and the genotype of the virus (Figure 10B). This is indicated by the nodes containing multiple samples originating from dogs with different vaccination statuses. This finding suggests that currently there is no specific genotype of the CPV-2 virus in New Zealand which can infect vaccinated dogs. While during the early emergence of the CPV-2c subtypes concerns were raised that CPV-2c may have the ability to infect vaccinated dogs, subsequent research has shown that CPV-2c infections in vaccinated dogs are no more common than infections in vaccinated dogs which are caused by other subtypes of CPV-2 (Hong *et al.* 2007).

Finally, while the majority of the samples originated from dogs under the age of six months, the absence of clustering of the samples which originated from dogs over the age of six months suggests the lack of a genotype of virus which favours dogs over the age of six months (Figure 10C). Had a CPV-2 mutant sufficiently distinct to the currently used vaccine strains and currently circulating field viruses been detected in New Zealand, clustering may have been seen around age for such a subtype. For example, during the initial emergence of CPV-2c the virus was thought to be able to more readily infect adult dogs than what had been seen for other subtypes.

3.4.2. Genetic Isolation of New Zealand Canine Parvovirus.

The network resulting from international sequences obtained from the NCBI database and the New Zealand samples showed that CPV-2 sequences were very closely related. Considering relative recent emergence of CPV-2 (Koptopoulos *et al.* 1986), this is perhaps to be expected. Alternatively, this low amount of divergence could represent a signal of strong selection on the VP2 gene preventing sequence divergence.

The reticulation noted in the central area of the international network (Figures 12, 13 and 14) indicates there was not enough data in this set of samples to accurately predict the exact pathway of evolution. Further to this, the network revealed some viruses of a more recent origin apparently giving rise to viruses of an older origin (see NZL001, USA009, USA010 and GER001 in the lower central region of the network). This could be a result of more recent viruses having responded to selection pressure in the same way that older viruses have. If that was true, the apparent close relationships between some of the viruses may be not because they were derived from the same most common recent ancestor, but because they have undergone convergent evolution as a result of positive selection pressure (Nielsen 2005). Furthermore, it is quite likely that a more chronologically comprehensive sample set may have revealed older sequences in some of the nodes which currently only contain recent sequences. There is a notable gap in the time series of this data set, with only a few samples pre-dating 2008. This lack of data has been an unfortunate short fall of this data set, as more data for the mid 1980's and 1990's may have aided in resolving some of the reticulation observed. A more complete time series of data may have allowed for robust statistical support for the results reported.

The results of the haplotype analysis including international CPV-2 data (Figure 11) suggest that the New Zealand population of CPV-2 originated from a single introduction of the virus. CPV-2 was first reported in New Zealand in 1979 (Horner *et al.* 1979), shortly after the emergence of CPV-2 worldwide (Koptopoulos *et al.* 1986). The absence of more recently identified CPV-2 subtypes (CPV-2b and 2c, see Chapter 2), together with the distinct clustering of all recent New Zealand CPV-2 sequences, suggests that CPV-2 viruses have not been regularly imported to New Zealand. The only New Zealand sequences that did not group together within one cluster were dated 1986 and 1990. The age of these sequences most likely explains their exclusion from the main cluster of contemporary New Zealand sequences. The absence of CPV-2 sequences from 1990 – 2009 is a limiting factor in the ability to fully elucidate evolution of CPV-2 in New Zealand. Although the data support a singular, or infrequent, introduction of CPV-2 to New Zealand, a more complete time series would be necessary to confirm this conclusion. As discussed in chapter 2, isolates of CPV-2b and CPV-2c were not observed in New Zealand in this study. These subtypes emerged more recently than CPV-2 and CPV-2a. The absence of CPV-2b and CPV-2c may suggest that the import of CPV-2 into New Zealand is hindered in some way. Cats and dogs entering New Zealand from countries other than Australia are subject to a minimum 10 day stay in quarantine. As the time of virus shedding in the faeces of a CPV-2 infected dog has been reported to be typically 4-7 days, up to maximum of14 days, (Meunier *et al.* 1981; Pollock 1982), even if a CPV-2 infected

dog entered New Zealand, it would most likely not to be shedding the virus by the time it is released from the quarantine to a general population. Further to this, strict vaccination requirement for dogs entering New Zealand may also play a role in the prevention of the importation of new CPV-2 genotypes.

As CPV-2 is a very stable virus and easily carried on fomites the entry of new CPV-2 genotypes into New Zealand via fomites would seem be a likely source of the importation of new CPV-2 genotypes. However, the results of the current study do not suggest this is occurring. This may be due to strict border requirements for the disinfection of soiled footwear and other personal items upon entry into New Zealand. Overall, our results suggest that the strict quarantine and border control measures in New Zealand not only prevent entry of diseases such as rabies, they may also aid in preventing the entry of new subtypes of CPV-2 and other viruses.

