Studies on the Molecular Epidemiology of Canine Parvovirus in the UK

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Memorandum

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author

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Abstract

Canine parvovirus (CPV) is a highly infectious gastrointestinal pathogen of all canids, the prevalence of which has remained high since it emerged in 1978, despite the development of highly efficacious vaccines with a long duration of immunity. Typing of CPV has historically been performed by mouse monoclonal antibodies which target specific amino acid changes, and has led to the definition of four antigenic types; type 2 which is no longer seen in the field, and types 2a, 2b and 2c, which are found co-circulating in various proportions in different countries. In order to evaluate how this relatively new virus emerged, spread with such speed, and how it evolves, we carried out a series of in depth studies of viruses in dogs seen at veterinary surgeries across the UK. We developed a sensitive long-range PCR to amplify the full VP2 gene which encodes the majority of the viral capsid protein, the major target of the host immune response.

Using this PCR, the prevalence of CPV seen in healthy dogs, but 58% of, often unvaccinated, animals hospitalised due to diarrhoea tested PCR positive, further indicating the severity of the virus infection.

To investigate the transmission and evolution of the virus both in the UK, and in relation to the global situation, we sequenced 150 VP2 genes from viruses from clinically ill animals over two years. Amongst these 150 viruses sequenced, 50 different DNA sequence types were identified, and apart from one case, all appeared unique to the UK. Phylogenetic analysis provided clear evidence for spatial clustering at the international level, and for the first time also at the national level, with the geographical range of some sequence types appearing to be highly restricted within the UK. The majority of predicted amino acid sequences were identical to those found elsewhere in the world, suggesting CPV VP2 has evolved a highly fit conformation. Phylogenetic analysis suggested complex antigenic evolution of this virus, with both type 2a and 2b viruses (the only types found in the UK) appearing polyphyletic. As such, typing based on specific amino acid mutations may not reflect the true epidemiology of this virus.

When the non-structural gene (NS) was sequenced for a representative sample of this population, it was found to be less variable than VP2. In addition, the NS gene sequence could not be used to classify a virus as type 2, 2a, 2b, 2c, but could be used to differentiate feline panleukopenia virus (FPLV) and CPV. In some cases, the same NS gene was seen in type 2a and 2b viruses, suggesting that recombination may be common.

As CPV has only recently emerged recently in the canine population, it was possible to investigate the evolution of the virus since it first emerged in the UK. Sequencing of 252 historical isolates collected between 1980 and 2010 showed a high evolution rate of 6.6×10^{-4} (95% confidence intervals 4.8-8.4) nucleotide substitutions per site per year in the UK. The time since the most recent common ancestor of CPV was 39.2 years (range 32.2-44.2), suggesting that the virus emerged in 1971, and may have circulated undetected in a dog or other susceptible population until it came to the attention of the scientific community in 1978. These chronological studies suggested that the evolution of CPV within the UK is complex and that there have been multiple introductions of new virus strains to the country over the past 30 years.

We showed for the first time that asymptomatic cats appear to be playing a significant role in the spread of CPV, with 32.5% (13/50) of clinically normal cats shedding CPV for up to six weeks in some cases. The VP2 sequence of these viruses were identical in 55% of cases to viruses found causing disease in dogs, suggesting that the risk of cross-species transmission of CPV from dogs to cats and vice versa may be a real possibility.

Despite the small size of the parvovirus genome, there are relatively few full sequences available, largely due to complex secondary structures at the genome termini making the genomes largely refractory to PCR amplification. Here we show for the first time that next generation sequencing using the Roche 454 platform can be applied to the small CPV genome, and this new technology allows for sequencing of the majority of the genome (up to 97.4%), including the termini, which we predict may be six nucleotides longer at its 3' ends than reported in previous studies.

The overall theme within this thesis is one of rapid evolution of CPV, allowing acquisition of a new host range, and an evolution which needs to be continually monitored to allow us to continue to prevent the disease via highly efficacious vaccines and to combat this potentially fatal, recently emerged virus. In the discussion, we conclude by questioning the currently accepted antigenic typing system and propose a new classification system largely based on host and phylogeny.

Section 1.1

General introduction to the parvoviruses

The *Parvoviridae* are a family of small (*parvus* = small in Latin) single stranded DNA containing viruses, known to cause disease in a wide range of animals, from higher mammals such as humans (Brown *et al*, 1993) and canines, to various insects (Hayakawa *et al*, 2000). Additional members are being increasingly recognised, for example, new viruses found in a gorilla, elephant, hedgehog and opossum (Kapoor *et al*, 2010, 2010b).

The viruses show T1 icosahedral symmetry (Agbandje *et al*, 1995), typically range from 18- 30 nm in size, and have a genome of around 5000 base pairs (Tijssen 2010).

Parvoviruses are known to require host cell factors and host DNA polymerase for viral replication, which only occurs in cells which are entering the S phase or in the G1 phase of the cell cycle (Rhode *et al*, 1974). Therefore the pathology caused by the virus is often observed in cells which have a high turnover, such as the gut epithelium (Fowler *et al*, 1970) and lymphoid tissue (Carlson *et al*, 1978).

There are currently 16 different genera (nine confirmed genera, and seven proposed genera) within the *Parvoviridae*. The most common, largest and well researched of these genera are shown in Table 1.1. In addition, the densoviruses, which infect insects, have recently been reclassified and now consist of nine smaller genera (Tijssen 2010).

Virus genus	Genome size range	Genome polarity	End termini	Example virus	References
Dependovirus	4680	Plus and minus	Identical	Adeno- associated virus -2	Zadori *et al*, (1995)
Erythrovirus	4986-5600	Plus and minus	Identical	B19	Yoo *et al* (1999), Brown *et al*, (1995)
Amdovirus	4592	Plus	Different	Aleutian mink disease virus	Bloom *et al*, (1988)
Bocavirus	5491	Minus	Identical	Bovine parvovirus	Chen *et al* (1986), Shade *et al* (1986)
Parvovirus	5081-5323	Minus*	Different	Minute virus of mice	Astell *et al*, (1983), Reed *et al* (1988), Rhode *et al*, (1983)
Densovirus	4176-6031	Plus and minus	Different	Galleria mellonella	Boublik *et al*, (1994), Bando *et al*, (1995)
Avetalvirus	5257	Plus	Identical	Chicken parvovirus	Day *et al* (2010)

Table 1.1. *Basic characteristics of the major genera within the parvovirus family. Not all genomes have been fully sequenced so some of the figures represent approximate genome sizes. This taxonomy has recently been reviewed by the International Committee for Virus Taxonomy (ICTV) (Tijssen et al, 2010).*

**LuIII is different to the rest of the parvovirus genus as it packages both plus and minus strands of the genome.*

Section 1.2

General characteristics of the *Parvoviridae*

Virus structure

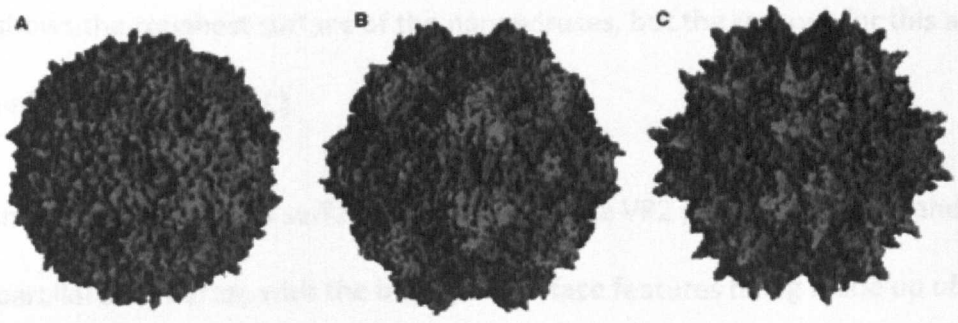

Figure 1.1. *Examples of the three main types of surface morphology observed in three parvoviruses: A. Galleria mellonella densonucleosis virus which has a smooth surface (densovirus genus). B. Minute virus of mice which is similar to canine parvovirus, showing the classical spike and cylinders on the surface (parvovirus genus). C. Adeno-associated virus-2 which has the roughest surface (dependovirus genus). (Taken from Fields et al, 2009)*

When the parvovirus icosahedral capsid is formed (see Figure 1.1), the DNA is then inserted into the preformed capsid (Xie *et al*, 1996), which can be composed of between one (as observed in recombinant B19 human parvoviruses) and six individual capsid proteins as observed in some insect parvoviruses (Kajigaya *et al,* 1991, Bando *et al,* 1995). In canine parvovirus, which has three viral proteins, VP1 is 143 amino acids longer than VP2 at the N terminus. VP3 is 17 amino acids shorter than VP2 at the N terminus (Wikoff *et al*, 1994).

Sixty of these capsid proteins lock together, in differing ratios, to form the capsid of the virus, the differing proportions of each protein producing capsids with slightly different morphology (Weichert *et al*, 1998). The densoviruses (Figure 1.1A) which

infect insects have a smooth surface, due to the lack of large insertions between the loops of the beta barrel in the viral proteins. Minute virus of mice, which shows many similarities to canine parvovirus, has got more surface structures than the densoviruses, such as a spike and cylinder (Figure 1.1B). The adeno-associated virus shows the roughest surface of the parvoviruses, but the reasons for this are unknown (Figure 1.1C).

In general, the capsid surface is made up of the VP2 protein's eight stranded anti parallel beta barrel, with the biological surface features being made up of insertions between the loops of the beta barrel. In the parvovirus genus, there is a threefold protrusion or spike which is 22 angstroms long, a 15 angstrom canyon which encircles a cylinder at each of the five fold axis and a 15 angstrom deep depression at each two fold axis (McKenna *et al*, 1999, Agbandje-McKenna *et al*, 1998).

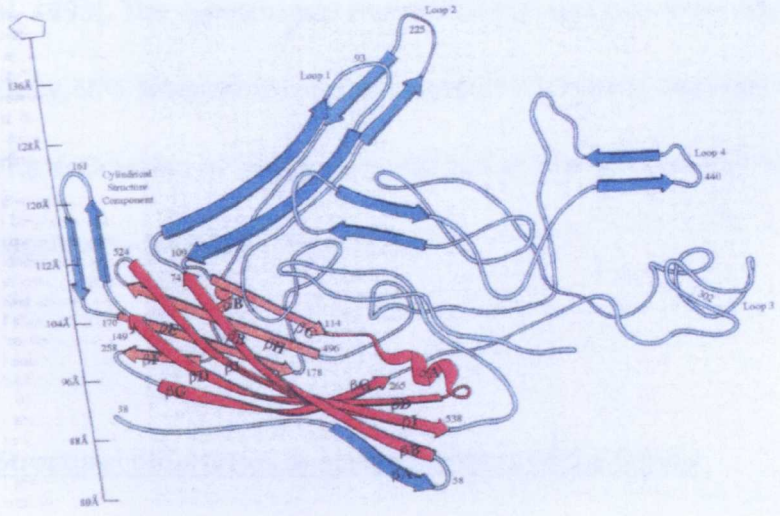

Figure 1.2. *The structure of a VP2 protein which makes up the capsid of CPV. It shows the beta barrel, which is a common feature of many viruses, highlighted in red. The loops are the rest of the structure (shown in the light blue) which are responsible for making up the surface structures such as the spike and cylinder. In total, 60 copies of VP2, as well as VP1 and VP3, form the capsid of the virus. (Figure from Tsao et al, 1991).*

The surface of the spike has been shown to be highly antigenic, containing many linear epitopes in the Parvovirus genera with one epitope being on the tip, and another one on the shoulder of the spike near residue 300 (Strasshein *et al*, 1994; Saikawa *et al*, 1993). Another epitope is located in the amino terminus of VP2 and the unique region of VP1 (Lopez de Turiso *et al*, 1991).

Parvovirus capsids also have areas which bind calcium ions which is important for virus infectivity and stability of the virion structure (Simpson *et al*, 2000). Eleven nucleotides in a specific motif occurring in several places within the genome bind to the interior of the capsid particles, but the significance of this is unknown (Tsao *et al*, 1992).

The five-fold cylinder is formed by the interlinking of five beta hairpins together, which leaves a narrow pore for exit or entry into or out of the virion particle (Wu *et al*, 1993). The cylinder acts as a portal for access in and out of the virion for DNA entry, VP1 N terminus exposure and VP2 terminal cleavage to VP3 (Farr *et al*, 2004). The depression of the virus capsid is also the thinnest and weakest point of the capsid structure (Wu *et al*, 1993).

Structural differences in other members of the family

Structurally there are no spikes on the surface of erythrovirus capsids such as B19, unlike the parvovirus genus, which is mainly due to the shortening of loops 3 and 4 of the jelly roll, although, the cylinder is not affected (Kaufmann *et al*, 2004).

Unlike most other parvovirus structures described to date, Kilham's rat virus, a member of the parvovirus genus was described as having a double capsid, with a highly hydrophobic capsid on the interior, and a highly hydrophilic capsid on the exterior (Wobbe *et al*, 1984).

Structural analysis of the buckeye butterfly densovirus (JCDNV) has shown that it has four capsid proteins (Bruemmer *et al*, 2005), five in the cockroach densovirus (Yamagishi *et al*, 1999), and six capsid proteins in the densovirus (Bando *et al*, 1995). The structure of the capsids of dependoviruses were found to be smooth, with no large spikes or depressions which are commonly found on the surface of other viruses such as canine parvovirus (Bruemmer *et al*, 2005; Simpson *et al*, 1998).

Proteins encoded by *Parvoviridae*

Most parvoviruses encode two non-structural (NS) proteins, and one to six viral proteins which form the capsid (discussed above) (Kajigaya *et al*, 1991, Bando *et al*, 1995). In the bocavirus genus only, there is also a third protein, known as the noncapsid (NP) protein (Chen *et al*, 1986).

Three mRNA transcripts are formed (Figure 1.4), known as R1, R2 and R3. The R1 mRNA transcript encodes NS-1 protein, R2 encodes the three forms of NS2, known as isoform P, L or Y, with L being the least common (Morgan *et al*, 1986). The VP proteins are formed from the R3 mRNA transcript. The VP1 transcript is then spliced

to form the VP2 protein, and this VP2 is then cleaved by proteases in the particle to form VP3 (Farr *et al*, 2006).

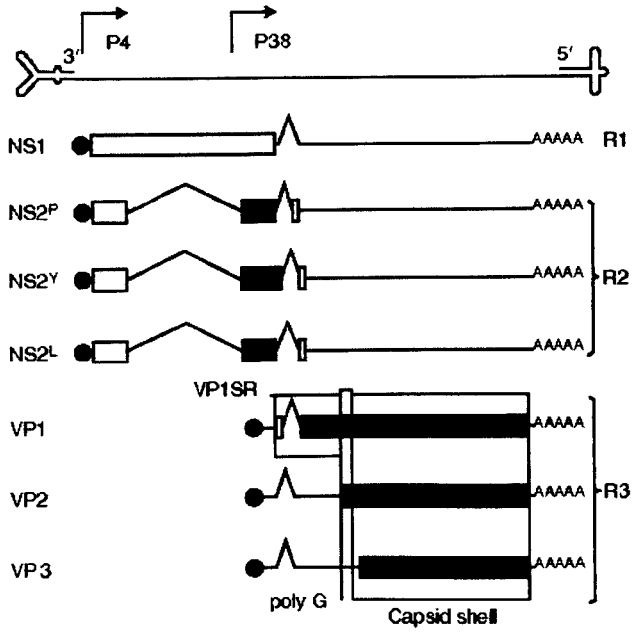

Figure 1.4. *The production of viral proteins in the parvovirus genus as exemplified by minute virus of mice (MVM). The genome and promoters are represented at the top, and the production of the NS-1 protein, the three isoforms of NS-2 and the three VP capsid proteins. (Taken from Cotmore et al 1995). This is described in more detail in the text above.*

Non-structural proteins (NS)

Most studies have concentrated on the capsid proteins, but both of the NS proteins are now receiving increasing levels of attention.

The NS proteins (NS-1 and NS-2) are formed first in infection (Naeger *et al*, 1993). NS-1 is required to transactivate the P38 promoter driving transcription for the VP proteins (Christensen *et al*, 1995), as well as co-activate its own P4 promoter, thereby increasing its own expression (Hanson *et al*, 1991). The NS-1 protein can

also down regulate proteins of cellular or other infectious agent origin (Legendre *et al*, 1992, Lorson *et al*, 1996). The NS-2 protein is required for protein synthesis and thus for a productive infection in their natural hosts (Li *et al*, 1991). In addition, NS-2 is required for efficient nuclear egress of the new progeny viruses from mouse cells (Eichwald *et al* 2002).

As well as this, the NS proteins have been found to interact with various cellular factors and proteins, such as the interation of NS-2 with the Smn (survivor motor neuron protein) (Young *et al*, 2002) and with the 14-3-3 protein family (Brockhaus *et al* 1996). This binding may be a link for the virus and cellular regulatory cascades. Activation of transcription has been shown to involve NS-1 and its interaction with Sp1 which is a common cellular transcription factor (Krady *et al*, 1995)

The N and C termini of the NS-1 protein have been shown to be cytotoxic when they accumulate in cells, and therefore are likely to be involved in viral exit from cells (Legendre *et al*, 1992).

NS-2 is also important for translation of viral mRNA, and in mutants or NS-2 deleted virions, the levels of mRNA production are decreased (Naeger *et al*, 1993).

NS-1 protein is inactive in its native form, and it is activated through phosphorylation of residues T435 and S473 by protein kinase C family in the cytoplasm of infected cells (Nuesch *et al*, 2003). This phosphorylation also activates the ATPase activity and the helicase activity of the protein, which are important for DNA unwinding and for DNA replication (Nuesch *et al*, 1998).

The N termini of NS-1 binds to the 5' end of the genome and remains intact with the DNA until after encapsidation, until after its removal by proteolytic or nucleolytic cleavage, which probably occurs after cell entry (Cotmore *et al*, 1989). NS-1 also acts as a nickase, and nicks the duplex origin sequence in the newly formed DNA, and thus allowing a 3' hydroxyl group to be free to prime de novo synthesis (Nuesch *et al*, 1995).

For nicking of the DNA to occur the NS-1 protein must form a precise ternary complex with origin DNA and host KWDK family of transcription factors to form the parvovirus initiation factor (PIF), which is important for viral infection and protein production (Christensen *et al*, 2001).

Viral capsid proteins (VP)

The viral capsid proteins make up the outer shell of the virus in all members of the *Parvoviridae*. In the parvovirus genus, there are three capsid proteins, VP1, VP2 and VP3 which are found in varying proportions (Tsao *et al*, 1991).

That said, VP2 can form particles on its own; but VP1 is required for infectivity of the virus particle (Xie *et al*, 1996). VP1 and VP2 are formed by alternative splicing of the R3 mRNA transcript to form the two separate proteins (Figure 1.4). Production of VP3 is different, occurring by proteolytic cleavage from the VP2 protein (Weichert *et al*, 1998) (as discussed above).

As the capsid proteins make up the outside of the virus, they are exposed to the immune response, and so maximal variation occurs here (Shackelton *et al*, 2005; Pereira *et al*, 2007). Many cellular receptors which interact with the capsid of different parvoviruses have been identified, for example the transferrin receptor for canine parvovirus (Palermo *et al*, 2003) and the erythrocyte P antigen for B19 erythrovirus (Brown *et al*, 1993). The dimple, (also known as the depression) on the capsid is involved in receptor binding (Lopez-Bueno *et al*, 2006) as well as in sialic acid binding. This is exploited by the haemagglutination test, which has been the gold standard diagnostic test for canine parvovirus since its emergence (Tresnan *et al*, 1995).

Within the N terminal region of the VP1 capsid protein (known as the VP1 unique region) is a nuclear localisation sequence which is important for nuclear transport of the virus for replication (Vihinen-Ranta *et al*, 1997). This region also has phospholipase A2 activity which is important for penetration of membranes by catalyzing the hydrolysis of phospholipid substrates (Zadori *et al*, 2001), and thus is required for parvovirus infectivity.

The VP1 unique region is not exposed on the surface of the virus when it forms, but can be exposed *in vivo* when exposed to low pH (Vihinen-Ranta *et al*, 2002) due to the plasticity of the capsid (Nelson *et al*, 2008). The virus enters the cell via clathrin mediated endocytosis and is transported within the cell through endosomes which allows the virus access to the low pH (Parker *et al*, 2000, Vendeville *et al*, 2009).

The capsid encapsidates the genome, and for the genome to be released, the capsid is not disassembled, although the mechanism of DNA exposure is not fully known (Ros *et al*, 2006).

Genome organisation and replication

In general, the protein coding regions of the *Parvoviridae* are encoded on a single strand (either the plus or minus strand) such as in the parvovirus and amdovirus genus (Astell *et al*, 1983). But, that said, some have coding sequences on both strands, for example the densovirus and erythrovirus genus (Dumas *et al*, 1992). These coding strands are flanked at both termini by palindromic repeats, which fold to form highly stable hairpins (Astell *et al*, 1979).

In the parvovirus genus, there are two large open reading frames (ORF's) in the DNA, one which encodes non-structural genes, and one encoding capsid genes (Vasudevacharya *et al*, 1992; Reed *et al*, 1988). These two ORFs alone are not sufficient for viral viability, and subsequent proteolytic cleavage of the viral proteins, and alternative mRNA splicing of the non-structural genes takes place to produce all the proteins required for viral replication (Jongeneel *et al*, 1986; Qiu *et al*, 2006; St Amand *et al*, 1991; Farr *et al*, 2006)

Parvoviruses usually have two promoters, one of which is found at 3.5 map units (mu) (known at the p4 promoter), producing the non-structural proteins, and the second at 38 mu (known as p38), producing the capsid proteins. Each promoter has the stable enabler regions, a cap site, a G/C rich activator and a TATA box (Diffoot *et*

al, 1993). In canine parvovirus (CPV) and minute virus of mice (MVM), both of which are members of the parvovirus genus, there are also two polyadenylation sites, observed at nucleotides 1580 and 4538 in CPV (Reed *et al*, 1988).

The viral DNA is replicated using a system known as a rolling hairpin replication mechanism (Figure 1.3). The light grey is the parental strand (indicated by the A and B), and the newly synthesised strand is shown in black (indicated by a and b) with the arrow at the 3' end. Steps (i) to (v) show the virus replicating to form a genome tetramer shown in (vi), in which there are three progeny genomes in addition to the parent strand. As shown in (vi) the genomes overlap on opposite strands and these are then excised to form the new genomes which are inserted into the preformed capsids. The hairpins snap together rapidly to form the new genomes.

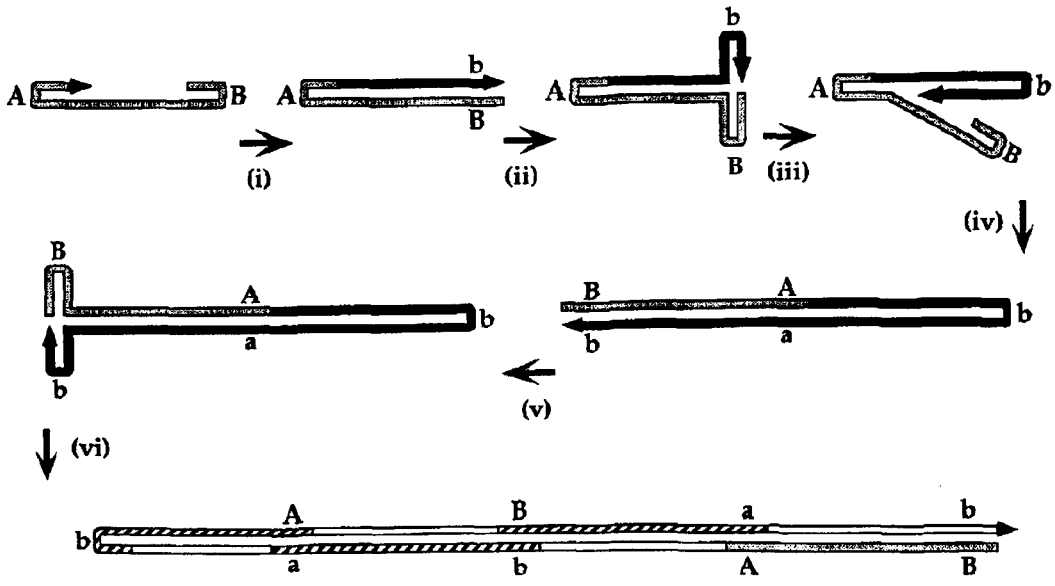

Figure 1.3. *The rolling hairpin model of replication for the DNA of parvoviruses. From Cotmore et al, (1995). The mechanism described in the text above.*

Section 1.3

Members of the *Parvoviridae*

Traditionally, parvoviruses have been divided into two large groups, known as autonomous parvoviruses which can replicate on their own and non-autonomous or defective (dependoviruses) parvoviruses which require a helper virus (such as adenovirus or herpes virus) to allow their replication (Atchinson *et al*, 1965; Buller *et al*, 1981).

The taxonomy of the viruses has been controversial, and has recently been revised (Tijssen 2010). Phylogenetic analysis of the highly conserved non-structural gene has grouped the viruses into 16 different genera within the family *Parvoviridae* (Figure 1.5) (Tijssen 2010). All of the genera are more similar in their non-structural proteins than their capsid proteins. Some of the more important and most commonly researched genera are discussed briefly below. (Viruses related to dogs or to CPV-2 are discussed in more detail than others).

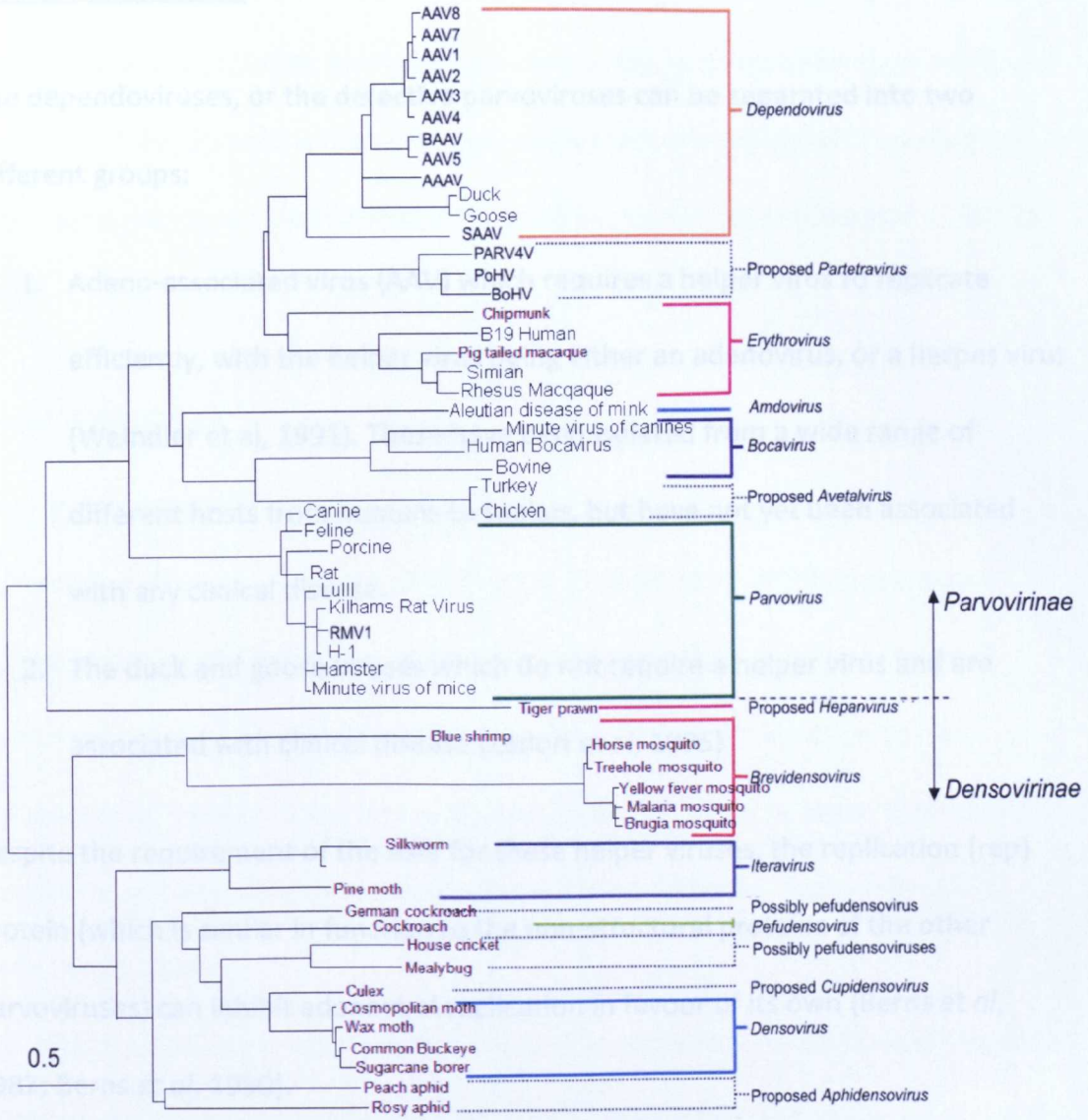

Figure 1.5. *Phylogenetic analysis of the members of the family Parvoviridae based on the non structural gene has grouped the viruses into two large sub-families, Parvovirinae and Densovirinae. Overall there are the 16 different genera described. The densovirinae sub-family genera has recently been broken down into nine different smaller genera, but for simplicity are grouped into one large group here. N.B. not all known parvoviruses are included as many have not yet been definitively grouped, but the main ones which are representative of the genera are shown. (Modified from Tijssen 2010).*

Genus Dependovirus

The dependoviruses, or the defective parvoviruses can be separated into two different groups:

1. Adeno-associated virus (AAV) which requires a helper virus to replicate efficiently, with the helper virus being either an adenovirus, or a herpes virus (Weindler et al, 1991). These have been isolated from a wide range of different hosts from humans to snakes, but have not yet been associated with any clinical disease.
2. The duck and goose viruses which do not require a helper virus and are associated with clinical disease (Zadori et al, 1995)

Despite the requirement of the AAV for these helper viruses, the replication (rep) protein (which is similar in function to the non-structural proteins of the other parvoviruses) can inhibit adenoviral replication in favour of its own (Berns *et al*, 1987; Berns *et al*, 1990).

Adeno-associated virus in humans is common in the human female genitourinary tract (Berns *et al*, 1990), and the respiratory tract. The AAVs can become latent, and, if a helper virus isn't present, can integrate into the q arm of human chromosome 19 (Samulski *et al*, 1991), commonly in muscle cells. This along with the apparent lack of pathogenicity caused by AAV has led to the virus being used as a potential human gene therapy vector (Russell *et al*, 1992).

Duck parvovirus causes up to 80% mortality due to signs similar to Derzsy's disease (Le Gall Recule *et al*, 1994), and goose parvovirus kills goslings under one month old (Kisary *et al*, 1978). Such is the severity of the goose parvovirus that a vaccine has been developed to protect farmers from large economic losses (Kisary *et al*, 1978).

Genus Erythrovirus

Human Parvovirus B19

B19 was first discovered and isolated in 1975 (Cossart *et al*, 1975), and is now a common virus within the human population, with over 50% of humans having contracted the virus (Cohen *et al*, 1988). B19 has several transmission routes, including from mother to the foetus, by bone marrow, blood or organ transplant/ transfusion or by the respiratory route (Anand *et al*, 1987; Broliden *et al*, 2001; Jordan *et al*, 1998).

The clinical pathology is mainly due to the ability of the virus to block erythropoiesis and increase inflammation (Ozawa *et al*, 1986). Signs in infected adults are generally mild, but can include fever, myalgia, coryza, headaches, nausea, and erythematous maculopapular exanthema (Tolfvenstam et al, 2009). In the foetus infected in utero by B19, more severe signs can occur, including hydrops fetalis (Brown et al, 1984), anaemia, liver inflammation, myocarditis (Garcia et al, 1998), heart arrthymia, cardiac arrest, erythema infantosium (Anderson, 1987; White et al, 1985; Reid et al, 1985) and even foetal death in 5-10% of cases infected in second trimester (Beigi et al, 2008; Riipinen et al, 2008; Enders et al, 2004; Syridou et al, 2008).

Immunocompromised people, such as those with HIV can develop chronic B19 parvovirus disease (Anderson et al, 1987; Kurtzman et al, 1987; Coulombel et al, 1989) and sickle cell disease patients can develop a transient aplastic crisis (Pattison et al, 1981; Young et al, 1988). Diagnosis generally uses PCR, serology, histopathology and or histochemistry (Anderson et al, 1986; Kleinman et al, 2007; Landolsi et al, 2009; Morey et al, 1992). To date, there is no specific vaccine or treatment for the disease (Tolfvenstam et al, 2009).

In addition to B19, three further erythroviruses have been described, namely rhesus, pig tailed macaque, and simian parvoviruses, of which relatively little is known. These viruses are known to cause anaemia in their hosts, but only animals which are immunosuppressed show any signs of disease, which differs slightly from B19 infection. Rhesus and pig tailed macaque viruses share more than 70% similarity with the B19 genome, and may provide animal models for B19 infection (Green *et al*, 2000).

Genus Amdovirus

The amdovirus genus has only one member, Aleutian virus of mink (AMDV).

Certain strains such as AMDV Utah are pathogenic for all mink (Alexandersen *et al*, 1994), whereas ADV-K is only pathogenic for Aleutian mink (Oie *et al*, 1996). ADV-G is a cell culture adapted virus, which is non-pathogenic for mink (Oie *et al*, 1996)

Mink kits develop severe interstitial pneumonia, as the primary target of the virus is alveolar type II lung cells, with lesions confined almost entirely to the lungs

(Alexandersen, 1986; Alexandersen *et al*, 1994). In adult mink, the disease and pathology is different, probably due to varying replication levels of virus in different tissues in young and old animals (Carlson *et al*, 1977).

Adult mink generally develop a slowly progressing, immune mediated disease characterised by hypergammagolobulinemia, arthritis, glomerulonephritis, and plasmacytosis (Burger *et al*, 1970).

As with other parvoviruses, transplacental infection can be teratogenic, causing foetal death and resorption if infection occurs during the first trimester of pregnancy (Broll *et al*, 1996). Infection in the second trimester causes a persistent infection in the offspring (Porter *et al*, 1977).

Serological studies revealed that antibodies to AMDV are quite common not only in wild European and American mink, but also in polecats, stone martens, pine martens and genets (Fournier- Chambrillon *et al*, 2004). Otters have also been shown to develop antibodies and may be susceptible to the disease (Manas *et al*, 2001, Wells *et al*, 1989). Ferrets and stoats may also be affected (McDonald *et al*, 2001).

Control of the disease is mainly by removal of any seropositive animals, as no vaccine is available.

Genus Bocavirus

This genus contains three members, a human bocavirus (HBov), the first parvovirus of canines discovered, minute virus of canines or canine parvovirus-1 (MVC), and a

bovine parvovirus (BPV). As discussed previously, bocaviruses encode another protein known as NP which is unique to this genus (Sun *et al*, 2009; Schwartz *et al*, 2002).

Human Bocavirus (HBoV)

Human Bocavirus was first described in 2005 (Allander et al, 2005) in nasopharyngeal aspirates of patients with respiratory tract infections, and many studies have detected HBoV in respiratory tract infections (Arden et al, 2006; Arnold et al, 2006; Choi et al, 2006; Manning et al, 2006). In addition, HBoV has been found in blood and faecal samples (Allander et al, 2007; Fry et al, 2007; Neske et al, 2007). HBoV is more common in childhood respiratory infections, with a prevalence of between 1.5 and 19% in respiratory tract infections (Arnold et al, 2006; Choi et al, 2006; Manning et al, 2006). By contrast, HBoV is rarely detected in asymptomatic individuals or in adults (Kesebir et al, 2006; Allander et al, 2007; Fry et al, 2007; Maggi et al, 2007).

Clinical signs associated with HBoV are in respiratory tract infections, with the virus persisting longer than other agents, but its presence in respiratory tract infections does not mean that it is acting as a pathogen (Allander et al, 2007). As it is a relatively new pathogen, its detection occurs mainly by PCR, but serology can also be used (Lu et al, 2006; Kleines et al, 2007; Neske et al, 2007; Simmonds 2008; Don *et al*, 2011; Pilger *et al*, 2011). Studies now suggest that four different genetic lineages circulate in the world (Kapoor *et al*, 2010c), although the significance of this is unclear.

Recently a virus which is similar to human bocaviruses has been found in gorillas (Kapoor *et al*, 2010b) and also in chimpanzees, which were also infected with B19 (Sharp *et al*, 2010).

Minute virus of canines (MVC or CPV-1)

This is referred to as MVC to avoid confusion with CPV-2 (the subject of this thesis).

Minute virus of canines was first isolated by Binn *et al* in 1970 from the faeces of a clinically normal non-diarrhoeic dog. The domestic dog is the only known host for this virus which is in contrast to CPV-2 (CPV), which infects all known canids, and felids (Carmichael *et al*, 1981). Virus isolation was achieved in Walter Reed canine cells, and this still remains the only cell line to support MVC growth.

Serological studies of dogs reveal infection is relatively common, with reported antibody prevalences of 18% in Turkey (Torun *et al*, 2005), 5- 15% in Japan (Mochizuki *et al*, 2002; Hashimoto *et al*, 2001), 36% in Korea (Jang *et al* 2003) and 50% in North America (Carmichael *et al* 1994). Interestingly a prevalence of 1.4% by virus isolation from diarrhoeic faeces of dogs in Japan, suggested MVC may be a possible agent for causing enteric disease (Mochizuki *et al*, 2002).

Despite this possible association with diarrhoea, and although seroconversion to MVC is common, the nature of any disease associated with MVC infection still remains controversial. Some reports suggest that infected dogs may show severe respiratory disease, such as bronchitis, interstitial pneumonia and sometimes

myocarditis (Carmichael *et al*, 1994; Harrison *et al*, 1992; Pratelli *et al*, 1999). In other studies, enteritis was the most prevalent sign (Jarplid *et al* 1996), although Carmichael *et al* (1994) found no enteric signs. Virus can be shed in the faeces from 3 to 9 days post infection (Macartney *et al*, 1988). MVC has also been described as teratogenic, and lead to foetal death, reabsorption and mummification if infected within first 30 days of gestation, whereas later infection leads to an immune response but no clinical signs (Carmichael et al, 1991, 1994).

Early comparison of CPV-2 and MVC revealed some similarities but many differences. The viruses have DNA of similar size, but had no similarities when digested with restriction enzymes (Macartney *et al*, 1988). Their capsid proteins were of a similar size, but shared no antigenic similarities. The genome of MVC has not been sequenced so more details of the sequence are unknown.

Bovine Parvovirus (BPV)

Bovine parvovirus (BPV) was first isolated in 1959 (Abinanti *et al*, 1961) and has only one antigenic type (Bates *et al*, 1972; Freeman *et al*, 1986).

Calves infected with BPV usually develop diarrhoea, but can remain clinically normal throughout, as occurs with most other parvoviruses. Diarrhoea of increased severity may develop with dual infection with other infectious agents, such as *Coccidia* spp and *Eimeria* spp (Durham *et al*, 1985). The virus can also cause growth retardation, which is a significant problem in economic terms (Storz *et al*, 1978). Antibodies to

BPV are common in most herds for both beef and dairy production (Storz *et al* 1972).

As for most parvoviruses, BPV has been shown to infect foetuses, and can lead to the development of an immune response within the foetus (Liggitt *et al*, 1982). Infection before 130 gestation can lead to abortion, or cerebellar, hepatic and adrenal lesions, following the teratogenic properties of the parvovirus family (Liggitt *et al*, 1982).

Genus Parvovirus

This group consists of the rodent parvoviruses, minute virus of mice (MVM) (the most studied example of parvoviruses), hamster parvovirus (HaPV), H-1 parvovirus, Kilhams rat virus (KRV), LuIII virus from hamsters and human cell lines, and rat parvovirus (RPV). Within this group is also PPV (porcine parvovirus), along with feline panleukopenia virus (FPLV) and canine parvovirus (CPV) which will be discussed in more detail later in this chapter.

Minute virus of mice (MVM)

MVM was first isolated in 1966 (Crawford 1966), and has become the prototype virus for work on the autonomous parvoviruses due to its ability to grow in cell culture and also to induce experimental infections in laboratory rodents.

Two genotypes of MVM have been described which have different host ranges; MVM(p) infects mouse fibroblasts whereas MVM(i), which is an immunosuppressive strain, can produce a productive infection only in mouse T lymphocytes (Gardiner *et al*, 1988).

The two genotypes have many similar biological properties and share over 85% of restriction enzyme sites (McMaster *et al*, 1981). Studies by Sahli *et al* (1985) showed 13 amino acid changes in the genome when comparing MVM(p) and MVM(i), which indicates that small amino acid changes can cause major host range and other biological changes (McMaster *et al*, 1981). The minor difference between the MVM(i) and MVM(p) capsids map to the fivefold axis, the shoulder of the three fold spike and the area surrounding the two fold depression, with the latter two regions being involved in the host tropism of MVM (Kontou *et al*, 2005).

Hamster parvovirus (HaPV and H-1 virus)

H-1 is another virus which has been isolated from human tissues such as embryonic tissues and tumours, but it has also been shown to grow in rat cells as well as in hamsters and hamster-derived cells (Hampton, 1970). Indeed, as H-1 can infect human cells without causing any known pathology, and like other parvoviruses has an affinity for transformed cancer cells, the virus has been used as a potential vector for possible cancer treatment (Cornelis *et al*, 1990)

Kilham's rat virus (KRV)

Kilham's rat virus (KRV) can cause disease in both newborn rats, and also in newborn hamsters, but older animals tend not to be affected (Kilham *et al*, 1964). When the virus is inoculated intercerebrellarly into newborn hamsters, it can cause cerebellar hypoplasia which may lead to chronic ataxia (Kilham *et al*, 1964). This is similar to the disease which can occur in kittens infected with FPLV *in utero* or shortly after birth (Kilham *et al*, 1971).

KRV, which was isolated from a rat nephroma cell, was shown to cause neonatal disease in rats, mice and hamsters, and can lead to physical abnormalities and mental retardation (Salzman *et al*, 1972).

LuIII

LuIII was first isolated as a contaminant of human cell lines (Hallauer *et al*, 1971). LuIII can infect human cells without causing any noticeable pathology, so the virus has been suggested for use as a vector for human gene therapy (Maxwell *et al*, 2004).

When LuIII is inoculated into newborn hamsters, it causes massive intestinal haemorrhage, and the virus can also cause a transplacental infection of hamster foetuses, causing abortion, with most severe signs being observed where infection occurs shortly after the start of gestation (Soike *et al*, 1976).

Porcine parvovirus (PPV)

Porcine parvovirus (PPV) is a common virus in pigs, where it is known to cause reproductive failures, foetal mummification (Mengeling *et al*, 1975), preweaning deaths (Morrison *et al*, 1985), and embryonic deaths (Rodeffer *et al*, 1975). However the stage of gestation of the sow is important in the disease manifestation, as infection in the first two thirds of pregnancy causes foetal deaths, whereas infection later in pregnancy causes no foetal death, but leads to an immune response in the piglets (Mengeling *et al*, 1981). This loss of reproductive potential leads to serious economic losses (Cartwright *et al*, 1967). Infection with these reproductive strains of PPV has not been associated with clinical signs either in sows, or in weaned pigs (Brown *et al*, 1980).

The clinical signs produced vary for different strains of virus, with some strains (e.g. NADL-7) causing high foetal mortality whereas others such as NADL-2 (a vaccine strain) showing little or no foetal pathogenicity (Mengeling *et al*, 1975b).

As well as the foetal signs, some strains of PPV have been associated with diarrhoea in pigs (Dea *et al*, 1984). However these have subsequently been shown to be serologically distinct from the PPVs typically associated with foetal signs (Yasuhara *et al*, 1989). A rare clinical manifestation appears to be skin lesions, and mild dermatitis (Choi *et al*, 1987, Kresse *et al*, 1985).

As with most viruses, high levels of maternally derived immunity are developed after pigs suckle colostrum and these antibodies can persist for up to nine months (Wrathall *et al*, 1987, Paul *et al*, 1980, 1982), although this is lost before the pigs

begin to mate. Therefore, protection in herds is achieved using various vaccines in gilts prior to mating (Paul *et al*, 1980b; Wrathall *et al*, 1984; Mengeling et al, 1981b).

Feline panleukopenia virus (FPLV)

Feline panleukopenia virus and mink enteritis virus (MEV) are the closest relatives of CPV, and share many similarities with each other. Although the variation between FPLV and CPV vary, an average of 115 nucleotide substitutions have been observed between the two viruses, and these lead to 40 predicted amino acid substitutions, mainly in the capsid proteins (Martyn *et al*, 1990).