As can be seen in the international network a sample of CPV-2c originating from Greece was found which was most closely related to CPV-2b isolates either from USA or South Africa (Figure 11 and 13). The CPV-2b isolate from the USA was also most closely related to another CPV-2c isolate which originated from China. The genetic relatedness of these samples suggests that CPV-2 may be moving relatively unhindered around the world. It also highlights the uniqueness of the findings with regard the closed genetic population of CPV-2 in New Zealand.

The evolution of CPV-2 subtypes has been considered to be the result of selection pressures exerted by the host's immune responses (Truyen *et al.* 1995; Decaro *et al.* 2008c; Yoon *et al.* 2009). One would expect such selection pressures, driven in part of vaccination, to be similar in New Zealand and overseas. Why these selection pressures have not resulted in the evolution of other CPV-2 subtypes in New Zealand is, therefore, unclear. Some authors suggested that wild carnivors may have played a role in the evolution of CPV-2 overseas (ref? e.g. Allison et al 2012). Thus, one possible explanation for the relative genetic stability of CPV-2 in New Zealand may be lack of wildlife hosts susceptible to infection with CPV-2 or related parvoviruses in this country.

3.4.3. Risk Factors Associated with Age and Vaccination Status

There was no apparent clustering of CPV-2 from dogs with different vaccination status (Figure 10b). This indicates that there was no one particular strain of CPV-2 circulating among dogs in New Zealand which was able to evade immune responses of a vaccinated dog (as described in Chapter 2). Thus, those dogs which did succumb to CPV-2 disease despite vaccination most likely did so as a result of factors other than the genetic make-up of the virus. These factors may include pre-

existing infection with another pathogen resulting in immune suppression, vaccine inefficacy or other host-related factors.

While it is uncommon to see dogs over the age of six months affected by CPV-2 enteritis, eight samples which originated from dogs over the age of six months were included in the analysis. There was a lack of genetic structure seen among the CPV-2 sequences from diseased dogs over the age of six months. The lack of structure in the network suggest that there was no specific CPV-2 genotype identified that would be more likely to cause disease in older, presumably better protected, dogs. As such, it is likely that other factors might have played a role in development of CPV-2 disease in those dogs. For example, unvaccinated dogs raised and kept in an isolated environment (e.g. a remote farm lands) may have not encountered CPV-2 antigens until later in life. Vaccination inefficiency and immune suppression in relation to another medical condition are also possible explanations for these cases. As a complete medical history from each case was not available these cases were not pursued further.

3.4.4. The Sub-typing of CPV-2 in Relation to the Genetic Evolution of CPV-2

Currently CPV-2 is subtyped based on the presence of specific amino acid at several antigenic sites within the capsid of the viral particle (Cavalli *et al.* 2008). Changes at these sites have been found to have impacts on the host range of the virus (Decaro *et al.* 2010) and the severity of disease seen in the infected host (Moon *et al.* 2008). Whilst typing based on the antigenic properties is a clinically relevant method of categorising the virus, it may not be informative with regard to the rate of evolution of the virus. The international network (Figure 12) suggests that the current method of CPV-2 subtyping does not reflect the evolution of the virus on a genomic level. This can be seen in the relationships found between CPV-2b and CPV-2c. Sequences of CPV-2c subtype appear to have arisen independently in distinct parts of the genetic network from different CPV-2b relatives (CHI008 and SOA002). It is not possible to connect the two CPV-2c sequences without back tracking through nodes containing CPV-2b and CPV-2a; this indicates that the two CPV-2c sequences evolved independently of one another. Considering the rapid rate of the emergence of the different subtypes of this virus (Parrish *et al.* 1988b; Parrish *et al.* 1991; Pereira *et al.* 2000), the close relationship between the sequences may initially not be surprising, although the distance in relationship between CPV-2c sequences is certainly intriguing. The network produced here reveals a possible explanation for the rapid emergence of the new subtypes in the apparent convergent

evolution of CPV-2c. Studies have found mutation rates in the VP-2 genome to be in favour of synonymous mutations (Truyen *et al.* 1995; Decaro *et al.* 2008c; Yoon *et al.* 2009).

3.5. CONCLUSIONS

In the analysis of CPV-2 sequences from New Zealand there was no structure in the population based on the parameters investigated.

It is possible that examining the correlation of survival, or disease severity, with virus genetics may have revealed a population structure. However, these measures both present unique challenges in their criteria. A number of dogs with clinical signs of CPV-2 are euthanized due to the high cost of treatment and variable outcome of treatment associated with this disease, therefore an analysis of survival may be confounded by population dynamics in different regions. Measurement of disease severity is also difficult, as a standardised set of criteria would need to be established and followed for this to be accurately recorded. Further to this, detailed and verified records of the animal's health in the weeks preceding infection would be required to rule out the possibility of pre-existing host related factors with may have affected the severity of disease.

The apparent convergent evolution of the CPV-2 subtypes suggested in the international network (Figure 12) may provide some insight into the rapid emergence of CPV-2 subtypes throughout the world. However, the population of CPV-2 in in New Zealand appears to be monophyletic, as is suggested by the international networks. Why other subtypes of CPV-2 have not evolved within New Zealand is not known.

4. CONCLUSIONS AND FUTURE RESEARCH

The continued monitoring of CPV-2 subtypes and genotypes is vital in order to continue to protect dogs effectively against this devastating and deadly virus. To date it has been found that the use of the original CPV-2 subtype in vaccines is still effective in protecting dogs against infection with all the currently known subtypes. However the rapid rate of evolution seen in this virus makes it a 'ticking time bomb' which may evolve to evade the currently available immunisations at any time. It is important to study not only the sites which have traditionally been used to subtype the virus but also to monitor the entire genome. The current focus of subtyping and evolutionary studies such as this one has been on the VP2 gene which encodes the major viral capsid protein. While changes in the VP2 protein may confer replicative advantages such as immune evasion and enhanced binding to the host cell it is still important to consider other the other proteins encoded in the genome.

The results of this survey suggest the control measures in place at New Zealand's boarders are largely effective at preventing the entry of CPV-2 into New Zealand. A more thorough search of universities, diagnostic laboratories and the laboratories of New Zealand's boarder control agencies for samples dating between 1980 and 2007 may uncover previously un-sequenced samples. The addition of these samples to a data set such as this one may shed more light on the frequency at which this virus is entering New Zealand and the evolution of the virus in New Zealand.

While the findings of the apparent convergent evolution of CPV-2c, as discussed in 3.4.4, may be clinically irrelevant with regard to disease progression, further investigation may aid in gaining a deeper understanding of the evolution of this virus. The presence of genetic markers in the CPV-2 genome which may act as indicators for upcoming antigenic shifts cannot be ruled out. If such markers were to be found they could aid in the ability to predict potential outbreaks and curb their impact on the canine population. The advancement of DNA sequencing technologies and subsequent reduction in the costs associated with these methods has resulted in a dramatic increase in number of studies investigating CPV-2 and many other microorganisms based on their genetic sequences. However, in the case of CPV-2 many of these studies are still very much focused on the pre-determined antigenically important sites. As such, the amount of sequence data available for other parts of CPV-2 genome is currently limited.

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6. APPENDICES

6.1. APPENDIX 1

6.1.1. LETTER DISTRIBUTED TO VETERINARIANS

Massey University
Veterinary Teaching Hospital

To provide outstanding education through clinical services and advancement of knowledge in an excellent environment.

A survey of canine parvovirus subtypes in New Zealand

February 2010

Dear Colleagues

We are asking for your help in conducting a study of canine parvovirus (CPV) subtypes present in New Zealand Very little is currently known about the different strains or subtypes of canine parvovirus that exist in New Zealand and their geographical epidemiology.

This study is funded by Intervet Schering Plough Animal Health. The initial aim of the research project is to identify what subtypes of CPV exist in New Zealand. It may be that the subtypes that exist in New Zealand are the same as those currently recognised elsewhere in the world, however the possibility remains that unique subyptes are present that have not yet been identified. If this is the case, this could have far reaching implications in terms of future prevention of CPV in New Zealand.

The Study

Over the next 12 months, we propose to define the common subtypes of CPV present in New Zealand using PCR and sequencing analysis. To do this, we require faecal samples from CPV ELISA positive dogs. We wish to obtain virus from as wide a geographic distribution as possible, and so, on bended knee, we ask for your help and support in this study. We are distributing sample pots and submission forms to up to 500 practices nationwide, using the sales representative network of Intervet Schering Plough. We wish to obtain around 200 ELISA positive samples, but will merrily and gratefully receive more than that.

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

1. Faecal sample from a CPV ELISA positive dog, placed in the pot provided
2. A completed specimen submission form
3. If possible, a sample of clotted blood in a plain tube. This is ideal, but not essential.

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

Yours sincerely

Nick Cave
Senior Lecturer
Small Animal Medicine and Nutrition

Magda Dunowska
Senior Lecturer
Veterinary Virology

6.1.2. SAMPLE SUBMISSION FORM

Massey University
Veterinary Teaching Hospital

Date: / /

To provide outstanding education through clinical services and advancement of knowledge in an excellent environment.

Canine Parvovirus Sample submission form

Project: A survey of canine parvovirus subtypes in New Zealand

Veterinary Practice: .....