FPLV was first isolated in 1957, (Bolin, 1957), but was reported many years before under different names, including feline agranulocytosis (Riser, 1946), feline distemper (Hindle *et al*, 1932) and feline infectious enteritis (Johnson *et al,* 1967).

The clinical signs and the pathogenesis of FPLV infection are similar to those occuring in CPV infection in dogs, with a few differences. Diarrhoea and vomiting are often more prominent in dogs infected with CPV, but are still present in FPLV infection; the leukopenia is more severe in cats infected with FPLV, involving neutrophils, eosinophils and monocytes and in severe or fatal cases, the leukocyte count can be less than 1000 cells/ cm (Goto *et al*, 1983). In the bone marrow, both myeloid and erythroid cell lineages are severely decreased during infection (Ichijo *et al*, 1976). FPLV has also been isolated from peripheral blood mononuclear cells in cats which have high virus neutralisation antibody titres, suggesting that the virus may persist inside them, allowing the development of an FPLV carrier state. The

virus has also been detected in urine and tissues in kittens infected *in utero*, or neonatally (Csiza *et al* 1971). No long term carrier state has so far been described for CPV (Miyazawa *et al*, 1999).

Kittens infected by FPLV *in utero* in seronegative queens, or those infected immediately postnatally, can develop cerebellar hypoplasia and ataxia (Kilham *et al*, 1971; Csiza *et al*, 1972a, 1972b). This is not fatal, although is life-long and there is no treatment.

In addition, FPLV has been reported to be associated with respiratory signs such as rhinitis and conjunctivitis, and also severe central nervous system lesions, but these seem relatively rare and may be due to co-infection with other pathogens such as feline calicivirus (FCV) (Csiza *et al*, 1971b). Hydraencephaly can also occur (Greene *et al*, 1982). Replication in the retina of cats has been known to cause retinal dysplasia and optic neuropathy, and these are often observed with the cerebellar hypoplasia syndrome (Brower *et al*, 2004).

Infection with FPLV is common in domestic cats, with an antibody prevalence of over 92% in cats which were unvaccinated in Costa Rica (Blanco *et al*, 2009). Indeed a study by Cave *et al* (2002), in the UK examining 274 kitten deaths at post mortem from 1986-2000 showed that 25% were attributable to FPL.

Although all parvoviruses are highly conserved, FPLV isolates are even more highly conserved. Decaro *et al* (2008) reported more than 99% similarity in Italian and British FPLV strains from clinically ill animals in the capsid proteins, but Battilani *et*

al (2006) reported that FPLV strains isolated in Italy form a unique FPL cluster found in Italy only. The reason for this difference remains unknown.

FPLV can be distinguished from CPV by its haemagglutination of pig red blood cells at different temperatures, a test which has been exploited for diagnosis of both CPV and FPLV. FPLV can haemagglutinate erythrocytes only at 4°C (Konishi *et al*, 1975) whereas CPV can haemagglutinate at a wider temperature and pH range (Senda *et al*, 1988). Non-haemagglutinating mutants of CPV have been reported (Parrish *et al* 1988). In addition, non-haemagglutinating mutants of mink enteritis virus (discussed later) have been reported (Rivera *et al*, 1984), and as such, it is possible that there will be non-haemagglutinating mutants of FPL too.

FPLV has been reported to have a seasonal increase in incidence between July and September, 8 to 16 weeks after the majority of kitten births (Reif, 1976), presumably due to the occurrence of a pool of susceptible kittens after the decline of maternally derived antibody.

Disease caused by FPLV has been observed for much longer than CPV, most of the cat population overall has now acquired good immunity to the virus. Vaccination is still highly recommended, and is often administered with other components including herpes virus and calicivirus (Dawson *et al*, 2001), and as a result, FPL is now rare in vaccinated cats.

Mink enteritis virus

The virus was first isolated in Fort William, Canada in 1947 and spread rapidly through Canada and the rest of the world (Burger *et al*, 1970). Mink enteritis virus (MEV) differs from the other parvovirus of mink ADV (discussed above) in that MEV replicates rapidly, whereas AMDV replicates relatively slowly (Storgaard *et al*, 1997).

The clinical signs of MEV infection are similar to those associated with FPLV and CPV, although the levels of vomiting are generally lower in MEV (Reynolds, 1969, 1970). As with FPLV, a carrier state has been described for mink, with virus detectable in the faeces for up to twelve months after recovery from an acute MEV infection (Bouillant *et al*, 1965).

Analysis of the sequence of the MEV whole genome revealed that it was 98% similar to the genomes of FPLV and CPV and both share the similar genome arrangements (Kariatsumari *et al*, 1991). So close is the relationship between MEV and FPLV, that infection of cats with MEV produces similar signs in cats as in mink, showing anorexia, vomiting and decreased leukocyte counts (Higashihara *et al*, 1981).

Sub family Densovirus

The densovirus, or densonucleosis viruses (DNV) infect invertebrates. This sub family contains nine newly suggested genera, all of which are grouped together here for simplicity.

These viruses can infect various orders of the insects, such as Lepidoptera, and Diptera. Little attention has been paid to pathology of these viruses in their respective hosts. Some appear to be apathogenic, whereas others are fatal, e.g. *Aedes albopictus* larvae parvovirus (Boublik *et al,* 1994) to their hosts, making them attractive potential biological pesticides (Jiang et al, 2007).

The densoviruses show similar structures and genome organisation to the sub family *Parvoviridae*, despite the obvious differences between mammals and insects. Apart from minor structural differences, there has been a report that the silkworm parvovirus structural protein has a DNA polymerase motif. This has never been reported in other parvoviruses to date and thus appears to be unlikely (Hayakawa *et al* 2000).

Genus Avetalvirus (Proposed)

The Avetalviruses contains two avian parvoviruses, chicken parvovirus (ChPV) and turkey parvovirus (TuPV) (Day *et al,* 2010). ChPV causes enteritis in chickens and thus causes a large economical loss to farmers. These viruses are different to the goose and Muscovy duck parvoviruses which have been identified previously (Brown et al, 1995b). Relatively little is known about these viruses, as they appear to be emerging in recent times, and so are not discussed in detail here.

Genus Partetravirus

This genus contains a newly discovered human pathogen, Parv 4, as well as Porcine Hokovirus (PoHV) and Bovine Hokovirus (BoHV). The latter two are not discussed here.

Parv 4

Parv 4, the most researched member of the Partetraviruses was first identified in 2005 (Jones et al, 2005), and is commonly found in patients with hepatitis C and people with HIV (Schneider et al, 2008; Manning et al, 2007; Longhi et al, 2007), but rarely found in healthy blood donors (Simmons et al, 2011). To date, three different genotypes of Parv 4 have been described, genotypes one and two are common in Western countries, whereas genotype three is only detected in Sub-Saharan Arica (Panning et al, 2010; Simmonds et al, 2008; Fryer et al, 2007; Botto et al, 2009). Transmission routes have not been firmly assigned to this virus, but it appears that parenteral transmission is the most common route, rather than sexual transmission (Sharp et al, 2009), but it is likely that other transmission routes exist (Sharp et al, 2010). There is generally no disease associated with Parv 4 infection, but it can cause complications for immunocompromised patients (Fryer et al, 2007). Virus has been detected using PCR (Tuke et al, 2010; Szelei et al, 2010), serology (Yang et al, 2011) and ELISA (Sharp et al, 2011).

Section 1.4

Canine parvovirus (CPV)

Emergence and evolution

A report in 1977 by Eugster *et al* reported the first isolation of parvovirus-like particles in the faeces of diarrhoeic dogs, but as the study used electron microscopy, it was not shown if the virus was MVC or the first discovery of CPV-2. In 1978, CPV was first reported and emerged almost simultaneously around the world: Britain (McCandlish *et al*, 1979), America (Fritz, 1979), New Zealand (Horner *et al*, 1979), Australia (Johnson *et al*, 1979), Germany (Osterhaus *et al*, 1980) and Canada (Gagnon *et al*, 1979). The virus was first noticed in collies in America, which had previously visited dog shows, and the close proximity of dogs in shows and veterinary surgeries made these ideal areas for virus spread (Appel *et al*, 1979).

Serological studies have failed to show CPV antibody in dogs before May 1978 in America (Pollock *et al*, 1984), or in Australia, suggesting that the virus emerged in two separate areas, or spread worldwide before clinical signs were observed (Walker *et al*, 1980). However, a report of antibody to CPV in a serum sample from a dog in Greece in 1974, four years before any disease was described with CPV (Koptopoulos *et al*, 1986), still raises the possibility that the virus was circulating at an albeit relatively low level prior to 1978.

The currently proposed evolutionary history of CPV is shown below (Figure 1.6) which was adapted from Truyen *et al*, (1998). The initial thought was that CPV had evolved from an FPL vaccine virus, but this was disproved by Truyen *et al* (1998) by

analysis of sequences from FPL vaccines and CPV field viruses, which showed that too many substitutions had accumulated in CPV for it to have come from the FPL vaccine. It has also been suggested that the virus has jumped from cats (FPLV) to dogs (CPV) via a wildlife intermediate such as a fox (Figure 1.6) (Truyen *et al*, 1998).

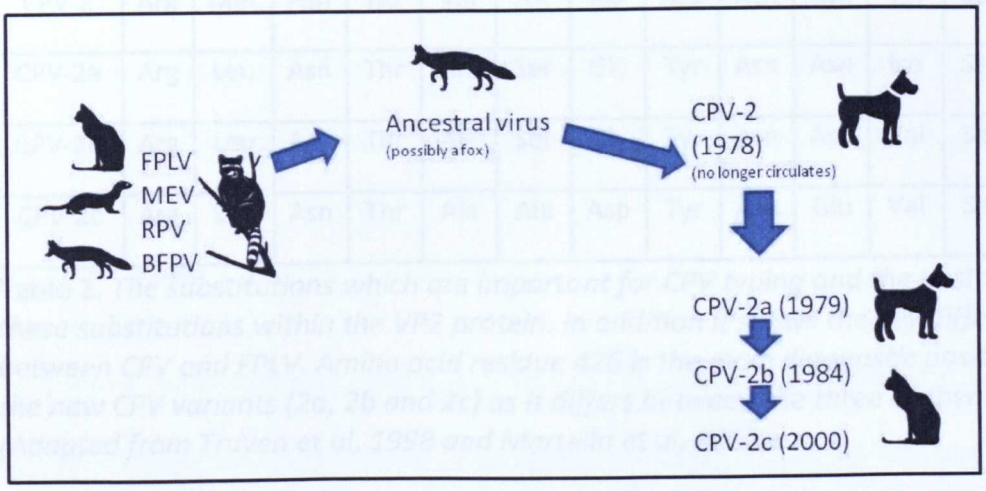

Figure 1.6. *The suggested evolutionary history of CPV, from its FPL ancestor, via a wildlife intermediate to the CPV-2 which spread around the world, and its subsequent evolution (Based on Truyen et al, 1998).*

Key: FPLV = feline panleukopenia, RPV= raccoon parvovirus, BFPV = blue fox parvovirus, MEV = mink enteritis virus.

Over the past 30 years, the virus has evolved into types 2a, 2b and 2c, which are observed in different proportions in different areas of the world, with the original CPV-2 no longer observed in field infections (Parrish *et al*, 1988b: Truyen *et al*, 1998). This typing system is based on antigenic changes associated with altered reactivity to mouse monoclonal antibodies (Parrish *et al*, 1985), with associated substitutions in the VP2 (Table 1.2).

Virus	VP2 Amino Acid residue												
	80	87	93	101	103	297	300	305	323	426	555	564	568
FPLV	Lys	Met	Lys	Iso	Val	Ser	Ala	Asp	Asp	Asn	Val	Asn	Ala
CPV-2	Arg	Met	Asn	Iso	Val	Ser	Ala	Asp	Asn	Asn	Val	Ser	Gly
CPV-2a	Arg	Leu	Asn	Thr	Ala	Ser	Gly	Tyr	Asn	Asn	Iso	Ser	Gly
CPV-2b	Arg	Leu	Asn	Thr	Ala	Ser	Gly	Tyr	Asn	Asp	Val	Ser	Gly
CPV-2c	Arg	Leu	Asn	Thr	Ala	Ala	Asp	Tyr	Asn	Glu	Val	Ser	Gly

Table 2. *The substitutions which are important for CPV typing and the positions of these substitutions within the VP2 protein. In addition it shows the key differences between CPV and FPLV. Amino acid residue 426 is the main diagnostic position for the new CPV variants (2a, 2b and 2c) as it differs between the three of them. (Adapted from Truyen et al, 1998 and Martella et al, 2006).*

Host range

The host range of CPV and FPLV is complex. *In vitro*, CPV has the ability to infect both canine and feline cells, whereas FPLV can only infect feline cells (Truyen *et al*, 1992). The *in vivo* host range of FPLV includes all felids, but the virus can only replicate in the thymus and bone marrow of dogs, causing no disease (Truyen *et al*, 1992). Cats inoculated with the original CPV-2 virus show no signs of infection (Truyen *et al*, 1992, Goto *et al* 1984). However, importantly, the newer CPV variants (2a, 2b and 2c) have regained a feline tropism, and are able to infect and cause disease in cats (Truyen *et al*, 1996, Ikeda *et al*, 2002).

The host range of CPV has been mapped to residues 93, 300, 301 and 323 of the VP2 protein (Parrish, 1999). Studies by Parker *et al* (1997) suggest that the structure of the ridge on the threefold spike where residues 300-323 are found is important

for infections. Alterations in 299 and 300 are also involved in host range, due to altering the binding of the viruses to the transferrin receptor (Hueffer *et al*, 2003) Amino acid residue 300 is important for control of the canine host range, and alteration at residue 300 from alanine to aspartic acid, abolished the canine host range of the virus. This was due to the aspartic acid at position 300 altering the structure of the virus by forming a salt bridge to argenine at position 81, which in turn alters the stability of the virion and alters the host range (Llamas-Saiz *et al*, 1996).

Outside of the laboratory, canine parvovirus has been isolated from cats, showing the clinical signs of FPL (i.e. fever, depression, anorexia, diarrhoea, vomiting and leukopenia less than 3000 cells per µl) (Gamoh *et al* 2003, Mochizuki *et al*, 1996). Studies by Ikeda *et al* (2000) suggested that over 80% of cat parvovirus infections in Japan were caused by CPV rather than FPLV, and a potential new variant was observed in leopard cats with a substitution at position 300 (Gly- Asp) which was initially named as 2c but is now not considered a new variant after testing with a monoclonal antibody panel (Parrish *et al*, 1982). CPV types 2a, 2b and 2c have been shown to cause disease in cats (Gamoh et al, 2003; Nakamura et al, 2001). CPV-2c has been isolated from cats which had an identical VP2 gene to others isolated from dogs has also been shown (Decaro *et al*, 2010). This suggests that in some countries CPV may be spreading more efficiently in cats than FPLV did, and that CPV is becoming the more common parvovirus in cats. As well as domestic cats, infection of various wild felids with CPV-2 have been reported, and attributed to cross species transmission from stray dogs to the cats in the zoo (Steinel *et al*, 2000).

Laboratory studies on CPV pathogenesis are very difficult to recreate the common clinical signs, and this may be due to using specific pathogen free (SPF) cats (and dogs) to study CPV pathogenesis, it has been suggested that the lower turnover of the tissues in SPF cats may not allow clinical signs to appear, such that the severity of disease may be underestimated. In support of this, although CPV-2b caused no signs in SPF cats, it was shown to induce severe signs of depression, anorexia, vomiting, diarrhoea and leukopenia in conventional (i.e. normal cats from breeders) cats, and one cat died of infection (Uchida *et al* 1988).

Taken together, these studies suggest that the CPV has the potential to become a problem in cats, and that there is a risk of spread between cats and dogs. However more work is required to determine how common cat infections by CPV are and the implications of this.

Because of this species cross over, some vaccine companies have tested their current FPL vaccine for cross protection against CPV infection in cats. A modified live FPL vaccine was shown to give full protection against a CPV 2b strain, with full prevention of lymphopenia and virus excretion (Chalmers *et al*, 1999). However, Nakamura et al (2001b) reported that low levels of neutralising antibodies were produced by cats which had been vaccinated against FPL and then infected experimentally with either CPV type 2a or 2c.

CPV as a risk to wild animals

As well as causing severe disease in domesticated dogs and cats, CPV has been found in many wild animals, and can potentially cause an extinction risk to certain wild canids.

CPV virus and associated pathology has been found in the following canids: coyotes (Evermann *et al*, 1980), raccoon dogs and foxes (Neuvonen *et al*, 1982, Frolich *et al*, 2005), wolves (Hedrik *et al*, 2003; Mech *et al*, 1997), maned wolves (Fletcher *et al*, 1979) and bush dogs (Janssen *et al*, 1982).

CPV antibody has been detected in the following canids: African wild dogs (Creel *et al*, 1997), San Joaquin kit foxes (Standley *et al*, 1997; Miller *et al*, 2000), wolves (Zarnke *et al*, 1987; Mech *et al*, 1995; Almberg *et al*, 2009), coyotes (Arjo *et al*, 2003; Cypher *et al*, 1998; Almberg *et al*, 2009; Sobrino *et al*, 2008), swift foxes (Miller *et al*, 2000), red foxes (Sobrino *et al*, 2008), wolverines (Dalerum *et al*, 2005), grey foxes (Riley *et al*, 2004), spotted hyenas (Harrison *et al*, 2004) and jackals (Shamir *et al*, 2001; Alexander *et al*, 1994).

As well as canids, it appears that other animals can be infected, including raccoons (Nettles *et al*, 1980; Kapil *et al*, 2010), giant pandas (Loeffler *at el*, 2007; Qin *et al*, 2010), porcupines (although it has not been stated whether it was CPV of FPLV) (Frelier *et al*, 1984), red pandas (Qin *et al*, 2007), skunks (Bakker *et al*, 2006), otters (Kimber *et al*, 2000), black bears (Dunbar *et al*, 1998; Madic *et al*, 1993), stellar sea lions (Burek *et al*, 2005), stone martens (Frolich *et al*, 2005), pine martens (Frolich *et al*, 2005) and brown bears (Marsilio *et al*, 1997).

Despite reports of natural CPV infections in raccoons, Appel *et al* (1982) and Barker *et al*, (1983) reported that CPV-2 was not infectious to raccoons in experiential infections; no reports are available on the newer CPV variants.

Most of the infections in wild animals are thought to be due to contact with domestic animals, or faeces from animals which live in nearby villages (Fiorello *et al*, 2004). Infection of CPV in red foxes is interesting, because of the isolation of a virus which appears to be an intermediate between FPLV and CPV, indicating that the species jump may have occurred via a wild animal, probably a fox (Truyen *et al*, 1998). Most work involving wild animals has involved CPV in wolves, and shows that the original CPV-2 as well as the newer variants may be responsible for a rapid decrease in wolf populations, probably due to deaths of young pups (Mech *et al*, 1993, 1995).

FPLV as a risk to wild animals

In a situation similar to CPV, antibodies to FPLV have been found in many different members of the felid species, including free ranging Florida panthers (Roelke *et al*, 1993), Californian mountain lions (Paul-Murphy *et al*, 1994), European wildcats (Leutenegger *et al*, 1999), Eurasian lynx (Ryser-Degiorgis *et al*, 2005), African Lions (Hofmann-Lehmann *et al*, 1996; Spencer 1991, Driciru *et al*, 2006), Canadian lynx (Biek *et al*, 2002), Rocky Mountain cougars (Biek *et al*, 2006), Namibian cheetahs (Munson *et al*, 2004) and Geoffroy's cats (Fiorello *et al*, 2007)

FPL disease has been described in Asian palm civet (Demeter *et al*, 2009, Komolafe, 1986), Eurasian lynx (Wasieri *et al*, 2009), leopards (Johnson 1964), lions (Studdert *et al* 1973) and cheetahs (Valicek *et al*, 1993).

As well as members of the felid family, FPLV has been described in other animals including raccoons (Junge *et al*, 2007). As with CPV, this apparent positive result for FPLV may be due to cross reactivity from a virus related to CPV known as raccoon parvovirus.

Typing of viruses by VP2 gene analysis

A wealth of information exists on the levels of the various antigenic types (CPV- 2, 2a, 2b and 2c) circulating within certain countries, despite there being no clear clinical meaning to the different types. CPV-2 is thought to no longer circulate in the field, but is still contained in some live vaccines (Gore *et al*, 2005). In contrast, the distribution of CPV types 2a, 2b and 2c seem to differ across different regions of the world (Figure 1.7). Viruses can be typed by a variety of methods, including monoclonal antibodies, sequence analysis and restriction fragment digestions using the key coding changes described in table 1.2.

These studies are generally based on relatively unstructured sampling strategies of national collections and / or are limited to using only partial VP2 gene sequence analysis or typing by key amino acid substitutions (Decaro *et al*, 2005, Davies *et al*, 2008, De Ybanez *et al*, 1995) rather than the full VP2 (Yilmaz *et al*, 2005, Costa *et al*, 2005, Kapil *et al*, 2007).

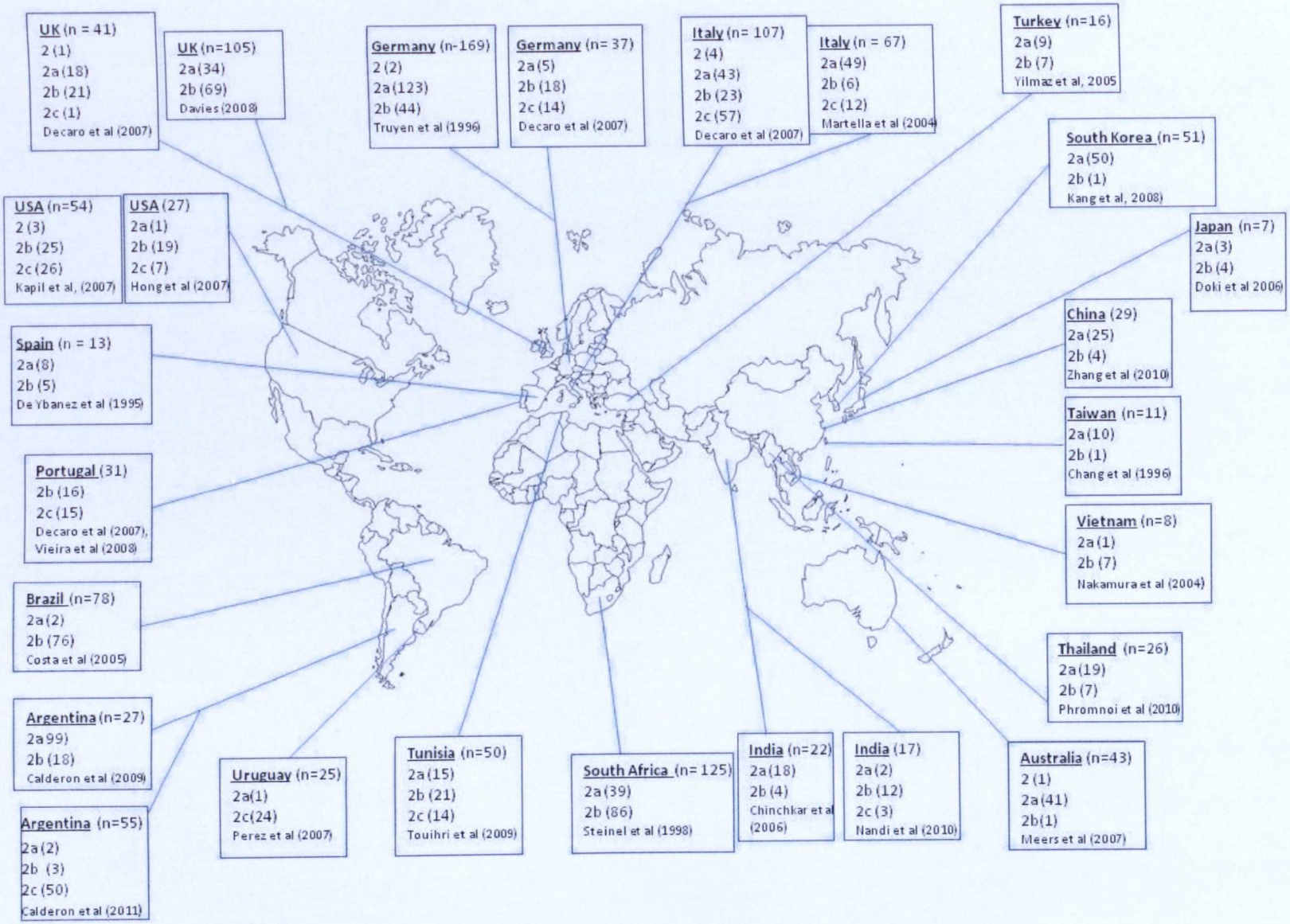
UK (n = 41)
2 (1)
2a (18)
2b (21)
2c (1)
Decaro et al (2007)
UK (n=105)
2a (34)
2b (69)
Davies (2008)
Germany (n-169)
2 (2)
2a (123)
2b (44)
Truyen et al (1996)
Germany (n= 37)
2a (5)
2b (18)
2c (14)
Decaro et al (2007)
Italy (n= 107)
2 (4)
2a (43)
2b (23)
2c (57)
Decaro et al (2007)
Italy (n = 67)
2a (49)
2b (6)
2c (12)
Martella et al (2004)
Turkey (n=16)
2a (9)
2b (7)
Yilmaz et al, 2005
South Korea (n= 51)
2a (50)
2b (1)
Kang et al, 2008)
Japan (n=7)
2a (3)
2b (4)
Doki et al 2006)
USA (n=54)
2 (3)
2b (25)
2c (26)
Kapil et al, (2007)
USA (27)
2a (1)
2b (19)
2c (7)
Hong et al (2007)
Spain (n = 13)
2a (8)
2b (5)
De Ybanez et al (1995)
China (29)
2a (25)
2b (4)
Zhang et al (2010)
Taiwan (n=11)
2a (10)
2b (1)
Chang et al (1996)
Portugal (31)
2b (16)
2c (15)
Decaro et al (2007),
Vieira et al (2008)
Vietnam (n=8)
2a (1)
2b (7)
Nakamura et al (2004)
Brazil (n=78)
2a (2)
2b (76)
Costa et al (2005)
Thailand (n=26)
2a (19)
2b (7)
Phromnoi et al (2010)
Argentina (n=27)
2a 99)
2b (18)
Calderon et al (2009)
Argentina (n=55)
2a (2)
2b (3)
2c (50)
Calderon et al (2011)
Uruguay (n=25)
2a(1)
2c(24)
Perez et al (2007)
Tunisia (n=50)
2a (15)
2b (21)
2c (14)
Touihri et al (2009)
South Africa (n= 125)
2a (39)
2b (86)
Steinel et al (1998)
India (n=22)
2a (18)
2b (4)
Chinchkar et al
(2006)
India (17)
2a (2)
2b (12)
2c (3)
Nandi et al (2010)
Australia (n=43)
2 (1)
2a (41)
2b(1)
Meers et al (2007)

Figure 1.7. *A summary of the reported distribution of CPV types around the world. Large gaps exist due to the areas where the virus has not been studied. Although sampling strategies, sampling sizes and methodologies vary, making it difficult to draw definitive conclusions, there is no obvious pattern of the distribution of the viruses, with 2a, 2b and in some cases 2c spread relatively widely around the world. The type 2 viruses in the studies are thought to be vaccine virus being shed.*

Clinical signs

Enteric form

Since the original worldwide epidemic, the majority of CPV infections have now become subclinical or produce only milder signs, particularly in older dogs, (Potgieter *et al*, 1981; McAdaragh et al, 1982), likely due to increased immunity, from previous exposure or vaccination in most animals (Walker *et al*, 1980; Carman *et al*, 1984; Jones *et al*, 1982). This said, CPV can still cause severe disease in young and adult dogs (Decaro *et al*, 2009; Hirayama *et al*, 2004). More severe disease tends to be most common in young pups, which have lost their maternally derived antibody but have not yet mounted a response to vaccination (Mockett *et al*, 1995). In experimental infections, severe signs of CPV may be difficult to reproduce, but can be made more severe with prior starvation before infection followed by re-feeding (Carman *et al*, 1982). In addition other infections such as giardiasis can increase the severity of CPV infection, presumably by increasing cell turnover in the gastrointestinal tract (Pollock, 1982).

Infection occurs via the faecal-oral route in a natural infection (Csiza *et al*, 1971b). The lymphoid tissues of the tonsils are infected first, which is followed by spread to other areas of the lymphoid tissue 2-3 days post infection in experimental cases (Carman *et al*, 1985; Carman *et al*, 1985b). Virus is then disseminated to the

gastrointestinal tract via the lymphoid tissues of Payers patches, and then infecting the epithelial cells and the villous crypts three days post infection. Infection of the small intestine coincides with viral shedding in the faeces (Pollock 1982).

In clinical infections, signs commonly consist of vomiting which is often noticed before severe diarrhoea (Azetaka *et al*, 1981; Nelson *et al*, 1979; Pletcher *et al* 1979). Diarrhoea tends to occur approximately six days post infection (Meunier *et al*, 1985), leading to severe dehydration (Parrish et al, 1980). Faeces often have a fetid foul odour (Pletcher *et al*, 1979), and may contain either blood (Nelson *et al*, 1979) and/or mucus (Carmen *et al*, 1982).

Pyrexia is common in most CPV infections (Carmen *et al*, 1982), and this usually coincides with the cessation of viremia and appearance of antibody (Pollock, 1982).

Weight loss is common in CPV infected dogs, due to enteric disease and decreased appetite / anorexia (Harcourt *et al*, 1980; Stann *et al*, 1984).

Leukopenia is common with both CPV and FPLV infection, FPLV usually causes panleukopenia, whereas CPV causes a relative lymphopenia and sometimes neutropenia (Parrish 1995; Stann *et al*, 1984).

The diarrhoeic fluid faeces are the main source of excretion of virus, and virus is typically shed in faeces from day 3 to 12 post-infection (Pollock, 1982; O'Sullivan *et al*, 1984), but rarely may last for up to 22 days (Carman *et al*, 1980).

Myocarditis form

Myocarditis is only described in pups under eight weeks of age which were infected either *in utero*, or immediately after birth (Robinson *et al*, 1980; Meunier *et al*, 1984), but is rare now due to vaccination and increased immunity in bitches

(Lenghaus *et al*, 1984). The mitotic rate of canine myocytes decreases rapidly after the first 2-3 weeks of life, which explains why infection of the myocardium and myocarditis is only described in pups under three weeks of age (Meunier *et al*, 1984), although clinical signs may show later.

In affected pups, death usually occurs without any clinical signs, or with a short period of respiratory distress, nasal frothing, gagging and listlessness (Burren *et al*, 1979). Due to this rapid death, it is generally not possible to diagnose CPV-induced myocarditis, and electrocardiogram results are often non-specific (Lenghaus *et al*, 1984), although tachycardia and arrhythmia have been reported (Carpenter *et al*, 1980, Robinson *et al* 1979).

Other unusual clinical manifestations

A dog presenting with severe necrotising vasculitis and encephalomalacia had CPV present in its brain, and it was suggested that this was causing the disease signs (Johnson *et al*, 1984).

CPV has also been described as causing infection of keratinocytes, which can lead to erythema multiforme, with skin ulceration, oral cavity vesicles and erythematous patches on abdomen and skin (Favrot *et al*, 2000).

In a case report of CPV infection causing myocarditis, mononuclear cell infiltration, cerebrum necrosis and focal haemorrhage in the cerebellum were also observed post mortem. These signs may be due to the viral infection, but may also be due to the myocarditis-induced ischemia and hypoxia (Agungpriyono *et al*, 1999).

Pathogenesis and Pathology

The pathology of parvovirus infection is closely linked to the viruses' requirement for rapidly dividing cells (Parrish, 1995) and falls into two main categories: myocarditis in neonatal pups and the enteric form, which is mainly observed in young pups but can also be in dogs of all ages.

Myocarditis

Parvoviral myocarditis, is usually recognised by pale streaks in the myocardium, and pups tended to die of severe left ventricular myocardial fibrosis, , or from severe, primary nonsuppurative myocarditis (Hayes *et al*, 1979; 1979b; Gagnon *et al*, 1980). Histopathological findings shows myocarditis characterised by the presence of basophilic (Mulvey *et al*, 1980, Robinson *et al*, 1980), or eosinophillic inclusion bodies (Hayes *et al*, 1979), degeneration and loss of cardiac myocytes, and areas of interstitial fibrosis (Meunier *et al*, 1984), with an infiltration of lymphocytes and plasma cells into the myocardium.

Gross pathological signs include pulmonary oedema, ventricle dilation, myocardium mottling, hydropericardium and fluid in the thorax and abdomen (Parrish *et al*, 1980).

Pulmonary oedema, haemorrhage and congestion are common, along with liver signs, such as fatty degeneration, congestion and coagulation necrosis (Jezyk *et al* 1979). The spleen, kidney and adrenal gland are congested, and the spleen and thymus show a reduction in lymphoid content. The intestine usually appears normal, without the enteric form of the disease (Jezyk *et al* 1979), but both have been reported together rarely (Van Rensburg *et al*, 1979, Hayes *et al*, 1979).

Some young dogs can survive the myocarditis which occurs within the first 2 months, but then died at five months of age due to congestive heart failure (Atwell *et al*, 1980). This is often due to the fibrosis caused by myocarditis.

Enteric disease

All four types of CPV (type 2,2a, 2b and 2c) are considered to cause severe enteritis (Decaro *et al*, 2006, 2007, Nandi *et al*, 2010, Davies *et al*, 2008, Vieira *et al*, 2008, Calderon *et al*, 2009) in susceptible animals. Moon *et al* (2008) reported that CPV-2a caused higher morbidity with increased severity of enteric disease than did CPV 2 or CPV-2b but CPV-2b caused higher mortality. The severity of leukopenia caused by CPV 2a was also shown to be more severe. This study did not test the pathogenicity of CPV 2c, and the report has not been substantiated elsewhere.

Initial reports suggested the newly emerged CPV-2c was associated with less severe disease than observed with the other newer variants, without hemorrhagic diarrhoea or vomiting observed (Decaro *et al*, 2005). However this now appears not to be the case (Vieira *et al*, 2008; Perez *et al*, 2007).

In addition, infection with CPV may not always be due to a singular type, as a dog was found to be co-infected with both a 2a and 2c variant (Battilani *et al*, 2007). In addition, there is some evidence from a study by Battilani *et al* (2007) that a quasispecies population may exist within one dog, similar to what occurs in mice infected with minute virus of mice (MVM) (Lopez-Bueno *et al*, 2003).

Infection is usually via the faecal-oral route, and immunofluorescence tests have shown that the viral antigen first appears within the tonsils, and then spreads via the lymph system to various other lymph organs including lymph nodes and spleen

within three days of infection (Meunier *et al*, 1985b). Four days post-infection antigen starts to appear within the small intestine, infecting the crypt epithelial cells of the duodenum, jejunum and ileum. As these tissues are rapidly dividing, they form the ideal environment for the virus to replicate, and histopathologically, a sign of this infection is the presence of intranuclear inclusion bodies (Parrish *et al*, 1980). Bone marrow and the liver also become antigen positive 4-5 days post-infection (Macartney *et al*, 1984).

The spread of virus around the body occurs by plasma viraemia, (i.e. mainly non-cell associated viraemia) which occurs from days 1-7 post-infection (Meunier *et al*, 1985).

The most common feature of CPV is necrosis of crypt epithelium, leading to the collapse and dilation of crypt lumina and villous atrophy (Nelson *et al*, 1979; Meunier *et al*, 1985; Miura *et al*, 1986). This is similar to the changes described in FPL and were thus used to suggest the link between the two diseases (Appel *et al*, 1979; Truyen *et al*, 1999). In severe cases of the disease, there is almost complete loss of crypts and villi but in milder or asymptomatic cases, less severe or no lesions are observed (Meunier *et al*, 1985).

Decaro *et al* (2007b) and Elia *et al* (2007) report the highest titres of virus being present in the organs with most severe pathology, such as lymphoid tissue (such as tonsil, reterophayngeal and mesenteric lymph nodes, thymus, bone marrow and spleen) and the jejunum, colon and rectum of the intestinal tract.

The lamina propria which is usually covered in normal intestine, can become inflamed in CPV-infected dogs (Macartney *et al*, 1984b). Despite severe intestinal changes, the stomach usually remains normal (Macartney *et al*, 1984c). Lymphocyte

numbers in lymph nodes and Peyer's patches in the intestine are greatly depleted with CPV infection. Lesions within the thymus may lead to partial or complete thymic atrophy 4-6 days post-infection (Meunier *et al*, 1985b). Lymphoid necrosis associated wtih a size alteration)either increase or decrease) may occur. The spleen may also become enlarged with possible hemorrhagic foci (Macartney *et al*, 1984c). In some cases, pulmonary and hepatic lesions may also occur. (Pletcher *et al*, 1979; Harcourt *et al*, 1980).

Bone marrow changes include alterations to the myeloid, erythroid and megakaryocytic cell line precursors, with the myeloid cell decreases particularly affecting neutrophils (Jacobs *et al*, 1980). Macrophages are often increased in numbers, and can be observed phagocytosing erythocytes and sometimes neutrophils. Bone marrow alterations may be due to a direct cytotoxic effect of CPV on bone marrow precursor cells, or due to the bacterial septicaemia which often results from infection (Boosinger *et al*, 1982). Other studies report hematopoietic cell depletion and lymphoid necrosis (Stann *et al*, 1984).

Because of the effects on the bone marrow and the lymphoid tissues, and the compromise of the small intestinal gut barrier, the risk of secondary infection is great, and can lead to the death of animals from septic shock (Goddard *et al*, 2008). Indeed several bacterial agents have been isolated from animals which died of CPV enteritis, including *Campylobacter* spp (Sandstedt *et al*, 1980), *Escherichia coli* (Isogai *et al*, 1989; Turk *et al*, 1990; Koutinas *et al*, 1998), *Clostridium perfringens* (Turk *et al*, 1992), Klebsiella, Enterobacter (Kreeger *et al*, 1984) and *Staphylococcus* spp (Koutinas *et al*, 1998). Other organisms isolated in CPV infected dogs include,

Haemobartonella canis (Gretillat 1981), canine distemper virus (Ducatelle *et al*, 1981) and Cryptosporidium (Denholm *et al*, 2001).

Regeneration of intestinal morphology and leucocyte counts typically occurs within nine days post-infection, and is almost complete by two weeks (Meunier *et al*, 1985).

Infection of pups and seronegative adults follows the above pattern. However, infection of neonatal pups can cause a generalised infection, allowing virus isolation from most organ tissues, and severe lesions are often present in the organs (Lenghaus *et al*, 1982)

Diagnostics

Tests for virus antigen

Acute, overt parvoviral disease usually requires rapid diagnosis, otherwise the result will arrive after the animal has succumbed to infection, or has recovered and been discharged.

Because of the ability of the virus to bind to sialic acid on erythrocytes, virus can be detected by haemagglutination (HA) and anti-CPV antibodies detected by haemagglutination inhibition (HAI) assays (Carmichael *et al*, 1980; Mathys *et al*, 1983) which are regarded as the gold standard test for CPV. However non-hamagglutinating mutants of CPV have been described, so other tests may be required (Parrish *et al*, 1988).

Early diagnostic methods used electron microscopy (Burtonboy *et al* 1979; Roseto *et al*, 1980), which is a slow, expensive, and insensitive test and the morphological similarities between CPV and MVC make differentiation difficult.

Virus isolation in cultured cells has also been used for diagnosis but requires viable virus and rapidly dividing cells to cause cytopathic effects (CPE). Slight CPE usually occurs, including rounding and clumping of the cells, and intranuclear inclusions may sometimes be visible (Joshi *et al*, 1998; Hirasawa *et al*, 1985). This method is susceptible to false negatives due to copro-antibody (i.e. antibody found within the gastrointestinal tract which is shed in the faeces) complexing with virus, and the tests can be contaminated, such as by trypsin containing parvoviruses of porcine origin (Hallauer *et al* (1971).

More recently, conventional polymerase chain reaction (PCR) has been used to detect CPV both in faeces and in paraffin preserved tissues (Truyen *et al* 1994: Hirasawa *et al* (1996): Ozkul *et al* (2002)). Variations of this test include a "touch down" PCR test (Schunck *et al*, 1995) and nested PCR, such as described by Hirasawa *et al* (1994), increase the sensitivity of viral DNA detection. However the clinical significance of PCR positive results is not necessarily clear, particularly after vaccination (Decaro *et al*, 2007c).

A benefit of PCR is that the product can be used for sequencing, or for restriction fragment length polymorphism (RFLP) (Sakulwira *et al* 2001; Greenwood *et al*, 1995), to type the virus and to allow differentiation of field viruses from some vaccine virus strains.

Using an internal control of ovine herpes virus, Decaro *et al* (2005b) developed a real time PCR (RT-PCR) for detection and quantification of CPV in faeces, further increasing diagnostic test sensitivity. However the specialised equipment required for this test sometimes, means it is not cost effective for diagnostic labs.

The use of RT-PCR has further been developed, by the use of minor groove binding probes, which allow for typing of the viruses, and differentiation of the field viruses from vaccine virus by using primers and probes which rely on single diagnostic substitutions within the viral genome (Decaro *et al*, 2005b, 2006, 2006b, 2006c). Although RT-PCR is very sensitive, it does not allow for genome sequencing of the product, and problems can be encountered with typing in this way due to other nucleotide substitutions (Calderon *et al*, 2009).

Several enzyme linked immunosorbent assays (ELISAs) have been developed which are rapid specific and sensitive tests which can be used in the veterinary surgery to detect all types of CPV (Lacheretz *et al* 2003; Esfandiari *et al*, 2000; Decaro *et al* 2009; Vieira *et al*, 2008). In addition, mouse monoclonal antibodies can also be used to distinguish the different variants of CPV, although to the authors knowledge these are not used in current diagnostic tests (Greenwood *et al*, 1996).

Due to the wide variety of tests available, questions have been raised as to which test is the best for diagnosis of CPV infection. Desario *et al* (2005) showed that both RT and conventional PCR were the most sensitive tests for virus, though such tests are not always readily available. Virus isolation and haemagglutination were less sensitive than either PCR test, and an immunochromatographic test was the least sensitive test examined. However, ELISA tests were shown to be more sensitive than haemagglutination for diagnosis, which allows use of the test in the clinic with reasonable certainty that the result is reliable (Mathys *et al*, 1983).

As CPV infection in dogs is commonly fatal, use of diagnostic tests at post mortem is useful. Nho *et al* (1997) reported an *in situ* hybridisation test which is used on tissues at post mortem. Immunohistochemistry using either CPV or related FPLV

probes is also used to detect CPV in fatal cases of the disease (Matsui *et al* (1993); McKnight *et al* (2007), Hullinger *et al* (1998)).

Tests for antibody

Haemagglutination inhibition tests (HAI) are often used to check for antibody to CPV, as titres of antibodies for CPV are known to be correlated with protection of infection (Pollock *et al*, 1982b). The HA test can also be used to detect CPV antibody of maternal origin which may interfere with vaccination (Olson *et al*, 1988). Any animal with an antibody titre over 1:80 is considered likely to be protected from infection (Wallace *et al* 1983, Pollock *et al*, 1984).

As ELISA tests have been used to detect viral antigen, others have been designed for antibody which can be used in the surgery, such as an indirect ELISA using monoclonal antibodies (Rimmelzwaan *et al*, 1991). This test, along with the one designed by Waner *at al*, (2006) specifically can be used prior to puppy vaccination, to test for residual maternally derived antibody which may prevent vaccination response.

Other ELISA's have been developed for detection of IgM which is indicative of recent infection with virus and thus can be used in a surgery, allowing for rapid treatment before appearance of clinical signs, or in early signs of infection (Waner *et al*, 2003, Florent 1986).

Increased sensitivity was achieved by Phukan *et al* (2005) by using an indirect ELISA rather than a sandwich ELISA and by using two monoclonal antibodies for both antigen and antibody detection (Rimmelzwaan *et al* 1990).

Epidemiology and transmission

Due to the ubiquitous nature of CPV and widespread vaccination, it is difficult to determine how prevalent the virus is within the dog population. Seroprevalence studies have shown that 60-100% of dogs tested are positive depending on the population sampled (Zupancic *et al,* 1987; Tennant *et al,* 1991; Helfer- Baker *et al,* 1980; McCaw *et al,* 1988; Levy *et al* 2008). In addition, 50% of wolves were shown to be seropositive for CPV despite no vaccination (Goyal *et al* 1986). However, as many dogs are vaccinated, (e.g approximately 70-80% of dogs in the UK (Gaskell *et al,* 2002) it is difficult to determine whether seropositivity is due to vaccination or exposure to a virulent field virus.

Sakulwira *et al* (2003) suggest that CPV is responsible for 63% of all canine diarrhoea at vet surgeries in Thailand. Indeed studies by Godsall *et al* (2010) (and see chapter three, this thesis), suggest that there is similar levels of CPV in severe enteritis cases presenting at the Peoples Dispensary for Sick Animals (PDSA) clinics within the UK. Nevertheless, despite high level vaccination, a number of reports indicate that infection and disease can still occur, particularly in situations where vaccination may be less common, such as shelters, charitable clinics, and also in young pups as they lose their MDA (Gooding *et al* (1982); Pollock *et al,* (1982b)). CPV infection tends to be more common in severe cases of enteritis (Godsall *et al,* 2010), but milder and subclinical infections may also occur (Potgieter *et al,* 1981, Walker *et al,* 1980).

Risk factors for CPV infection

There is evidence to suggest that breeds may play a part in genetic susceptibility of dogs to CPV. Glickman *et al* (1985) reported an increased risk of CPV infection in Rottweillers, Doberman Pinschers and English Springer Spaniels. Houston *et al* (1996) reported that, in addition to the above three breeds, American Pit Bull Terriers and German Shepherds were at increased risk of CPV infection, but Toy Poodles and Cocker Spaniels are at a lower risk of becoming infected with CPV. Houston also reported that intact males were more likely than females to be infected with CPV. No report of breed susceptibilities was reported in the recent study of severely diarrhoea in dogs from a largely unvaccinated population by Godsall *et al* (2010), despite the study including many high risk breeds including Rottweillers, and Staffordshire bull terriers. Dogs which had been neutered, and ,unsurprisingly, vaccinated dogs, were at less risk of presenting with CPV enteritis. Other factors such as young age, stress, intestinal parasites such as *Giardia* spp or helminth infections can worsen the prognosis for CPV and increase the severity of signs (Brunner *et al*, 1985).

Other reports have suggested that there may be an association between disease occurring and weather effects (Shakespeare *et al* 1999) or a seasonal influence with more cases observed between July and September, possibly due to increased socialisation and exercise during this period (Houston *et al*, 1996; Battersby *et al*, 2006; Sakulwira *et al*, 2003).

Treatments

Diarrhoea and vomiting caused by CPV lead to dehydration, particularly in young pups (Battersby *et al*, 2006). The treatments are usually supportive, consisting of fluid therapy and electrolyte supplementation to prevent dehydration (Battersby *et al*, 2006). Serotherapy with hyperimmune CPV sera has also been used for treatment of CPV enteritis (Ishibashi *et al*, 1983).

Food and liquid is usually withheld until vomiting and diarrhoea have ceased, to decrease intestinal cell turnover rate (Carman *et al* 1982; Carlson *et al*, 1977). However Mohr *et al* (2003) suggest that early enteral nutrition allow dogs to show earlier clinical improvement.

Recombinant feline interferon (type omega) is a new treatment which has been shown to improve clinical signs and decrease CPV mortality (Martin *et al*, 2002; Minagawa *et al*, 1999; De Mari *et al*, 2003). Interferon works by displaying antiviral, antiproliferative and immunomodulatory effects against viruses (Adolf, 1995), as well as enhancing the cellular immunity of normal dogs (Kuwabara *et al*, 2006), and stimulating antibody production, leading to increased immunity duration (Paltrinieri *et al*, 2007). Interferon treatment has also been shown to be useful in cats, for treatments of viruses such as FPLV, calicivirus, and coronavirus (infectious peritonitis) (Mochizuki *et al*, 1994).

Recombinant human granulocyte colony stimulating factor (rhG-CSF) has also been used for treatment of canine parvovirosis. However, Rewerts *et al* (1998) and Mischke *et al* (2001) report that rhG-CSF does not decrease hospital stay, or alter the neutrophil count associated with CPV, so questioning its use for treatment.

Antiemetics are commonly used for controlling vomiting in CPV infections (Mantione *et al*, 2005), with Metoclopramide being the most commonly used (Mantione *et al* 2005).

As secondary infections due to gram negative bacteria and endotoxins are major contributing factors for death in parvovirus infected animals, anti-endotoxin antisera has been used successfully to reduce mortality (Dimmitt, 1991). Recombinant bactericidal/ permeability- increasing protein has shown no effect against parvovirus infection (Otto *et al*, 2001).

Treatments usually involve a cocktail of drugs including antibacterials to reduce secondary infections, anti-emetics to prevent vomiting and IV fluids to prevent dehydration for maximal survival.

Immunity and maternally derived antibody

There is good correlation between antibody titre and protection from CPV infection. Pollock *et al* (1982b) reported that a serum haemagglutination inhibition titre of 1:80 post-vaccination or post-challenge was protective against CPV clinical signs, plasma viraemia (Meunier *et al*, 1985), and virus shedding. It has been reported that there was prevention of virus replication in the pharynx (the first known infection site for the virus) after successful vaccination (Eugster, 1980; Wallace *et al* (1985, 1985b).

In a more recent study, a haemagglutination inhibition titre of 1:80 was shown to prevent signs, but still allowed virus shedding and an increased antibody titre indicative of active infection. Titres of 1:160 still allow the virus to replicate to low titres and for a shorter period than lower titres of antibodies (Decaro *et al*, 2005c).

Dogs which develop local gut mucosal antibody often have a more favourable outcome suggesting that local antibody production is more important than humoral antibody for CPV resistance (Rice *et al*, 1982).

CPV disease tends to occur in younger animals at the stage at which their MDA has declined to levels which are too low to provide resistance to infection but sufficient to suppress the response to vaccination (O'Brien, 1994). This is known as the immunity gap (McCaw *et al* 2006). The levels of MDA in the serum of a puppy depends on the amount of antibody received from colostrum during nursing, and this in turn depends on the antibody levels in the dam, and also the litter size: antibody levels in the dam are in turn related to vaccination status and possible exposure to virulent virus (O'Brien, 1994). The puppy usually receives antibodies which are equivalent to about 50% of the dam's titre, which decay with a half life of around 9.5 days (Pollock *et al*, 1982).

The failure of a small number of dogs to develop an adequate immune response to vaccination is usually due to MDA, and much attention has been paid to development of vaccines which can overcome MDA and allow earlier vaccination and thus protection. In general, MDA declines to non-interfering levels by 10-12 weeks of age but can persist for up to 16 weeks or more in some cases (Gooding *et al*, 1982; Decaro *et al*, 2005c; Hoare *et al*, 1997; Buonavoglia *et al*, 1994), and it is reported that a MDA titre of 1:10 will prevent vaccination response but not infection (Pollock *et al*, 1984). However, this is likely to vary for different vaccines and vaccine types.

As for most viral infections, IgM is produced rapidly post infection with parvoviruses, but decays to undetectable within 20 days of infection. IgG is produced at a similar rate and time, but remains elevated for long periods after infection with minimal decay (Nara *et al*, 1983). Post-infection, immunity from IgG is thought to be life-long; but this has never been proven (Carmichael *et al* 1983; Bohm *et al*, 2004; Schultz *et al*, 2006). Secretory antibody IgA has also been reported in the small intestine and faeces, and if not present, then the disease usually lasts longer and is more severe (Brunner *et al*, 1985; Rice *et al*, 1982).

Early studies suggested that CPV vaccination and infection were immunosuppressive, mainly due to lymphopenia causing direct suppression of lymphocytes (Kesel *et al*, 1983). Indeed, Krakowka *et al* (1982) reported that a dog infected with CPV shortly after receiving a modified live virus (MLV) trivalent vaccine developed canine distemper encephalitis, due to the immunomodulatory effect of the CPV immune response. Mastro *et al* (1986) and Miyamoto *et al* (1992) reported that CPV vaccination caused a decrease in lymphocyte blastogenesis, which was of short duration but was present for all vaccination administration. This only occurs in some animals, with other animals showing no such immunomodulatory signs.

Despite these reports, Phillips *et al* (1987) reported that neither vaccine nor virulent field virus are immunosuppressive despite development of a transient leukopenia. It was thus concluded that the immune system is not suppressed by CPV, and therefore does not play a role in the disease pathogenesis.

Vaccination

Due to the severity of CPV disease, there was an urgent need for vaccines to be developed. The development of vaccines for CPV protection was rapid, and progressed from heterologous vaccines (FPL and MEV), to killed CPV vaccines and finally to highly effective modified live virus (MLV) vaccines.

Heterologous vaccines

Several studies have reported that the antibody titres generated in dogs vaccinated with either inactivated, or FPL MLV vaccines were generally of low levels and short duration, lasting only a few months (Chapek *et al* 1980; Thompson *et al*, 1988; Wierup *et al*, 1982; Gordon *et al* 1982). Antibody titres in dogs vaccinated with a MEV vaccine were shown to be of even shorter duration (Carman *et al*, 1982b). Thus dogs needed to be revaccinated relatively frequently (i.e. every six months to one year) to maintain some level of protection. Pollock *et al* (1983) found that antibody levels would develop more quickly and last for up to one year, if higher titres of FPL virus (up to $10^{5.7}$ $TCID_{50}$) were used for dogs compared to those used in cat vaccines.

Despite the fact that FPL vaccines were not very effective against CPV in dogs, they were the only method of protection available when CPV first emerged and so were useful at the time until other vaccines were developed.

Killed CPV vaccines

In order to try to increase antibody tires in dogs, killed CPV vaccines were developed, and several reports indicate that these vaccines were more efficacious in dogs than the previously used FPLV vaccines, providing immunity for over one year (Smith *et al*, 1986; Wallace *et al*, 1985; Povey *et al*, 1982; Pollock *et al*, 1982c). Despite the benefits of longer duration and higher antibody titres, problems still existed in overcoming residual MDA, and slow increase in antibodies, preventing early socialisation of puppies (Wallace *et al*, 1985b). In addition, the chemicals used to produce the killed vaccines left the possibility that there could be serious adverse clinical reactions (Eugster 1980; Wallace et al, 1985).

Modified live CPV vaccines

Modified live virus vaccinations are highly immunogenic and safe when used alone, or in combination with other antigens, including canine distemper virus, canine adenovirus, canine parainfluenza and *Bordetella bronchiseptica* (Kennel cough) (O'Brien *et al*, 1994; Miyamoto *et al*, 1995; Abdelmagid *et al*, 2004; Mouzin *et al*, 2004; Gore *et al*, 2005). Despite relative efficacy compared to earlier vaccines, there are still three major issues which are currently being addressed: the ability to overcome low levels of maternally derived antibody and reduce the immunity gap, the increased duration of immunity (DOI) and cross protection from different vaccines to all three field virus types (2a, 2b and 2c) and the ability of these viruses to prevent viral shedding

A range of studies have shown that MLV vaccines can overcome residual MDA, at antibody titres of 1:64 (Burtonboy *et al*, 1991) and at 1:160 (Buonavoglia *et al* 1994;

Martella *et al* 2005) allowing vaccination of pups to occur earlier. In order to overcome low levels of MDA, higher titre MLV vaccines were developed (Hoare *et al* 1997, O'Brien *et al*, 1986), allowing vaccination of puppies to occur earlier.

Some MLV vaccinates shed virus (Carmichael *et al*, 1981), which can interfere with diagnostic tests, and may lead to a slight risk of reversion to virulence of vaccine virus in the dog population. However, in the licensing process for vaccines, a number of studies are carried out to test for such a possibility including back passage studies in experimental dogs.

Current arguments suggest that as a population, we may be vaccinating our dogs and cats more often than is necessary (O'Driscoll et al, 2010), so much effort has been concentrated on increasing the DOI from vaccines. Abdelmagid *et al* (2004) reported that the three above mentioned core viral antigens had DOI of four years for the Schering Plough CPV 2b vaccine. Gore *et al* (2005) report a three year duration of immunity for Intervet's type 2 trivalent vaccine. As these animals are kept in total isolation, it is probable that vaccination DOI is longer in the field due to the likelihood of environmental challenge.

Studies by Bohm *et al* (2004) showed that 95% of 144 dogs which presented at veterinary clinics in England which had not been vaccinated for between three and 15 years still had protective titres against CPV (although lower percentages were protected against canine distemper virus (CDV) and canine adenovirus (CAV). In addition, similar high titres were observed in animals presenting at clinics for revaccination in Nigeria (Eghafona *et al*, 2007), and in the USA (McCaw *et al*, 1998). This suggests that the vaccine induced antibody may persist well in the dog population without re-vaccination but it is also possible that natural boosting

against CPV also occurs, although it appears that CDV and CAV natural challenge was low.

Due to the recent emergence of three antigenic types (2a, 2b and 2c), concern has been raised about the effectiveness of cross protection in the currently available vaccines, particularly type 2 based vaccines. Vaccine companies have tested their vaccines to confirm that they are protective against all known types (Spibey *et al*, 2008, Appel *et al*, 1987).

Despite this, recent reports (Decaro *et al*, 2009) have suggested that type 2c, particularly in Italy was able to cause disease in older animals which had been fully vaccinated (both as puppies and annually boosters) with a CPV type 2 vaccine.

Pratelli *et al* (2001) studied the responses of dogs to type 2 and 2b (the main vaccine types) to both heterologous and homologous challenge. Vaccination with a type 2 based vaccine followed by homologous challenge (which is no longer observed in the field) lead to a higher neutralising antibody titre, than when challenged with a type 2b virus. This suggests that the immunity induced by a type 2 vaccine is not as strong as that induced by a type 2b vaccine

In conclusion, MLV vaccines against CPV have been shown to highly effective biological products with good efficacy and long DOI, high seroconversion rates and high antibody titres. The issue now regards the level of protection offered by type 2 and 2b vaccines in heterologous and homologous challenges.

Aims of thesis

The overall aim of this thesis was to investigate the evolution of canine parvovirus, a severe gastrointestinal pathogen of dogs. More specifically the aims of the thesis are

- To develop a highly sensitive and specific PCR assay to encompass the entire VP2 gene in order to investigate virus evolution
- To investigate the phylogenetic relationships and spatial dynamics of CPV viruses which currently circulate within the UK using sequence analysis of the VP2 capsid gene.
- To further examine phylogenetic relationships and spatial dynamics of these viruses using the non-structural (NS-gene): a gene of paramount importance to the virus, but often not investigated
- To examine the evolution of CPV within the UK over the thirty years since its emergence and to ascertain if 2-2a intermediates exist.
- To determine the risk which asymptomatic cats may play in the transmission of CPV to dogs
- To obtain full genome sequences using next generation sequencing (454) of CPV 2 and 2a viruses to try and expedite sequence of the terminal hairpin regions

Chapter 2

Development of a PCR assay suitable to amplify full capsid gene to allow for sequencing of amplicons for virus typing

Introduction

Definitive and rapid diagnosis is particularly important for diseases which carry high morbidity and mortality such as canine parvovirus (CPV). Of all canine gastrointestinal viruses, CPV is the main one which requires prompt correct diagnosis, due to its severity and persistence in the environment (German *et al*, 2001). In addition to diagnosis, viruses can also be typed into four different antigenic types (CPV-2, 2a, 2b and 2c) using key amino acid residues, to ascertain the epidemiology of CPV within a country (Yilmaz *et al*, 2005; Costa *et al*, 2005; Kapil *et al*, 2007; Decaro *et al*, 2005; Davies *et al*, 2008; De Ybanez *et al*, 1995). For this reason, studies on typing and virus evolution target the VP2 gene (Costa *et al*, 2005; Phrimnoi *et al*, 2010; Kang *et al*, 2008; Hong *et al*, 2007).

As previously discussed (Chapter 1), many different diagnostic techniques have been used to diagnose CPV infection, ranging from the rapid in house ELISA test kit used in veterinary surgeries (Esfandiari *et al*, 2000; Rimmelzwaan *et al*, 1991), to the more complicated and sensitive laboratory diagnostic tests such as PCR (Decaro *et al*, 2005b, 2006, 2006b, 2006c). Real time PCR is a highly sensitive test, it requires specialised lab equipment which is expensive, making it unsuitable for routine diagnostics. Studies by Desario *et al* (2005) showed that PCR was the most sensitive test available for the detection of CPV in faeces, but that ELISA tests were also

generally sensitive, more so than haemagglutination and virus isolation. Desario *et al* (2005) and Decaro *et al*, (2005) used minor grove binding probes for diagnostics by real time PCR which rely on specific single substitutions. However, some viruses have failed to type using this method (Calderon *et al*, 2009).

The sensitivity of PCR can be a negative point for use in diagnostics, for example, after vaccination with some modified live vaccines against CPV, small amounts of vaccine virus can be shed in the faeces (Pollock 1982). This would lead to a positive PCR result, which could then be interpreted incorrectly. It is therefore important that the vet interprets the results in addition to the clinical signs presented by the animal.

The gold standard diagnostic test for CPV from faeces has been haemagglutination (HA) (Carmichael *et al*, 1980) which utilises the ability of the virus to bind sialic acid on the surface of erythrocytes (Tresnan *et al*, 1995). A haemagglutination inhibition test is run alongside the HA test to confirm its specificity. Haemagglutination, along with virus isolation techniques have been used for diagnostics but, are affected by copro (faecal) -antibody, and also requires infectious virus (Joshi *et al*, 1998).

Diagnostics are often limited by the sensitivity (ability to detect small amounts of virus) and specificity (specific for one virus or family) of the test used. It was our aim here to develop an assay, which encompassed the entire VP2, which can be used reliably to screen samples, even those where there are only low levels of virus present. In addition, we also wanted to sequence VP2 amplicons to investigate the evolution and diversity of the viruses, which would not be possible with real time PCR.

Methods and results

Primer design- Methods

For this study, it was important to develop a PCR which could amplify the entire VP2 gene in one product to allow for sequencing of the full VP2 gene, enabling evaluation of the evolution of the virus. Therefore it was important to design two primers which were external to the VP2 capsid gene, to ensure that good high quality sequence was obtained for the full gene including both ends.

Primers were designed to flank the VP2 gene, using a range of previously published primers (Table 2.1) (Meers *et al,* 2007, Decaro *et al,* 2006, Hirasawa *et al,* 1996, Doki *et al,* 2006). Another pair of primers were also designed (Table 2.1) using a DNA alignment in Chromas Pro (Technelysium Pty Ltd) using 20 genomes which contained all VP and NS coding regions available in Genbank (Genbank accession numbers: M38246, AJ564427, X55115, FO11664, D26079, EU659116, EU659117, EU659118, EU659119, EU659120, EU659121, NC_001539, EU310373, AY742932, AY742934, AY742935, AY742936, M19296 and M38245). As feline panleukopenia (FPL) and CPV are both closely related, the primers were designed to be universal, and thus detect both viruses as they can easily be differentiated by sequencing if required. As any two combinations of primers were to be used together, it was important that the annealing temperatures of both forward and reverse primers were similar, so both could be used in the same reaction at the same time (table 2.1).

Results

In total four forward and four reverse primers were either designed here or have been used in previous studies which could be used for PCR of the full VP2 gene. These are shown below in table 2.1

Primer name	Direction	Primer sequence	Position	Source
EF	Forward	GCCGGTGCAGGACAAGTA	2748-2765	This thesis
VP2 rev	Reverse	ATTTTCTAGGTGCTAGTTGAGA	4511-4532	This thesis
JS1F	Forward	AGCTACAGGATCTGGGAACG	2843–2862	Meers *et al* (2007)
JS2R	Reverse	CCACCCACACCATAACAACA	4799–4818	Meers *et al* (2007)
P33	Forward	ATGGCACCTCCGGCAAAGA	2286-2304	Decaro *et al* (2006)
P34	Reverse	TTTCTAGGTGCTAGTTGAG	4513-4531	Decaro *et al* (2006)
VP2F	Forward	ATGGCACCTCCGGCAAAGA	2285-2303	Hirasawa *et al* (1996)
VP2R	Reverse	CCAAGACTTGTAAATGCACC	4224-4247	Doki *et al* (2006)

Table 2.1. *Primers designed, or from other previous studies, which would allow for full VP2 amplification in a single PCR reaction. The positions of these primers as supplied by the publication from which they were extracted based on the full genome (Reed et al, 1988) are also shown in the table.*

Primer testing- Methods

To ascertain which primer set was best, each forward primer was tested with each reverse primer using two known HA positive CPV faecal samples (kindly supplied by Intervet Schering Plough), a CPV vaccine sample (Nobivac modified live CPV vaccine, Intervet Schering Plough- Type 2), and unknown CPV status faecal sample (sent to us by the PDSA), a horse faecal sample (as a faecal sample negative control) and

molecular water as an additional negative control. The DNA was extracted using a simple boil preparation (discussed below).

Each primer was tested using a gradient block with thermo-cycling temperatures suggested by the manufacturers of the master mix (Abgene), and the average annealing temperature suggested by the manufacturers of the oligonucleotide primers (MWG Eurofins) (table 2.1).

Results

No false positives occurred with the horse faecal sample or the water due to cross contamination by any other faecal material, such as E. coli. The CPV faecal sample and CPV trivalent vaccine always gave a band of the expected size (approximately 2000 base pairs expected), although the brightness of these varied when tested with all the primer combinations. This was using a high titre vaccine, so it was also of interest to test the primers using canine faecal samples of unknown titres.

When tested with canine faecal samples, six primer sets (Lane numbers: 3, 4, 7, 9, 15 and 19, Figure 2.1, table 2.2) did not give a band at the expected size and so were not used again. Another, six primer sets (Lane numbers: 6, 11, 14, 21, 22, 26, figure 2.1, table 2.2) produced only faint bands which would have proved difficult to sequence, and so were not analysed further. Five primer sets (Lane numbers: 2, 10, 12, 23 and 24, figure 2.1, table 2.2) produced a band at the expected size, but also had numerous other bands which could have lead to the contamination of the sequencing, and so were not used any further. Only lanes 5 and 17 (Figure 2.1, table

2.2) produced a single band at 2000 base pairs. The primers used in lane 5 (figure 2.1, table 2.2) gave the brightest product and were chosen to be the ideal primer set. Lane 5 used primers EF and JS2R, and were chosen for further testing for use in these epidemiological studies.

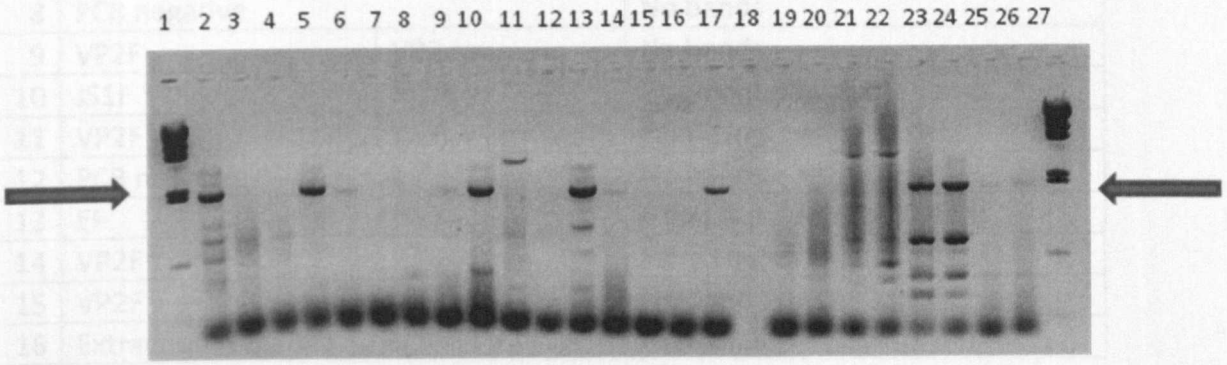

Figure2.1. *A 1% agarose gel stained with ethidium bromide of the known CPV positive sample only tested using different primer pairs tested to develop the PCR reaction. Lanes 1 and 27 are the Lambda HindIII DNA marker. The primers used for each lane are shown in table 2.2.*

Using this information (Figure 2.1 and table 2.2) the primer pair chosen were EF and JS2R as these gave high DNA levels, both through band brightness and nanodrop results (nanodrop reading of >300 g/ng), showed only one band in the lane, and were shown to be specific to CPV when sequenced.

Lane	Forward Primer	Reverse Primer	Results
1	Marker		
2	EF	VP2 rev	Good, but additional bands
3	JS1F	VP2 rev	No bands
4	P33	VP2 rev	No bands
5	EF	JS2R	Good, single band
6	P33	JS2R	Faint single band
7	JS1F	P34	No bands
8	PCR negative		No bands
9	VP2F	VP2 rev	No bands
10	JS1F	JS2R	Good but additional bands
11	VP2F	JS2R	Faint single band
12	PCR negative		No bands
13	EF	P34	Good but additional bands
14	VP2F	P34	Faint single band
15	VP2F	VP2R	No bands
16	Extraction Negative		No bands
17	JS1F	VP2 rev	Good, single band
18	Blank		
19	EF	VP2R	No bands
20	Extraction negative		No bands
21	JS1F	VP2R	Faint, with several bands
22	P33	VP2R	Faint, with several bands
23	P33	P34	Good, but additional strong bands
24	VP2F	VP2R	Good, but additional strong bands
25	PCR negative		No bands
26	JS1F	VP2R	Faint, with several bands
27	Marker		

Table 2.2.*Primer sets used for each of the above PCR products. The lane number corresponds to figure 2.1, with the primers used, and also a comment regarding the brightness of bands and number of bands per lane.*

Test of different PCR master mix- Methods

Two CPV positive faecal samples, extracted by boil preparation were used to test two different PCR master mixes using primers EF and JS2R chosen previously. A common PCR master mix which is used for products of around 500-1000 base pairs was compared to an extensor PCR master mix which is designed for long PCR

products of up to 40 kilo base pairs. The stated composition of the master mixtures are:-

Extensor PCR master mix (Thermo Scientific) – 1.25 units of total DNA polymerase, 2.25 mM MgCl, 350 uMdNTP's,75 mMTris HCL, 20 mM (NH4)2SO4 and 0.01% Tween 20.

PCR master mix (Thermo scientific) - 1.25 units thermoprime plus DNA polymerase of *Thermus aquaticus* origin, 75 mMTris HCL, 20 mM (NH4)2SO4, 1.5 mM MgCl, 0.01% Tween 20 and 0.2 mM of each dNTP (dATP, dGTP, dTTP and dCTP).

Results

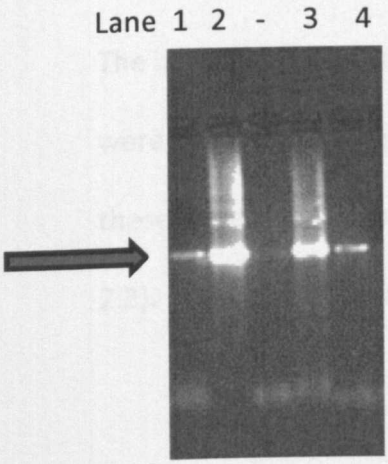

Figure 2.2. *A 1% agarose gel stained with ethidium bromide showing the brighter bands from the PCR using the extensor PCR master mix (lanes 2 and 3), rather than the common PCR master mix (lanes 1 and 4). The arrow indicates the size of the expected band. The minor bands which are also visible only appeared rarely and did not affect subsequent sequencing of the band*

The extensor master mix, gave the brightest band, suggesting maximal DNA level from a single PCR reaction (lanes 2 and 3 on figure 2.2). This was confirmed by the use of the nanodrop to confirm that the brightest band did contain more DNA than the less bright band (Data not presented). Therefore the extensor master mix was used throughout the rest of the experiments.

Primer temperature gradients- Methods

Using a CPV positive faecal sample the optimal primer temperature was estimated using EF and JS2R and the melting temperature provided by the oligonucleotide manufacturer with 5°C subtracted. This was used as the central point of a temperature gradient ranging from 47°C to 58°C.

Results

As can be observed (Figure 2.2), the lowest (47 and 48 °C) and highest temperatures (57-58 °C) did not show any band, possibly as the temperature did not allow specific primer binding. Weak bands were observed at temperatures 49 °C (lane 3, Figure 2.3), 50°C (lane 4), 55 °C (lane 9), and 56 °C (lane 10) figure 2.3). The brightest bands and the highest DNA concentrations as judged by nanodrop were observed at temperatures 52 °C and 53 °C (lanes 6 and 7), suggesting that these were the optimal temperatures for the primers to be used in the PCR (figure 2.3). A temperature of 52°C was thus used for the remaining PCR tests.

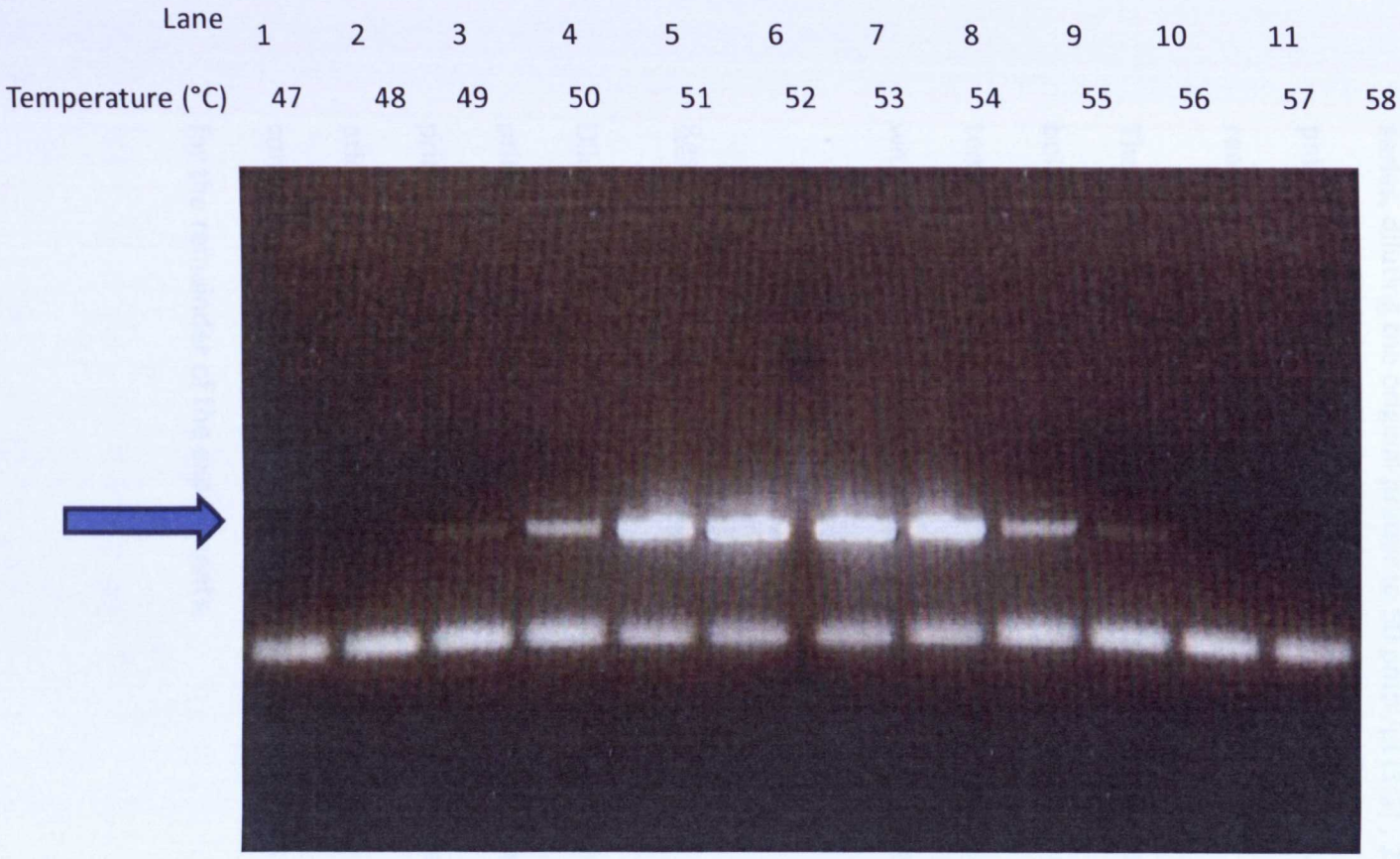

***Figure 2.3**. A 1% agarose gel stained with ethidium bromide showing the primer temperature gradient. The lanes are labelled with the temperature they were ran at. The arrow indicates the position of the expected band size (around 2000 base pairs).*

Primer dilution gradients - Methods

Primers were diluted upon arrival using molecular grade water to 100 pmol/µl, aliquoted and frozen at -20°C. One of these aliquots was used to produce a dilution series, diluting the original primer to 50 pmol/µl (1:2) , 25 pmol/µl (1:4), 12.5 pmol/µl (1:8), 6.25 pmol/µl (1:16) and 3.125 pmol/µl (1:32). In each 50 µl PCR reaction, 1.5 µl of each primer was used.

The PCR reaction used two known CPV positives and a vaccine virus extracted by boil prep, with a single negative sample for each primer set with an annealing temperature of 52 °C. The primer concentration which gave the brightest band, with least primer wastage was chosen for remainder of the studies.

Results

Dilution of PCR primers gave the expected result, with lots of unincorporated primers and nucleotides in the low dilutions, but this gradually decreased as the primers were diluted. Based on primer band brightness and amount of residual primer, the optimal dilution of the primers was found to be 1:8-1:32. A primer concentration of 1:8, equivalent to 12.5 pmol/µl (2.5µl in a 50 µl reaction) was used for the remainder of the experiments.

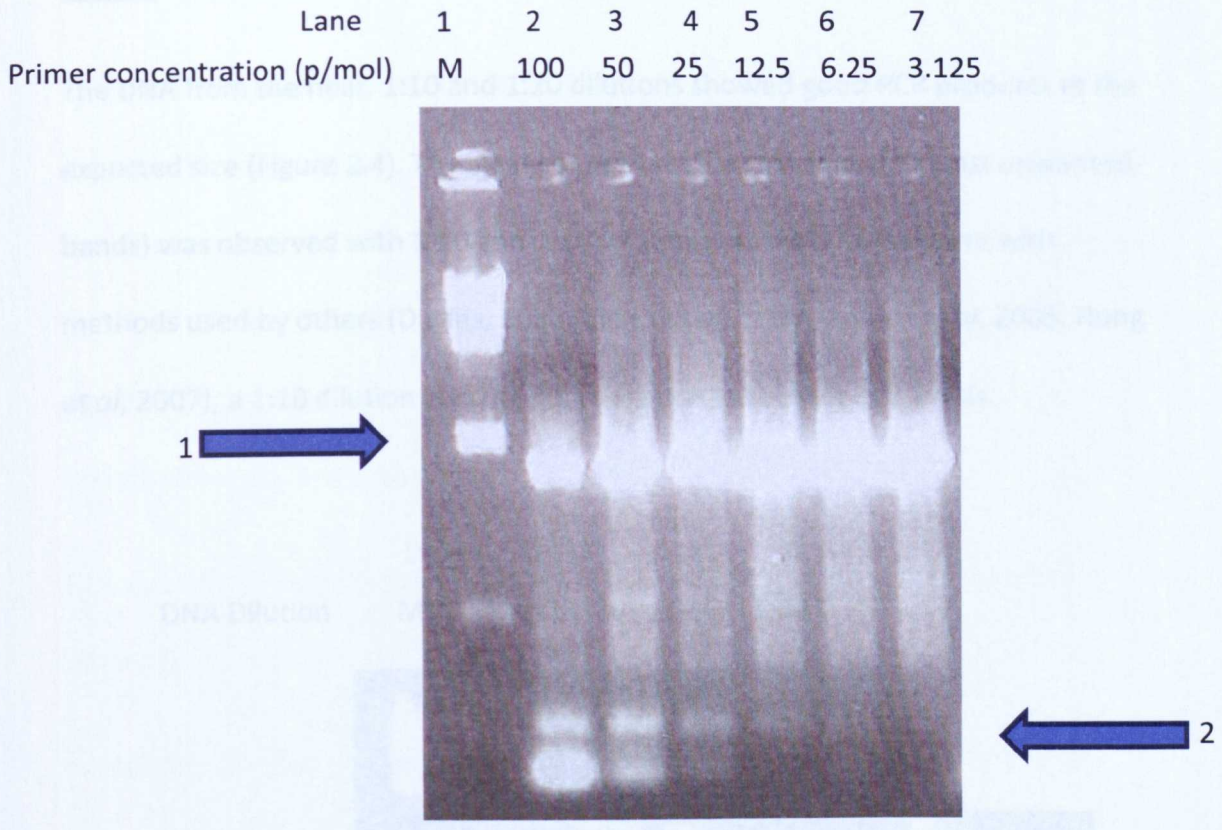

Figure 2.4. *A 1% agarose gel stained with ethidium bromide of the primer dilutions. The primer band, indicated by arrow 2 probably reflects unincorporated primers, which decreases as the primer is diluted. Arrow 1 shows the position of the expected product size (2000 base pairs). Gel loaded with the same sample, using different primer concentrations, shown with each lane*

DNA dilutions - Methods

Schunck *et al* (1995) suggested that CPV DNA extracted from faeces by boil preparation should be diluted 1:10 prior to use in PCR. To ascertain if this was true, DNA of unknown concentration was used as PCR template direct from the extraction process, at 1:10 as suggested by Schunck *et al* (1995), at 1:20 and 1:30.

Results

The DNA from the neat, 1:10 and 1:20 dilutions showed good PCR products at the expected size (Figure 2.4). The cleanest product (i.e. the one with least unwanted bands) was observed with 1:10 and 1:20 dilutions. In order to conform with methods used by others (Davies, 2008, Vieira *et al*, 2008, Decaro *et al*, 2006, Hong *et al*, 2007), a 1:10 dilution was used throughout the rest of this thesis.

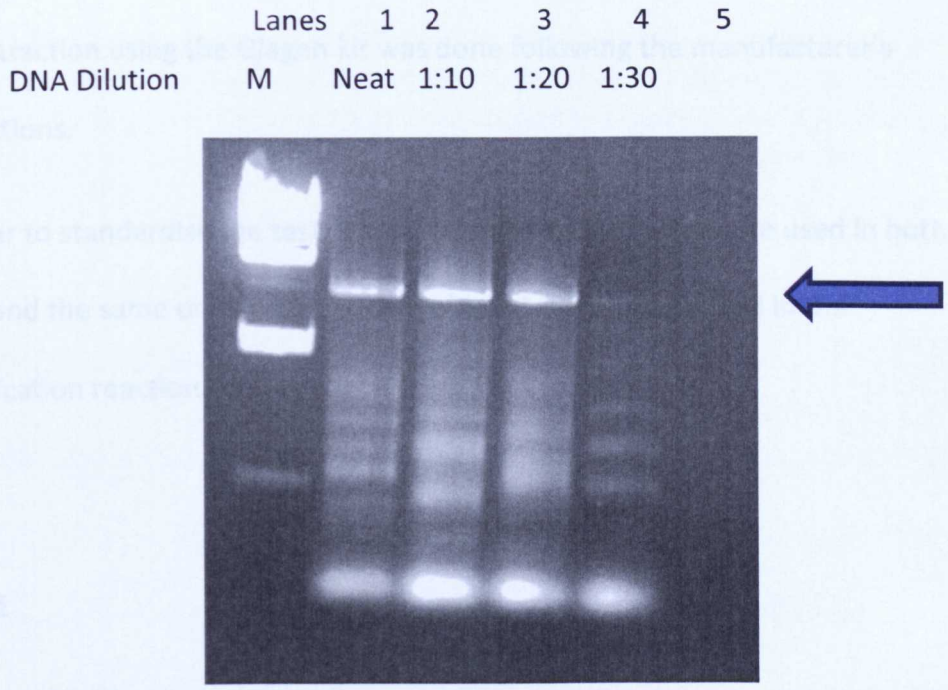

Figure2.5. *A 1% agarose gel stained with ethidium bromide for the dilution of the DNA. The dilution of the DNA in each lane is indicated. The arrow indicates the position of the expected band (approximately 2000 base pairs).*

DNA extraction - Methods

Two methods were tested for viral DNA extraction, a simple boil prep reaction, or use of a Qiagen kit (QiaAMP DNA Stool kit). For the boil prep reaction, samples were homogenised (10% W/V) in phosphate buffered saline (PBS) and centrifuged for 15 minutes at 9300 rpm. The supernatant was boiled for 15 minutes chilled on ice, then centrifuged again as before for 5 minutes. The supernatant was removed, and stored at 4°C until use.

The extraction using the Qiagen kit was done following the manufacturer's instructions.

In order to standardise the test, the same volumes of faeces were used in both tests, and the same overall percentage of 'raw' faeces were used in the amplification reaction.

Results

Using the PCR developed here, the two DNA extractions were tested. It was shown that maximal DNA was obtained from the boil preparation method (figure 2.6, lane 2), rather than the Qiagen QiaAMP DNA Stool kit (figure 2.6 lane 1), The Qiagen QiaAMP mini kit produced a cleaner colourless sample, whereas the boil preparation method gave a light brown sample, which could potentially inhibit or contaminate PCR reactions

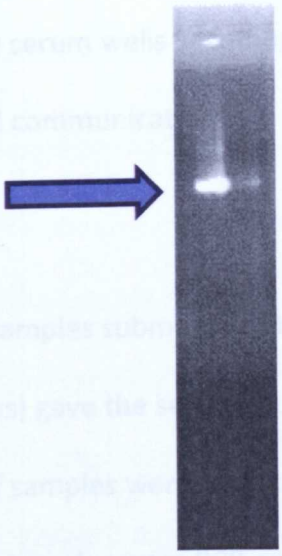

***Figure 2.6**. A 1% agarose gel stained with ethidium bromide showing a brighter band produced by the boil prep extraction method (lane 2). Although there is an additional band in lane 2 it is not sufficient to lead to a false diagnostic reading, or to interfere with the sequencing protocol, and does not appear every time*

Comparison of PCR to haemagglutination- Methods

Faecal samples which were collected from clinically ill animals, (chapter 3; Godsall *et al*, (2010) for sample information), were submitted directly to a commercial laboratory by the veterinarian for a routine faecal diagnosis of CPV using haemagglutination/ haemagglutination inhibition tests (HA/HAI). Methods for the HA are as described previously (Carmichael *et al,* 1980). In addition, a HAI was run alongside the HA to confirm the HA was CPV specific. The HA results from the commercial laboratory were compared to the long range PCR designed here using primers EF and JS2R. The commercial laboratory report results as either positive or negative, with no report of titres. For a positive result to be reported there should be haemagglutination of red blood cells at two or more dilutions higher in the

negative serum wells in comparison to the positive serum (Laboratory personnel, Personal communication).

Results

Of 411 samples submitted, 294 samples (comprising 143 HA positives and 151 HA negatives) gave the same result when tested by PCR or HA (table 2.4). However 117 (29%) of samples were reported as negative by the HA test, but were positive by PCR. No samples were PCR negative but HA positive suggesting that the HA/ HAI test carried out by the commercial lab is specific.

	PCR +	PCR -	Total
HA+	143	0	143
HA -	117	151	268
Total	260	151	

Table 2.4 The number of samples which were PCR positive and negative, compared to HA positive and negative results reported by the commercial laboratory. In total, 117 results were reported incorrectly as negative by a HA test, which were later found to be PCR positive.

Using these results, it can be observed that the PCR test and HA results agree in 72% of cases (i.e. HA positive and PCR positive, or vice versa) but disagree in 28% (i.e. PCR positive but HA negative). These results indicate that the HA test is specific for parvovirus as no samples were HA positive but PCR negative, but also suggests that the HA test is less sensitive that the PCR test developed here, with the PCR test detecting parvovirus in 28% (n=117) of cases.

Development of internal primers for sequencing- Methods

Internal sequencing primers were designed to give full, double stranded coverage of the VP2 gene, in addition to the external primers used for the PCR. As the average sequencing read is around 800 base pairs, it was important to make sure each product overlapped, and would not be more than 750 -800 base pairs in length. The internal primers were developed using the same alignment for the external primers, developing the primers 800 bases apart (approximately) in regions which were conserved in all the viruses.

The amplicons from the EF and JS2R primers were sent for sequencing using 6 primers (including the two external PCR primers). The sequence of the four internal primers, along with the two PCR primer sequences and positions within the genome are shown below in table 2.5.