Samples submitted: CPV positive faeces ☐ Clotted blood ☐ (if available)

Patient Details

Animal ID (name/owner): .....

Breed:..... **Age:** **Date of birth:** / /

Sex: Entire male ☐ Castrated male ☐ Entire female ☐ Speyed female ☐

Clinical history (please attach clinical records if available)

.....

.....

.....

.....

Parvovirus status

Faecal ELISA positive YES ☐ **ELISA Test** brand:.....

Vaccinated: YES ☐ NO ☐

Date(s) vaccinated: .....

Brand of vaccine: .....

6.1.3. SAMPLES TESTED FOR CPV-2 BY PCR

Sample Number	Date Collected	Source	Location	Rapid CPV Antigen Detection Kit Result	PCR Result
CPV001	2/10/2009	Participating Vet Clinic	Unknown	Positive	Positive
CPV002	10/08/2009	Participating Vet Clinic	North Island	Negative	Positive
CPV003	6/08/2009	Participating Vet Clinic	Unknown	Positive	Positive
CPV004	6/08/2009	Participating Vet Clinic	Unknown	Negative	Positive
CPV005	Unknown	Participating Vet Clinic	South Island	Unknown	Positive
CPV006	27/08/2009	Participating Vet Clinic	Unknown	Positive	Positive
CPV007	Unknown	Participating Vet Clinic	South Island	Unknown	Positive
CPV008	Unknown	Participating Vet Clinic	South Island	Unknown	Positive
CPV009	Unknown	Participating Vet Clinic	South Island	Unknown	Positive
CPV010	Unknown	Participating Vet Clinic	South Island	Unknown	Positive
CPV011	13/01/2010	Intervet Schering Plough	North Island	Positive	Negative
CPV012	10/01/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV013	29/12/2009	Intervet Schering Plough	North Island	Positive	Positive
CPV014	10/10/2006	Historic	Unknown	Unknown	Positive
CPV015	10/10/2006	Historic	Unknown	Unknown	Positive
CPV016	10/10/2006	Historic	Unknown	Unknown	Negative
CPV017	7/11/2008	Historic	Unknown	Virus Isolate	Positive
CPV018	4/10/1979	Historic	Unknown	Virus Isolate	Negative
CPV019	13/02/2010	Intervet Schering Plough	North Island	Positive	Positive
CPV020	19/02/2010	Participating Vet Clinic	South Island	Unknown	Negative
CPV021	22/02/2010	Participating Vet Clinic	South Island	Unknown	Positive
CPV022	25/02/2010	SPCA	North Island	Positive	Positive
CPV023	15/03/2010	Intervet Schering Plough	North Island	Positive	Positive
CPV024	17/03/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV025	18/03/2010	Participating Vet Clinic	North Island	Negative	Positive
CPV026	17/03/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV027	22/03/2010	Participating Vet Clinic	North Island	Negative	Positive
CPV028	24/03/2010	Participating Vet Clinic	North Island	Negative	Positive
CPV029	27/03/2010	Intervet Schering Plough	North Island	Positive	Positive
CPV030	26/03/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV031	26/03/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV032	30/03/2010	Participating Vet Clinic	North Island	Unknown	Negative
CPV033	25/03/2010	Participating Vet Clinic	South Island	Positive	Negative
CPV034	31/03/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV035	25/03/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV036	9/04/2010	Participating Vet Clinic	North Island	Unknown	Positive
CPV037	8/04/2010	SPCA	Unknown	Positive	Negative
CPV038	9/04/2010	SPCA	North Island	Positive	Positive
CPV039	9/04/2010	Participating Vet Clinic	Unknown	Unknown	Positive
CPV040	13/04/2010	Intervet Schering Plough	North Island	Negative	Positive
CPV041	13/04/2010	Participating Vet Clinic	North Island	Unknown	Positive
CPV042	14/04/2010	Participating Vet Clinic	North Island	Positive	Negative
CPV043	16/04/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV044	25/04/2010	Participating Vet Clinic	North Island	Negative	Negative
CPV045	19/04/2009	Intervet Schering Plough	North Island	Positive	Positive
CPV046	27/04/2010	Participating Vet Clinic	North Island	Positive	Positive

Sample Number	Date Collected	Source	Location	Rapid CPV Antigen Detection Kit Result	PCR Result
CPV047	29/04/2010	Participating Vet Clinic	North Island	Negative	Positive
CPV048	29/04/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV049	29/04/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV050	29/04/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV051	29/04/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV052	27/04/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV053	11/05/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV054	7/05/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV055	5/05/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV056	13/05/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV057	12/05/2010	SPCA	North Island	Positive	Positive
CPV058	17/11/2009	Intervet Schering Plough	South Island	Positive	Positive
CPV061	19/05/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV062	21/05/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV063	8/06/2010	Participating Vet Clinic	South Island	Positive	Negative
CPV064	9/06/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV065	23/06/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV066	12/06/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV067	10/06/2010	Miscellaneous	North Island	FELINE ORIGIN* (FPV)	Positive
CPV068	9/07/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV070	9/07/2010	SPCA	North Island	Positive	Positive
CPV071	12/07/2010	Participating Vet Clinic	North Island	Unknown	Positive
CPV072	10/07/2010	SPCA	North Island	Positive	Positive
CPV073	26/05/2010	SPCA	North Island	Positive	Positive
CPV074	11/08/2010	SPCA	South Island	Positive	Positive
CPV075	Unknown	Participating Vet Clinic	South Island	Positive	Positive
CPV076	13/07/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV077	4/07/2010	SPCA	North Island	Positive	Positive
CPV078	25/08/2010	Intervet Schering Plough	North Island	Negative	Positive
CPV079	27/08/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV080	24/05/2010	Participating Vet Clinic	North Island	Negative	Positive
CPV081	10/09/2010	Participating Vet Clinic	North Island	Negative	Positive
CPV082	2/09/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV083	25/08/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV084	10/09/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV085	26/10/2010	Participating Vet Clinic	North Island	Positive	Negative
CPV086	14/10/2010	Participating Vet Clinic	North Island	Positive	Positive
CPV087	29/11/2010	Participating Vet Clinic	South Island	Positive	Positive
CPV088	14/12/2010	Participating Vet Clinic	North Island	Positive	Positive
MAF.WV.1	19/05/2009	Historic	Unknown	Unknown	Positive
MAF.WV.2	3/04/1990	Historic	Unknown	Unknown	Positive
MAF.WV.3	27/08/1986	Historic	Unknown	Unknown	Positive
MAF.WV.4	9/04/1980	Historic	Unknown	Unknown	Positive
NOBIVAC	Unknown	Vaccine	Unknown	N/A	Positive
PROTECH C3	Unknown	Vaccine	Unknown	N/A	Positive
VANGUARDF	Unknown	Vaccine	Unknown	N/A	Positive

6.1.4. Details for Survey Dogs That Tested Positive for CPV-2 by PCR

Sample Number	Vaccinated	Number of Vaccine Doses	Sub-type	Breed	Sex	Age in months	Recovered?
CPV001	No	None	CPV-2a	Cross Breed	Male, Entire	3	Unknown
CPV002	No	None	CPV-2a	Cross Breed	Male, Entire	6	Yes
CPV003	Yes	1 Dose	CPV-2a	Pig dog	Male, Unknown	4	Unknown
CPV004	Yes	1 Dose	CPV-2a	Pig dog	Female, Entire	4	Died
CPV005	Unknown	Unknown	CPV-2a	Unknown	Unknown	Unknown	Unknown
CPV006	Yes	1 Dose	CPV-2a	Cross Breed	Unknown	4	Unknown
CPV007	Unknown	Unknown	CPV-2a	Unknown	Unknown	Unknown	Unknown
CPV008	Unknown	Unknown	CPV-2a	Unknown	Unknown	Unknown	Unknown
CPV009	Unknown	Unknown	CPV-2a	Unknown	Unknown	Unknown	Unknown
CPV010	Unknown	Unknown	CPV-2a	Unknown	Unknown	Unknown	Unknown
CPV012	Unknown	Unknown	CPV-2a	Cross Breed	Unknown	4	Euthanised
CPV013	Yes	2 Doses	CPV-2a	Rottweiler	Female, Entire	10	Unknown
CPV019	Yes	2 Doses	CPV-2a	Labrador	Male, Entire	4	Yes
CPV021	Unknown	Unknown	CPV-2a	Border Collie	Male, Entire	5	Yes
CPV022	Yes	2 Doses	CPV-2a	Cross Breed	Male, Neutered	Unknown	Unknown
CPV023	Yes	1 Dose	CPV-2a	Pit Bullterrier	Male, Entire	16	Yes
CPV024	No	None	CPV-2a	Pig dog	Male, Entire	10	Yes
CPV025	No	None	CPV-2a	Dogue de Bordeaux	Male, Entire	6	Yes
CPV026	No	None	CPV-2a	Cross Breed	Male, Entire	2	Yes
CPV027	Yes	1 Dose	CPV-2a	Huntaway	Male, Entire	4	Euthanised
CPV028	Yes	1 Dose	CPV-2a	Rottweiler	Male, Entire	11	Yes
CPV029	Yes	1 Dose	CPV-2a	Fox terrier	Male, Neutered	6	Died
CPV030	No	None	CPV-2a	Heading Dog	Male, Entire	2	Unknown
CPV031	No	None	CPV-2a	Cross Breed	Male, Entire	2	Unknown
CPV034	No	None	CPV-2a	Cross Breed	Female, Entire	6	Yes
CPV035	Yes	1 Dose	CPV-2a	Rottweiler	Female, Entire	3	Died
CPV036	No	None	CPV-2a	Cross Breed	Female, Entire	4	Yes