Primer	Position	Sequence	
EF	2748-2765	GCCGGTGCAGGACAAGTA	PCR primer
ER	3581-3558	TGTTCCTGTAGCAAATTCATCACC	Internal
MF	3441-3466	TACCATCTCATACTGGAACTAGTGG	Internal
MR	4140--4118	GTATAGTTAATTCCTGTTTTACCTCC	Internal
555F	4003-4022	CAGGAAGATATCCAGAAGGA	Internal
JS2R	4818-4799	CAACCCACACCATAACAACA	PCR primer

Table 2.5. *Position and sequences of the six primers used for the sequencing of the full VP2 gene. EF and JS2R (Meers et al, 2007) are the primers used for the PCR reaction, and the remaining primers are internal, allowing for full double stranded sequencing of the VP2 gene.*

Results

The results of assembling the six sequences into a single consensus sequence are shown below (Figure 2.7). As predicted, each sequence was overlapped to produce a final amplicon of 1755 base pairs, covering the full VP2 sequence, with complete coverage of both strands.

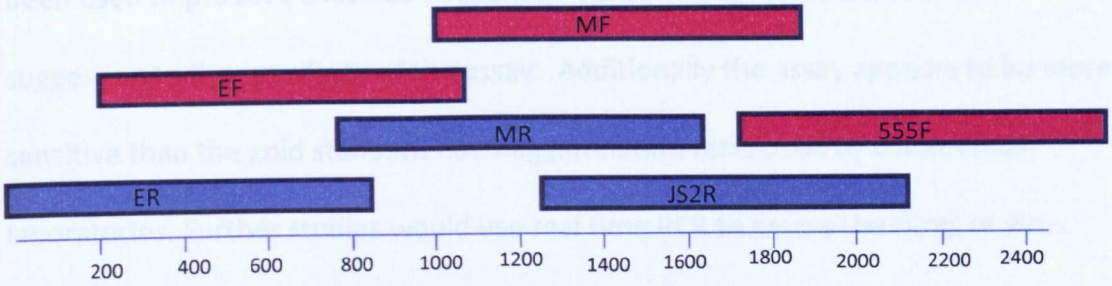

Figure 2.7. *A schematic diagram of the sequencing primers used for sequencing of the VP2 gene of CPV. The entire gene is sequenced in both directions, and the gene does not start until full good quality double stranded sequence is available. Red blocks indicate sequence generated using forward primers, whereas blue blocks indicate sequence generated using reverse primers.*

Discussion

PCR has been used in many studies to detect canine parvovirus (CPV) (Doki *et al*, 2006, Pereira *et al*, 2000, Perez *et al*, 2007, Hirasawa *et al*, 1994) with varying success. This study has developed a PCR assay which was specific for both CPV and FPLV (the latter shown in chapter five), in faecal samples. Using two primers, one developed in this study (known as EF) and one from a study carried out by Meers *et al* (2007) (known as JS2R), a PCR which can amplify the full VP2 gene was developed. To allow for full double stranded sequencing, four internal primers were also developed. This appears to be a good quality, reliable PCR assay which was

used successfully for detection of CPV and FPLV VP2 from tissues (chapter 3), faecal samples and vaccines.

The PCR developed here appears to be specific, as samples which were positive for canine coronavirus, canine distemper virus, canine adenovirus, *E. coli, Campylobacter* spp or *Salmonella* spp showed no false negatives. This assay has been used to produce over 500 sequences from different samples, further suggesting to the specificity of the assay. Additionally the assay appears to be more sensitive than the gold standard haemagglutination tests used by commercial laboratories. Further studies would use real time PCR to assess the titres of virus which can be detected by the conventional PCR assay.

The long range 'extensor' PCR master mix was shown to give a higher DNA concentration than the conventional PR master mix. The concentration of the primers at 12.5 pmol/ ul allowed for significant dilution of the primers at an optimal temperature of 52-53 °C as discovered in the gradient block. .

Despite the DNA used here being extracted by a crude boil preparation, it appears that this does not lead to any loss of DNA, which appears to occur when using commercial kits for extraction. This could potentially affect the diagnostics of low titre samples, which would be negative if a commercial kit was used, but positive by boil preparation reaction. A potential problem with extraction by a crude method is that potential inhibitors of PCR reactions which could be found in hemorrhagic faeces, including calcium and magnesium ions, bacteria, haemoglobin, and blood breakdown products such as heme (Moreira, 1998). These did not appear to cause any issues in this study.

The results from the HA/HAI test are a concern, not only to vets but also to epidemiologists as the prevalence of CPV may be being mis-represented. Of the results, 29% were reported as being negative by HA, when they were positive by PCR. Fortunately the diagnosis of CPV by commercial laboratories is only important for epidemiology and clinic hygiene as the result is likely to arrive after the animal has been discharged from the surgery or has succumbed to infection. Elisa tests can give a rapid result (within 10 minutes) in a veterinary surgery (Lacheretz *et al* 2003; Esfandiari *et al*, 2000, Decaro *et al* 2009; Vieira *et al*, 2008), which has been shown to be relatively reliable (Desario *et al*, 2005). This allows veterinarians to treat the animals with interferon if a positive Elisa result is returned (Martin *et al*, 2002; Minagawa *et al*, 1999; De Mari *et al*, 2003).

However the incorrect HA results may have been due to the presence of copro-antibody in the faeces which would prevent virus HA (Joshi *et al*, 1998). If the sample was taken late in an infection, then there may have only been little virus remaining, so the HA test may not be positive (Pollock 1982).

Desario *et al* (2005) reported that real time PCR, including minor groove binding probes such as that employed by Decaro *et al* (2005b, 2006b, 2006c) in a number of studies was more sensitive than conventional PCR such as that used here and elsewhere (Sakulwira *et al*, 2001, Vieira *et al*, 2008, Yilmaz *et al*, 2005). Desario *et al* (2005) also reported that both PCR tests were more sensitive than HA, ELISA, and virus isolation tests, suggesting that the PCR test should be offered by most laboratories for detection of parvoviruses. In this study we decided against using real time PCR as we wanted to investigate the evolution and diversity of the viruses,

which could only be done using conventional PCR and sequencing protocol. This method allows for typing of viruses easily, and also provides much more information regarding the virus diversity and evolution than simply typing with real time PCR. However it is possible that our PCR, though optimised, may be less sensitive than the real time PCR reported by Decaro *et al* (2005b).

Chapter three

Molecular epidemiology and phylogeny reveals complex spatial dynamics of in areas where canine parvovirus is endemic

Introduction

Sequence analysis has revolutionised our knowledge of the spatial and temporal dynamics of infection, allowing a greater understanding of the evolution and molecular epidemiology of pathogens. This is particularly important for rapidly evolving pathogens such as RNA viruses (e.g. feline calicivirus (Coyne *et al*, 2007), foot and mouth disease virus (Cottam *et al*, 2008), and influenza virus (Rambaut *et al*, 2008), and also for certain single stranded DNA viruses with high substitution rates, such as canine parvovirus (Shackelton *et al*, 2005).

Viruses are typed (as type 2, 2a, 2b or 2c) using key amino acid residues in VP2 (CPV2a - Met-87-Leu, Ile-101-Thr, Ala-300-Gly, Asp-305- Tyr and Val-555-Ile ; CPV2b – Asp-426-Asn and Ile-555-Val reversion; CPV2c – Asp-426-Glu) (Buonavoglia *et al*, 2001; Truyen *et al*, 1998; Martella *et al*, 2006*)*. Substitutions in VP2 have also been shown to influence host range (Parker *et al*, 1997), haemaglutination spectrum (Parrish *et al*, 1988) and affinity of receptor binding (Palermo *et al*, 2006).

Currently, the original CPV type 2 is thought not to circulate in the general dog population although it is present within certain live vaccines. The distribution of CPV types 2a, 2b and 2c seem to differ across different regions of the world (Decaro *et al*, 2007; de Ybanez *et al*, 1995; Greenwood *et al*, 1996; Decaro *et al*, 2005; Pereira *et al*, 2000; Sagazio *et al*, 1998; Wang *et al*, 2005). It has recently been suggested that the initial rapid global spread of CPV-type 2 was a feature of the

naïve dog population it gained access to, and is in contrast to the current more endemic phase of disease, where the international range of new strains is more restricted (Hoelzer *et al*, 2008).

Whilst these studies are collectively improving our knowledge of the spatial and temporal dynamics of CPV transmission, they are generally based on relatively unstructured sampling strategies of national collections and / or are limited to using only partial VP2 gene sequence analysis or typing by key amino acid substitutions (Costa *et al*, 2005; Davies 2008; Decaro *et al*, 2005; de Ybanez *et al*, 1995; Kapil *et al*, 2007; Yilmaz *et al*, 2005). In this study, we have used a cross sectional study of clinically ill dogs, and full VP2 sequence analysis, to investigate in depth the evolution and spread of virus at national and local levels, and in relation to the global situation, to specifically test the hypothesis that currently there is limited international spread of CPV.

Methods

Samples

Faecal samples were obtained from 25 People's Dispensary for Sick Animals (PDSA) Petaid hospitals across mainland UK (Figure 1) from 373 clinically ill dogs presenting with diarrhoea of unknown aetiology that required more than conservative treatment. Clinical information and signalment were supplied with the samples and presented elsewhere (Godsall *et al*, 2010). In addition, 16 CPV positive samples from 11 different locations within the UK (one post mortem sample, 12 from a

commercial diagnostic laboratory and three potential vaccine breakdowns), and viruses from the six commercial vaccines currently used within the UK (coded A-F), were also obtained. All samples and vaccines were collected over a two year period (2006-2008). Samples were stored at -80 C until used.

Amplification and sequence of VP2

Primer design and sequence of the two PCR primers used here to amplify full VP2 gene are discussed previously (Chapter 2 page 66). DNA was extracted as described previously (Decaro *et al*, 2005; chapter 2 page 79).

DNA amplification was carried out in 25 µl reactions, consisting of 12.5 µl extensor PCR master mix (Abgene), 8.5 µl of molecular water (Sigma), (12.5 pmoles of primer EF, 12.5 pmoles primer JS2R) (Meers *et al*, 2007) and 2 µl DNA. Negative controls were processed alongside faecal samples throughout all stages (1 negative control (water or PBS) per 2 faecal samples). All samples were processed in order of arrival at the laboratory and not batched according to location. The PCR cycling conditions were 1 min at 94°C, followed by 40 cycles of denaturation at 94°C, annealing at 52°C and extension at 72°C, followed by a final extension phase of 68°C for 10 minutes.

Amplicons were purified using Qiaquick PCR purification kit (Qiagen) according to manufacturer's instructions. Sequencing of full VP2 was generated using internal sequencing primers, which are discussed earlier (chapter 2 page 79). All sequences were aligned into a double-stranded consensus sequence using Chromas Pro 1.41

(Technelysium Pty Ltd). All external primer sites were removed, giving a final consensus sequence of 1755 base pairs. All sequences were homozygous, with no evidence to suggest dual or mixed infections.

Sequence analysis

Consensus sequences were aligned by Clustal W. These alignments included sequences available for feline panleukopaenia virus (Genbank accession number M24004), and also included published sequences for two CPV type2 (M23255 and U22186). The most appropriate evolution model was predicted using MODELTEST as implemented in Topali (Milne *et al*, 2008). The final model for nucleotide substitutions chosen was the TrN (Tamura-Nei) model (Tamura *et al*, 1993), which was used to infer bootstrapped maximum likelihood trees using PHYML implemented on the ATGC bioinformatics platform (Guindon *et al*, 2003). Amino acid trees were drawn using Mega 4, rooted using FPV and drawn using the Dayhoff PAM matrix. The final alignment was screened for evidence of recombination and selection using GARD and SLAC available through the Datamonkey web server (Pond *et al*, 2005).

To seek correlations between the geographical origin of a particular sequence and its position within the phylogeny, a posterior set of trees was obtained through Bayesian MCMC analysis using BEAST v1.4 (Drummond *et al*, 2007). This analysis implemented the most-favoured model identified in the earlier step (TrN + γ + I); a comparison of alternative MCMC models (HKY + γ + I ; GTR + γ + I) (GTR- General

Time Reversible, HKY- Hasegawa-Kishino-Yano) by Bayes' factor (Suchard *et al*, 2001) confirmed the TrN model was also the most appropriate in a Bayesian MCMC context. We also compared the fit of the strict and 'relaxed' (uncorrelated exponential distribution; 'UCED') molecular clock models; the UCED model provided a better fit. Similarly, the constant population-size model was preferred over an exponential-growth model (available on request). The MCMC trace was inspected in TRACER v1.5 (Rambaut *et al*, 2007) for convergence. This posterior set of trees was subjected to Bayesian Tip-association Significance testing implemented by BaTS (Parker *et al*, 2008). In addition, Simpson's equitability index (E) was also used as a measure of population diversity (Begon *et al*, 1990; Simpson, 1949), where P_i is the proportion of identical sequence types within the population and S is the total number of distinct sequence types within the population. An E value of 1 equates to maximum diversity (all sequences different), whereas E tends toward zero as the diversity decreases and the number of sequence types increases.

$$E = \frac{1}{\sum_{i=1}^{S} P_i^2} \times \frac{1}{S}$$

In order to test for geographical clustering we examined the proportion of each sequence type in each sampling location. Since many sites were negative for a given sequence we used a non-parametric analysis, and performed a separate Fisher Exact Test for each sequence type, using Bonferroni's method to correct for multiple tests. Analyses used Stata 11 (StataCorp, College Station, Texas, USA) and significance was set at $P < 0.05$.

UK sequences were compared to worldwide sequences using the Blast software (Altschul et al, 1990).

Analysis of clinical signs

As signalment and clinical details were available with all the samples, we were able to evaluate possible associations between CPV type (CPV- 2a, 2b or 2c) and clinical outcome (death or survival), severity of clinical signs (as reported by the clinician), breed, age, sex, colour, presence of vomiting, and presence of hemorrhagic diarrhoea.

Results

Of the 373 samples obtained from the PDSA, 255 (68%) were PCR positive (Table 3.1). Of these, consensus VP2 sequence was obtained for 134 samples, selected to include one or more sequences from each of the 25 hospitals that submitted a positive sample. Sequence was also obtained from the 16 samples obtained from others sources (post mortem, commercial lab, and potential vaccine breakdowns), and from the six vaccines (A-F) currently used in the UK.

2b Sequence Type Number													PDSA PetAid Hospital			2a Sequence Type Number									N	Ratio
single	2	3	5	6	9	12	13	15	18	23	27	29	Code	Hospital	prevalence	34	37	42	46	47	48	50	Single		2a
20, 30, 1					1				2		3*	1	ST	Stoke	79% (23/29)		1							11	0.09
11, 24							1		3*	3*			BW	Bow	51% (22/43)								39	10	0.1
16								4*					WV	Wolverhampton	52% (9/17)								31	6	0.17
14,17,26						1	2	1			1		BH	Birmingham	71% (23/32)			1	1			1		12	0.25
	2						2	1					LE	Leicester	73% (11/15)	1							43	7	0.29
4, 28							2		1				NX	New Cross	39% (11/28)			2						7	0.29
10,7			3	3								2	EV	Everton	85% (49/57)	15*							33	26	0.62
					3								CV	Coventry	82% (14/17)	1		4*					45	9	0.67
21											1		CF	Cardiff	76% (10/13)			4*						6	0.67
			1										HU	Huyton	82% (24/29)	10*							36	12	0.92
													BM	Bournemouth	33% (3/9)						1		40	2	1
	1												GL	Glasgow	45% (5/11)								44	2	0.5
													NW	Newcastle	50% (1/2)								49	1	1
											1		BR	Bristol	61% (8/13)									1	0
										4*			BS	Basildon	63% (7/11)	1								5	0.2
	1												GH	Gateshead	66% (4/6)									1	0
									1				MB	Middlesboro	66% (4/6)					2				3	0.67
													PL	Plymouth	66% (2/3)				1					1	1
25													NT	Nottingham	71% (5/7)									1	0
		4*											HL	Hull	75% (6/8)									4	0
													TM	Thamesmead	75% (3/4)							1		1	1
	1												CR	Croydon	80% (4/5)									1	0
						2							SF	Sheffield	80% (4/5)									2	0
	2												HD	Hendon	100% (2/2)									2	0
													SD	Sunderland	100% (1/1)						1			1	1
16	7	4	4	3	4	3	7	6	7	7	6	3	Sub Total for PDSA		68% (255/373)	28	1	11	2	2	2	2	9	134	0.43
19 HP 8 CW 22. AS	2 SH BL					1 LV					2 WO StH	1 LV	Others (non-PDSA samples)		100% (16/16)	2 StH LV	1 LV	1 CA					32 BA 38 CA 41 BP 35 StH	16	0.5
18	9	4	4	3	4	8	3	6	7	7	8	4	Grand TOTAL of each sequence type			30	2	12	2	2	2	2	13	150	
	9	1	2	1	2	5	2	3	4	2	6	3	Number of hospitals per sequence type			7	2	5	2	1	2	2			

Table 3.1. *The origin of samples and the samples contained within each DNA sequence type. Origin of samples are described down the centre of the table, with the code on the left (for position of these codes relating to UK geography, see Figures 1 and 2). Sequence type numbers are located across the top (and correspond to those on the tree in Figures 1 and 2). The origin of each sample within this group is located underneath in the column under the sequence type (for example sequence type 3 has 4 samples from Wolverhampton, 1 from Birmingham etc). Single sequence types are shown towards the ends of the rows. Total number of sequences from an area is shown on the right (N) and the proportion of these which are 2a are shown on the far right. Samples of non-PDSA origin are shown at the bottom, with the total number of sequences per sequence type underneath. The number of hospitals where a sequence type was isolated is at the very bottom of the table. Sequence types which showed significant evidence of clustering in certain areas are indicated with an *, though numbers were small (see text).*

(Origin of non PDSA samples: HP= Hartlepool, CW = Crewe, LV = Liverpool, StH = St Helens, BL = Blyth, WO = Worcester, CA = Cambridge, BA = Barrow, BP = Blackpool, SH= Shrewsbury)

Prevalence of CPV types in the UK

Based on key amino acid substitutions, the 150 viruses were typed as 2a (43%; n = 65) or 2b (57%; n= 85) (Table 3.1). No type 2 or 2c were found. For those hospitals for which more than five sequences were available, the proportion of 2a/2b sequences ranged from 92% type 2a in Huyton (11 of 12) to 91% type 2b in Stoke (10 of 11) (Table 3.1), suggesting type 2a and 2b may have different distributions in different areas of the UK. The vaccines consisted of two CPV- type 2b vaccines (A-B) and four CPV-type 2 vaccines (C-F) (Figure 3.1-3.6).

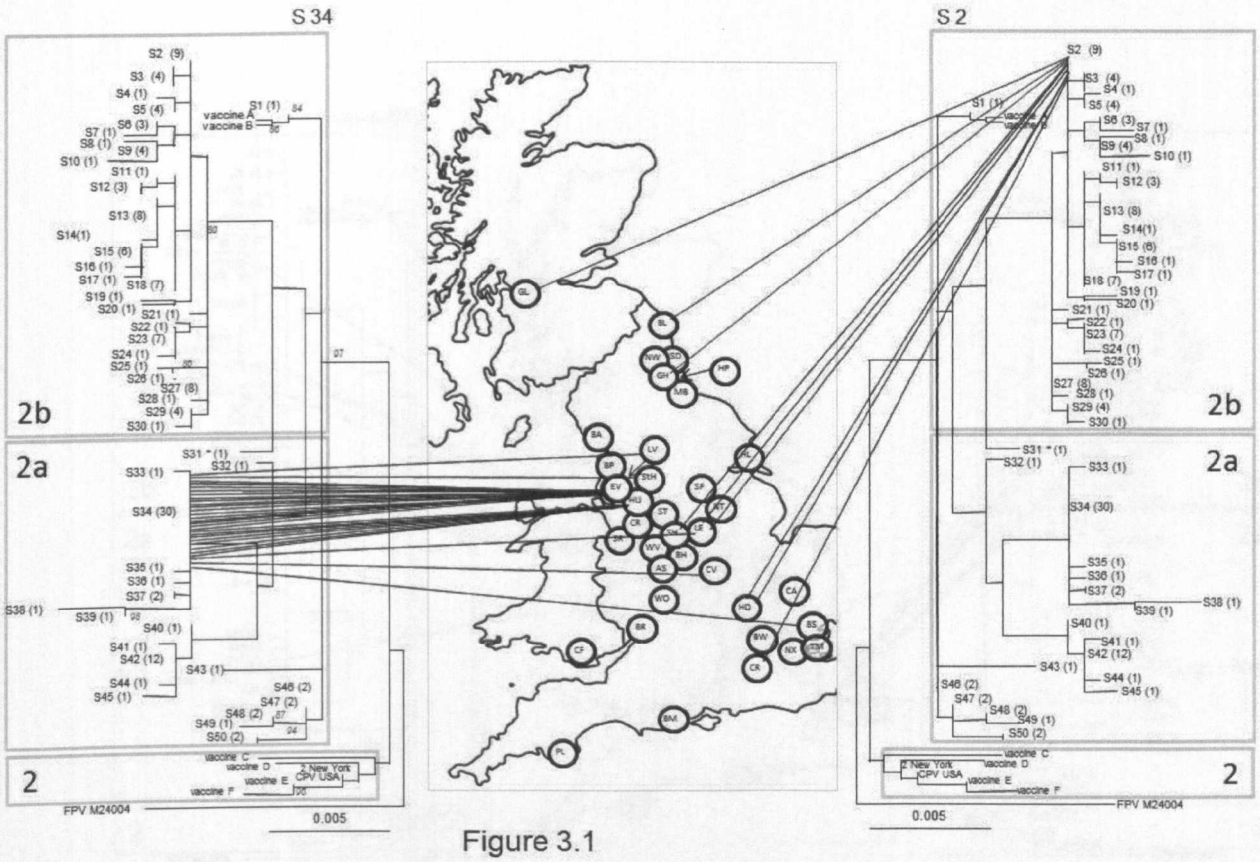

Figure 3.1

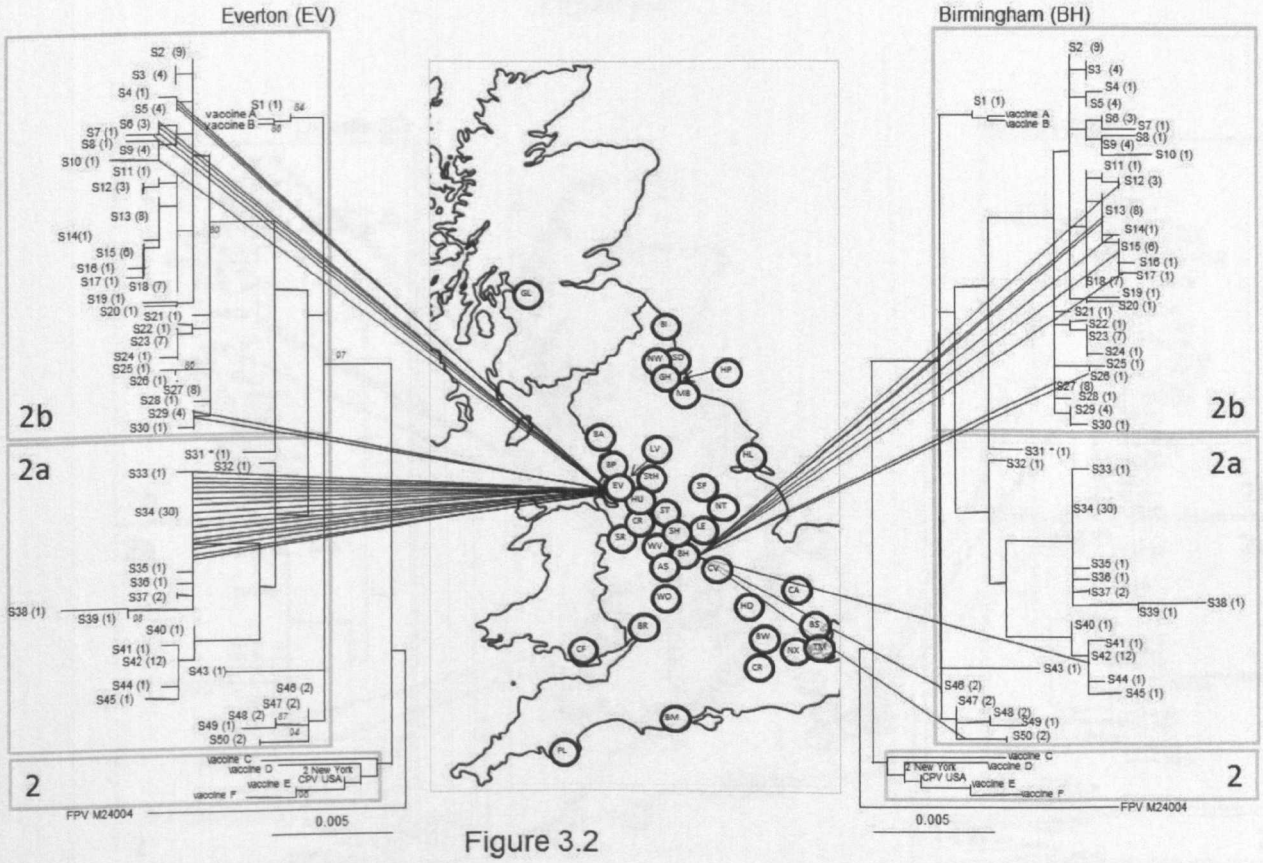

Figure 3.2

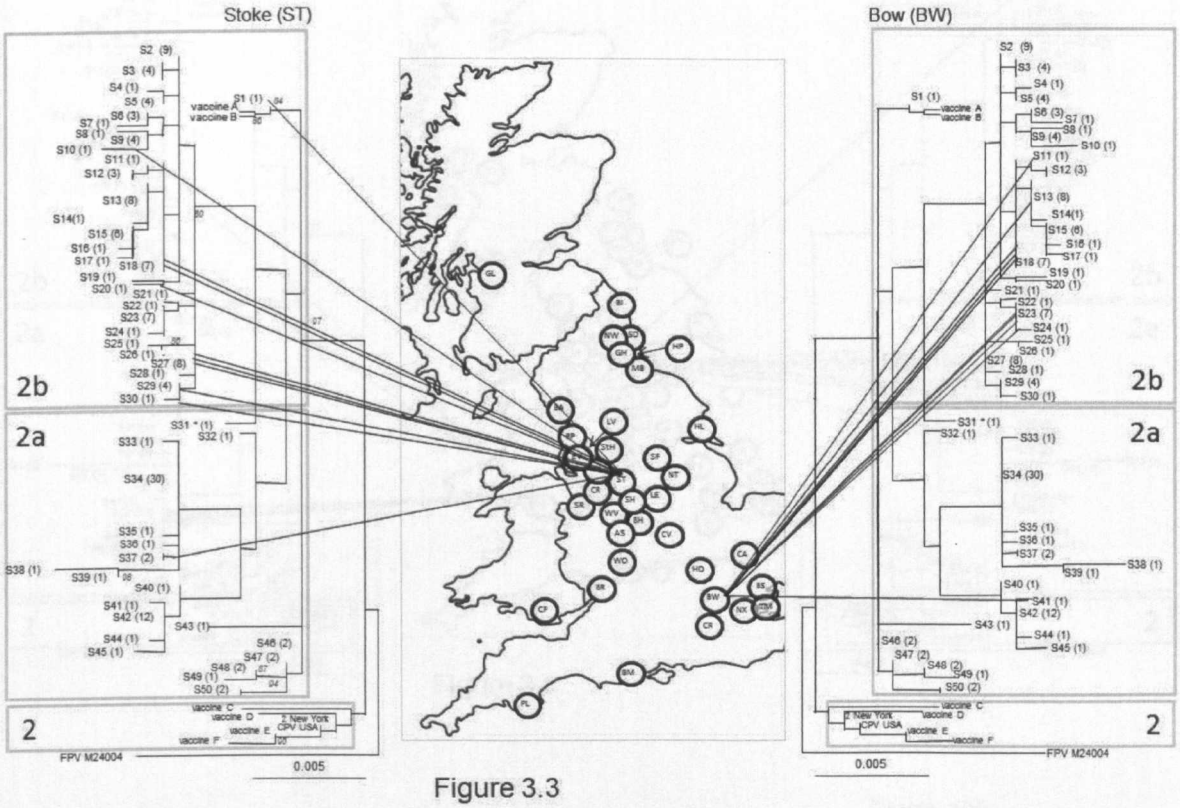

Figure 3.3

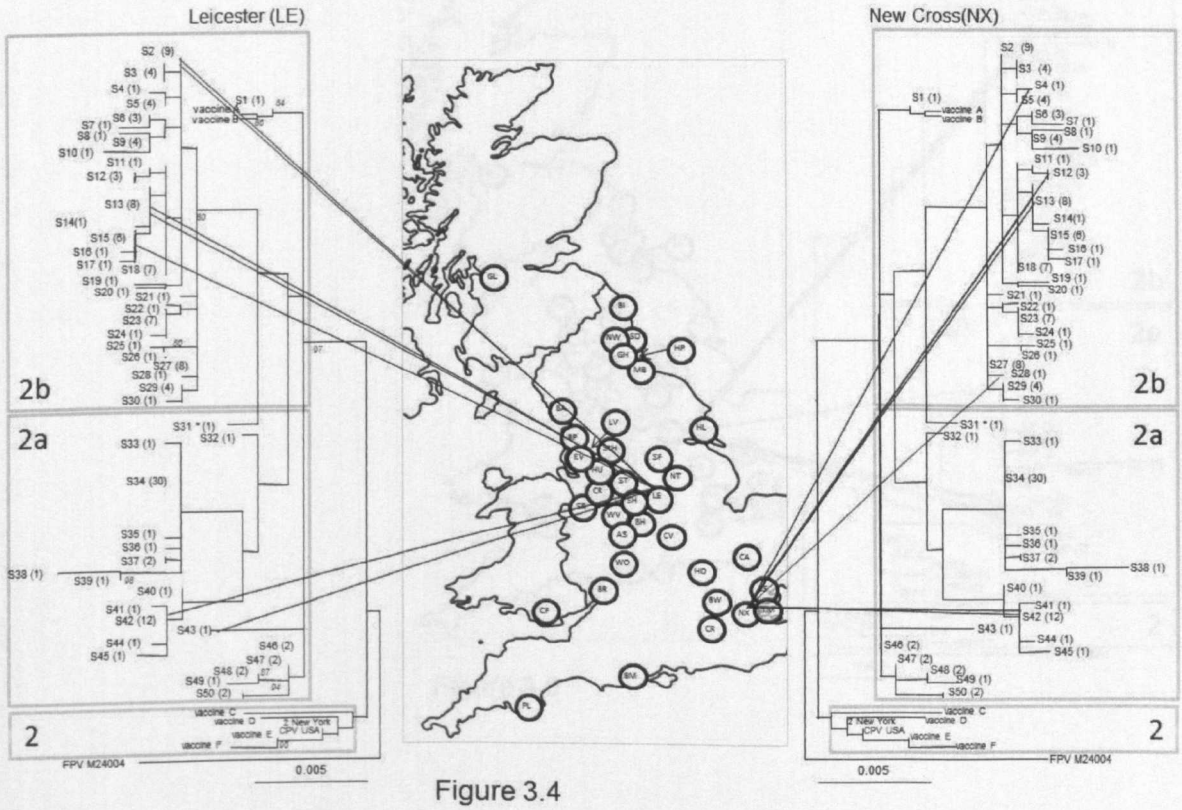

Figure 3.4

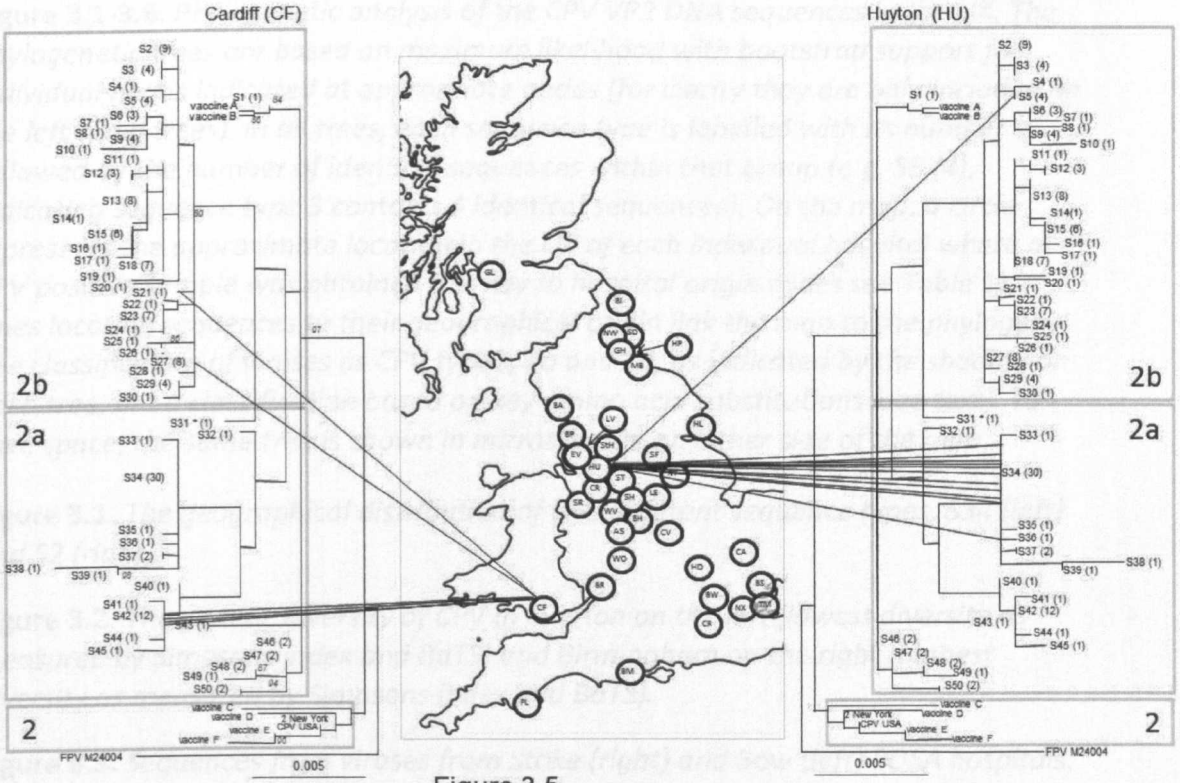

Figure 3.5

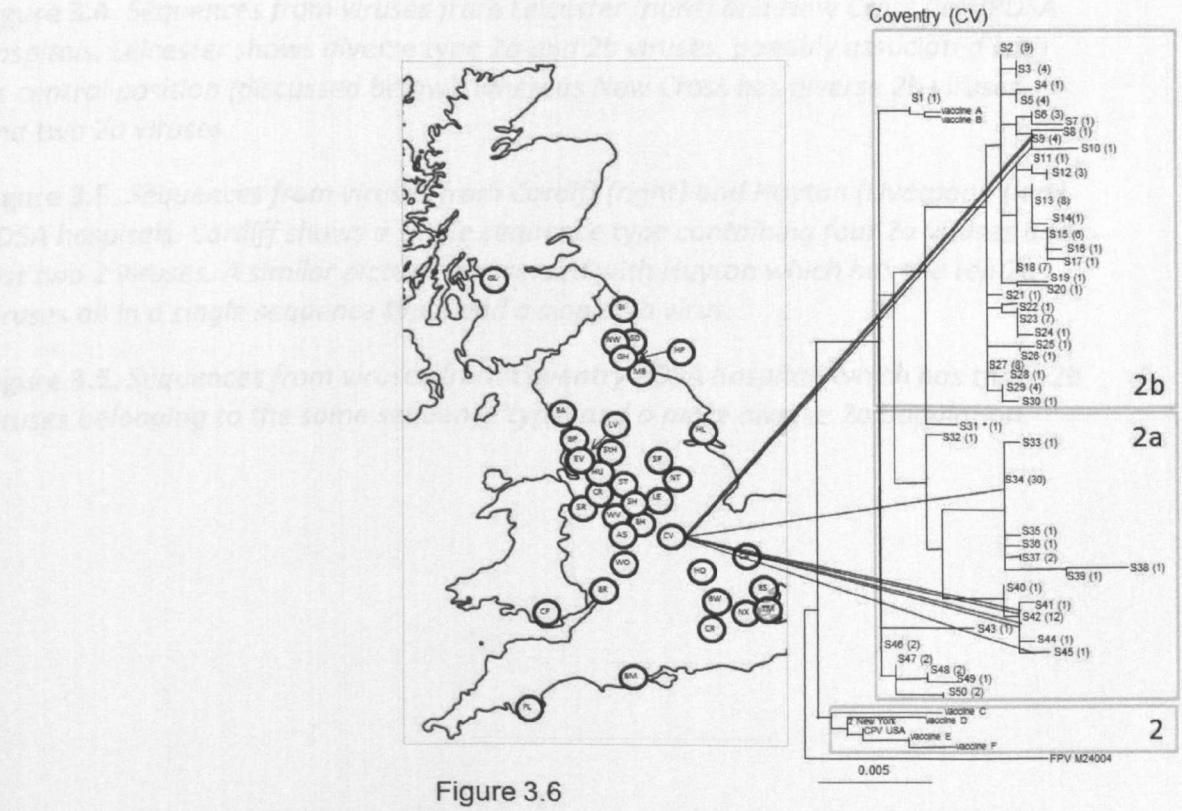

Figure 3.6

Figure 3.1-3.6. *Phylogenetic analysis of the CPV VP2 DNA sequences in the UK. The phylogenetic trees are based on maximum likelihood with bootstrap support for individual nodes indicated at appropriate nodes (for clarity they are only included on the left-hand trees). In all trees, each sequence type is labelled with its number followed by the number of identical sequences within that group (e.g. S3 (4), indicating sequence type 3 contains 4 identical sequences). On the map, a circle represents the approximate location in the UK of each individual hospital where a CPV positive sample was obtained (for key to hospital origin codes see Table 1). Lines locating sequences to their geographical origin link the map to the phylogeny. The classification of viruses as CPV type2, 2a and 2b, as indicated by the shading on each tree, are a classification based on key amino acid substitutions (see text). To save space, the same tree is shown in mirror image on either side of the map.*

Figure 3.1. *The geographical distribution of two different sequence types, S34 (left) and S2 (right).*

Figure 3.2. *The genetic diversity of CPV in Everton on the left (lowest diversity as measured by Simpsons index and BaTS) and Birmingham on the right (highest diversity as measured by Simpsons index and BaTS).*

Figure 3.3. *Sequences from viruses from Stoke (right) and Bow (left) PDSA hospitals. Stoke shows diverse type 2b and a single 2a virus, whereas Bow has similar 2b viruses, again with a single 2a virus.*

Figure 3.4. *Sequences from viruses from Leicester (right) and New Cross (left)PDSA hospitals. Leicester shows diverse type 2a and 2b viruses, possibly associated with its central position (discussed below), whereas New Cross has diverse 2b viruses, and two 2a viruses.*

Figure 3.5. *Sequences from viruses from Cardiff (right) and Huyton (Liverpool) (left) PDSA hospitals. Cardiff shows a single sequence type containing four 2a viruses and just two 2 viruses. A similar picture is observed with Huyton which has the ten 2a viruses all in a single sequence type, and a single 2b virus.*

Figure 3.6. *Sequences from viruses from Coventry PDSA hospital, which has three 2b viruses belonging to the same sequence type, and a more diverse 2a population.*

DNA sequence analysis

Considerable diversity was found within the VP2 DNA sequences we obtained, with 50 genetically distinct sequences, or sequence types (S), identified amongst the 150 clinical samples sequenced (Figure 3.1-3.6 and Table 3.1). Thirty-one of the 50 different sequence types were found only once, whereas some sequence types were quite common; for example eight sequence types contained more than five sequences, and the most common (S34) comprised 30 sequences (Table 3.1 and Figure 3.1). Of the 50 different sequence types, 20 comprised type 2a viruses and the remaining 30 were type 2b viruses (Table 3.1 and Figure 3.1), suggesting type 2b might be more variable in the UK.

The majority of sequences clustered separately depending on their type (2a or 2b). However, neither CPV2a nor 2b was monophyletic: S31 which typed as a 2a virus based on key amino acid substitutions, grouped phylogenetically within the 2b virus sequences (Figure 3.1). In addition, S1 (type 2b) clustered close (2-8 nucleotide substitutions) to S46-50 (type 2a). This may suggest possible 2a/2b intermediates or parallel evolution.

Spatial range of sequence types in the UK

In order to explore the spatial range of individual sequence types, we calculated the number of hospitals in which each of these sequences was found (Table 3.1). Some sequence types were geographically restricted as exemplified by S34 which was found at seven hospitals mostly clustered in the North West of England including

Liverpool, Everton, Huyton and St Helens (Left side of Figure 3.1). Other sequence types were geographically dispersed as exemplified by S2 which was again found in seven different hospitals ranging from the North (Glasgow) to the South (Croydon and Hendon) (Right side of Figure 3.1). There was statistical support ($p<0.05$) for geographical clustering for S34 (Everton and Huyton), S3 (Hull), S23 (Basildon and Bow), S42 (Coventry and Cardiff). However, for many areas, sample numbers were small.

The finding of evidence of clustering for some sequence types in particular geographic locations was confirmed using Simpson's index of diversity (Table 3.2). For example, Everton had the highest number of viral sequences (n=26) but showed the lowest level of DNA (E=0.1) and second lowest amino acid diversity (0.08). In other areas, sequence diversity was much higher, for example in Birmingham (E=0.86 and 0.3 for DNA and amino acid sequences respectively) (Table 3.2). Results of BaTS analysis also suggested in some cases there was an association between the hospital of isolation and phylogenetic clustering (Everton, Stoke, Bow, Coventry), whereas in other hospitals there was no evidence for this (Birmingham, Leicester, New Cross, Cardiff) (Table 3.2). These extremes of diversity for Everton and Birmingham can also be represented phylogenetically (Figure 3.2-3.6), and for the other hospitals in table 3.2.

Type / city	N	DNA		Protein		(p) BaTS
		S	E	S	E	
2a	65	20	0.2	10	0.04	-
2b	85	30	0.54	14	0.02	-
Birmingham	12	11	0.86	5	0.3	1
Stoke	12	8	0.8	5	0.04	0.04
Leicester	7	5	0.64	4	0.37	1
New Cross	7	5	0.64	3	0.33	1
Bow	10	6	0.45	4	0.19	0.03
Coventry	9	4	0.33	4	0.33	0.05
Cardiff	6	3	0.33	3	0.33	1
Huyton	12	3	0.12	3	0.12	0.06
Everton	26	7	0.1	3	0.08	0.01

Table 3.2. *Support for geographical clustering. Simpsons index of diversity, where N = number of sequences, S is the number of different sequence types and E is the equitability index (where 1 is maximum diversity). This is shown for CPV type 2a and 2b at the top, and then for hospitals with more than five sequences below. P values calculated by BaTS (significant at <0.05) suggesting correlation between phylogeny and PDSA origin are also indicated.*

Simpson's index also showed that at the DNA level, the 2b viruses had a higher level of diversity than the 2a viruses (E= 0.54 and 0.20 respectively) (Table 3.2). However, these relative diversities were reversed at the amino acid level, due to the merging of the majority of the 2b viruses into a single amino acid sequence (amino acid group A) (Figure 3.7). As these samples were collected over a two year period it is unlikely that these results are due to time of sample collection.