Sample Number	Vaccinated	Number of Vaccine Doses	Sub-type	Breed	Sex	Age in months	Recovered?
CPV038	Unknown	Unknown	CPV-2a	Cross Breed	Female, Entire	3	Died
CPV039	No	None	CPV-2a	Cross Breed	Female, Entire	3	Unknown
CPV040	Yes	2 Doses	CPV-2a	Bichon Frisé	Female, Entire	8	Died
CPV041	No	None	CPV-2a	Cross Breed	Male, Entire	2	Yes
CPV043	No	None	CPV-2a	Cross Breed	Female, Entire	12	Yes
CPV045	Yes	1 Dose	CPV-2a	Beagle	Male, Neutered	7	Yes
CPV046	No	None	CPV-2a	Cross Breed	Male, Entire	5	Euthanised
CPV047	No	None	CPV-2a	Pig dog	Female, Entire	5	Euthanised
CPV048	No	None	CPV-2a	Pit-bull Terrier	Female, Entire	2	Died
CPV049	No	None	CPV-2a	Cross Breed	Female, Entire	2	Died
CPV050	No	None	CPV-2a	Cross Breed	Male, Entire	5	Yes
CPV051	No	None	CPV-2a	Cross Breed	Female, Entire	4	Yes
CPV052	No	None	CPV-2a	Papillion	Male, Entire	7	Yes
CPV053	No	None	CPV-2a	Cross Breed	Female, Entire	4	Yes
CPV054	No	None	CPV-2a	Cross Breed	Male, Entire	3	Euthanised
CPV055	No	None	CPV-2a	Jack Russell	Male, Entire	2	Euthanised
CPV056	No	None	CPV-2a	Cross Breed	Male, Entire	3	Euthanised
CPV057	Unknown	Unknown	CPV-2a	Cross Breed	Male, Unknown	Unknown	Euthanised
CPV058	Yes	3 Doses	CPV-2a	Shar Pei	Female, Entire	12	Euthanised
CPV061	No	None	CPV-2a	Cross Breed	Female, Entire	2	Unknown
CPV062	No	None	CPV-2a	Heading Dog	Male, Entire	5	Yes
CPV064	No	None	CPV-2a	Cross Breed	Female, Entire	4	Euthanised
CPV065	No	None	CPV-2a	Pit-bull Terrier	Male, Entire	3	Died
CPV066	Yes	1 Dose	CPV-2a	German Shepherd	Male, Entire	3	Yes
CPV068	Yes	1 Dose	CPV-2a	Bull mastiff	Female, Entire	6	Yes
CPV070	Yes	2 Doses	CPV-2a	Cross Breed	Female, Entire	3	Euthanised
CPV071	No	None	CPV-2a	Cross Breed	Female, Entire	4	Yes
CPV072	Unknown	Unknown	CPV-2a	Cross Breed	Female, Speyed	3	Euthanised

Sample Number	Vaccinated	Number of Vaccine Doses	Sub-type	Breed	Sex	Age in months	Recovered?
CPV073	Unknown	Unknown	CPV-2a	Cross Breed	Female, Entire	4	Euthanised
CPV074	Yes	1 Dose	CPV-2a	Cross Breed	Female, Entire	2	Euthanised
CPV075	No	None	CPV-2a	Cross Breed	Male, Entire	3	Euthanised
CPV076	No	None	CPV-2a	Cross Breed	Female, Entire	3	Yes
CPV077	Yes	3 Doses	CPV-2a	Cross Breed	Female, Speyed	4	Euthanised
CPV078	Yes	1 Dose	CPV-2	Cross Breed	Male, Entire	12	Unknown
CPV079	No	None	CPV-2a	Huntaway	Male, Entire	2	Yes
CPV080	Yes	1 Dose	CPV-2a	Rottweiler	Male, Entire	6	Died
CPV081	No	None	CPV-2a	Pig dog	Male, Entire	2	Died
CPV082	Yes	1 Dose	CPV-2a	Labrador	Male, Entire	3	Yes
CPV083	Yes	1 Dose	CPV-2a	Cross Breed	Male, Entire	6	Yes
CPV084	No	None	CPV-2a	Heading Dog	Male, Entire	4	Unknown
CPV086	No	None	CPV-2a	Huntaway	Female, Entire	1	Yes
CPV087	Yes	2 Doses	CPV-2a	Cross Breed	Female, Speyed	18	Unknown
CPV088	Yes	2 Doses	CPV-2a	Cross Breed	Female, Entire	2	Unknown