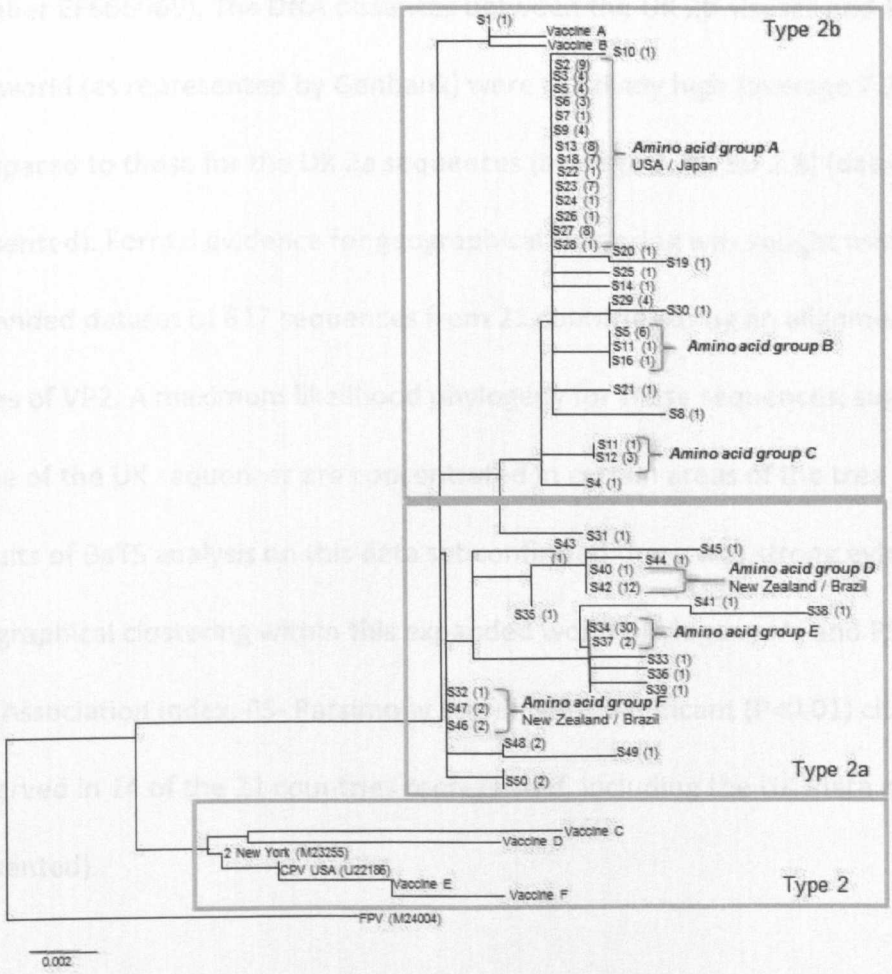

Figure 3.7

Figure 3.7 *.A neighbour-joining amino acid tree of the same samples as used in Figure 1. Samples are labelled with the same sequence type number as is used in the DNA tree. Brackets are used to indicate large amino acid groups which are referred to in the text (marked A-F). For A, D and F, where these sequences have been identified both in this country (this study) and in other countries (based on Genbank data), the other countries are listed next to the taxa name.*

Comparison of UK and worldwide sequences

Almost all the sequence types we identified were unique to the UK at the DNA level, with only S40 being found outside the UK, in China (Genbank accession

number EF666069). The DNA distances between the UK 2b viruses and the rest of the world (as represented by Genbank) were relatively high (average 7.23, SD 1.4), compared to those for the UK 2a sequences (average 3.75, SD 2.8) (data not presented). Formal evidence for geographical clustering was sought using an expanded dataset of 617 sequences from 21 countries using an alignment of 1386 bases of VP2. A maximum likelihood phylogeny for these sequences, suggests that some of the UK sequences are concentrated in certain areas of the tree (figure 3.8). Results of BaTS analysis on this data set confirmed there was strong evidence for geographical clustering within this expanded world phylogeny (AI and PS<0.001) (AI- Association index, PS- Parsimony score), with significant (P<0.01) clustering observed in 14 of the 21 countries represented, including the UK (data not presented).

Figure 3.8. *A ML tree (TN93 model) including 617 partial CPV VP2 sequences from around the world obtained in this study and from Genbank. The distribution of the UK sequences we obtained across the phylogeny is indicated by pie charts next to appropriate regions of the tree. Those regions of the tree without a pie chart contained no UK sequences.*

COUNTRY OF ORIGIN	NUMBER
BELGIUM	1
BRAZIL	31
CHINA	106
FINLAND	2
FRANCE	61
GERMANY	20
GREECE	2
INDIA	18
ITALY	71
JAPAN	19
KOREA	11
NZE	1
POLAND	1
SOUTH AFRICA	4
SOUTH KOREA	25
SPAIN	2
TAIWAN	18
THAILAND	29
UK	130
USA	35
VAC	2
VIETNAM	8

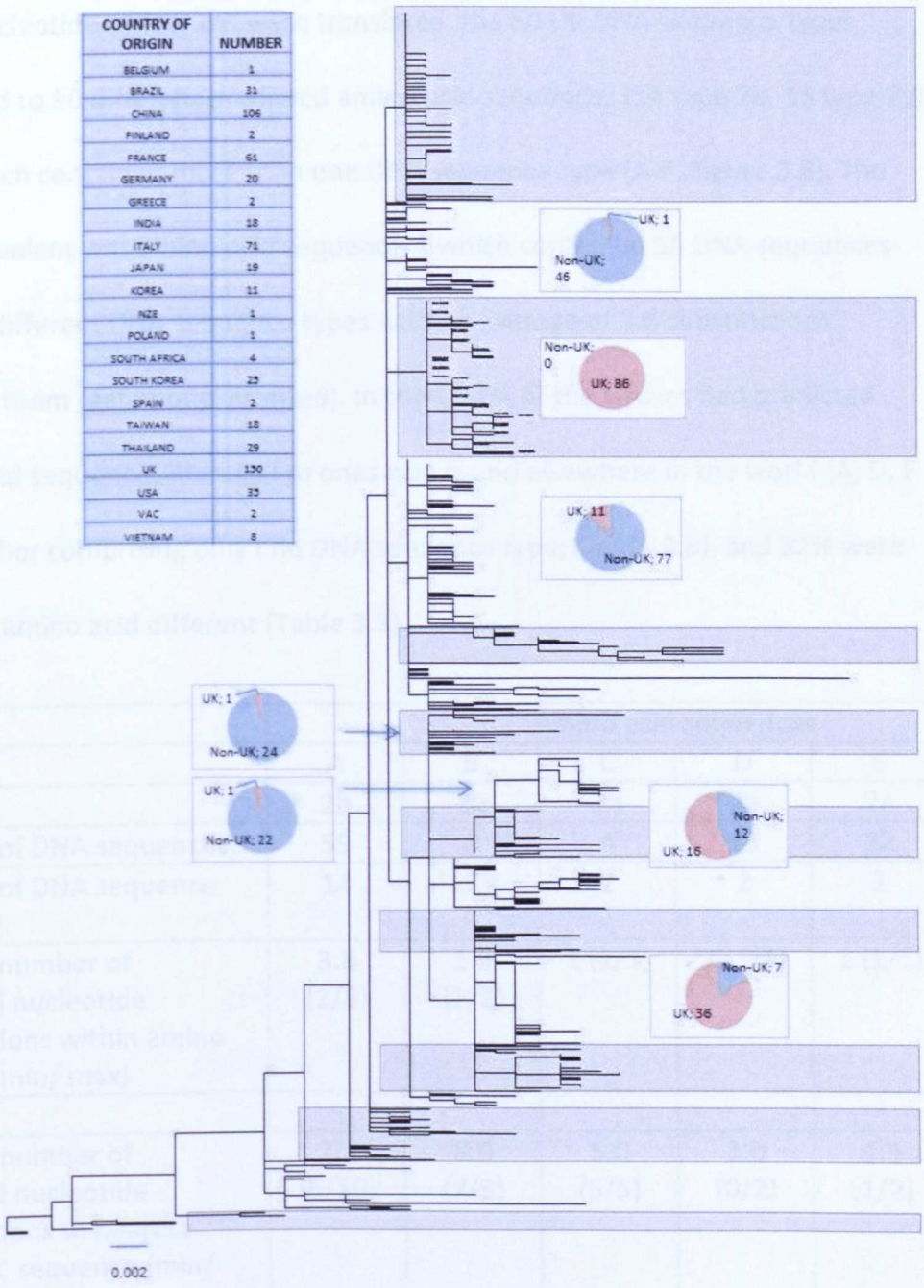

Figure 3.8

Amino acid variability

When nucleotide sequences were translated, the 50 UK DNA sequence types converted to 30 different predicted amino acid sequences (14 type 2b, 16 type 2a), six of which contained more than one DNA sequence type (A-F, Figure 3.8). The most prevalent was amino acid sequence A which contained 59 DNA sequences from 14 different DNA sequence types with an average of 3.8 substitutions between them (data not presented). In total, 57% of the viruses had predicted amino acid sequences identical to ones also found elsewhere in the world (A, D, F and another comprising only one DNA sequence type; Figure 3.8), and 32% were only one amino acid different (Table 3.3).

	Amino acid sequences					
	A	B	C	D	E	F
CPV type	2b	2b	2b	2a	2a	2a
Number of DNA sequences	59	8	4	13	32	5
Number of DNA sequence types	14	3	2	2	2	3
Average number of observed nucleotide substitutions within amino acid ST (min/ max)	3.8 (1/8)	1.3 (1/2)	1 (1/1)	1 (1/1)	1 (1/1)	3.3 (1/5)
Average number of observed nucleotide substitutions to nearest Genbank sequence (min/ max)	7.2 (5/10)	8.0 (7/9)	5.0 (5/5)	1.0 (0/2)	1.5 (1/2)	1.3 (1/2)
Number of observed amino acid substitutions to nearest Genbank sequence	0	1	1	0	1	0
Country	USA, Japan	USA, Japan	USA, Japan, Italy	New Zealand, Brazil	China, Brazil	New Zealand, Brazil

Table 3.3. *Amino acid sequences (A-F) containing greater than one DNA sequence type. The numbers of DNA sequences and DNA sequence types making up each amino acid sequence are indicated. The number of observed nucleotide (synonymous) differences within each amino acid sequence were calculated for the UK, and then with the rest of the world using sequences available on Genbank. The countries containing identical amino acid STs (or one amino acid different) to those found in the UK are shown at the bottom of the table.*

When full VP2 consensus sequences were analysed, two specific substitutions, Val – 139- Iso (n = 35) and Arg- 274- Lys (n = 8) were only identified within the UK samples when compared to those from the rest of the world via Genbank. The substitutions at position 139 is responsible for the formation of nucleotide sequence type 34.

Clinical outcome in relation to sequence type

There appeared to be no association between clinical signs and sequence either at the level of virus type (CPV 2a or 2b), or DNA sequence type, or at the level of individual specific amino acid substitutions (data not shown). Neither type 2 nor type 2b vaccine virus were detected in faecal samples, although one type 2b field virus (S1) was only two bases different from vaccines A and B (Figures 3.1 and 3.2).

Discussion

Spatial and temporal dynamics of CPV-2 spread, based largely on unstructured sampling techniques, may lead to a potentially biased view of the diversity and types of virus circulating both within and between countries. Here, we have used a

cross-sectional sampling strategy over two years, together with sequence analysis of the full CPV capsid gene (VP2, 1755 base pairs) to investigate the spread and evolution of CPV-2 at both local and national levels, and to relate this to the global transmission of the virus.

We have found considerable diversity of CPV strains obtained from across the UK, with 50 different DNA sequence types identified within the 150 viruses analysed. Interestingly, apart from one sequence previously identified in China, all VP2 sequence types appeared to be unique to the UK. Phylogenetic analysis and Bayesian tip-association significance testing on an expanded set of published global sequences showed some evidence for geographical clustering at an international level, suggesting that currently there are limited opportunities for global transmission, as has previously been suggested by others (Hoelzer *et al,* 2008). Despite this observation, sequences from individual countries, as exemplified by the UK, were generally not monophyletic, implying national diversity is produced by a combination of local evolution occasionally supplemented by importation of new sequence types. The relative significance of these two processes remains to be determined.

There was less diversity at the amino acid level than at the DNA level with only 32 unique amino acid sequences identified. In contrast to the DNA sequences, the amino acid sequences were similar in the majority of instances (57% identical; Figure 3) to those found elsewhere in the world. This apparent relative stability of the virus at the amino acid level is likely due to high structural and functional requirements of the capsid gene in this small virus, and, in contrast to previous

studies (Hoelzer *et al*, 2008), was associated with a lack of evidence for positive selection in VP2. Two unique amino acid substitutions were identified within the UK samples. Residue 139 was mutated from valine to isoleucine in 35 samples, and forms part of the beta barrel inside the virus (Reed *et al*, 1988). Residue 274 was mutated from arginine to lysine in eight samples, and is found on the threefold spike, a region of high antigenicity (Parrish *et al*, 1988b). At this point, we have no evidence to indicate whether these substitutions have any biological significance.

The variability in the full VP2 gene sequence enabled us to investigate in depth the molecular epidemiology of these viruses at the national level. We showed that within the background of countrywide diversity, there was also evidence of significant geographic clustering in some areas, as exemplified by S34 (Figure 1) which was not only the most predominant virus in the Liverpool region (i.e. Everton, Huyton, St Helens, Liverpool), but was also rarely found elsewhere. As this sequence type was present over a period of two years, it suggests we were not observing a short term epidemic. Such spatial clustering suggests some CPV sequence types may have restricted opportunities for spread, even within countries. This geographical restriction of certain virus types highlights the importance of rigorous, epidemiologically representative sampling strategies for the study of viral molecular epidemiology.

Of the potential vaccine breakdowns which were included in our study, none were identical to the sequences obtained from vaccine used in the UK, suggesting live vaccines are not causing disease in this population. However, one sequence (S1) was only two nucleotides different in the VP2 region from two type 2b vaccines (A

and B on figure 1-2), and several viruses classified as antigenic type 2a (S46-50) were also phylogenetically close to these vaccine sequences (equivalent to 2-8 nucleotide substitutions). Whether these field viruses represent the ancestor strains for these vaccines, or whether S1 may have evolved from the vaccine either in that individual dog or in the wider dog population, is unknown. In addition, all of the potential vaccine breakdowns were distinct and showed no consistent substitutions, suggesting that there is no group of viruses circulating within the UK which is specifically capable of evading vaccine-induced immunity. This is in agreement with a range of studies that have shown that currently available vaccines protect against the full range of antigenic types identified to date (Siedek *et al*, 2007; Spibey *et al*, 2008).

The traditional typing of the viruses based on key amino acid positions of CPV types 2a, 2b and 2c showed that only CPV types 2a and 2b were circulating within the UK, and is in agreement with a previous study (Davies, 2008). Decaro *et al* (2007) found only one 2c circulating within the UK, and this remains the only 2c identified in the UK to date. Since many countries have high levels of CPV 2c circulating (Davies, 2008), it might be expected that CPV 2c would have reached the UK by now, unless the virus has some restrictions on its global spread (Hoelzer *et al*, 2008). None of the ancestral CPV type 2 was found in our study, confirming that it no longer appears to be circulating as an important cause of disease.

Although the majority of viruses clustered on the basis of these key amino acid substitutions, neither 2a or 2b viruses appeared to be monophyletic. Indeed there was some evidence to suggest that in the UK at least, the 2a/2b phenotype is not

that stable and may have evolved on several occasions as indicated by the S46-50 group, S31 and S32-S45. Although 2a and 2b (and indeed 2c) clearly do have some antigenic differences largely based on monoclonal antibody reactivity (Parrish *et al*, 1986), such lack of stability together with no clear evidence for clinical differences may suggest this classification system needs revising. In this regard, it will be important to further explore the historical diversification of this virus by obtaining sequences for older viruses. In addition, as neither 2a or 2b type is monophyletic, results by real time PCR typing generally targeting a small part of the VP2 gene may give a false impression of the epidemiology of this virus (Decaro *et al*, 2005b, 2005c, 2006, 2006b, 2006c).

Chapter Four

Prevalence of canine parvovirus (CPV) in different populations of dogs

Introduction

All four different types of canine parvovirus (CPV) (i.e. CPV-2, 2a, 2b and 2c) are associated with severe vomiting and enteric disease in young pups (Pollock, 1982; Decaro *et al.*, 2006d; Nandi *et al.*, 2010; Davies, 2008; Vieira *et al.*, 2008), and still causes a problem worldwide, particularly in unvaccinated populations, or in pups which have lost their maternally derived antibody and have not yet responded to vaccination. Current CPV vaccines are based on modified live viruses, which are known to induce a long duration of immunity, and are both safe and highly efficacious, preventing both infection and viral shedding in some cases (O'Brien, 1994; Miyamoto *et al.*, 1995; Abdelmagid *et al.*, 2004; Mouzin *et al.*, 2004; Gore *et al.*, 2005)

Despite the severity of CPV infection in susceptible individuals, some animals can have subclinical infections (Potgieter *et al.*, 1981), and shed virus in faeces for short periods, without any noticeable clinical signs or pathology. Occasional, persistent, subclinical infections have been observed in infections with feline panleukopenia virus (Miyazawa *et al.*, 1999; Czisa *et al.*, 1971) where virus can be shed long term in urine and remain in tissues, following *in utero* or neonatal infections.

Previous studies have shown that the prevalence of CPV in cases of gastroenteritis from around the world, over a period of 27 years, have ranged from 35.5 to 54.0% (Yesilbag *et al.*, 2007; Hammond *et al.*, 1983; Nandi *et al.*, 2010; Hirasawa *et al.*, 1996; Castro *et al.*, 2007). However, there appear to be no reports of CPV in

clinically normal or mildly ill animals, and most studies on CPV detection in clinical cases have concentrated on the type and phylogeny of the virus found.

Another common viral cause of canine gastroenteritis is canine coronavirus (CCoV), an RNA virus, which infects the villus tips of the intestinal epithelium (Binn *et al.*, 1974), which is in contrast to CPV which infects the crypts of the villi (Fowler *et al.*, 1970; Carlson *et al.*, 1977). Vaccines based on either canine or feline coronavirus are available and may be useful for decreased signs and pathology of CCoV disease particularly in puppies (Pratelli *et al.*, 2004). It has been suggested that the effects of CCoV and CPV together can increase the morbidity and mortality observed, compared to infection with one of the viruses (Evermann *et al.*, 1980; Pratelli *et al.*, 1999b; Appel *et al.*, 1988). Both viruses can cause clinical signs which are very similar, so differentiation can be difficult (Carmichael *et al.*, 1981).

As there is are few data on the prevalence of these two viruses in populations with varying levels of clinical disease or in clinically healthy animals, this study was designed to determine the prevalence of CPV and CCoV in samples from three different populations of dogs, with varying levels of disease.

Methods

Faecal sampling for populations one (kennels) and two (case control) were co-ordinated by Dr. J. Stavisky. Faecal samples for population three were collected in collaboration with S. Godsall (PDSA). All RNA extractions, PCR and sequencing related to CCoV were carried out by Dr. J. Stavisky. All DNA extractions, PCR and sequencing related to CPV were carried out by S. Clegg.

Population one: Kennelled dogs (clinically normal)

The study of this population was done in collaboration with Dr J Stavisky (manuscript in preparation).

The population comprised four premises (Figure 1), two of which were boarding kennels, and thus kept pet dogs for short periods of time (in Cheshire and Solihull) and two of which were rescue kennels (in Merseyside and Cambridge). The rescue kennel in Merseyside was visited weekly for eleven consecutive weeks as part of a longitudinal study, and all faecal samples were screened for both CPV and CCoV . The remaining three kennels were visited on only one occasion. The two rescue kennels experience high turnover of animals, rendering the population susceptible to viral spread (Sokolow *et al*, 2005), and all dogs were vaccinated within a few days of arrival. In contrast to the rescue shelters, the two boarding kennels appeared to have better hygiene and biosecurity, and both kennels required dogs to be fully vaccinated before they were accepted. The turnover of the boarding kennels was not known.

All kennels were visited early in the morning prior to the morning cleaning, and faecal samples were collected from the floor for all dogs which had defecated overnight,.

For all dogs, their date of entry into the kennels and their location within the kennel was recorded. Due to ethical constraints, as participating owners in the boarding kennels could not be individually asked for consent, no further details could be obtained for privately owned dogs. For the rescue shelter dogs, further information, including estimated age, breed, sex and any known clinical disease were ascertained.

Population two: Case control study of dogs presenting with diarrhoea (cases) and clinically normal animals (controls)

A matched case-control study design was used, in order to enable comparison between dogs with and without diarrhoea. Details are given elsewhere (Stavisky *et al*, 2011)

Briefly, the staff of thirteen different, first opinion veterinary surgeries across the UK (Figure 1) were recruited on the basis of their willingness to participate in the study. Two surgeries were located in South-West Scotland (Lanarkshire), seven in North-West England (Merseyside, Cheshire, Manchester, West Yorkshire) and four in Southern England (Buckinghamshire, Berkshire, Dorset, Wiltshire).

Cases comprised any dog presenting at the surgery with diarrhoea, defined as an increased fluidity, volume or frequency as noticed by the owner. For every case, two controls (ideally the next two dogs) which presented at the same surgery without enteric disease were collected. Controls were removed if they had any history of enteric disease two weeks prior to presentation. None of the cases or controls were hospitalised.

Signalment was collected from the attending veterinary surgeon in the first instance, and then via a questionnaire conducted over the telephone with the owner within a week of receipt of sample.

Population three: Hospitalised animals (severely ill animals)

For details of the sampling for this population, see details in Godsall et al, 2010.

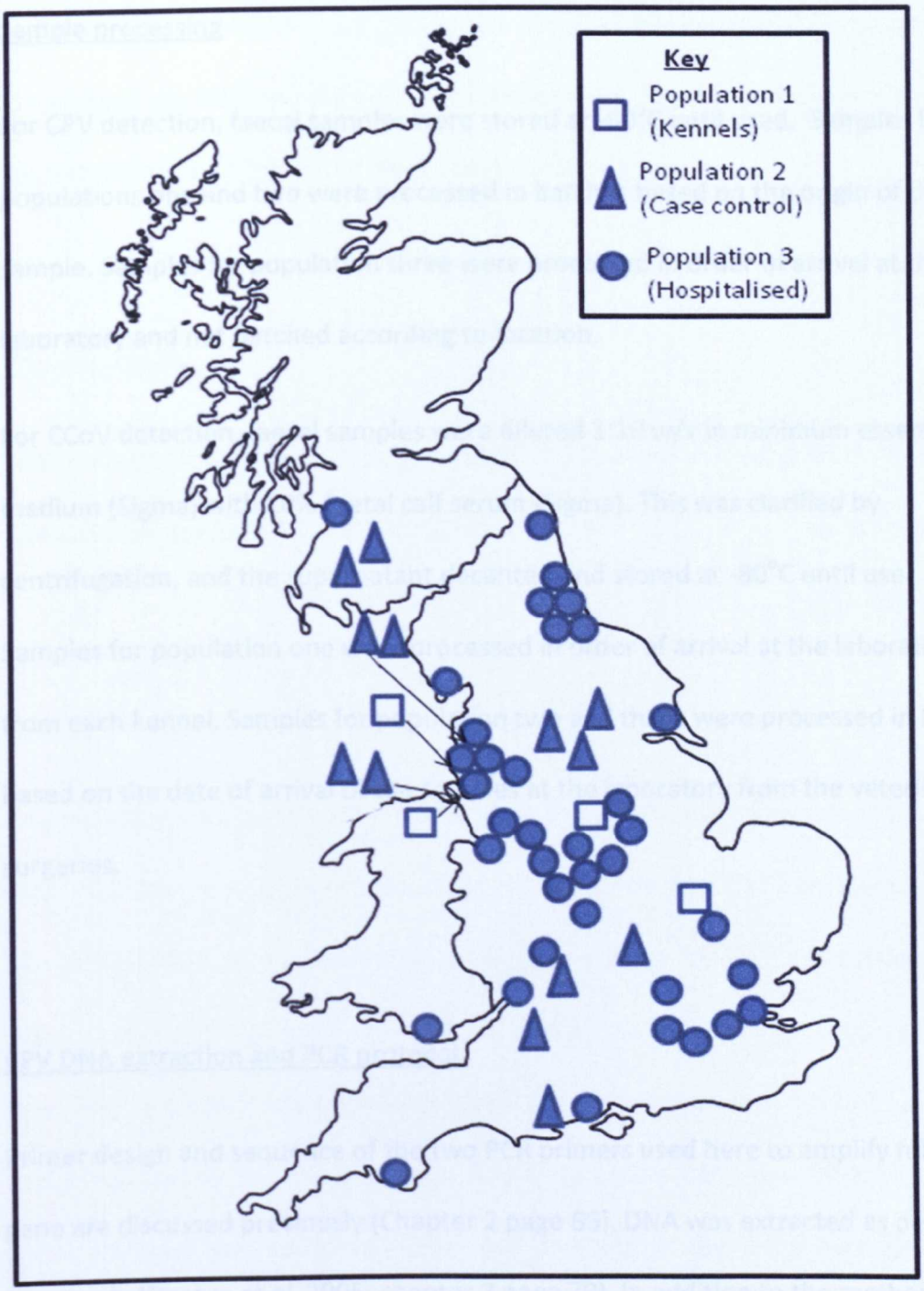

Figure 4.1. *The approximate location within mainland UK of the kennels and veterinary surgeries used in this study. Kennels used in population one are shown with a square, surgeries used in population two are shown with a triangle, and circles indicate the location of the PDSA hospitals used in population three.*

Sample processing

For CPV detection, faecal samples were stored at -80°C until used. Samples for populations one and two were processed in batches based on the origin of the sample. Samples for population three were processed in order of arrival at the laboratory and not batched according to location.

For CCoV detection, faecal samples were diluted 1:10 w/v in minimum essential medium (Sigma) with 10% foetal calf serum (Sigma). This was clarified by centrifugation, and the supernatant decanted and stored at -80°C until use. Samples for population one were processed in order of arrival at the laboratory from each kennel. Samples for population two and three were processed in batches based on the date of arrival of the samples at the laboratory from the veterinary surgeries.

CPV DNA extraction and PCR protocol

Primer design and sequence of the two PCR primers used here to amplify full VP2 gene are discussed previously (Chapter 2 page 66). DNA was extracted as described previously (Decaro *et al*, 2005; chapter 2 page 79). In addition to the methods discussed in previous chapters, an FPV vaccine strain was initially used as a positive control to confirm that the PCR was working correctly. Due to the low levels of CPV observed in samples from populations one and two, the PCR positive control was run in addition to the samples to confirm the PCR had worked. This FPV vaccine positive control could easily be differentiated from any CPV isolate by sequencing.

As it emerged that there was a high level of CPV in samples from population three, the positive control was removed and samples were processed without a positive control to decrease the risk of cross contamination.

CPV sequencing

Sequencing of full VP2 was generated using internal sequencing primers, which are discussed earlier (chapter 2 page 79).

CCoV RNA extraction

One hundred and twenty six microlitres of thawed faecal supernatant was spiked with 14µl Bovine Viral Diarrhoea Virus (BVDV) strain NADL (kindly supplied by Dr Kim Willoughby) as an internal positive control (IPC) for RNA extraction, reverse transcription and PCR (Willoughby *et al*, 2006). This 140µl mixture was used as the template for nucleic acid extraction, using the Qiamp Viral RNA mini kit (Qiagen) as recommended by the manufacturer. One negative control, consisting of molecular water (Sigma), was added for every five samples at the nucleic acid extraction stage.

Reverse transcription was carried out using Moloney murine leukaemia reverse transcriptase enzyme (M-MLV) (Superscript III RT, Invitrogen Life Technologies) according to the manufacturer's instructions. Six µl of RNA was used as a template,

and the reaction was primed with 100ng random hexamers (Abgene Ltd). cDNA was stored at 4°C until used.

CCoV PCR

For CCoV, real-time RT-PCR was carried out using the primer/ probe combinations as previously described (Decaro *et al*, 2005d), with the addition of a modified internal control consisting of a real-time BVDV assay as described above (Willoughby *et al*, 2006). Assays were carried out separately for type I and type II CECoV, and were performed in triplicate.

A calibration curve for quantification was run in triplicate on each plate using manufactured positive control template molecules (Eurofins MWG) in tenfold dilutions ranging from 10^2-10^7 copies per µl of template. Cycling conditions consisted of activation of Thermo-start™ DNA Polymerase at 95°C for 15 minutes, followed by 38 cycles of denaturation at 95°C for 15s, annealing at 53°C (type I-specific assay) or 48°C (type II specific assay) for 30s, and extension at 60°C. All reactions were carried out in triplicate. In addition to the negative controls from the RNA extraction stage, a further six negative controls consisting of molecular water (Sigma) instead of cDNA were used for each 96 well plate.

Primer/ probe	Sequence 5' to 3'	Sense	Amplicon size (bp)
CCOVI-F	CGTTAGTGCACTTGGAAGAAGCT	+	111
CCOVI-R	ACCAGCCATTTTAAATCCTTCA	-	
CCOVI-Pb	FAM-CCTCTTGAAGGTACACCAA-MGBNFQ	+	-
CCOVII-F	TAGTGCATTAGGAAGAAGCT	+	105
CCOVII-R	AGCAATTTTGAACCCTTC	-	
CCOVII-Pb	FAM-CCTCTTGAAGGTGTGCC-MGBNFQ	+	-
BVDV1_2 F	CCA TGC CCT TAG TAG GAC TAG C	+	95
BVDV1_2R	TGA CGA CTA CCC TGT ACT CAG G	-	
BVDVPb	VIC-AACAGTGGTGAGTTCGT-MGBNFQ	+	-

Table 4.1: *Real-time PCR primers used for the diagnosis of canine coronavirus (CCoV)*

Conventional PCR was carried out on all samples found to be positive by real-time RT-PCR, using the primers CCOV1 (TCCAGATATGTAATGTTCGG) /CCOV2 (TCTGTTGAGTAATCACCAGCT) (Pratelli *et al*, 1999c), which detect both types I and II CECoV and are targeted at the conserved M gene region of the genome. The purpose of performing the conventional PCRs was to obtain sequence fragments for phylogenetic analysis.

CCoV sequencing

As the M gene PCR amplified both type I and type II CECoV, it was not possible to generate reliable sequence from mixed type I and II CECoV infections. Therefore, additional primers were designed from alignments of the antigenically important S gene region from sequences available on Genbank. The two pairs of S gene primers were designed to differentiate between type I and type II CECoV and could therefore be used in samples with mixed infections. All conventional primers are listed in table 4.3.

Primer	Target	Sequence 5' to 3'	Sense	Amplicon size (bp)
CCOV1	Type I & II CECoV	TCCAGATATGTAATGTTCGG	+	409
CCOV2		TCTGTTGAGTAATCACCAGCT	-	
S1For	Type I CECoV	CTA GTG GAC TTG GCA CTG TTG ATG AAG AC	+	779
S1RevR		TCA CCT CTT CCC ATT CGG TTG GAA GC	-	
S2ForR	Type II CECoV	GCT TTT TGA TAA GGT TGT AAC ATC	+	796
S2RevAR		GTT TCA TAA GCT GTT GGT AAT AGC	-	

Table 4.2: *Conventional PCR primers used for the typing of canine coronavirus (CCoV)*

Sequencing and sequence analysis

Products from conventional PCR on samples collected from population one (kennels) were sequenced using the Applied Biosystems Genetic Analyzer with Bigdye terminator. Consensus sequences were produced using ChromasPro version 1.32 (Technelysium Ptl Ltd) and sequence alignments were performed using MEGA version 4.0 (Tamura *et al*, 2007).

Results

		Number of Samples	CPV	CCoV
Population 1: Kennels	Boarding	109	0%	(21/109) 18.80%
	Rescue	169	0%	(83/169) 48.80%
Population 2: Case Control	Cases	80	(4/80) 5%	(7/80) 8.80%
	Controls	174	0%	(3/174) 1.40%
Population 3: Hospitalised		373	(217/373) 58%	(30/373) 7.90%

Table 4.3. *Prevalence of canine parvovirus (CPV) and canine coronavirus (CCoV) in the three different populations tested. Population one- Kennels, both boarding and rescue; population two- Case control study of non-hospitalised, milder cases of diarrhoea: population three- Hospitalised animals with more severe diarrhoea*

Samples

In total, 966 samples were collected from the three study populations. Three hundred and forty faecal samples were collected from clinically healthy dogs from the four kennels in population one; 86 non-hospitalised cases of diarrhoea were collected along with 167 controls from the case control study for population two, and 373 samples from hospitalised animals with enteric disease (population three) (Table 4.3).

The non-hospitalised cases in population two were typically milder than those of the hospitalised cases in population 3. For the cases in population two, the owner of the dog described the nature of the diarrhoea as mild (slightly soft) in 4 (5%), moderate (cow-pat like) in 25 (31%), and severe (very watery) in 51 (64%). However, 39% of these cases were described as having signs such as lethargy or had collapsed, with 30% being described the owners as 'a little quiet', and the remaining 31% being classified as normal. 40% of animals were described as having various amounts of blood in the faeces, with only 9% classified by the owner as being 'very bloody'. For population 3, all dogs were hospitalised with severe diarrhoea, and in many cases vomiting (87%), and 70% of the cases the diarrhoea was haemorrhagic.

CPV prevalence

Overall, the prevalence of CPV in population one was 0% from both the rescue and boarding kennels as well as the controls in population two, whereas there was a

prevalence of 5% in the cases of population two. Population three, which were hospitalised animals, and were selected specifically to have a high level of CPV, had a prevalence of 58% (Table 4.3).

Population one, which was from four different kennels, had no severe signs of clinical enteric disease during collection. Occasionally mild diarrhoea was present, but this may have been due to stress, or alteration of diet associated with entry into the kennel. No CPV (0%) was observed in this population from 340 samples.

Population two was the case control study, where controls were all clinically healthy with no evidence of diarrhoea in the preceding two weeks, and cases which presented at the surgery with diarrhoea of varying severity. In total, 2% (4/80) of the case samples were CPV positive, with 0% (of 174 samples) of the controls being CPV positive.

The third population consisted of clinically ill animals which had been hospitalised due to diarrhoea which required more than conservative treatment (see above). In this population, 58% of 373 samples were CPV positive.

These same samples from all three populations were also tested for coronavirus (table 4.3). Population one showed a prevalence of 18.8% CCoV positive in the boarding kennels, and a higher percentage (48.8%) CCoV positive in rescue kennels. Population two had a prevalence of CCoV of 8.8% in the cases, and 1.4% in the controls. Population three prevalence of CCoV was 7.9% (Table 4.3).

Dual infections

No CPV was detected in population one so there was no opportunity for dual infection. In the case control study for population two, there was no evidence of dual infections, but there was only a low level of CPV prevalence (5%).

In the hospitalised animals from population three, only seven (2%) had a dual infection with both CPV and CCoV.

CPV types

Sequence analysis of the four CPV isolates from the cases presenting in population two, showed that there were three type 2b viruses, and one type 2a viruses, with no type 2 or 2c found.

Due to the large number of positives in the hospitalised animals, a selection of these were chosen to be sequenced. Selection was based on several criteria; including the concentration of DNA present after the PCR, and also to cover all available breeds, and clinical syndromes, as well as to give full coverage of the mainland UK (as much as possible) with at least one sample sequenced from each hospital which submitted a positive sample. Of the 150 viruses sequenced in the PDSA study (population three), 65 (43%) were type 2a viruses, 85 (57%) were type 2b viruses, with again no type 2 or 2c being found (see chapter 3, and Davies *et al*, 2008).

Typing of canine coronaviruses

As mentioned previously, two types of CCoV are known to circulate in the population, and can infect dogs either individually or together. Due to the highest prevalence of CCoV being observed in the kennelled population (population one) these viruses were typed using PCR primers targeted at the antigenically important S gene region. The products were then sequenced (table 4.4).

Kennel	Number of samples	Total CCoV positive	% positive	Type I	Type II	Mixed
Boarding	109	10	18.8	9	1	0
Rescue	169	53	48.8	24	12	17

Table 4.4. *The typing of canine coronaviruses(CCoV) from the kennelled population (population one). The kennels consisted of two types: the boarding kennels and the rescue kennels. As the results for both boarding kennels, and both rescue kennels were similar, the results were merged.*

It was of interest that the rescue kennels contained both a higher prevalence of CCoV and a larger percentage of mixed infection when compared to the boarding kennels infection.

In the case control study (population two), where cases were non-hospitalised and generally showed milder enteric disease, the CCoV isolates were typed as type I (3 cases and 2 controls), three were type II (all cases) and one case has a mixed infection of type I and II viruses.

Discussion

The aim of this study was to investigate the prevalence of CPV in three different canine populations; a kennelled population of clinically normal dogs (population one), a case control population made up of non-hospitalised cases presenting with diarrhoea, and controls which were animals with no enteric disease (population 2), and a hospitalised population which were animals severely ill with enteric disease (population 3).

Canine parvovirus prevalence.

The prevalence of CPV ranged from 0% in the kennelled animals (population one) and the controls in population two, to 5% of the cases in population two, and 58% in the hospitalised animals in population three.

The highest prevalence (58%) was found in population three (hospitalised animals) is likely due to the fact that CPV is known to cause severe illness, so prevalence is likely to be highest in cases selected on this basis (Decaro *et al*, 2006d; Nandi *et al*, 2010; Davies, 2008; Vieira *et al*, 2008). The prevalence of CPV observed in the hospitalised animals (population three) is similar to that recently reported in other studies of severe diarrhoea (Yesilbag *et al*, 2007; Hammond *et al*, 1983; Nandi *et al*, 2010; Hirasawa *et al*, 1996; Castro *et al*, 2007). Thus it appears that CPV is still causing a problem in certain populations, such as the PDSA clinics, where many animals appear not to be vaccinated; up to 80% as reported by Godsall *et al* (2010).

There is difficulty in timing of the first CPV vaccine to coincide with the decrease in maternally derived antibody below the level which prevents infection, but still prevents vaccination response (Gooding *et al*, 1982; Decaro *et al*, 2005c; Hoare *et al*, 1997; Buonavoglia *et al*, 1994). This said, vaccination is, in general, highly successful in protecting animals from this disease, and is the mainstay of disease control (Day *et al*, 2010b). In addition, the high level of disease seen here may be due to reactivation of latent virus, which has stayed within the dog from a previous infection, but we have no data to confirm this.

The prevalence of CPV in the cases of population two (case control study), which generally had only mild diarrhoea, was relatively low (n=4; 5%) and again confirmed the general severe nature of the gastrointestinal disease associated with CPV infection. Indeed, three unvaccinated cases from this population, one of which was a puppy, showed typical clinical signs typical of CPV. In the 4th case, the dog was over ten years old, and was fully vaccinated. However the owner reported frequent diarrhoea and the presence of CPV was surprising. Animals which have been fully vaccinated have previously been reported to be infected with CPV, but these cases appear to be rare (Decaro *et al*, 2009). Such animals highlight the fact that it may be beneficial to test the antibody response of dogs post vaccination, to confirm that they have mounted a suitable response to the vaccine, and will thus be protected (Olsen *et al*, 1988). However, this is likely to be impractical under most field conditions.

CPV was not detected in the healthy dogs (population one and controls of population two). The absence of CPV in these populations may be due to the

majority of dogs having up to date previous vaccination, as is required for acceptance into these boarding kennels, and vaccination occurs prior to admission in the rescue shelters.

The lack of positive faecal samples from animals which had been recently vaccinated indicates that the vaccine virus was either not shed, or perhaps less likely, was being shed at low titres below the level of detection of the assay used. MLV vaccine virus can be shed in the faeces of vaccinated dogs for up to a week post vaccination at low titres, which can result in a positive result at a diagnostic laboratory if the animal presents with gastroenteritis in this period (Decaro *et al*, 2007c). This shedding is prevented if there is some antibody present, whether this be from previous vaccination, or field infection (Larson *et al*, 1997). Despite this, some of the newer high titre viruses are not shed in faeces, regardless of the antibody status of the vaccinate (Gore *et al*, 2005).

Rescue kennels like those sampled in population one can offer an ideal environment for virus spread, because of varying levels of sanitation and high animal turnover (Sokolow *et al*, 2005). The source of infection during an outbreak is often the introduction of an infected animal which is shedding the virus but not showing any clinical signs (Potgieter *et al*, 1981; Appel *et al*, 1979). During the period of sampling, there was no evidence of widespread enteric disease in any of the kennels in this study. However, there had been severe outbreaks of CPV enteritis in the past at the rescue kennels associated with high morbidity and mortality (personal communication, kennel personnel). The absence of CPV in this population suggests that animals which have been vaccinated have mounted a

suitable response to the vaccine, and that sufficient disinfecting of the premises has been done after cases had occurred.

Canine coronavirus prevalence.

The prevalence of canine coronavirus (CCoV) also differed between the three populations, but contrasted to the levels of CPV. In the hospitalised population, which showed high (58%) CPV levels, low levels of CCoV were observed (7%), suggesting that in contrast to CPV, CCoV is not a major cause of severe enteric disease. In comparison, CCoV prevalence appears to be high in kennelled animals (18%) suggesting that CCoV can be carried asymptomatically by animals in a kennel. Although most infected dogs appear to show no signs, animals which are either infected with other viruses, or are immunosuppressed may be more likely to develop disease signs. Infection with CCoV in conjunction with CPV (Evermann *et al*, 2005) can cause a more severe disease, and it has also been shown that CCoV infection subsequent to CPV infection can cause severe disease and death (Pratelli *et al*, 1999b, Appel *et al*, 1988). Godsall *et al* (2010) suggest that dual infection with CPV and CCoV is unlikely, and if an animal was infected with CCoV, it was less likely to be infected with CPV. Dual infection was rare in this study, with only 7 (2%) animals having CCoV and CPV infection, and all these were noticed in population three (hospitalised animals). This may be a reflection of the very low prevalence of CPV in the other two populations.

As CCoV vaccines are now available, vaccination of dogs prior to entry into kennels with the coronavirus vaccine may reduce level of infection, and morbidity due to CCoV (Pratelli *et al*, 2004b).

Chapter 5

Analysis of the non-structural gene 1 of CPV of dog origin

Introduction

As discussed in the introduction (chapter 1) canine parvovirus, encodes two different non-structural proteins, known as NS-1 and NS-2 (Wang *et al*, 1998, Cotmore *et al*, 1997, Morgan *et al*, 1986. NS-1 is a nuclear phosphoprotein, involved in DNA replication, and activates promoters within the host cell (Nuesch *et al*, 1995). It has been shown in minute virus of mice (MVM) that NS-2 is produced in infection prior to the viral capsid proteins (Naeger *et al*, 1993), and it is thought that the NS-1 protein co-activates the p38 promoter, allowing production of the viral capsid (VP1, VP2 and VP3) genes (Christensen *et al*, 1995b, Hanson *et al*, 1991). For this reason, NS-1 has been shown to be vital for effective viral replication in all host cell types (Naeger *et al*, 1993). It has also been suggested that NS-1 co-localizes with DNA replicating bodies (Cziepluch *et al*, 2000). The CPV NS-1 is a site specific binding protein, that recognizes the sequence ACCA (Tattersall *et al*, 1976). NS-1 initiates viral replication by creating a nick, which is site specific and on a single strand (Nuesch *et al* (1995). This nick is located at a consensus sequence (CTWWTCA), and is created after NS-1 binds to the ACCA sequence (Nuesch *et al*, 1995). NS-1 is also shown to interact with several cellular factors, and is thought to have multiple functions but as yet, these are poorly defined (Cotmore *et al*, 1995)

It is widely considered that the motifs required for the functions of NS-1 are conserved among all parvoviruses (Yeung *et al*, 1991). The C terminus of the NS-1 gene has also been linked to the cytotoxic effect of the virus and thus its pathogenicity (Legendre *et al*, 1992).

The NS-1 protein is generally not exposed to the immune system of the host, but may be externalised when it is bound to the 5' end of the DNA (Cotmore *et al*, 1988, 1989), so it is possible that the immune response may also drive the evolution of the NS-1 gene. Indeed antibodies to the NS-1 gene have been reported for some parvoviruses (Cotmore *et al*, 1995; Yeung *et al*, 1991), providing further evidence to support this suggested evolution mechanism.

Despite the importance of the NS-1 protein in the life cycle of the virus, previous studies have not examined the levels of variation in the NS-1 gene of the CPV genome, with the NS-1 gene often overlooked in favour of typing only using the VP gene. Conclusions are often made regarding the viruses being the same based on the VP gene alone, without consideration of the NS-1 gene where differences may alter the efficiency with which the viruses replicate. In addition, the NS-1 gene has not previously been assessed for their potential for typing of CPV, and no studies have examined the differences between the NS-1 protein of the different CPV types (2, 2a, 2b and 2c).

Therefore the aim of this study was to investigate the variation of the NS-1 protein in CPV, to ascertain if the protein is as highly conserved as previously suggested, and to confirm that the important motifs are conserved. In addition, the potential for the NS-1 gene to be used in virus typing was also examined.

Methods

Samples

Fifty eight of the 150 samples which were collected from clinically ill animals from the PDSA hospitals, and for which the VP2 gene has been sequenced (chapter 3; Godsall *et al*, 2010), were chosen for NS-1 gene sequencing in this study. The choice of the samples selected for NS-1 gene sequencing was based on several criteria including different VP2 sequences, different clinical signs and a full UK geographical spread.

In order to compare levels of variation within the NS-1 gene, all available NS-1 genes on Genbank were aligned along with those sequenced in this study. However, in order to compare typing by NS-1 gene, it was important that the VP2 gene was also available, so only the Genbank sequences with the VP2 available, and those sequenced here were included in the analysis.

For details of the methods used for VP2 design primer, DNA Extraction, VP2 PCR and sequencing of both VP2 and NS1 genes, please see pages 66 and 79 of this thesis

NS-1 gene PCR

Primers were chosen to amplify the full NS-1 gene region, using both full genomes of CPV and FPV, and NS-1 sequences available on Genbank, aligned using CLUSTAL as implemented in Mega 4 (Tamura *et al*, 2007). Unfortunately the PCR for the VP2 and NS-1 gene products did not overlap, and attempts to bridge the gap proved unsuccessful (data not presented).

The primers used were two external primers NS For: 5' ACC GTT ACT GAC ATT CGC TTC- 3' and NS Rev :5' CCT TAC CTC TCC TGG CTC- 3'. Four internal primers were then used to give full overlapping sequence of the NS-1 gene. These were, CPV 3: 5'- CGG GAG AAT TCA AAC TAA AAA GG- 3', CPV 4:5' GGG CGG AGC CTA AAA TAC AA- 3',CPV5 : 5'- GAG GAT TGC TTG CCG CTT – 3' and CPV6 : 5' – CAG TCT TCA GGT GAT GTT ACT- 3'.

The PCR cycling conditions were 1 min at 94°C, followed by 40 cycles of denaturation at 94°C, annealing at 53°C and extension at 72°C. A final extension phase of 68°C for 10 minutes was used to terminate the PCR. The final predicted amplicon size was approximately 2200 base pairs and contained the full NS-1 gene and more than 90% of NS-2. However, because of complex splicing of NS-2, and the lack of sequence at the N terminus of NS-2 we analysed NS-1.

Sequence analysis and phylogeny

All sequences were aligned into a double-stranded consensus sequence using Chromas Pro 1.41 (Technelysium Pty Ltd). All NS-1 gene consensus sequences were aligned and analysed using Mega 4 (Tamura *et al*, 2007). All external primer sites were removed to give a final consensus sequence of 2007 base pairs. The differences within the sequences of the VP2 and NS-1 genes were compared.

The most appropriate evolution model was predicted using MODELTEST as implemented in Topali (Milne *et al*, 2008), and this was used to infer bootstrapped maximum likelihood trees (1000 bootstraps) using PHYML implemented on the ATGC bioinformatics platform

(Tamura *et al*, 1993). Amino acid trees were drawn using Mega 4, rooted using FPLV and drawn using the Dayhoff PAM matrix.

Analysis of mutations with clinical signs and haemagglutination results

As clinical details (such as vaccination status, clinical signs (including vomiting severity, and severity of enteritis) and presence of lethargy), signalment (such as sex, breed and pedigree status) and haemagglutination results were available for the samples sequenced here, we were able to evaluate possible associations between NS-1 gene mutations and clinical outcome, and to assess if a mutation affected the result of the diagnostic haemagglutination test.

Results

In total 56 NS-1 genes were sequenced from isolates collected from dogs which were attending PDSA hospitals across mainland UK. Samples were selected to cover most PDSA practises across the mainland UK, along with different clinical patterns and breeds of dog and different VP2 gene sequences. Previous studies had typed these viruses by sequencing the VP2 gene and indicated that the 59 samples were made up of 21 type 2a viruses and 35 type 2b viruses (chapter 3).

Where a sample was positive for VP2, and then tested for NS- gene, it was always positive for both. In no cases was it negative for one and positive for the other, suggesting that both PCR reactions work well.

Phylogenetic grouping of viruses

Phylogenetically, using sequences from this study, and all available NS-1 genes from canine and feline parvoviruses on Genbank, FPLV NS-1 genes grouped separately from the CPV NS-1 genes at both the DNA (Figure 5.1a), and amino acid level (Figure 5.1b) which is similar to that seen with the VP2 gene. This suggests that the two viruses are different to each other in both genes, but have evolved from a common ancestor.

Sample A15 (labelled with a * on figure 5.1, 5.2 and 5.3) is an isolate of particular interest as although it was collected as part of the study with the PDSA (chapter 3 this thesis), it was sent to us in error as it was from a cat. It appears that this represents an intermediate sequence between FPLV NS-1 genes and CPV NS-1 genes.

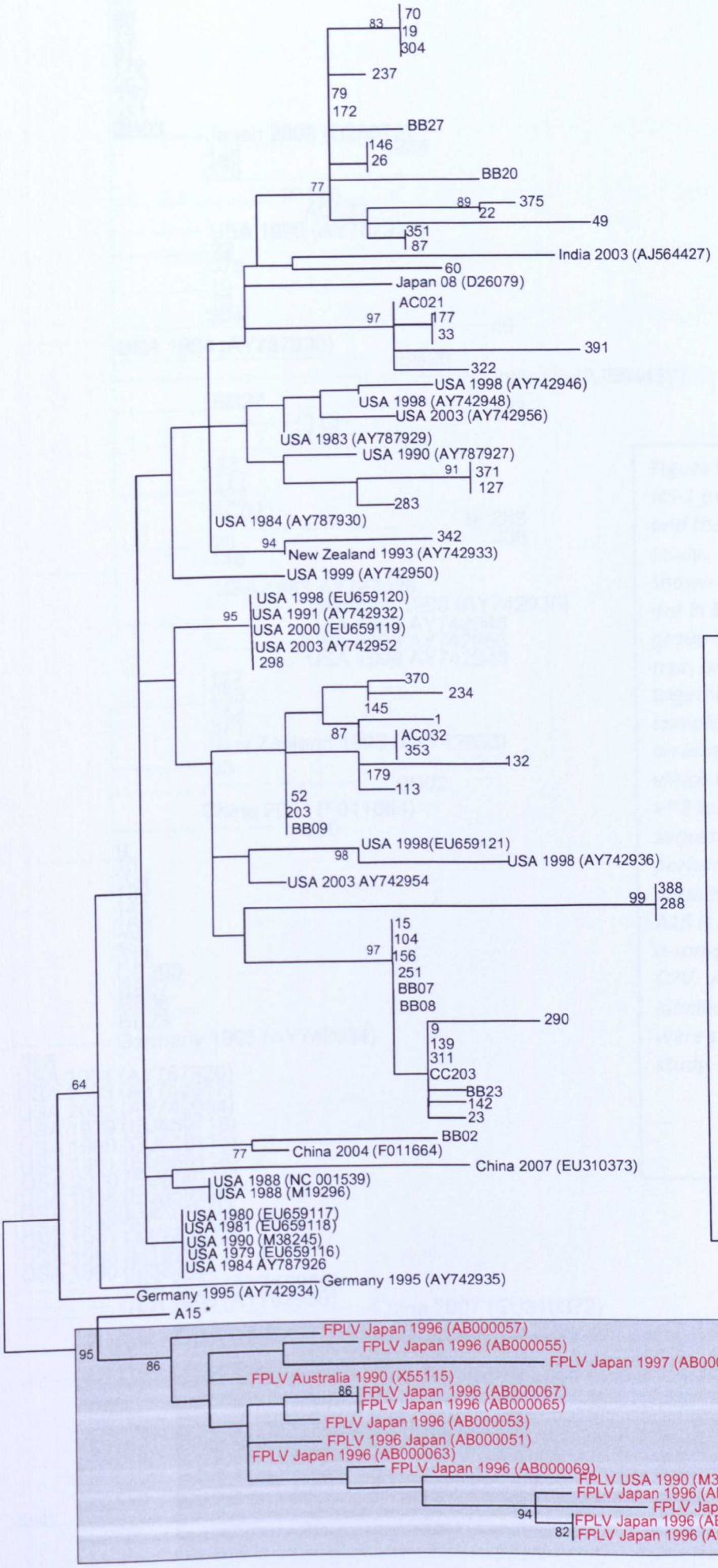

Figure 5.1a.*A DNA tree of all NS-1 genes available on Genbank and those sequenced as part of this study. The FPLV sequences are shown in red; CPV sequences are shown in black. The NS-1 genes of FPL viruses are grouped together at the bottom of the tree, while the CPV sequences group together at the top of the tree. Bootstrapping was performed 1000 times, and values greater than 75 are shown. Sample A15 is labelled with an *, as this was a sample from a cat, infected with CPV. All samples which are not labelled with a country of origin were sequenced as part of this study.*

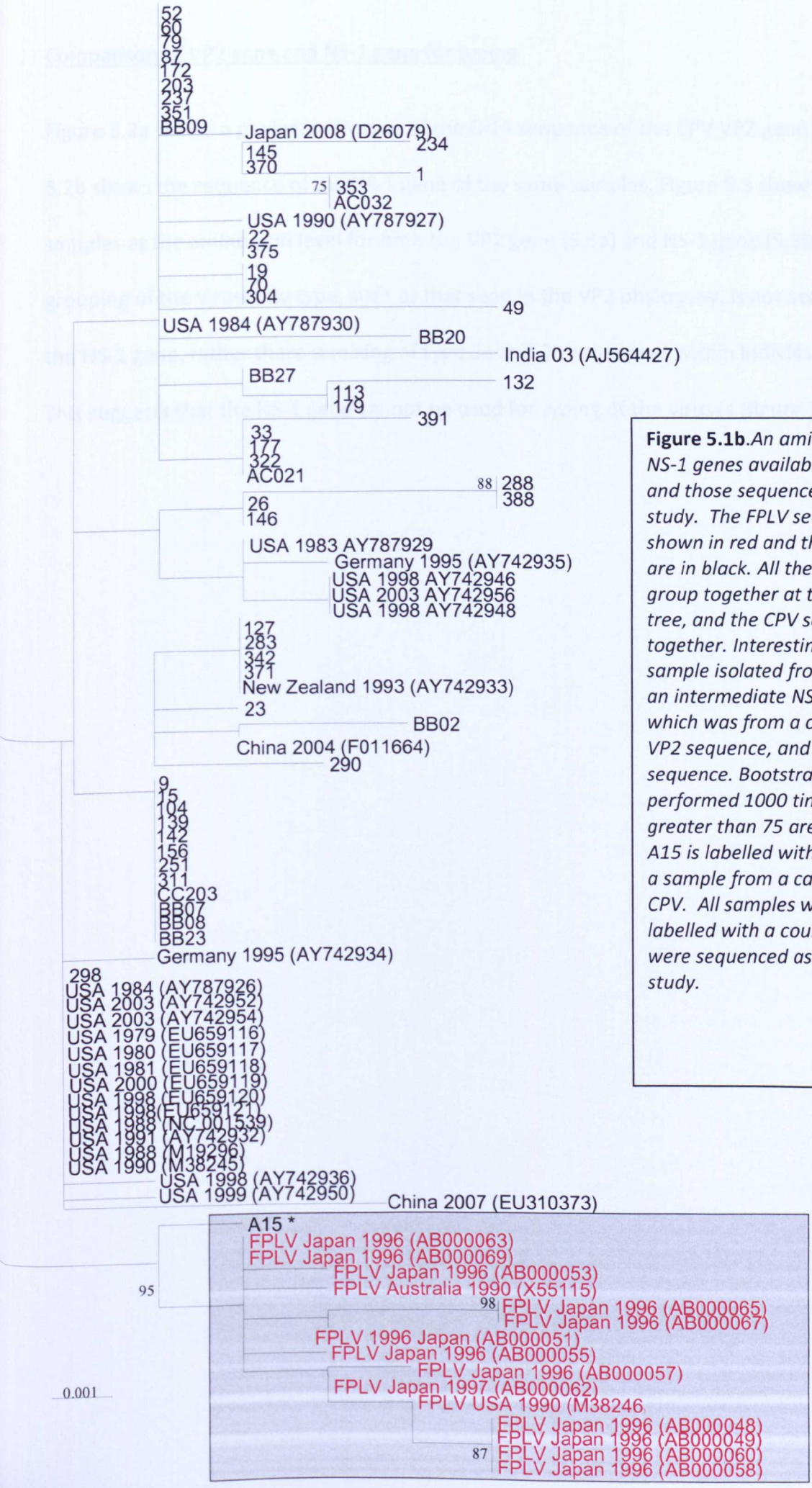

Figure 5.1b.*An amino acid tree of all NS-1 genes available on Genbank and those sequenced as part of this study. The FPLV sequences are shown in red and the CPV sequences are in black. All the FPLV sequences group together at the bottom of the tree, and the CPV sequences group together. Interestingly, A15 (a sample isolated from a cat) showed an intermediate NS-1 sequence, which was from a cat, had a CPV like VP2 sequence, and a FPLV like NS-1 sequence. Bootstrapping was performed 1000 times, and values greater than 75 are shown. Sample A15 is labelled with an *, as this was a sample from a cat, infected with CPV. All samples which are not labelled with a country of origin were sequenced as part of this study.*

Comparison of VP2 gene and NS-1 gene for typing

Figure 5.2a shows a phylogenetic tree of the DNA sequence of the CPV VP2 gene and figure 5.2b shows the sequence of the NS-1 gene of the same samples. Figure 5.3 shows the same samples at the amino acid level for both the VP2 gene (5.3a) and NS-1 gene (5.3b).The grouping of the viruses by type, such as that seen in the VP2 phylogeny, is not seen within the NS-1 gene, rather there is mixing of type 2a and 2b sequences within individual clusters. This suggests that the NS-1 gene cannot be used for typing of the viruses (figure 5.2).

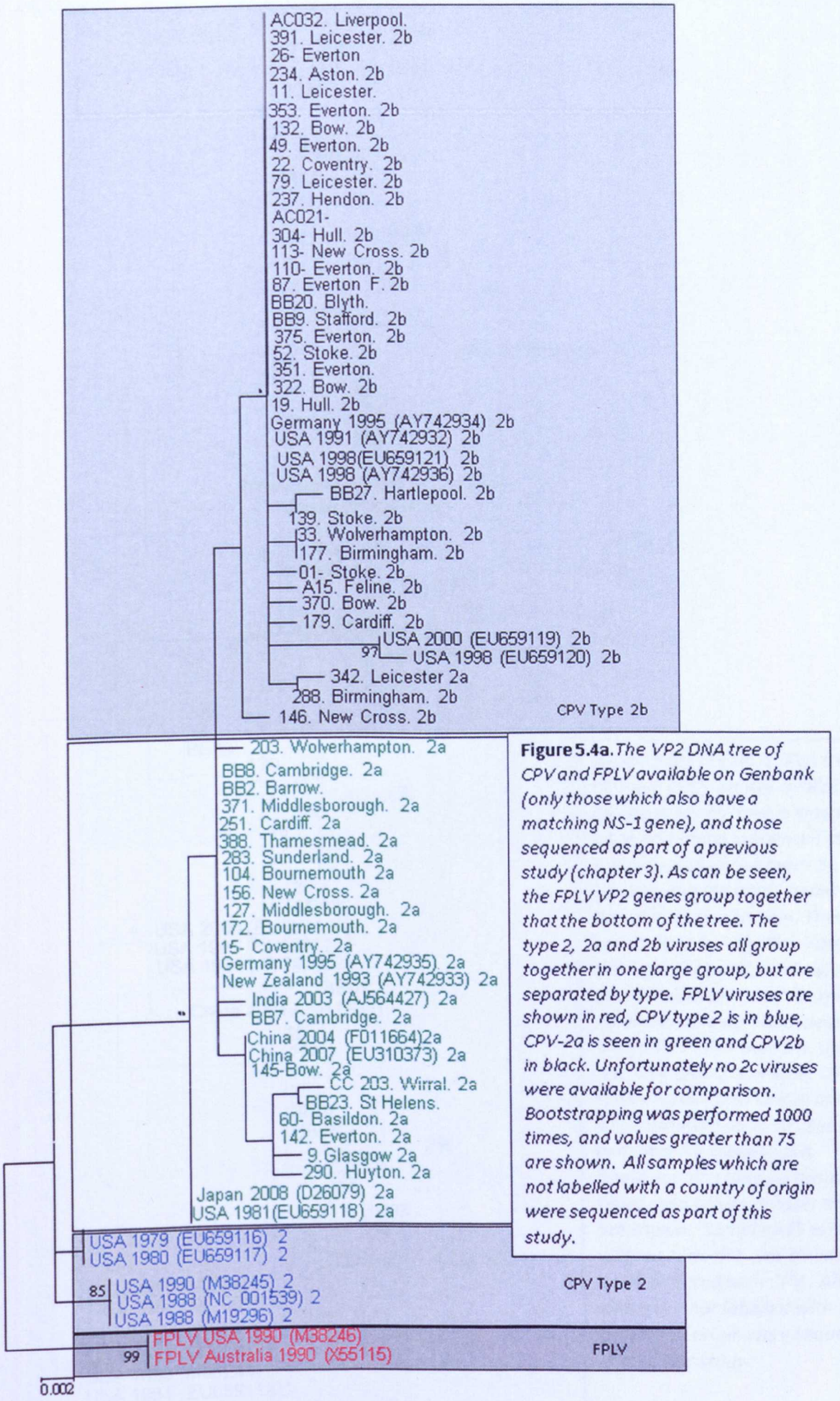

Figure 5.4a. *The VP2 DNA tree of CPV and FPLV available on Genbank (only those which also have a matching NS-1 gene), and those sequenced as part of a previous study (chapter 3). As can be seen, the FPLV VP2 genes group together that the bottom of the tree. The type 2, 2a and 2b viruses all group together in one large group, but are separated by type. FPLV viruses are shown in red, CPV type 2 is in blue, CPV-2a is seen in green and CPV2b in black. Unfortunately no 2c viruses were available for comparison. Bootstrapping was performed 1000 times, and values greater than 75 are shown. All samples which are not labelled with a country of origin were sequenced as part of this study.*

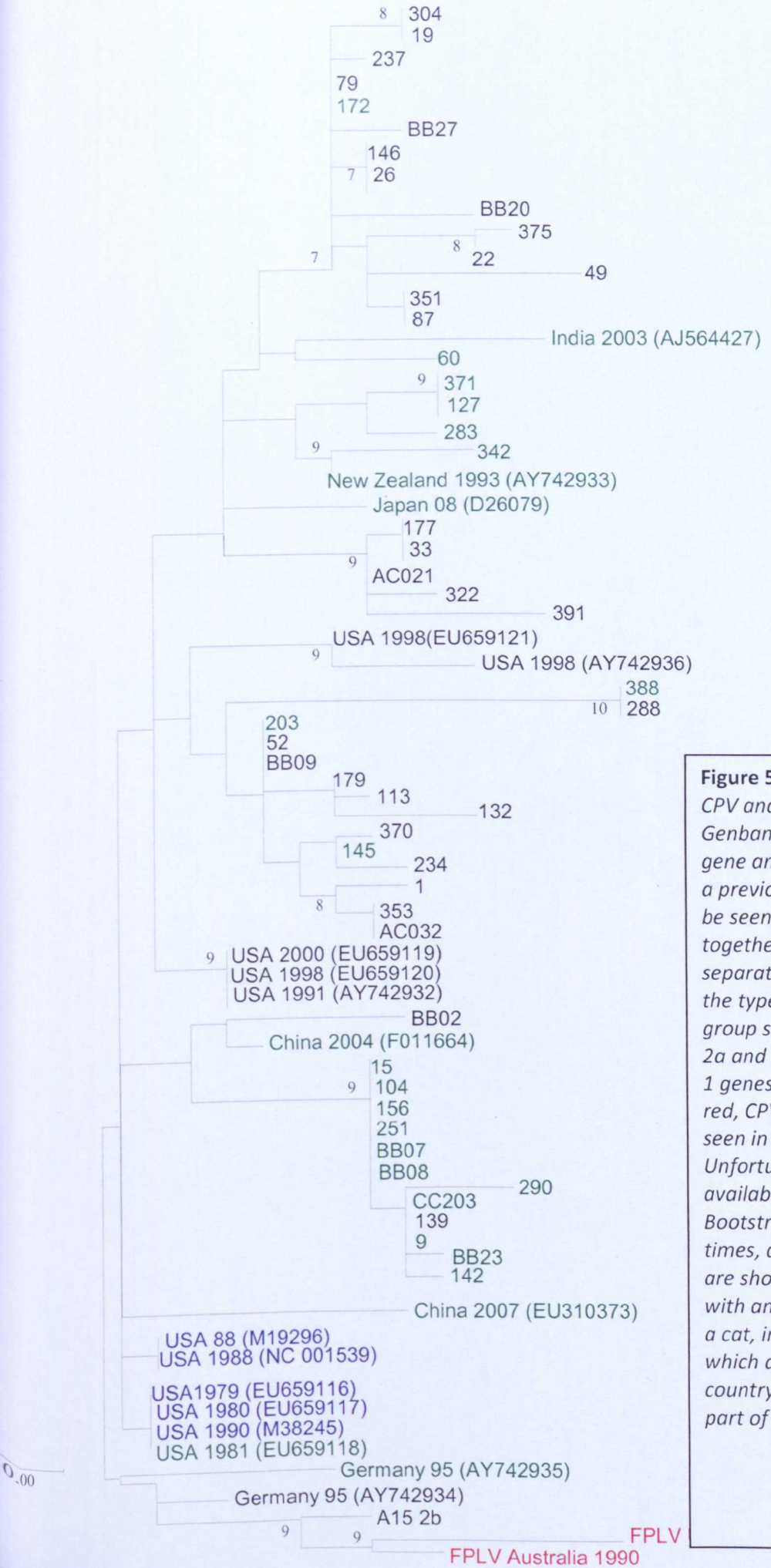

Figure 5.3b.*The NS-1 DNA tree of CPV and FPLV viruses available on Genbank which have a matching VP gene and those sequenced as part of a previous study (chapter 3). As can be seen, the FPLV VP2 genes group together at the bottom. There is a separate group of CPV viruses, but the type 2, 2a and 2b viruses do not group separately, and in some cases 2a and 2b viruses have identical NS-1 genes. FPLV viruses are shown in red, CPV type 2 is in blue, CPV-2a is seen in green and CPV2b in black. Unfortunately no 2c viruses were available for comparison. Bootstrapping was performed 1000 times, and values greater than 75 are shown. Sample A15 is labelled with an *, as this was a sample from a cat, infected with CPV. All samples which are not labelled with a country of origin were sequenced as part of this study.*

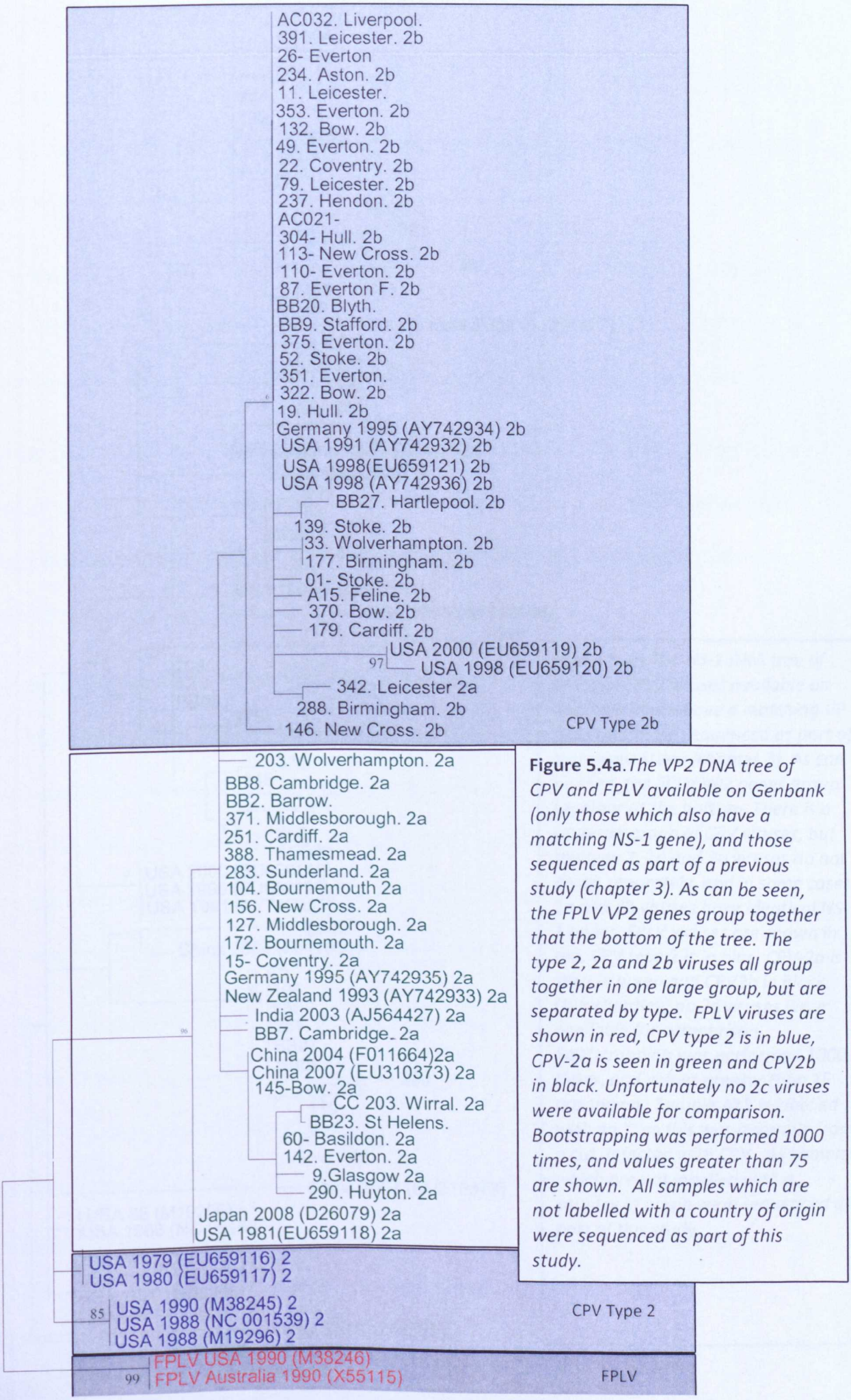

Figure 5.4a.*The VP2 DNA tree of CPV and FPLV available on Genbank (only those which also have a matching NS-1 gene), and those sequenced as part of a previous study (chapter 3). As can be seen, the FPLV VP2 genes group together that the bottom of the tree. The type 2, 2a and 2b viruses all group together in one large group, but are separated by type. FPLV viruses are shown in red, CPV type 2 is in blue, CPV-2a is seen in green and CPV2b in black. Unfortunately no 2c viruses were available for comparison. Bootstrapping was performed 1000 times, and values greater than 75 are shown. All samples which are not labelled with a country of origin were sequenced as part of this study.*

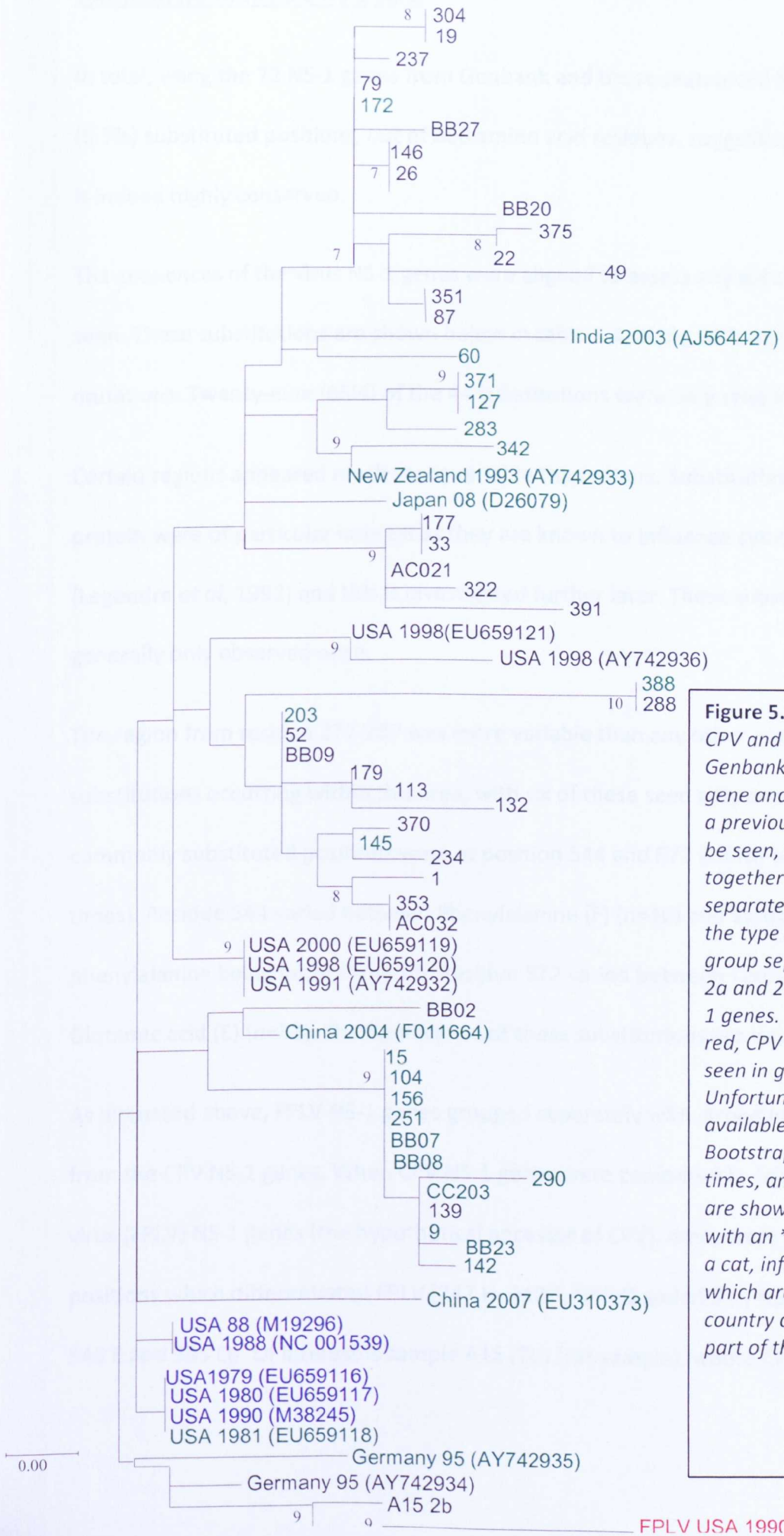

Figure 5.3b.*The NS-1 DNA tree of CPV and FPLV viruses available on Genbank which have a matching VP gene and those sequenced as part of a previous study (chapter 3). As can be seen, the FPLV VP2 genes group together at the bottom. There is a separate group of CPV viruses, but the type 2, 2a and 2b viruses do not group separately, and in some cases 2a and 2b viruses have identical NS-1 genes. FPLV viruses are shown in red, CPV type 2 is in blue, CPV-2a is seen in green and CPV2b in black. Unfortunately no 2c viruses were available for comparison. Bootstrapping was performed 1000 times, and values greater than 75 are shown. Sample A15 is labelled with an *, as this was a sample from a cat, infected with CPV. All samples which are not labelled with a country of origin were sequenced as part of this study.*

Substitutions within the NS-1 gene

In total, using the 72 NS-1 genes from Genbank and those sequenced here, there were 44 (6.5%) substituted positions, out of 668 amino acid residues, suggesting that the NS-1 gene is indeed highly conserved.

The sequences of the virus NS-1 genes were aligned to assess any substitutions which were seen. These substitutions are shown below in table 5.4, along with the frequencies of these mutations. Twenty-nine (65%) of the 44 substitutions were only seen in one sequence.

Certain regions appeared much more variable than others. Substitutions at the start of the protein were of particular interest as they are known to influence cytotoxicity of the virus (Legendre *et al*, 1992) and this is investigated further later. These substitutions were generally only observed once.

The region from residue 277-287 was more variable than any other area, with eight substitutions occurring within this area, with six of these seen only once. The two most commonly substituted positions were at position 544 and 572 (which was mutated 19 times). Residue 544 varied between Phenylalanine (F) (n=40) and Tyrosine (Y) (n=32), with phenylalanine being more common. Residue 572 varied between Lysine (K) (n =19) and Glutamic acid (E) (n=53). The significance of these substitutions are unknown.

As discussed above, FPLV NS-1 genes grouped separately with strong bootstrap support from the CPV NS-1 genes. When CPV NS-1 genes were compared to feline panleukopenia virus (FPLV) NS-1 genes (the hypothetical ancestor of CPV), there were four amino acid positions which differentiated FPLV (247 H, 248 T, 545 Q and 595 H) from CPV(247 Q, 248 I, 545 E and 595 Q). Of interest is sample A15 (2b) (cat sample), where CPV was isolated based

on the virus VP2 sequence (indicated with an * on figures 5.2, 5.3 and 5.4). This cat CPV shares three of the four (not 545 Q) of the FPLV specific amino-acids. Interestingly it also groups as an intermediate phylogentically between the FPLV and CPV genotypes.

Table 5.4. *Table of substitutions seen within the NS-1gene. The residue which is substituted is shown down the left, with the sample number on the top line. The residues indicated in red are where the virus is substituted to a residue different from that commonly seen. In addition, residues 544 and 572 were the most commonly substituted positions and are discussed above, but not mentioned in this table.*

	FPLV (X55115)	FPLV (M38246)	CPV (AJ564427)	388_2a	375_2b	322_2b	BB27_2b	BB20_2b	BB02_2a	AC021_2b	A15_2b	391_2b	304_2b	290_2a	288_2b	234_2b	177_2b	145_2a	146_2b	132_2b	113_2b	49_2b	33_2b	26_2b	22_2b	19_2b	1_2b	CPV (D26079)	CPV (EU310373)	CPV (AY742934)	CPV (AY742935)	CPV (AY742936)	Frequency
3	G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	1
7	T	T	T	T	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	1
10	V	V	V	V	V	V	V	V	I	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	1
23	N	D	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	1
48	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	I	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	1
115	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	A	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	1
132	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	I	V	V	V	V	V	V	V	V	V	V	1
221	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	L	H	H	H	H	H	1
222	M	M	M	M	M	M	M	M	M	M	M	M	I	M	M	M	M	M	M	M	M	M	M	M	M	I	M	M	M	M	M	M	2
226	S	S	S	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	1
247	H	H	Q	Q	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	3
248	T	T	I	I	I	I	I	I	I	I	T	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	3
249	V	V	G	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	1
252	L	L	L	L	L	L	L	L	L	L	V	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	1
277	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	N	I	I	I	I	I	I	I	I	I	1
278	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	S	Q	Q	Q	Q	Q	Q	Q	Q	Q	1
279	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	N	T	T	T	T	T	T	T	T	T	1
280	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	R	K	K	K	K	K	K	K	K	K	K	K	K	1
281	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	R	R	K	K	K	K	K	K	K	K	K	K	K	3
282	E	E	G	E	E	E	K	E	E	E	E	E	E	E	E	E	E	E	E	K	K	E	E	E	E	E	E	E	E	E	E	E	5
286	K	K	Q	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	1
287	C	C	C	C	C	C	C	C	I	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	1
350	D	D	D	N	D	D	D	D	D	D	D	D	D	D	N	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	N	D	3
351	N	N	N	N	N	S	N	N	N	S	N	S	N	N	N	N	S	N	N	N	N	N	S	N	N	N	N	N	K	N	N	6
360	A	A	A	A	V	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	V	A	A	A	A	A	A	A	2
443	I	V	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	1
545	Q	Q	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	2
573	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	S	1
575	I	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	1
579	C	C	C	C	C	C	C	W	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	1
582	L	L	L	S	L	L	L	L	L	L	L	L	L	L	S	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	2
583	E	E	E	K	E	E	E	E	E	E	E	E	E	E	K	E	E	E	K	E	E	E	E	K	E	E	E	E	E	E	E	E	4
584	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	A	T	1
590	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	T	P	T	P	T	P	P	P	P	P	P	T	P	P	P	P	P	7
595	H	H	Q	Q	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	3
597	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	H	L	L	L	1
598	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V	T	T	T	1
599	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	R	P	P	P	1
600	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	L	1
655	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	N	T	T	T	T	T	1
656	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	C	S	S	S	S	S	1
661	E	E	E	E	E	E	E	E	E	E	E	R	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	1

Comparison of UK NS-1 genes to the rest of the world NS-1 genes

It has previously been shown in chapter 3, that the sequences of the viruses at the DNA level of the VP2 genes are, in general, unique to the UK. However, at the amino acid level, the sequences were more similar to those seen around the rest of the world.

Unfortunately not many NS-1 sequences have been sequenced, so there is limited analysis power available; however the sequences from this study which were identical to others available on Genbank are discussed below.

Of the viruses sequenced in this study, only one virus (virus 298) was identical at the DNA level to others found on Genbank. This was identical to several isolated in the USA from 1991-2003 (accession numbers EU659120, AY742932, EU659119 and AY742952) (figure 5.2a).

At the amino acid level, only two viruses sequenced in this study were identical to others found on Genbank. The same virus as discussed above (298) was again identical to the NS-1 sequences of the four viruses listed above and to eight other viruses (AY787926, AY742954, EU659116, EU659117, EU659118, EU659121, NC_001539 and M19296), all of which were isolated within the USA over various years.

In addition, at the amino acid level, sample numbers 127, 283, 342 and 371 sequenced here were identical to a NS-1 gene sequence from a virus previously isolated in New Zealand (Genbank accession no: AY742933).

Association of NS-1 mutations with clinical parameters.

There were no clear links between an isolate's NS-1 sequence and clinical parameters associated with infection including sex, breed, pedigree status, vaccination status, ability to haemagglutinate as judged at diagnosis, clinical signs and outcome (survival or death). Although NS-1 gene has been linked for example to cytotoxicity, and variation in this gene may link to altered pathogenicity, other factors than their sequence may also influence the outcome of infection.

Selection pressures on NS-1

As it has been reported previously that the NS-1 gene can be attached the 5' end of the genome (Cotmore *et al*,1988), and thus be exposed to the external environment, it was of interest to ascertain if any pressures were on the CPV NS-1 gene.

Using Datamonkey (Pond *et al*, 2005), it was found that ten amino acid residues were under negative selection, suggesting that these are absolutely necessary for the function of the gene. These residues were 8, 15, 107, 114, 172, 369, 408, 422, 430 and 641. In contrast, no residues were shown to be under positive selection.

Discussion

Despite the importance of the NS proteins for viral replication, there have been no studies analysing their variation, with all other variation studies in CPV focusing on the VP2 capsid gene. This is particularly evident in the numbers of NS-1 genes (N=41, 11 full CPV NS-1, 27

from full genomes (17 CPV, 10 FPLV), 3 FPLV NS-1) available on Genbank (http://www.ncbi.nlm.nih.gov/genbank/, accessed June 20th 2011) compared to 740 (384 full VP2, 356 Partial) VP2 genes available. Of these, only the 27 full genomes had both NS-1 and VP2 gene sequenced.

In this study of 56 viruses, to the author's knowledge the largest survey of NS-1 gene variability in canine parvovirus, we confirmed that the gene is highly conserved, being less variable than VP2, showing high similarity to other parvoviruses, and generally evolving under purifying selection. This clearly suggests that the sequence of NS-1 is tightly restricted to allow the protein produced to fulfil its function. Despite this, there were some areas of interesting diversity. In particular, two amino acid mutations, amino acid residues 544 (mutated to either tyrosine (n=32) or phenylalanine (n=40) and 572 (mutated 19 times) appear to be highly variable, and are located close together. The significance of these mutations is unknown.

In this study, we found considerable evidence to suggest that recombination has played an important part in the evolution of this population of viruses, as evidenced by the lack of clustering of different CPV antigenic types seen in the NS-1 phylogeny. A stark example of this is where exactly the same NS-1 gene was carried by both a CPV 2a (sample 288) and a CPV 2b (sample 388) (see figure 2). This lack of clustering was conserved regardless of the methods used to produce the phylogram, and is in contrast to the VP2 gene where generally, viruses of the same type group relative to their VP2 gene, with only rare exceptions. Recombination has been reported previously for different CPV types (Shackelton *et al*, 2007) and between CPV and FPLV (Ohshima *et al*, 2009). For recombination to occur both viruses must infect the same call at the same time, and the

high level of recombination here suggests that infection of the same cell with a CPV 2a and 2b virus must be more common than previously suggested. Unfortunately the two PCR assays used here for VP2 and NS-1 genes, did not overlap, despite several attempts by the author to generate amplicons bridging the predicted recombination site somewhere between NS-1 and VP2. Therefore the observed results could be explained by differential amplification form a mixed infection. This seems unlikely because we found no evidence of mixed infections as judged by the purity of the sequence obtained throughout this thesis for both the NS-1 and VP2 genes. In addition, the conserved nature of these parvovirus genes makes it unlikely that a PCR assay would preferentially amplify one antigenic type over another. That said, we clearly have not categorically proven this recombination, and this would be a priority for any ongoing research.

In addition, the evolution rates of the VP2 gene and the NS-1 gene have been shown to be different, suggesting that they evolve at different rates (Hoelzer *et al*, 2008). This may be another explanation for the different NS sequences.

An interesting case of potential recombination was seen in a feline faecal sample (A15) collected from a clinically normal (i.e. no gastroenteric signs) cat The VP2 gene was typical of a CPV type 2a but phylogenetic analysis of this sample's VP2 gene suggests the cat was infected with a typical canine type virus. However, interestingly, phylogenetic analysis of the NS-1 gene of this virus grouped it most closely to FPV viruses, suggesting recombination between a CPV like VP2 and an FPLV like NS-1 gene. Recombination between FPLV and CPV has previously been reported (Ohshima *et al*, 2009). This raises the interesting possibility that recombination between dog and cat parvoviruses may have contributed to the species jump from canine back to felines. Such recombination would likely to have to occur in a cat,

since cats can be infected with both FPLV and CPV whereas dogs have never been shown in the field to be infected with FPLV, and experimentally, FPLV only replicates poorly in dogs (Truyen *et al*, 1992). The extent of recombination in parvoviruses in cats is tested further in chapter 7.

When the NS-1 genes of CPV and FPLV viruses were analysed phylogenetically, there appeared to be four residues in the FPLV NS-1 gene which are specific to FPLV sequences and are different in the CPV gene: 247 H, 248 T, 545 Q and 595 H. These amino acids are important in the ability to use the NS-1 gene to differentiate FPLV from all CPV types, and this may be useful to differentiate viruses isolated from cats which can be infected by both CPV and FPLV and ascertain if they are true CPV or a CPV/FPLV recombinant (Gamoh *et al* 2003, Mochizuki *et al,* 1996, Ikeda et al, 2002). When the NS-1 gene of the A15 sample, isolated from the clinically normal cat discussed above was analysed, three of the four FPLV specific amino acid residues (not 454 Q) were seen, suggesting that it may represent a rare intermediate in the evolution of canine and feline parvoviruses.

The low number (n= 41) of NS-1 gene sequences available on Genbank means it is difficult to compare world NS-1 sequences to those generated here. However, the small number of sequences available were generally different to those produced here, with only one virus sequenced here being identical to others around the world (USA). However, despite the number of mutations, none appeared to occur within motifs which are conserved in most parvoviruses and therefore must be of importance to the virus, and this suggests that this gene is under a selection pressure to keep these important motifs including those responsible for functions such as nicking of the replicative form DNA (RF-DNA) (Nuesch *et al*, 1995), ATP binding (Gottschalck *et al*, 1994), nuclear translocation (Nuesch *et al*, 1993),

nuclear localisation sequences (Eichwald *et al*, 2002) and for production of NS-2 (Naeger *et al*, 1990).The conservation of these motifs suggest that these motifs are important in both CPV function as well as MVM.

Despite the fact that the NS-1 protein is often external to the virion particle (Cotmore *et al*, 1988), when tested for selection pressures, it was shown that only negative selection influences the evolution of the CPV NS-1 gene. Ten residues were identified as being under negative (purifying) selection, suggesting these may have a particularly important role in the function of this protein. Despite this, none of the negatively selected sites identified here appear to be in important conserved motifs. Therefore it is possible that these residues are important in keeping the structure of the protein, allowing for its exposure or positioning of the important motifs.

Chapter 6

Evaluation of the chronological evolution of CPV

Introduction

Evolution requires genetic diversity created by random substitution (Abraham *et al*, 2007) or recombination (Lim *et al*, 2011), and selection pressures such as that which occurs due to the immune response (Liang *et al*, 2010). Evolution is important as it leads to the regular emergence of new viruses and new virus strains in naive populations (Li *et al*, 2007; Tapper 2006). The survival and transmission of these new viruses are generally rare events, but where a virus gains access to a new host species, they are often associated with high morbidity and mortality. Important examples of such species jumps include HIV from chimpanzees to humans (Sharp *et al*, 2010), canine parvovirus from its feline ancestor (Truyen *et al*, 1998) and occasional antigenic shifts such as the recent H1N1 influenza virus pandemic (Chowell *et al*, 2011; Comas-Garcia *et al*, 2011; Zepeda Lopez *et al*, 2010). Even when viruses stay within the same host population, evolution can allow evasion of host immunity from antibodies or vaccines, and again cause high morbidity and mortality, such as is observed with influenza virus antigenic drift each winter (Boyd *et al*, 2006, Parrish *et al*, 2005)

It is very rare to have the opportunity to study the emergence of an organism, due to the fact that many organisms emerged many years ago, and thus actual samples from its earliest history are either completely absent or extremely limited. In addition, for many organisms, their evolution rate is so slow that changes do not

accumulate within the time scale of modern microbiological sampling. There are but notable exceptions, most typically within the RNA viruses where their high evolution rate and regular emergence has provided the ability to study the molecular evolution of outbreaks. Some examples include severe acute respiratory syndrome (SARS) (Holmes *et al*, 2004, Wang *et al*, 2005b), human immunodeficiency virus (HIV) (Fryer *et al*, 2010, Batorsky *et al*, 2010) and rabbit haemorrhagic disease virus (RHDV) (Kerr *et al*, 2009, Alda *et al*, 2010).