6.1.5. SAMPLES INCLUDED IN INTERNATIONAL NETWORK ANALYSES

Code in Network	Country of Origin	Accession Number	Subtype	Year
ARG001	Argentina	JF346754	CPV-2a	2003
ARG002	Argentina	JF414826	CPV-2c	2010
ARG003	Argentina	JF414825	CPV-2c	2010
ARG004	Argentina	JF414817	CPV-2b	2003
ARG005	Argentina	JF414818	CPV-2c	2008
ARG006	Argentina	JF414819	CPV-2c	2008
ARG007	Argentina	JF414820	CPV-2c	2009
ARG008	Argentina	JF414821	CPV-2c	2009
ARG009	Argentina	JF414824	CPV-2c	2010
ARG010	Argentina	JF414823	CPV-2c	2009
ARG011	Argentina	HQ413322	CPV-2c	2008
BRA001	Brazil	DQ340404	CPV-2a	1980
BRA002	Brazil	DQ340434	CPV-2a	2000
CHI001	China	GU569948	CPV-2a	1986
CHI002	China	JF767492	CPV-2a	2009
CHI003	China	JF767494	CPV-2a	2009
CHI004	China	HQ651237	CPV-2a	2010
CHI005	China	HQ883273	CPV-2a	2010
CHI006	China	GU380301	CPV-2a	2009
CHI007	China	JF795456	CPV-2a	2010
CHI008	China	GU380305	CPV-2c	2009
CHI009	China	GU380299	CPV-2b	2009
CHI010	China	JN403045	CPV-2a	2011
CHI011	China	GU452715	CPV-2a	2009
CHI012	China	GU569946	CPV-2a	2001
CHI013	China	JF767493	CPV-2a	2009
CHI014	China	GU452713	CPV-2a	2009
GER001	Germany	AY742934	CPV-2a	1995
GER002	Germany	AY742935	CPV-2a	1995
GER003	Germany	AY742942	CPV-2a	1997
GRE001	Greece	GQ865519	CPV-2c	2009
GRE002	Greece	GQ865518	CPV-2c	2010
ITA001	Italy	FJ005249	CPV-2c	2008
ITA002	Italy	FJ005250	CPV-2c	2008
ITA003	Italy	FJ005251	CPV-2c	2008
ITA004	Italy	FJ005256	CPV-2a	2008
ITA005	Italy	FJ005257	CPV-2a	2008
ITA006	Italy	FJ005258	CPV-2a	2008
ITA007	Italy	FJ005264	CPV-2b	2008
ITA008	Italy	GU362933	CPV-2a	2008
ITA009	Italy	FJ005259	CPV-2a	2008
ITA010	Italy	GU362934	CPV-2a	2008
ITA011	Italy	AF306449	CPV-2b	1995
ITA012	Italy	AF306450	CPV-2b	2006
ITA013	Italy	FJ005254	CPV-2a	2005
ITA014	Italy	AF306447	CPV-2a	1995
ITA015	Italy	AF306445	CPV-2a	1996
NIG001	Nigeria	HQ602990	CPV-2a	2010
NIG002	Nigeria	HQ602992	CPV-2a	2010
NIG003	Nigeria	HQ602993	CPV-2a	2010
NIG004	Nigeria	HQ602994	CPV-2a	2010
NIG005	Nigeria	HQ602995	CPV-2a	2010
NIG006	Nigeria	HQ602991	CPV-2a	2010
CPV001	New Zealand	CPV001	CPV-2a	2009
CPV003	New Zealand	CPV003	CPV-2a	2009
CPV006	New Zealand	CPV006	CPV-2a	2009
CPV009	New Zealand	CPV009	CPV-2a	2009