Interestingly the same is also true for canine parvovirus (CPV). Unlike most DNA viruses, CPV has a relatively rapid evolution rate (Hoelzer *et al*, 2008; Shackelton *et al*, 2005;Pereira *et al* 2007) similar to that in RNA viruses, such as feline calicivirus (Coyne *et al*, 2006) or foot and mouth disease virus (Wright *et al*, 2010). As a result, since its emergence, and as discussed in chapter one, further diversification has occurred such that there are now at least three new and different antigenic types of CPV (2a, 2b and 2c) which now circulate in different proportions worldwide (Decaro *et al*; 2005, Davies, 2008; De Ybanez *et al*, 1995; Yilmaz *et al*, 2005, Costa *et al*, 2005, Kapil *et al*, 2007). The mechanism for the evolution of these antigenic types remains uncertain since intermediates between them have never been reported. As well as the emergence of new antigenic types, the virus has also undergone a complex series of host switches after it first emerged in the dog population, such that early CPVs only infected dogs, whereas newer variants can infect both cats and dogs (Truyen *et al*, 1996, Ikeda *et al*, 2002).

The recent emergence of this virus, followed by further ongoing antigenic and host tropism changes, means this virus offers an almost unique opportunity to examine

the historical emergence and evolution of a relatively new DNA virus. This study was undertaken to evaluate the evolution of CPV since its emergence in 1978 within the UK, and was carried out by sequencing the VP2 capsid gene over 200 archived viruses isolated between 1979 and 2010.

Methods

Sample collection

The samples were obtained from the UK since the emergence of the virus in 1978, up to the present day (2010). The samples were obtained by convenience from a variety of sources, including potential vaccine breakdowns, viruses used in a previous study of CPV types circulating within the UK (Greenwood *et al*, 1995), and samples sent in for diagnosis of potential CPV infection from various veterinary surgeries and dog shelters. These were from areas covering the majority of Great Britain (i.e. England, Scotland and Wales) and were obtained from either our laboratory or from Intervet-Schering Plough laboratories.

In addition, 68 samples previously sequenced from the PDSA study (chapter 3) were also included.

Where possible, sequence was obtained direct from faeces. However, due to the lack of faecal sample available on some occasions, sequence was obtained from viruses passed on either Crandell feline kidney cells (CRFK) or A72 dog cells. Any samples which had been passaged more than twice were discarded from the study so as to minimise the effect of evolution/attenuation due to cell culture passage.

In addition, since CPV 2c is rarely found in the UK (Davies, 2008; Decaro *et al*, 2007; Chapter 3), four samples of 2c were obtained from Italy and were included for comparison, and to confirm that the primers used for PCR amplification could detect 2c.

DNA extraction and VP2 amplification

DNA was extracted as described previously page 96. VP2 primer design, amplification and sequencing are discussed previously (page 104).

Sequence analysis

All sequences were aligned into a double-stranded consensus sequence using Chromas Pro 1.41 (Technelysium Pty Ltd). All external primer sites were removed, giving a final consensus sequence of 1755 base pairs. All sequences were homozygous, with no evidence to suggest dual or mixed infections.

Estimates of evolution rates and the time since most recent common ancestor (TMRCA) were estimated using a Bayesian Markov Chain Monte Carlo (MCMC) approach as implemented in BEAST v1.4 (Drummond *et al*, 2007). In addition, a TMRCA was also calculated for the large NS dataset using all available NS genes from Genbank and those sequenced in chapter 5.

The most appropriate evolution model was predicted using MODELTEST as implemented in Topali (Milne *et al*, 2008), and this was used to infer bootstrapped

maximum likelihood trees (1000 bootstraps) using PHYML implemented on the ATGC bioinformatics platform (Tamura *et al*, 1993). Amino acid trees were drawn using Mega 4 (Tamura *et al*, 2007), rooted using FPLV, and calculated using the Dayhoff PAM distance matrix.

Results

In total, 180 CPV and FPLV samples from the UK, collected between 1979 and 2010 were sequenced, chosen with an average of four samples per year, and from different veterinary practices, suggesting different geographical locations. Four 2c samples obtained from Italy were also sequenced. In addition, 68 samples which were sequenced as part of the PDSA study (chapter three) were also included in the analysis as samples from 2006-2008. This gave a total of 252 samples.

These samples were typed using key amino acid positions (Truyen *et al,* 1998; Martella al, 2006) as FPLV (n=19), CPV type 2 (n=36), CPV 2a (n=100), CPV-2b (n=93) and 4 CPV 2c of Italian origin.

The majority of the FPLV samples were from 1979-1980 and 1986 (16 of 19) (see figure 6.1). The first type 2 sample in this study was isolated in 1981, and the final one was isolated in 1990. Type 2a, which was the second antigenic variant of CPV to emerge, evolving from the initial CPV type 2, showed a surprising infection pattern in this study. It appears that type 2a emerged initially in 1980, (labelled 3 on figure 6.4). The next CPV-2a was found in 1987 (single sample) with the majority of 2a sequences seem from 1990. This suggests three possible waves of infection, the

first in 1980 which failed to persist (or only attained a relatively low prevalence below the ability of this study to detect it), as did the second wave in 1987. Only the 2a viruses sequenced from 1990 persisted at high prevalence in this dog population. Type 2b samples were isolated continuously from 1984 to 2008. No samples were obtained in 2009. The largest numbers of samples were from 2007 and 2008, and this is due to our large PDSA study (chapter 3).

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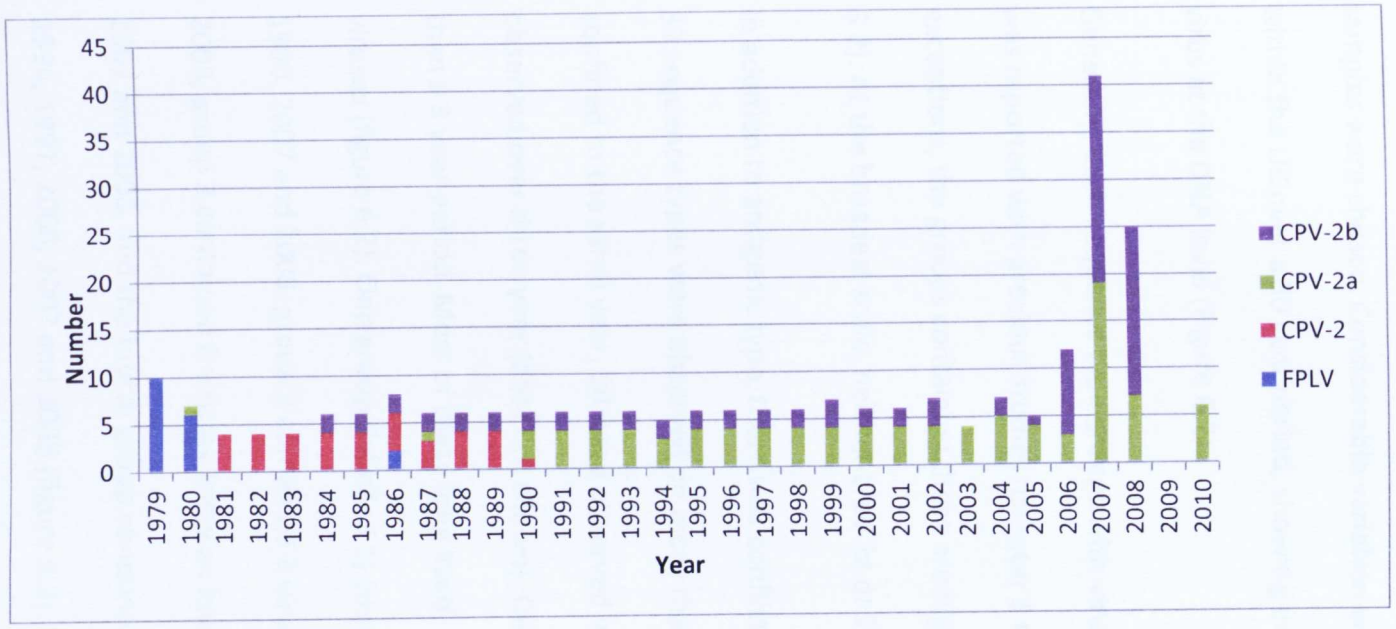


Figure 6.1.*A summary of the samples used in this study with the year which they were obtained. This is discussed in more detail in the text above. The 2c samples were not included as these were all isolated from Italy.*

Maximum likelihood DNA Tree

In total, there were 179 distinct DNA sequence types isolated within this study, of which nine were FPLV (with between 1-9 sequences in each type), 22 were type 2 (1-4), 83 were 2a (1-8), 63 were 2b (1-8) and two were 2c (1-3). Although there were more type 2a viruses sequenced in this study, this cannot be extrapolated to indicate that this type was more prevalent in the UK, due to the way which the samples were chosen. Considerable variation was observed in viruses isolated within the UK over a 30 year period, showing evidence of rapid evolution of the virus at the DNA level (Figure 6.2).

Generally, each sequence type groups with viruses of a similar antigenic type, as was reported with previous studies (chapter 3 this thesis), with just two single exceptions, the groups containing these are highlighted in red on the tree (figure 6.2). At the broadest scale, neither type 2a or 2b was monophyletic.

In addition to antigenic type, there was conflicting evidence of temporal clustering: 39 sequence types were observed on more than one occasion. 12 of these were confined to the same year, 18 were observed in two consecutive years, and 2 were observed over three year (data not shown). Only seven were observed over longer than a 3 year period. Most of these long lived, stable sequence types were type2b viruses (figure 6.2). One group (labelled 1) contained 3 viruses and was observed in 1990, 2007 and 2008, group 2 contained 3 viruses and was found in 1992, 2007 and 2008, group 3 contained 8 viruses and was found in 1995, 1997, 1998, 1999 (3), 2007 and 2008, and the fourth group contained four viruses and was observed in 1996, 1997, 2006, 2007 and 2008 (figure 6.2). It suggests that these viruses were

observed in the mid 1990's, then disappeared, or were not sampled, and then re-emerged in the late 2000's. Interestingly, and in contrast to the 2b viruses discussed above, all the 2a viruses which grouped together were observed in similar years, with the longest period that a CPV 2a virus type was observed being six years (1994-2000) (see figure 6.4).

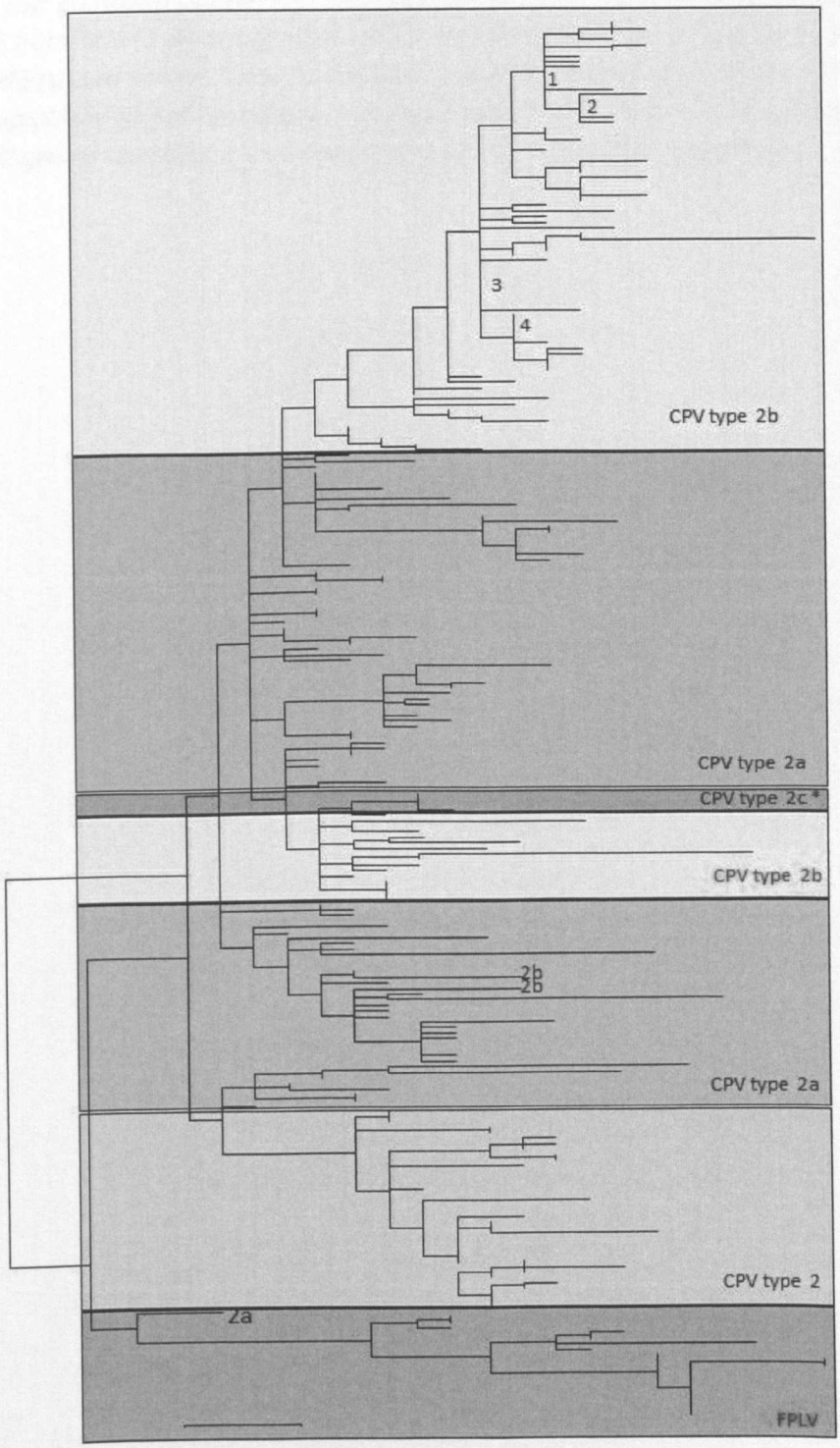


Figure 6.2.*A DNA maximum likelihood tree of the historical sequences isolated within the UK, as well as those isolated as part of the study with the PDSA (Chapter 3 this thesis). The viruses are labelled with their respective type, but as discussed previously, both 2a and 2b are not monophyletic. Due to the large size of the tree, the branch taxon labels have been removed, the groups have been labelled with their respective predicted antigenic type. The two groups highlighted in red do not*

contain singular types, with the FPLV group appearing to contain a single 2a virus, and the 2b group containing two type 2a viruses. The four groups which are labelled with numbers (1-4) are groups which are observed over a long period of time, and are discussed below. Type 2c (marked with an asterisk) are of non-UK origin. The groups highlighted in red are groups which do not contain a single type (i.e. the FPLV group contains a 2a sequence as well as the FPLV sequences).

Maximum Likelihood amino acid tree

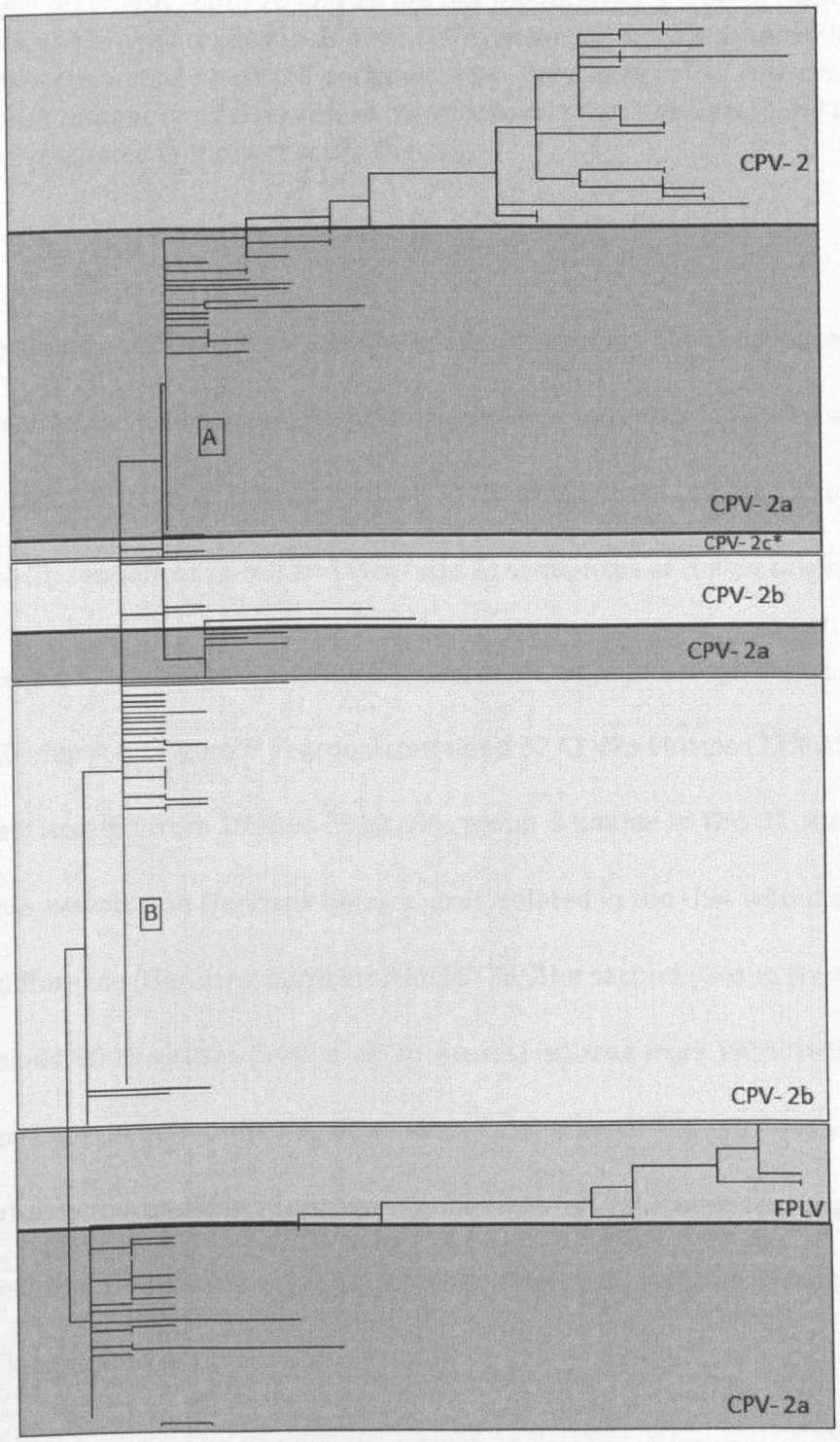


Figure 6.3.*An amino acid maximum likelihood tree of the historical sequences isolated within the UK, as well as those isolated as part of the study with the PDSA (Chapter 3 this thesis). The viruses are labelled with their respective type, but as observed previously, both 2a and 2b are not monophyletic. Due to the large size of the tree, the branch taxon labels have been removed, the groups have been labelled with their respective predicted antigenic type. Type 2c (marked with an asterisk) are of non-UK origin. A and B represent the main amino acid groups found in this study and are discussed in more detail in the text.*

At the amino acid level, the 188 different DNA sequence types collapsed into 97 different amino acid sequences including six FPLV sequences (each of which contained 1-9 sequences in each type), 18 2b sequences (1-6), 41 2a sequences (1-32), 30 2b sequences (1-50) and two type 2c sequences of Italian origin (1-3).

Within the 97 amino acid sequence types, there were two large amino acid groups. One (Group A on Figure 6.3) group contained 32 CPV2a viruses (32% of all 2a viruses) isolated from 1996 to 2010. This group is unique to the UK, with the closest relative available on Genbank being a virus isolated in the USA with a single amino acid difference (Genbank number: AAV36776).The second (Group B on figure 6.3) contained 50 2b viruses (54% of all 2b viruses) isolated from 1985 to 2008. This group is not unique to the UK, as an identical sequence had previously been reported within the USA (Genbank number AAV36771). These two large groups suggest that their amino acid sequences are highly fit, and thus remain circulating for a long period of time.

Beast tree

Bayesian analysis of the samples from the UK using BEAST (figure 6.4) showed that both FPLV and CPV type 2 were monophyletic.

For type 2a and 2b, the phylogeny was more complicated. When both 2a and 2b viruses first appear on the phylogeny in 1980 and 1984 respectively, they are quite distinct. Subsequently, between 1986-1993, both 2a and 2b viruses are found clustered together (marked with an asterisk on figure 6.4). Following this, and up to the most contemporaneous part of tree, there appear to be more clusters which contain 2a and 2b viruses mixed together, and suggesting the 2a / 2b phenotype has emerged on more than a single occasion in this data set.

Despite sequencing continuously over a 30 year period, there appeared to be at least four long branch lengths within the tree suggestive of unsampled diversity. These ranged in length from approximately 5 years (labelled Y on figure 6.4), 8.5 years (X), 12 years (W), to a maximum of 14 years (Z).

Using these historical samples, we were able to evaluate the evolution of the virus within the UK in detail. The common ancestor of the UK CPVs sampled here was predicted to be approximately 39.2 years before 2010 (i.e. 1971) (range 32.2-44.2) (labelled 1 on figure 6.4). However, this ancestor may not have physically been within the UK, and appears not to have been associated with any clinical disease in dogs. The common ancestor for the original CPV type 2 was 31.2 (range 29-33) years ago (i.e. approximately 1979), (labelled 2 on figure 6.4), which lead to the initial outbreak of parvovirus disease in the UK. This fits with most estimates of

virus emergence. Using the NS gene sequenced in chapter 5, the TMRCA for the NS-genes (obtained and discussed in chapter 5) was calculated to be 84 years ago from 2010 (i.e. 1926, range 1873-1964).

For FPLV the time since the most recent common ancestor was approximately 1965 years (from 2010) (95% confidence 40-79 years) (data not shown). The first of the antigenic variants (2a) to be observed within the UK is also shown (labelled 3 on figure 6.4).

The majority of clinical cases occurred due to viruses which have evolved from a common ancestor which appeared approximately 31 years ago (i.e. 1979) (labelled 4 on figure 6.4) within the UK. This is when the general replacement of CPV type 2 viruses occurred, leading to the current viruses observed in CPV infections from 1984 to 2010.

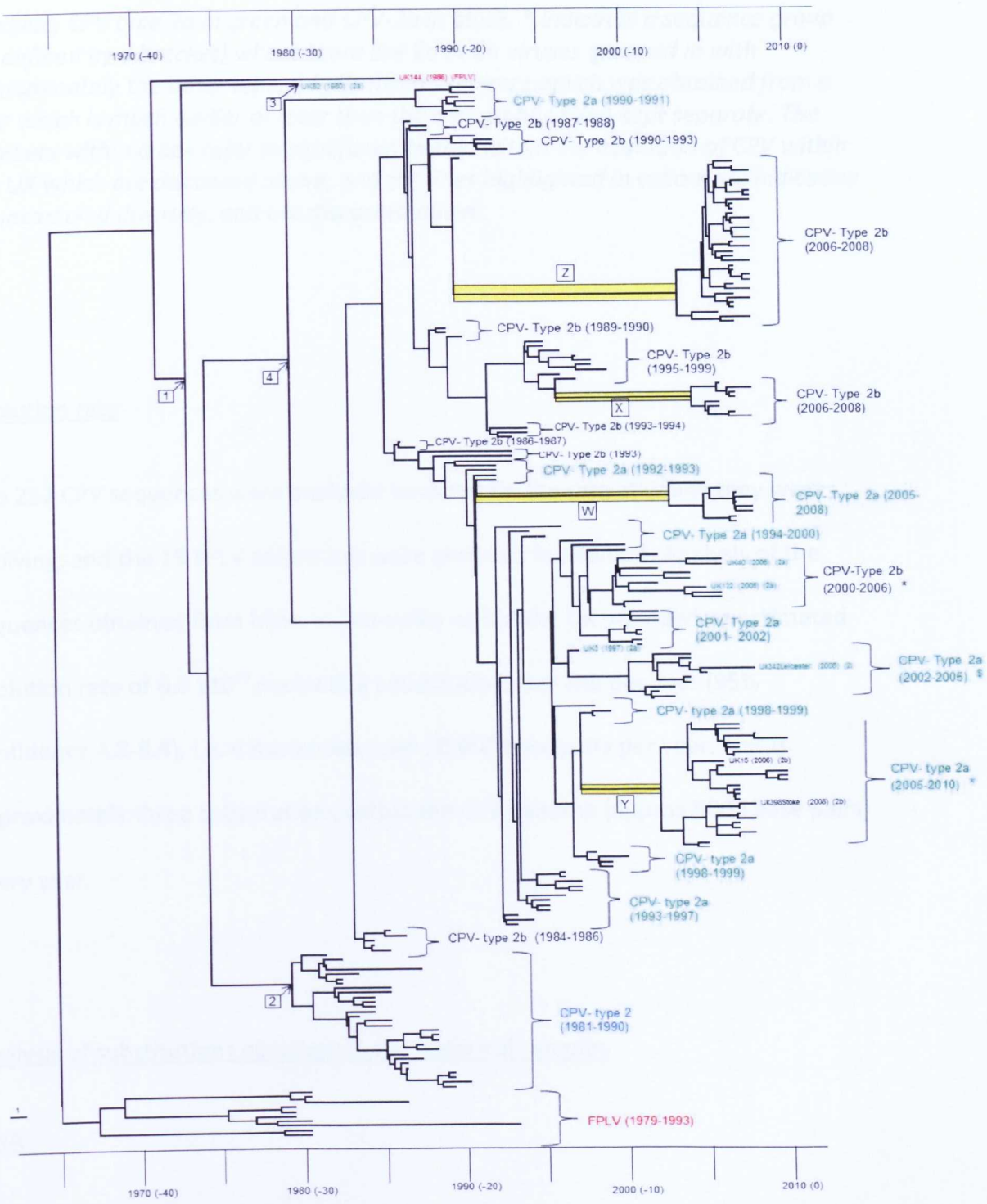


Figure 6.4. *A maximum clade credibility tree produced by Beast, to allow for analysis of viruses isolated at different time points. All sequences which are of the same time, and isolated at a similar time point are grouped together using a bracket for simplicity. Although FPLV isolates all group separately, as do the original CPV type 2 viruses, the type 2a and 2b viruses do not group separately, in some cases groups have both types of virus grouped together, suggesting the typing system is poor. Of all the viruses examined here, only CPV type 2 are phylogenetically monophyletic, with the others being polyphyletic. All FPLV sequences are indicated in red, CPV type*

*2 in blue, CPV type 2a in green and CPV-2b in black. * indicates a sequence group (as defined by a bracket) where there are 2a or 2b viruses grouped in with predominately the other type. $ indicates a sequence which was obtained from a year which is much earlier or later than the rest, so has been kept separate. The numbers within a box refer to significant events within the evolution of CPV within the UK which are discussed above, and the lines highlighted in yellow are indicative of unsampled diversity, and are discussed above.*

Evolution rate

The 252 CPV sequences were analysed to ascertain the rate at which they were evolving, and the 19 FPLV sequences were analysed in addition. Analysis of the sequences obtained from historical samples within the UK provided an estimated evolution rate of 6.6×10^{-4} nucleotide substitutions per site per year (95% confidence 4.8-8.4), i.e. 6 nucleotides per 10,000 base pairs per year. This is approximately three substitutions within the CPV genome (approx 5000 base pairs) every year.

Analysis of substitutions observed in the historical samples

DNA

Using the 252 sequences from UK canine and feline parvoviruses sequenced here, substitutions were analysed at the DNA and amino acid level. In total there were 239 substituted nucleotide positions out of 1755 (13.6%) in the VP2 gene, of which 46.4% (111/239) of these substitutions were only found once. Fourteen of these substitutions were observed in more than 40 different sequences suggesting that

these substitutions are common in the UK dog population (nucleotide numbers: 36, 259, 302, 415, 720, 889, 899, 913, 1221, 1276, 1291, 1602, 1662 and 1695).Fifty one of the substitutions occurred more than six times(data not shown).

Amino acid

There were 115 substituted amino acid residues out of 585 (19.7%) in the VP2 gene, of which 80 (69.6%) of the substitutions occurred twice or less. Of the remaining 35 substitutions, 25 of them were not at key positions known to differ between FPLV and the CPV types. Thirteen of the substitutions were observed on three to five occasions, suggesting that they are not common within the dog population within the UK.

Residue 13, which is located in a B cell epitope, was altered from a proline residue (n=224) which is most common, to a serine residue which is observed in 21 2a viruses and five 2b viruses, isolated from 1993 to 2008. In addition, this position is also found to alter to an arginine residue (a single 2a virus in 2008) and a histidine residue (a single 2b virus in 1993). Due to its position this may alter the antigenicity of the virus.

Residue 85 is an isoleucine in the early FPLV isolates (observed in 1979-early 1980), but is then an asparagine in all CPV and FPLV viruses isolated from mid 1980. Residue 139, which is located in the beta barrel, is a valine residue in 211 viruses, but substituted to an isoleucine in a single FPLV virus and a single CPV-type 2 virus, and also in six 2b viruses and 33 2a viruses.

Residue 232, which make up the three fold spike is observed as an isoleucine in all CPV and in all FPLV isolated after 1980. Prior to 1980 it exists as a valine residue in all 16 isolates.

Several of the amino acid substitutions are found within the beta barrel. Residue 324 is substituted only in some type 2a viruses. This is generally a tyrosine (Y) residue in all FPLV, CPV2, CPV2b and 2c, but varies between a tyrosine (n=85), asparagine (n=3) and Isoleucine (n=12)in type 2a viruses. Residue 347, is generally an alanine residue, but is substituted in some CPV-2 (n=12) and some early type 2a (n=3) viruses. It is also substituted to a serine residue in one type 2 virus. Residue 375, is substituted in 32 type 2 viruses from an asparatic acid residue in most FPLV and CPV viruses, to an asparagine. Additionally two type 2 viruses had a glutamic acid residue at this position. Residue 386 is a glutamine residue in all FPLV viruses and late CPV 2, 2a, 2b and 2c viruses, but appears as a lysine residue in all CPV type 2 viruses isolated up to 1985, when it reverts back to the glutamine residue in 1985. Residue 389 is a threonine residue in all CPV viruses and late FPLV viruses, but is an asparagine residue in all FPLV viruses isolated 1in 1980 and before. A similar situation occurs in residue 566 which is found on the two fold depression.

Comparison of historical samples to those observed in other parts of the world

As discussed in chapter 3 (this thesis), more recent isolates of the virus were found to be distantly related to viruses from other countries, suggesting that viruses may be unique to each country. It was thus of interest to assess if this was the case for

other isolates from historical cases, as it was predicted that older strains maybe more similar to those present in other countries.

Each sequence obtained here was compared to those available on Genbank, and the number of substitution to this nearest Genbank neighbour was compared over the years, to ascertain if more substitutions occurred in time from the earlier sequences to the later sequences (table 6.2).

Virus year	Number of sequences	Average number of substitutions
FPL	19	4.79
1980-1985	26	2.12
1986-1990	30	3.97
1991-1995	29	6.20
1996-2000	32	3.53
2001-2005	28	4.11
2005-2010	66	4.77

Table 6.2.*Analysis of sequence of the VP2 gene of CPV isolated from the UK compared to those previously sequenced from around the world as available on Genbank. The number of substitutions between those observed within the UK, and those observed on Genbank was noted, and the average number of substitutions was then used to calculate if there were more substitutions observed in currently circulating viruses than in historical viruses.*

As can be observed from table 6.2, overall there is a gradual increase in the number of substitutions since the emergence of CPV in the UK starting in 1980. However, this is not a uniform rise, in that there is an apparent peak in 1991-1995 during which the viruses appeared to be at their most diverse, followed by a dip in 1996-2000.This suggests that viruses are becoming more localised within the UK, and distant from those in other countries as they evolve, further suggesting a country-specific CPV strains discussed in chapter 3.

The early viruses in 1980-1983 were more similar to those observed in the Far East (South Korea and China), whereas the later samples from the 1980's were more similar to the USA and French sequences (data not presented). Throughout the 1990's, the sequences were more similar to those isolated previously in the USA, and the Far East (Japan, China, Thailand, and South Korea). The samples isolated since 2000 show a wider variation of countries with the most similar virus sequences, varying from the Far East, USA, Poland, Italy and Brazil. This may just be representative of the countries where CPV has been investigated.

Of the 252 samples sequenced here, only 16(6%) of them were identical to those previously reported on Genbank. Of these 16, nine were type two viruses, which had previously been reported in South Korea, France and the USA. Only a single FPLV and a single 2b virus with identical sequence had been reported previously. Five 2a sequences with identical sequence available on Genbank had also been reported previously. Thirty-four were only a single nucleotide different than one previously reported. Such is the conserved nature of these viruses, that across the 1755 nucleotides which make up the entire VP2 gene, the maximum number of nucleotide changes from one in this study to the closest neighbour on Genbank was 12 nucleotide residues.

Discussion

Canine parvovirus provides a relatively unique opportunity to examine viral evolution, since it only emerged in 1978, it evolves quickly, particularly for a DNA virus, and samples exist from the very first infections to the present day. Here we have conducted a large study of 252 viruses, from the UK between 1980 and 2010, to characterise the evolutionary dynamics of this important virus through its VP2 sequence, since it first emerged in the UK.

Overall, the VP2 gene was well conserved, with only 13.6% of 1755 nucleotide positions and 19.7% of 585 amino acid changes substituted in the 252 samples analysed here. The majority of these substitutions were singletons, so are unlikely to be of importance in the overall evolution of the virus, but some, such as residue 13, and numerous substitutions within the beta barrel of VP2 appear to be common within the dog population. Unfortunately the significance of these substitutions are unknown, but it is clear that these viruses can still cause disease, as they were isolated from clinical cases of parvovirosis. Our studies raise several new ideas about the evolution of the virus within the UK.

The first key event to consider is the common ancestor of all feline and canine parvovirus. Hoelzer *et al* (2008) predicted the time since the most recent common ancestor (TMRCA) of this virus to be 98 years ago (~1910) (range 1840-1953) from 2008. The reasons why the figure for FPLV is 1965, we report is more recent is unknown, but it has been suggested that CPV emerged in Europe, possibly within Europe (Parrish *et al*, 1988b; Koptopoulos *et al*, 1986), so the close location of the UK to Europe may explain how this virus has emerged earlier within the UK.

Alternatively, CPV may have emerged within the UK years before it emerged elsewhere, but due to the isolated location of the UK, the virus never left to infect dogs elsewhere. It is then possible that a further substitution existed which allowed the virus to cause a severe clinical disease and rapid spread. This cannot be confirmed and there is no serological evidence to support this theory.

By comparison, the TMRCA for the NS-genes (obtained and discussed in chapter 5) was calculated to be 84 years ago from 2010 (i.e. 1926, range 1873-1964). This figure is similar to that observed for the VP2 gene, and is comparable with the results obtained by Hoelzer *et al* (2008).

A second key event in the evolutionary history of CPV was its emergence as a canine pathogen, first described in 1977-78 (Eugster *et al* ,1977; McCandlish *et al*, 1979; Fritz, 1979; Horner *et al*, 1979; Johnson *et al*, 1979; Osterhaus*et al*, 1980; Gagnon *et al*, 1979). The common ancestor of the UK CPVs sampled here was predicted to be approximately 39.2 years before 2010 (i.e. 1971) (range 1966-1978). This date is slightly earlier than that predicted by Hoelzer *et al*, (2008), who suggested this event occurred around 1976 (range 1973- 1981), similar to the predicted emergence date of CPV in 1977-78 (Eugster *et al*, 1977; McCandlish *et al*, 1979; Horner *et al*, 1979; Gagnon *et al*, 1979). Thus the virus may have emerged earlier in the UK than it did elsewhere but the confidence intervals for these estimates are wide ranging.

The next notable event in the history of CPV emergence was the arrival of new antigenic and host range variants, which effectively appeared to replace the original emerging strain CPV-2. Interestingly, we observed type 2 viruses from 1981-1990,

which is after the time when CPV type 2 was reported to have disappeared from the field, although it was still present in some live vaccines (Gore *et al*, 2005). Therefore it is possible that some of these viruses were shed from previously vaccinated animals (Decaro *et al*, 2006b, 2007c). The emergence of the antigenic variants in our study populations that replaced CPV-2 appeared to follow a different pattern from that elsewhere in the world where 2a and 2b appeared to emerge and persist. Canine parvovirus 2a first appeared in 1980, causing clinical disease, but then no samples were identified again until 1990, apart from a single sample in 1987. This may be due to the number of samples in our collections, or the lack of 2a causing clinical disease in the 1980-1990 period. Alternatively, the sample collection method, using viruses collected as part of vaccine breakdown studies may have excluded this type of virus. In contrast to the fragmented emergence of CPV-2a, CPV 2b was first isolated in 1984 and was consistently identified from then until 2008, after which no 2b samples were identified. Whether this apparent difference in the emergence of 2a and 2b is purely an artefact of the study design and sampling frequency, or whether it is a true representation of these events are uncertain.

Early in the emergence of the virus, CPV 2a and 2b generally separate phylogenetically. However, in the more recent parts of the tree, this tends to breakdown, suggesting the antigenic variants may now be generated by parallel evolution. In addition, there are four long branch lengths on the Bayesian tree, varying in length from 5 to 14 years, which suggests that the viruses in this period

were unsampled. The reason for this unsampled diversity is unknown, but may reflect the importation of viruses into the country from abroad.

As would be expected, the 179 DNA sequence types of CPV are generally grouped by year of isolation. However there were some notable exceptions particularly for CPV2b, exemplified by at least four groups of viruses observed over long periods of time, suggesting that this is a particularly fit virus type (1-4 on figure 6.2). This is surprising as the evolution rate suggests approximately three substitutions per year within the genome, so for the virus not to alter over 15 years would appear to be unlikely; the reason for this is unknown. Clearly, it could be due to laboratory contamination of samples, or mixing of tubes as has been reported recently for influenza virus (Worobey, 2008), but due to the fact that, in some cases, these sequences were determined in two independent studies (the PDSA study covering 2006-2008 and the samples sequenced in this chapter including the earlier sequences), this appears unlikely. Other possibilities include the repeated introduction into the UK dog population from some other stable source e.g. from a vaccine, or accidental release of virus from a laboratory, such as occured with foot and mouth disease virus in 2007 (Cottam *et al*, 2008). Sequence evolutionary stasis could also be due to viral latency, which has been reported previously for humans infected with adeno-associated viruses (Samulski *et al*, 1991), but has not been reported for CPV. Understanding these persistent nucleotide sequences, and especially seeing if they persist in the population now, may give new insights into the biology of this virus in its host population.

As well as the apparent long-term persistence of these nucleotide sequences in this population, two amino acid types were also extremely common over long periods of time, suggesting that these viruses evolved to produce an amino acid sequence which is fit, with ideal conformation, and one which does not alter a great deal.

Despite the long persistence of some nucleotide sequences, and the common nature of two specific amino acid sequences, it was somewhat surprising that at the DNA level, only a single 2b virus was identical to one previously reported, particularly when the amino acid group which contained 50 2b viruses, was identical to others observed around the world. This suggests that there are more silent substitutions which do not affect the amino acid sequence of the VP2 gene. The low number of substitutions observed across the 252 samples (maximum 12 nucleotides differences to Genbank) is further suggestive of the conserved nature of parvoviruses. The nine identical type 2 viruses may suggest that the same original virus spread around the world, but, as reported previously (chapter 3 and Hoelzer et al, 2008) the virus has now begun to evolve into country specific sequences.

The evolution rate reported here of 6.6 $x10^{-4}$ nucleotide substitutions per site per year (95% HPD 4.8-8.4) was higher than that reported by Hoelzer *et al* (2008) of 2.2 (95% HPD 1.7-2.7) x 10^{-4}nucleotide substitutions per site per year. It is also higher than the evolution rates suggested by Pereira *et al* (2007) of $1.2x10^{-4}$ and that proposed by Shackelton et al, (2005) of 1.2-2.3 $x10^{-4}$. All the evolution rate estimates suggested for CPV are similar to that suggested for RNA viruses known to cause foot and mouth disease, for which an evolution rate of $7.8x10^{-4}$was suggested

(Wright *et al*, 2010). It was lower than that proposed for feline calicivirus where an evolution rate of 3.84×10^{-2} was suggested (Coyne *et al*, 2006).

Chapter 7

An assessment of the burden of parvovirus infection in clinically normal cats; canine parvovirus persistence in asymptomatic feline carriers

Introduction

Cats have been identified as being able to carry and become infected with canine parvovirus (CPV) (Ikeda *et al*, 2002), This is often manifested with signs similar to those observed in feline panleukopenia (Mochizuki *et al*, 1996, Gamoh *et al*, 2003) and high levels of virus are shed within the faeces as reported for CPV in dogs (Mochizuki *et al*, 1996). As well as causing disease, a recent pilot study has suggested that clinically normal cats may also shed CPV (Whitby *et al*, 2010). Taken together, this suggests some types of CPV are capable of replicating within cat tissues. As CPV is thought to have evolved from FPLV via a wildlife intermediate, it is possible that a substitution back to certain FPLV residues with a CPV backbone is a possibility (Ohshima *et al*, 2009).

Despite the importance of FPL, there is little data available on its prevalence. In a study of 274 dead kittens from private households and rescue shelters between 1986-2000, 25% of the mortality was attributed to parvovirus infection by post-mortem and histopathological examination (Cave *et al*, 2002). Other data on prevalence is largely anecdotal. Prevalence of FPLV in the UK are generally perceived to be low (author's unpublished observations). In rescue shelters, sporadic outbreaks do still occur in what are high turnover populations of cats (Cats Protection staff, personal communication). Some of these rescue shelters may act as an ideal place

for viruses to spread among animals, both within species, and also between species, due to relatively low hygiene and biosecurity and high rapid animal turnover (Sokolow *et al*, 2005). In addition, as parvoviruses are highly stable, the virus can persist in infected areas, and infected clothes and fomites for over 12 months (Gordon et al, 1986). As such, opportunities also exist for cross species transmission by personnel working with both cats and dogs, as well as vector transmission by rodents and flies (Burger *et al*, 1970).

More recently, relatively high prevalence of shedding of CPV by clinically normal cats has been reported in a small pilot study of 20 cats in a rescue shelter (Whitby *et al*, 2010). Therefore the potential for both dogs and cats to shed CPV without showing clinical signs related to gastroenteric disease appears to be quite high.

Although parvoviruses are generally considered to cause acute infection, cats have been shown to carry FPLV for long periods following infection *in utero* or neonatally (Csiza *et al* 1971: Bouillant *et al*, 1965). No such carrier state has ever been reported in dogs infected with CPV, with the longest shedding time reported being 22 days post infection (Carman *et al*, 1980). Whether cats can similarly develop a CPV carrier state is unknown.

It is now clear that cats could potentially be a risk factor for infecting other dogs and cats and it raises many questions, including the unknown prevalence, unknown levels of shedding, and also the significance of the infection of cats with CPV. This chapter describes the first in depth longitudinal study of parvovirus prevalence in cats, and viral shedding as a result of natural asymptomatic infection in two local

rescue shelter populations. Viral sequencing of the VP2 gene allowed us to assess the potential for cross species transmission from cats to dogs and vice versa.

Methods

Samples

Two sampling strategies were employed within this study, longitudinal and a cross sectional study.

Longitudinal study

Cat faecal samples were obtained from an RSPCA shelter in North West England, every week for an eight week period from January to March 2010. The shelter had 20 cages for cats, including four in an isolation ward where new arrivals and ill cats (as judged by kennel and veterinary personnel) were placed (Figure 7.1). Cats and dogs were housed separately in two distinct accommodation blocks; the shelter personnel and shelter visitors were able to travel freely between the two blocks.

At each visit, faecal samples were obtained from the litter tray or the cage floor from each resident cat. Clinical information and signalment such as approximate age, sex, breed and vaccination regime (often as judged by rescue shelter personnel and/ or vets), as well as the date the animal was admitted, were obtained for each cat from which a faecal sample was collected. None of the animals had any history of gastroenteric illnesses and no history of FPL was reported within the shelter over the previous few years.