Code in Network	Country of Origin	Accession Number	Subtype	Year
CPV019	New Zealand	CPV019	CPV-2a	2009
CPV022	New Zealand	CPV022	CPV-2a	2010
CPV026	New Zealand	CPV026	CPV-2a	2010
CPV030	New Zealand	CPV030	CPV-2a	2010
CPV031	New Zealand	CPV031	CPV-2a	2010
CPV036	New Zealand	CPV036	CPV-2a	2010
CPV038	New Zealand	CPV038	CPV-2a	2010
CPV039	New Zealand	CPV039	CPV-2a	2010
CPV040	New Zealand	CPV040	CPV-2a	2010
CPV041	New Zealand	CPV041	CPV-2a	2010
CPV068	New Zealand	CPV068	CPV-2a	2010
CPV071	New Zealand	CPV071	CPV-2a	2010
CPV075	New Zealand	CPV075	CPV-2a	2010
CPV079	New Zealand	CPV079	CPV-2a	2010
CPV080	New Zealand	CPV080	CPV-2a	2010
CPV081	New Zealand	CPV081	CPV-2a	2010
CPV082	New Zealand	CPV082	CPV-2a	2010
CPV084	New Zealand	CPV084	CPV-2a	2010
MAFWV1	New Zealand	MAFWV1	CPV-2a	2009
MAFWV2	New Zealand	MAFWV2	CPV-2a	1990
MAFWV3	New Zealand	MAFWV3	CPV-2a	1986
NZL001	New Zealand	AY742933	CPV-2a	1993
SOA001	South Africa	HQ602989	CPV-2	2010
SOA002	South Africa	HQ602987	CPV-2c	2010
SOA003	South Africa	HQ602985	CPV-2b	2010
SOA004	South Africa	HQ602969	CPV-2b	2010
SOA005	South Africa	HQ602970	CPV-2b	2010
SOA006	South Africa	HQ602971	CPV-2a	2010
SOA007	South Africa	HQ602972	CPV-2a	2010
SOA008	South Africa	HQ602973	CPV-2b	2010
SOA009	South Africa	HQ602974	CPV-2a	2010
SOA010	South Africa	HQ602975	CPV-2a	2010
SOA011	South Africa	HQ602977	CPV-2a	2010
SOA012	South Africa	HQ602978	CPV-2a	2010
SOA013	South Africa	HQ602981	CPV-2b	2010
SOA014	South Africa	HQ602982	CPV-2b	2010
SOA015	South Africa	HQ602986	CPV-2b	2010
SOA016	South Africa	HQ602983	CPV-2b	2010
SOA017	South Africa	HQ602979	CPV-2b	2010
SOA018	South Africa	HQ602984	CPV-2b	2010
SOA019	South Africa	HQ602980	CPV-2b	2010
SOA020	South Africa	HQ602976	CPV-2b	2010
SKO001	South Korea	EU009201	CPV-2a	2003 - 2006
SKO002	South Korea	EU009203	CPV-2a	2003 - 2006
SKO003	South Korea	EU009205	CPV-2b	2003 - 2006
SKO004	South Korea	FJ197833	CPV-2a	2007
SKO005	South Korea	FJ197835	CPV-2a	2007
SKO006	South Korea	FJ197837	CPV-2a	2007
NOBIVA	Unknown	NOBIVA	CPV-2	Unknown
PROTEC3	Unknown	PROTECH C3	CPV-2b	Unknown
REFSEQ	Unknown	M38245	CPV2a	Unknown
URG001	Uruguay	JF906788	CPV-2a	2010
USA001	USA	JN867603	CPV-2b	2009
USA002	USA	JN867605	CPV-2b	2009
USA003	USA	JN867607	CPV-2b	2008
USA004	USA	JN867609	CPV-2b	2008
USA005	USA	EU659116	CPV-2	1979

Code in Network	Country of Origin	Accession Number	Subtype	Year
USA006	USA	EU659117	CPV-2	1980
USA007	USA	EU659118	CPV-2a	1981
USA008	USA	EU659119	CPV-2b	2000
USA009	USA	EU659120	CPV-2b	1998
USA010	USA	EU659121	CPV-2b	1998
USA011	USA	JN867602	CPV-2b	2008
USA012	USA	JN867606	CPV-2b	2009
USA013	USA	JN867608	CPV-2b	2009
USA014	USA	JN867604	CPV-2b	2008

6.2. APPENDIX 2

6.2.1. RECIPES

Electrophoresis Gel (1% TBE) (100 mL)

Agarose LE (Axygen)	1g
TBE 0.5%	100 mL
Ethidium Bromide	5 µL

Electrophoresis Gel (1% TAE) (100 mL)

Agarose LE (Axygen)	1g
TAE 0.5%	100 mL
Ethidium Bromide	5 µL

Tris/Borate/EDTA Buffer (TBE Buffer) x5 (1 L)

Tris-Base	54 g
Boric acid	27.5 g
0.5M EDTA (pH 8)	20 mL
Distilled H_2O	800 mL

Mix and adjust volume to 1L with distilled water. For making 0.5x TBE working solution, dilute 1L of 5x solution to 10L distilled water.

0.5M EDTA (pH 8.0) (100 mL)

Disodium EDTA	18.6 g
MQ H_2O	70 mL

Adjust pH with NaOH pallets. Allow buffer to equilibrate for 5 minutes (minimum), re-check pH. Make volume to 100mL and autoclave.

Phosphate Buffered Saline (PBS) (5 L)

NaCl	40 g
KCl	1g
Na_2HPO_4	5.75 g
KH_2PO_4	1 g

Adjust pH to 7