Between each of the cages, there was a solid plastic partition which prevented contact between adjacent cages. However, cats were allowed to freely roam in the communal area between cages, usually on their own, when their cage was being cleaned out, increasing the risk of potential infection spread. Cages were cleaned and litter trays were emptied daily. All RSPCA cats were vaccinated with a trivalent modified-live vaccine (Feligen RCP, Virbac), either upon arrival, or within a few days of entry to the shelter.

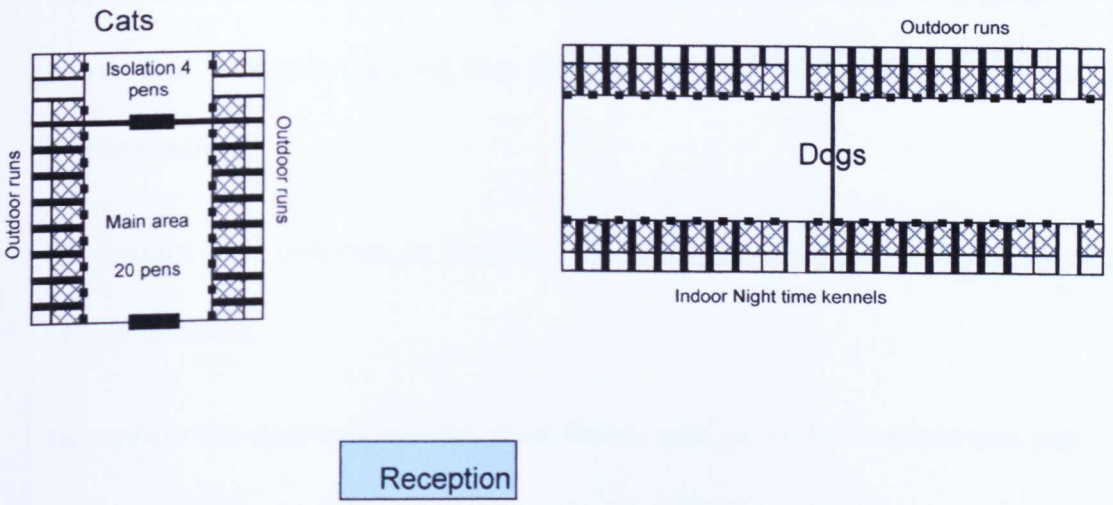


Figure 7.1. *The organisation of the RSPCA shelter involved in the longitudinal study where the cats and dogs were housed.*

As well as cat faecal samples, dog faecal samples (n= 122) were also obtained from the same shelter on three occasions (weeks 4 (n=42), 6 (n=36) and 8 (n=44)) and were collected and tested by different lab personnel on three occasions (weeks 4, 6 and 8). Samples were collected from dogs which had defecated overnight, or early in

the morning, and were collected before the morning clean. All dogs were vaccinated upon arrival or within a few days with a trivalent dog vaccine (Vaccine unknown).

Cross sectional

In addition to the longitudinal study, 50 cat faecal samples were also collected from a Cats Protection shelter which housed only cats. These were either strays or surrendered as unwanted pets. Cats were vaccinated (vaccine unknown) as for the RSPCA shelter.

All samples were collected as described previously, and stored at room temperature until processing.

To confirm the virus was present in the faeces and not in the environment, wet swabs, each dipped in its own unique tube of distilled water were taken from every cage, testing toys, feeding and water bowls, floors, ramps, bed blankets, litter trays and sleeping trays at the RSPCA shelter used in the longitudinal study. No environmental samples were taken from the Cats Protection shelter.

Sample processing

Samples were stored as neat faeces and frozen at -80°C. The sample which was used for PCR was taken from the faeces and the DNA was extracted on the same day as it arrived in the lab.

DNA extraction, amplification and sequence of VP2

DNA was extracted as described previously page 96. VP2 primer design, amplification and sequencing are discussed previously (page 104).

Analysis of Non Structural (NS) gene

DNA for the NS gene PCR was extracted as described previously (page 96). The NS gene PCR protocol and sequencing protocol are discussed previlusly (page 129)

Sequence analysis

All sequences were aligned into a double-stranded consensus sequence using Chromas Pro 1.41 (Technelysium Pty Ltd). All external primer sites were removed, giving a final consensus sequence of 1755 bp for the VP2 gene, and 2004 base pairs for the NS gene. All sequences were homozygous, with no evidence to suggest dual or mixed infections. Consensus sequences were aligned by Clustal W and phylogenetic trees were drawn using Kimura 2 parameter distances as implemented in Mega 4 (Tamura *et al*, 2007). Trees were rooted to sequences available for FPLV (Genbank accession number M24004), and also included published sequences for two CPV2s (M23255 and U22186). Amino acid trees were also drawn using Mega 4, rooted using FPLV and drawn using the PAM matrix (Dayhoff) method. Maximum likelihood (ML) trees were inferred using PHYML implemented on the ATGC bioinformatics platform (Guindon *et al*, 2003), with bootstrapping performed 500 times.

Cat sequences were compared to worldwide sequences using the Blast software (Altschul *et al*, 1990).

Virus Isolation

All PCR positive faecal samples from the longitudinal study, which had been previously frozen at -80°C, were homogenised in PBS and inoculated onto either Crandell Rees feline kidney (CRFK) cells or A72 dog cells to ascertain if infectious virus was being shed. Cells were examined daily for the appearance of cytopathic effect (CPE) typical of CPV infection, and if none had appeared in three days, the same cells were re-passaged onto new cells. The CPE which is typical of CPV infection often involves total destruction of the cell monolayer. Earlier CPE evidence includes cell rounding and disassociation of the cells from the flask surface (Mochizuki *et al*, 1986).

Immunofluorescence

Infected cells were also tested using CPV fluorescent antibody to confirm the presence of CPV antigen in cell nuclei, typical of natural dog CPV infection. Cells (CRFK and A72) were infected with CPV, and observed after 24 hours. Cells were stained using a CPV specific mouse monoclonal antibody conjugated with fluorescein isothiocyonate (FITC) (Kindly supplied by Neil Greenwood, Intervet-Schering Plough)

known to recognise the capsid of the virus. The cells were viewed by fluorescent microscopy (Leica model DM1L).

Haemagglutination (HA)

Viruses were recovered from the cell cultures and tested for the ability to haemagglutinate swine red blood cells according to published methodologies (Mathys *et al*, 1983; Carmichael *et al*, 1980). Haemagglutination can be used to differentiate FPLV from CPV, as FPLV will only HA at 4°C whereas CPV has a wider HA spectrum (Carmichael *et al*, 1980). All haemagglutination results were compared to the sequence results.

Briefly, swine red blood cells (supplied by Intervet-Schering Plough) were washed three times with PBS, and re-suspended in PBS with 1% bovine serum albumin. Red blood cells and virus preparations were added to wells in equal volumes, and were observed for signs of haemagglutination after 60 minutes by the author, and confirmed by a second operator.

Results

In total, 46 cats (37%) of the 124 sampled (34/74 (46%) from the RSPCA shelter and 13/50 (26%) from the Cats Protection) tested positive at some point during the study.

Prevalence of infection in cats- longitudinal RSPCA study

As the rescue shelter received different numbers of animals every day, the numbers of samples available on each day varied (Table 7.1). The rescue shelter appeared to have a relatively high turnover with different numbers of animals arriving each week. In total 74 different cats were tested during the 8 week period. On any given day, the average number of samples obtained was 24.5 (range 20-27). Over the eight weeks tested, PCR gave an average prevalence of parvovirus infection of 33.9% (61 positives among 180 samples) in the RSPCA shelter (range 25.0% - 40.9% over the different weeks) (table 7.1), with 34/74 (46%) cats positive at some point.

Week	1	2	3	4	5	6	7	8	Total
Number of Samples	23	20	22	27	23	20	23	22	180
Number positive	7	8	9	10	7	5	8	7	61
Prevalence (%)	30.4	40.0	40.9	37.0	30.4	25.0	34.8	31.8	

Table 7.1. *The prevalence of parvovirus positive faecal samples obtained from healthy cats housed at the local RSPCA.*

Prevalence of infection in cats- cross sectional (Cats Protection) study

The PCR prevalence was 26% (13/50) of the samples collected from the 50 cats in the Cats Protection shelter.

Environmental samples

Eight environmental swabs were taken from each of the 20 cages (n=160) within the RSPCA shelter, consisting of swabs taken from toys, feeding and water bowls, floors, ramps, bed blankets, litter trays and sleeping trays at the RSPCA shelter used in the longitudinal study. No environmental samples were taken from the Cats Protection shelter. As all swabs taken from the environment were negative, it strongly indicates that the virus was present in the faeces of the animals and not in the surroundings.

Prevalence of infection in dogs

Dogs (n=122) were tested on weeks 4 (n=42), 6 (n=36) and 8 (n=44) at the same RSPCA shelter as the cat samples in the longitudinal study to ascertain the levels of prevalence in dog faecal samples. All dogs tested on each of the three weeks were negative by the same PCR test as used for the cat faecal samples. As all PCR results were negative, no dog samples were used for virus isolation studies.

Longitudinal shedding patterns

Shedding patterns by individual clinically normal cats at the RSPCA shelter are shown below (table 7.2). As can be observed, there appear to be three distinct patterns of infection; some cats which are consistently infected every week when tested, some cats which tested intermittently positive, and others which remained negative throughout.

Thirty-one percent of the cats (23 of 74) were consistently positive for parvoviruses (i.e. every week sampled they were positive), although 16 were only tested on a single occasion. The remaining seven were sampled two or more times (average 3.5, range 2-6), and within this group, five cats (46, 48, 57, 58 and 69) were positive for four or more weeks (table 7.2).

Forty of the 74 cats (54%) were consistently negative when sampled.

Eleven (15%) of cats were intermittently positive. Five cats (L45, L46, L48, L50 and L51) were positive on arrival and subsequently stopped shedding the virus. The remaining six cats (L41, L42, L43, L44, L47 and L49) tested negative on arrival at the shelter, and subsequently tested positive at a later sample, suggesting they may have become infected in the shelter, either from another cat, or from other sources such as fomites or dogs.

Viral typing

Based on sequence analysis, none of the positive samples from either shelter were FPLV. In the RSPCA shelter, CPV type 2a made up 77% of the 61 parvoviruses identified (47 sequences from 24 different cats; shaded grey boxes in table 2), and the remaining 23% of parvoviruses were type 2b (14 sequences from 10 different cats; table 7.2). No type 2 or 2c were detected.

In contrast, in the 13 sequences from the Cats Protection 31 % (n=4) were 2a, and 69% (n=9) 2b. Again, no type 2 or 2c were detected.

Cat	Weeks							
	1	2	3	4	5	6	7	8
1	-							
2	-	-	-					
3	-							
4								-
5								-
6								-
7	-	-						
8	-	-						
9	-	-						
10	-							
11	-	-						
12	-	-	-	-	-	-		
13	-							
14	-							
15	-	-						
16		-	-	-				
17		-	-	-				
18			-	-				
19			-	-				
20			-	-	-	-	-	-
21			-	-	-			
22			-	-	-			
23			-	-				
24			-					
25				-	-	-	-	-
26				-	-	-	-	
27				-	-			
28				-	-	-	-	
29					-	-	-	
30					-			
31					-	-	-	
32					-	-	-	
33						-	-	
34						-	-	-
35						-	-	-
36							-	-
37							-	
38								-
39								-
40								-

	1	2	3	4	5	6	7	8
41	-	2a						
42	-	-	2a					
43	-	-	-	2b	-			
44	-	-	-	-	2b			
45		2a	2a	-	-	-		
46		2a	2a	2a	2a	2a	-	-
47			-	-	-	-	-	2a
48			2a	2a	2a	2a	2a	-
49				-	-	-	2a	2a
50					2a	-	-	-
51							2a	-

	1	2	3	4	5	6	7	8
52							2b	
53							2b	
54								2b
55								2a
56								2a
57	2a	2a	2a	2a				
58	2b	2b	2b	2b				
59	2a							
60	2a							
61	2a							
62	2a	2a	2a					
63	2a							
64				2a				
65				2a				
66		2b						
67		2b						
68			2b					
69			2a	2a	2a	2a	2a	2a
70				2a	2a			
71				2b	2b			
72						2a	2a	2a
73						2a		
74							2a	

Table 7.2. *The shedding patterns of cats with parvoviruses based on PCR in the longitudinal study at the RSPCA shelter. An empty box is indicative that no sample was collected that week, either due to the cat being re-homed, or at the vets, or that the cat hadn't yet arrived. Positive results were all CPV, and thus could be typed as 2a, or 2b. As some cats were rehomed or removed during the study, not all lines are complete for the full eight week study period. 2a isolates are indicated with a white box, whereas 2b isolates are indicated with a shaded box. The table is split into three sections, cats which were consistently negative, cats which were intermittently positive, and cats which were constantly positive.*

Analysis of cat virus VP2 genes

Full VP2 gene sequence was obtained from all positive cats at the RSPCA shelter and the Cats Protection shelter. Figure 7.2 shows a maximum likelihood tree as an indication of levels of variation, both at the nucleotide (figure 7.2a) and amino acid levels (figure 7.2b).

In total, there were 32 different unique DNA sequence types observed within the cats, 25 in the RSPCA, and 9 in Cats Protection. Interestingly, two of the sequence types were observed at both shelters. Of these 32 sequence types, 6 were 2a and 26 were 2b. Seventeen of these sequence types were unique to this study having not been observed in the dogs in chapter 3. Together with the high reproducibility of the sequencing methodology developed and employed in this study (chapter 2 and 3), this provided strong evidence that these positive samples were not the result of laboratory contamination.

These 32 DNA sequence types formed 15 amino acid sequence types, of which six were observed at the Cats Protection and 10 at the RSPCA shelter, and again, some of these sequence types were at both shelters.

Cats which were infected for two or more weeks appeared to shed the same virus throughout infection as judged by sequence of the VP2 gene of the virus in the longitudinal study at the RSPCA shelter (shown in Red on Figure 7.2a).

Of the 32 sequence types in the two shelters, 25 were restricted to individual animals. Of the remaining seven, two were found in both shelters (group A CS2,3, 11, L65 and group B CS6,CS7,L44, L58,L71), one was found just in the Cats protection shelter (CS 4 and 5,

Group C), and four were found in more than one cat in the longitudinal study (Groups D-G). Four of these seven cases contained cats (L43, 44, 47 and 49, indicated with an astericks on Figure 7.2a) that were negative on arrival at the shelter, becoming positive during their stay, and therefore possibly suggesting transmission events within the shelter (Figure 7.2a).

At the amino acid level, there was decreased diversity, particularly within the 2b sequences of the cat capsid proteins, compared to the 2a, with most 2b viruses collapsing into one group.

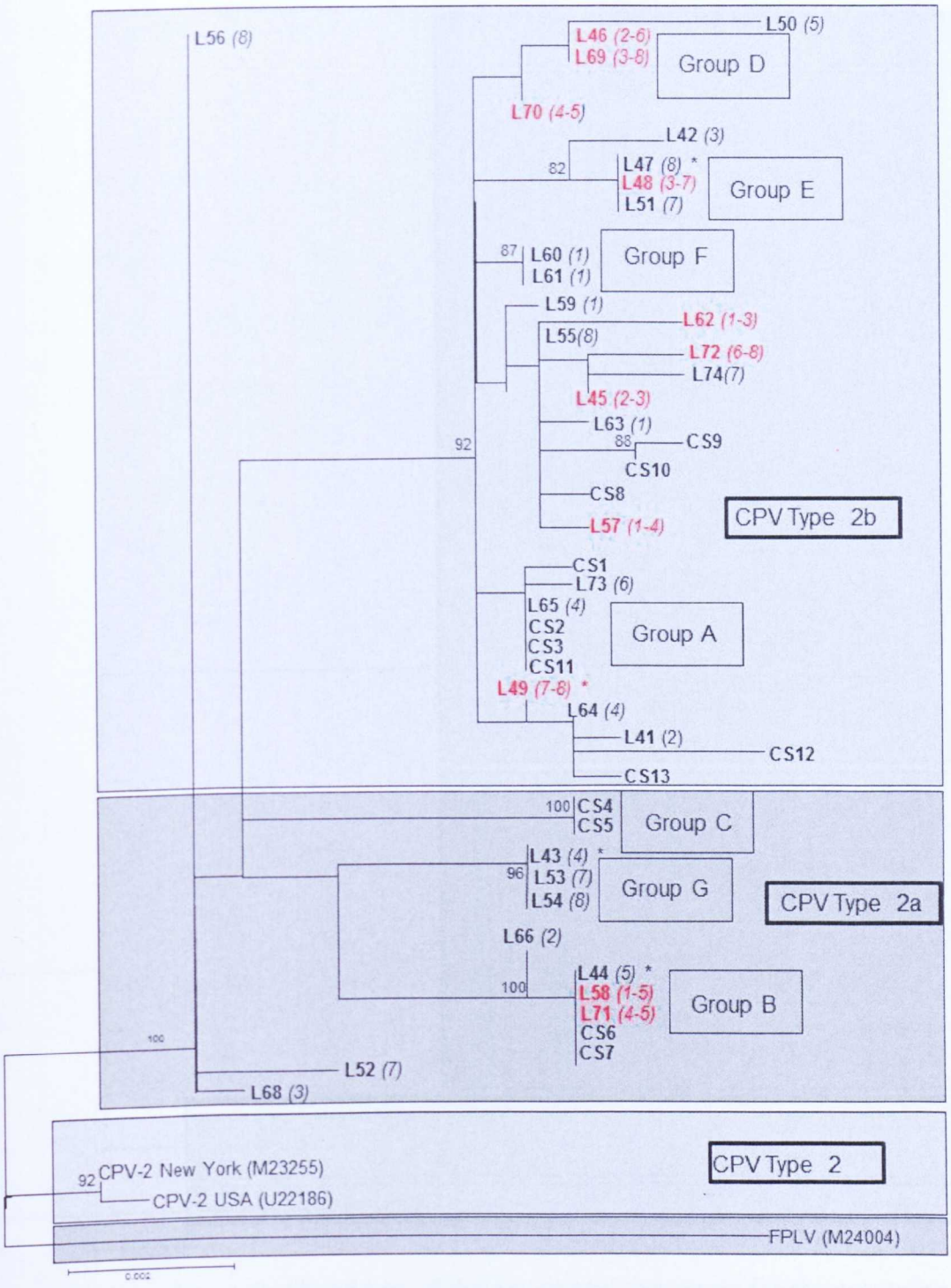


Figure 7.2a. *A maximum likelihood DNA tree of the cat viral sequences from the two studies. The trees are rooted to an FPLV sequence available on genbank (Accession number M24004). Each tree is labelled with the number of the cat (in table 1) in bold, with the week/ weeks at which it was positive in brackets in italics. L indicates that the virus was isolated from the longitudinal study, and CS indicates the cross sectional study Sequences highlighted in red are long lived sequences, occuring over several different weeks.*

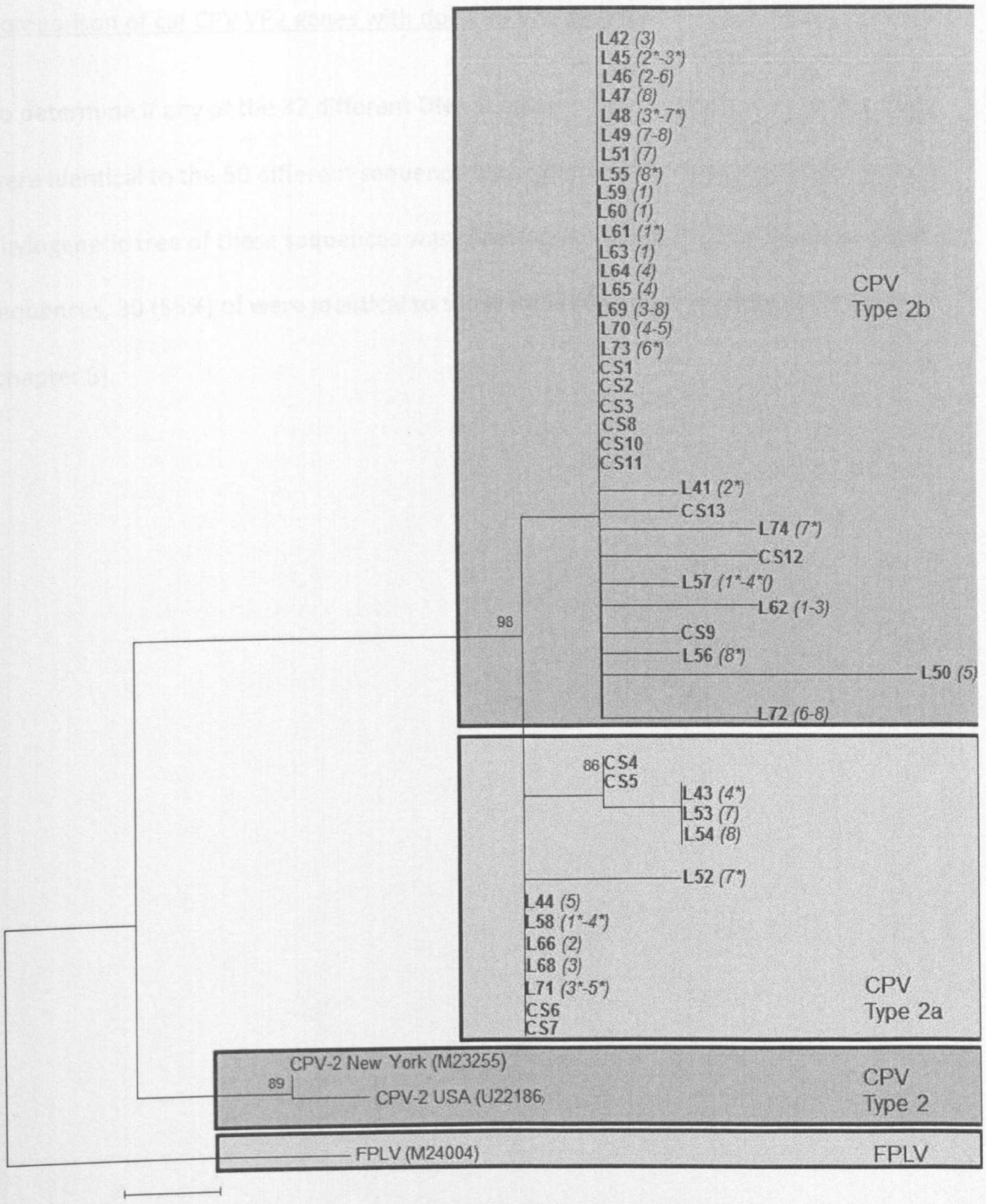


Figure 7.2b. *A maximum likelihood tree of the amino acid sequences for the cats using the same samples used on figure 7.2a. The trees are rooted to an FPLV sequence available on genbank (Accession number M24004). Each tree is labelled with the number of the cat (in table 1) in bold, with the week/ weeks at which it was positive in brackets in italics. L indicates that the virus was isolated from the longitudinal study, and CS indicates the cross sectional stud. The asterisks indicate the viruses which the NS gene was also sequenced.*

Comparison of cat CPV VP2 genes with dog CPV VP2 genes

To determine if any of the 32 different DNA sequence types found in cats in this study were identical to the 50 different sequence types identified in dogs in chapter three, a phylogenetic tree of these sequences was constructed (figure 7.3). Of the 54 positive cat sequences, 30 (55%) of were identical to those isolated in dogs in another UK study (chapter 3).

Figure 7.3b. *An amino acid maximum likelihood tree of CPV VP2 sequences which were isolated from clinically healthy cats at the RSPCA and cat's protection and compared with sequences from clinically ill dogs in a previous study in the UK with the PDSA (chapter 3). Cat sequences are shown in black, with the name of the cat it was isolated from in bold (L= longitudinal study, CS = cross sectional study) and the week/ weeks it was isolated in brackets and italics. Dog sequences are shown in red, and the FPLV sequence is shown in green.*

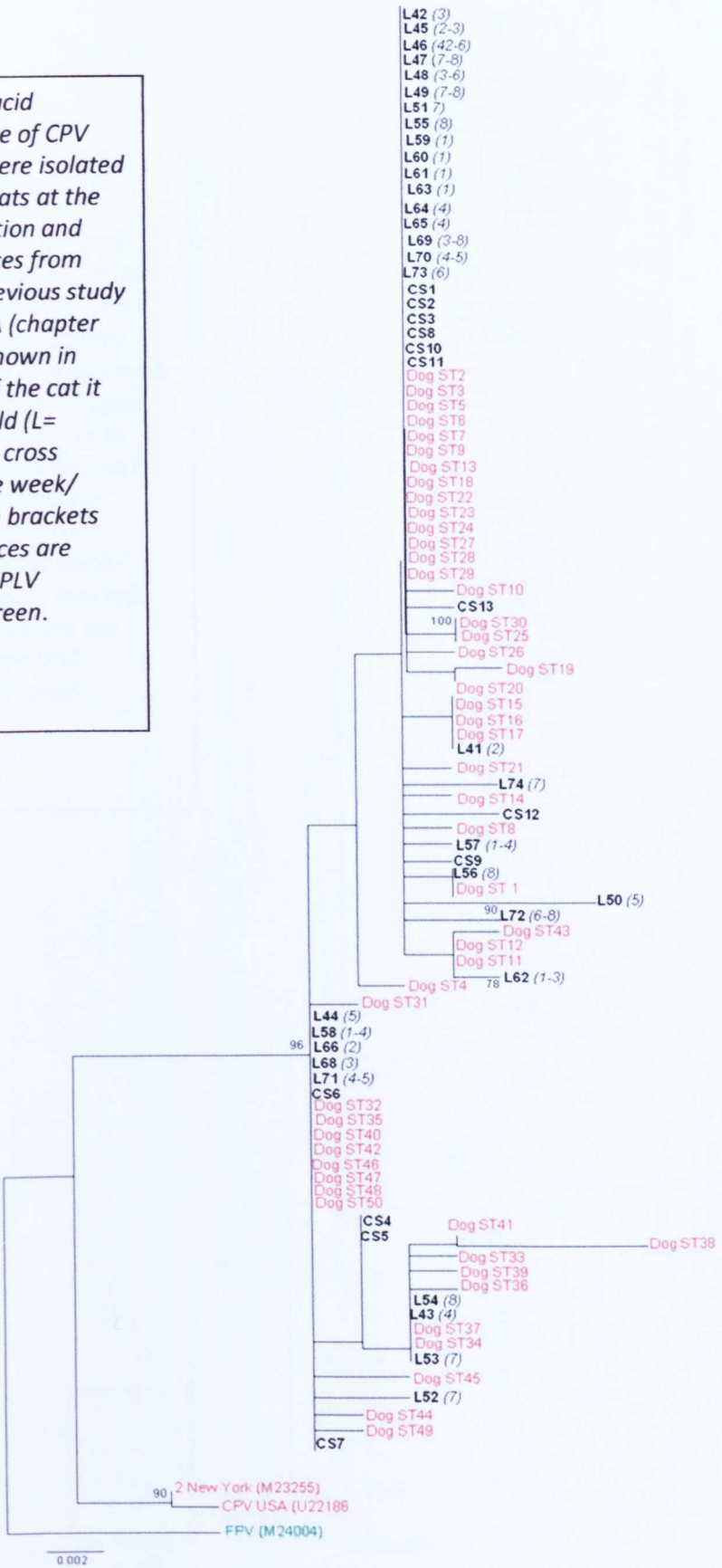
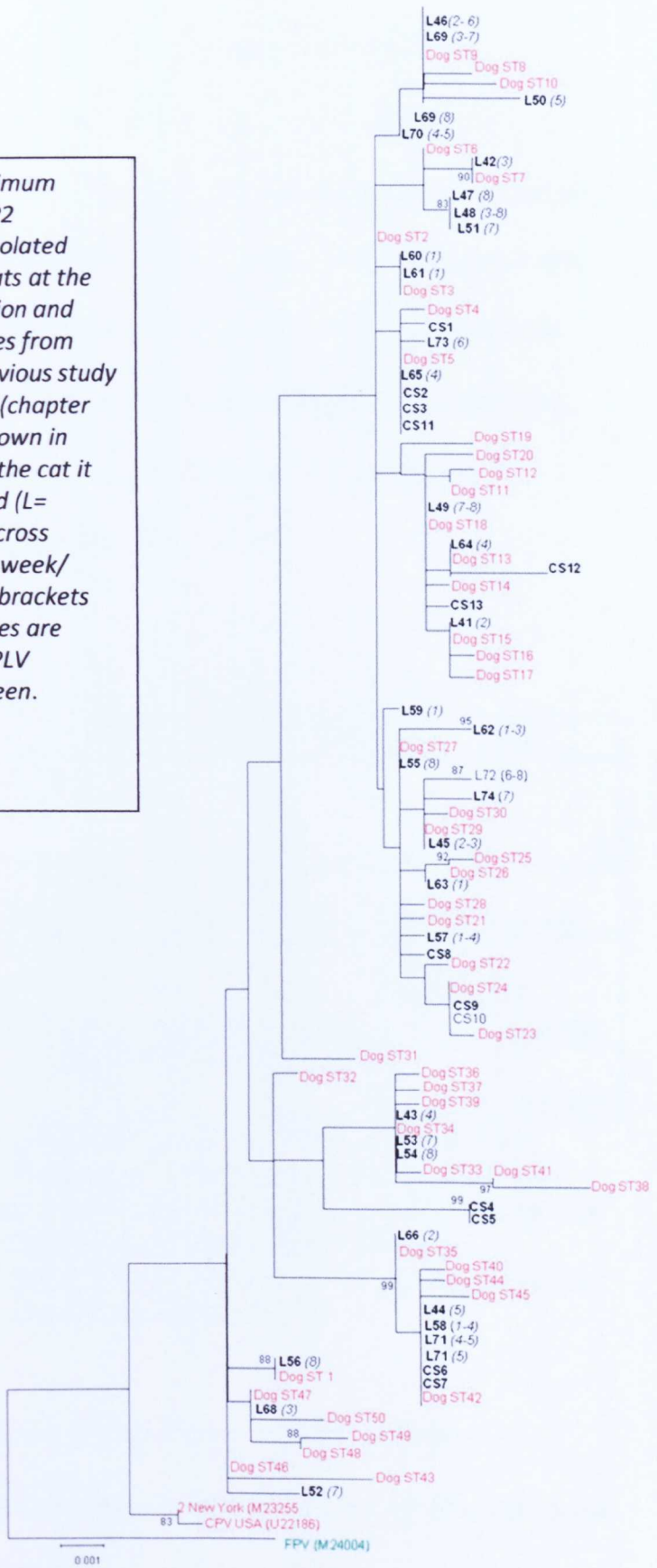


Figure 7.3a. *A DNA maximum likelihood tree of CPV VP2 sequences which were isolated from clinically healthy cats at the RSPCA and cat's protection and compared with sequences from clinically ill dogs in a previous study in the UK with the PDSA (chapter 3). Cat sequences are shown in black, with the name of the cat it was isolated from in bold (L= longitudinal study, CS = cross sectional study) and the week/ weeks it was isolated in brackets and italics. Dog sequences are shown in red, and the FPLV sequence is shown in green.*



Comparison of cat VP2 genes with dog VP2 genes available on Genbank

To ascertain if the cat sequences were identical to other sequences which have previously been identified, cat sequences were compared to those available on Genbank. Out of the 33 different DNA sequences, six of them (cat numbers L54, L56, L66, L68, CS4 and CS7) were within one nucleotide of a sequence which had been previously identified and published on Genbank. However, only CS4 was identical to a sequence published on Genbank (DQ025997), which was isolated from a dog with CPV disease in 2005 (table 3). The remaining 27 sequences were found to be more than three nucleotides different to Genbank sequences (range 3-11).

Cat number	L54	L56	L66	L68	CS4	CS7
Number of nucleotide differences to nearest genbank	1	1	1	1	0	1
Country of origin of nearest sequence	China	Italy	China	Brazil	France	China
Nearest genbank accession number	GQ 169543	FJ 222822	EF 666069	DQ 340426	DQ 025997	EF 666069
Nucleotide substitution (UK- Residue- World)	C-1695-T	A- 1708-G	G-814-A	A-660-G		G-814-A

Table 7.3. *The cat sequences which were identical or one nucleotide different when compared to genbank. L= longitudinal study at the RSPCA shelter, CS = cross sectional study at the Cats Protection shelter. The country where this nearest sequence was found and its Genbank accession number is also shown, with the position where the substitution occurs, expressed as the substitution in the UK sequence- the residue which is substituted- the residue found in the nearest genbank sequence.*

At the amino acid level, greater similarity was observed in relatedness of the cat sequences isolated in the UK, and those available on Genbank. Of the 19 different amino

acid groups containing cat sequences, two were identical, and seven were within one amino acid of others identified on Genbank (table 7.4). The others were two to four amino acid residues different.

The group which contains cat 69 had 22 separate amino acid sequences contained within it, was identical to 14 of the compared dog sequences, and was identical at this amino acid level to sequences previously found in the USA. The group which contains cat 58, and cat 25, was identical to fifteen of the fifty dog sequences, and was also found in New Zealand. This is shown below in table 7.4.

Cat number	No of AA differences to nearest genbank	Country of origin of nearest sequence	Genbank accession no	Substitution
L41	1	Japan	BAH10515	K-273-R
L54	1	China/ Brazil	ABK06752	I-139-V
L56	1	USA	AAV36771	K-321-N
L57	1	Japan	BAH10515	A-9-D
L58	0	New Zealand	AAV36776	
L69	0	USA	AAV36771	
CS4	1	Brazil/China	ABD04006	I-139-V
CS9	1	USA	AAV36771	R-271-P
CS13	1	Japan	BAH10515	F-6-V

Table 7.4. *The amino acid relatedness of cat sequences identified in this study, and how similar they were to other sequences identified around the world. The Genbank number of the nearest virus is shown in the table, along with the position where the viruses differ in the form of UK substitution- residue substituted- world residue.*

Common cat substitutions

To assess substitutions occuring within the cat sequences, all cat sequences, along with those from dogs in chapter 3, an FPLV sequence, and a sequence from CPV type 2 were compared. In an alignment of the 20 CPV VP2 amino acid sequences identified in this study in cats (16 of which were from the RSPCA shelters and four of which were from the cats protection), a total of 13 amino acid positions were substituted, six of which were substituted in more than one sequence. These substitutions were at residues 9, 13, 139, 226, 324 and 426 (which is a position which differs between types 2a, 2b and in the newly discovered 2c). These substitutions are shown below in table 7.5.

Two substitutions are observed in FPLV which are not observed in the CPV isolates from cats, further suggesting that the cat VP2 sequences are not of FPLV origin.

	6	9	13	87	93	101	103	112	139	182	185	190	226	297	300	305	323	324	375	426	434	551	564	568
L48 (3)	V	D	P	L	N	T	A	V	V	T	F	M	S	A	G	Y	N	Y	D	D	D	M	S	G
L52 (7)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	N	*	L	*	*
L53 (7)	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	I	*	N	*	*	*	*
L54 (8)	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	I	*	N	*	*	*	*
L72 (7)	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	*	*	*
L72 (6)	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	*	*	*
L50 (5)	*	*	*	*	*	*	*	*	*	*	V	R	*	*	*	*	*	*	*	*	H	*	*	*
L43 (4)	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	I	*	N	*	*	*	*
L62 (3)	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*
L62 (1)	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*
L57 (2)	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
L57 (1)	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
L62 (2)	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*
L72 (8)	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	*	*	*
L57 (4)	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
L57 (3)	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CS4	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	*	*	N	*	*	*	*
CS5	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	*	*	N	*	*	*	*
CS12	*	*	*	*	*	*	*	L	*	L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CS13	F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Dog_ST33	*	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	I	*	N	*	*	*	*
Dog_ST41	*	*	S	*	*	*	*	*	I	*	*	*	*	*	*	*	*	I	*	N	*	*	*	*
Dog_ST11	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
FPLV	*	*	*	M	K	*	V	*	*	*	*	*	*	S	A	D	D	*	*	N	*	*	N	A
CPV- 2	*	*	*	M	*	I	*	*	*	*	*	*	*	S	A	D	*	*	N	N	*	*	*	*

Table 7.5. *Substitutions observed within the cat CPV isolates in this study and the residue which they are substituted at. The dot indicates that the residue in that sample is same as that in the first line of that column. Samples are named as in figure 7.3. (L= longitudinal study, CS = cross sectional study) and the week/ weeks it was isolated in brackets. In addition, three dog sequences from chapter 3 are also used, showing that some cat substitutions are also observed in dog sequences previously isolated. For completeness and comparison, an FPLV and CPV-2 sequence is also included.*

Virus isolations

In all the samples testing positive by PCR (74, from 47 cats, some of which were observed for more than one week), virus was isolated on cells of either dog or cat origin, indicating that cats are shedding potentially infectious CPV particles. The CPE was similar to that which is observed by CPV isolated from dogs (data not presented).

Haemagglutination

All viruses which were isolated were shown to haemagglutinate pig red blood cells in a fashion similar to CPV isolated from dogs. This indicates that the viruses were all CPV rather than FPLV, a result which is in agreement with the sequence results.

Analysis of NS genes

The NS genes of 18 cat parvovirus samples covering all 8 weeks tested from different cages were selected for sequencing. These are indicated on figure 7.2b with an asterisk. All the NS genes tested were similar to CPV genes rather than those from FPLV, indicating that these viruses at least, were true CPV, rather than an FPLV/CPV recombinants (data not shown). No samples tested NS gene positive but VP2 negative.

Correlation of results

All results were in correlation with each other, and if a sample was positive by PCR, it was positive by HA and VI as well. However, 25 µl more virus preparation of unknown titre (75µl compared to 50µl) was added to the HA wells than in a usual diagnostic test, as the aim was to test if the virus does still haemagglutinate, rather than for diagnostic purposes. Any wells which contained no virus were negative for haemagglutination throughout.

Immunofluorescence

One virus isolated from cat 58 was used for immunoflourescence on CRFK and A72 cells, using a CPV specific monoclonal antibody, as the virus had been shown to be CPV using sequencing and haemagglutination tests. The pictures taken of the cells are shown below in figure 7.4.

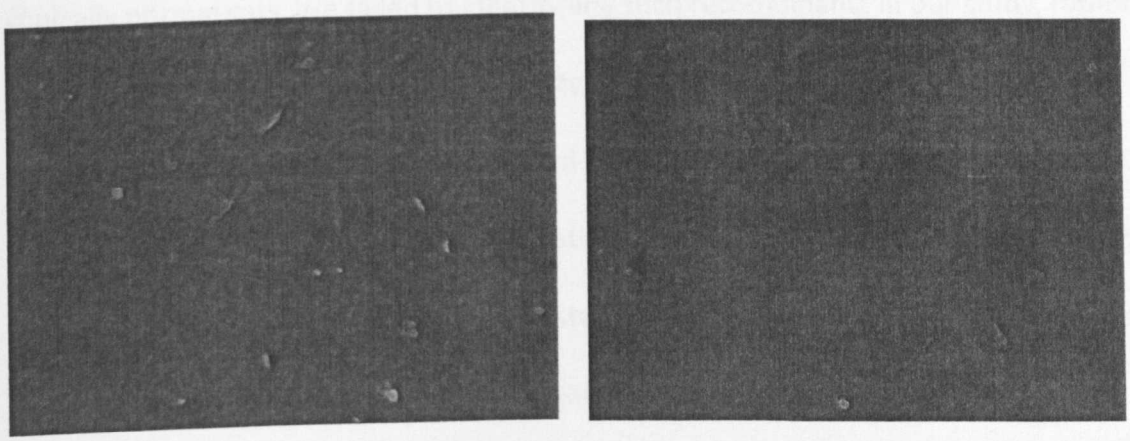


Figure 7.4. *A photo of the immunofluorescence of a CPV isolated from cat 58, shown on A72 dog cells (left) and CRFK cat cells (right). The photo shows the fluorescence of the nucleus, in a pattern similar to that observed with CPV.*

Discussion

In this study, PCR, phylogenetic analysis, and HA together suggested a high prevalence (37%) of CPV in 124 clinically normal healthy domestic cats in two rescue shelters. Longitudinal sampling showed some cats tested positive for a periods of four to six weeks suggesting some may act as long term carriers. Virus was isolated from all PCR-positive samples suggesting the samples contained infectious virus. In contrast, samples from the environment and dogs in the same shelters all tested negative. Although we cannot state conclusively that the cats in this study were actually infected with CPV without using invasive procedures such as tissue biopsies for mRNA or immunofluorescence on tissues, these data suggest for the first time that CPV infection, or CPV carriage, may be common in some populations of clinically normal cats.

Studies by Ikeda *et al* (2000) suggested that over 80% of cat parvovirus infections in Japan were caused by CPV rather than FPLV. In addition, Ohshima *at el*, (2009) identified recombination between FPLV and CPV using sequences available on Genbank. However, our study has for the first time identified CPV at high prevalence in two populations of clinically normal cats. We failed to identify any such recombinants in our study, rather all 74 VP2 and 18 NS sequences were of CPV type. Taken together, this suggests these positive cats were likely to be truly infected with canine parvovirus. The high level of CPV carriage shown here in clinically healthy cats is both surprising, and alarming. A previous short report (Whitby *et al*, 2010) and this study indicate that there is a high prevalence of CPV in clinically normal cats and this highlights the potential for cross species transmission of the virus between these animals. In addition, although we could not associate infection with disease, it will be important to determine whether such clinically

silent infections are compromising the host immune response, as is common in more overt parvovirus disease which decrease the number of circulating leukocytes both in CPV (Parrish *et al*, 1995: Stann *et al*, 1984) and in FPLV (Goto *et al*, 1983: Ichijo *et al*, 1976).

Comparing the CPV sequences from cats obtained here to those identified elsewhere in this thesis in dogs (chapter 3), provides further evidence that these cats are shedding CPV capable of being transmitted between cats and dogs. Specifically, the VP2 gene sequences from 55% of the cats, including the two sequence types found in both shelters, were identical to those isolated from dogs within the UK (Chapter 3). These results cannot be explained by sample contamination, since the samples in this chapter were processed at a separate time to those from the study described in chapter 3, and because we made rigorous use of negative controls which always tested negative. Rather, this strongly suggests these viruses are common in both sick canine and healthy feline populations in the UK.

Within this study, we found a large diversity of CPV sequences in each shelter sampled, similar to that described in canine populations (chapter 3). There were some instances where transmission may have occurred; six cats that tested negative for 1-5 weeks after first arriving in the shelter, subsequently started shedding virus, and four of these cats were shedding virus with identical VP2 sequence to others in the shelter. Despite these rare possible transmission events, the overall high level of diversity suggests that there are limited opportunities for transmission of CPV within these shelters, and that the diversity observed is a reflection of the diversity observed in the general population. Biosecurity, which is a common feature of shelter design and management, appears to

be, in these two shelters, providing at least some level of control against CPV transmission between cats within the shelter. In addition, there was no evidence that cats sequentially inhabiting the same cage were similarly infected, suggesting that the cages were cleaned suitably when vacated. This is consistent with previous studies in such shelters showing limited transmission of feline calicivirus, an important respiratory pathogen of cats (Radford *et al*, 2001; Coyne *et al*, 2007b).

Within this overall high prevalence, there was some evidence for different infection patterns in individual cats, with some appearing to shed virus intermittently, some tested consistently negative, whilst others were able to shed virus for over many weeks. This situation is reminiscent of that observed for other small animal infections (Coyne *et al*, 2006).

It is of particular concern that clinically healthy cats are able to shed CPV for long periods, acting as a potential reservoir of infection. In dogs, CPV is generally shed for up to twelve days in faeces when tested using virus isolation (Pollock, 1982, O'Sullivan *et al*, 1984), although up to 22 days has been reported in one study (Carman *et al*, 1980). To the author's knowledge, the duration of CPV shedding in dogs has not been evaluated by more sensitive methods such as PCR. (Desario *et al*, 2005). Here we identified cats that shed CPV for four weeks (n=2), five weeks (n=2) and six weeks (n=1). Virus was isolated in cell culture from each cat at each time point confirming this increased duration of shedding was not just an artefact of high PCR sensitivity. Each of these cats shed the same virus VP2 sequence throughout their infection. Together with the large number of sequence types present in the shelter, this suggests that these cats were persistently infected rather than undergoing repeated rounds of infection-clearance-reinfection.

Despite this apparent infection, there was no evidence of overt clinical disease in any of these cats. It will be interesting to determine the mechanism of this persistence, and to compare it to other parvoviruses, particular to FPLV which has been shown to persist in mononuclear cells in occasional cats (Miyazawa *et al*, 1999). Such clinically normal long term shedders are likely to be important to identify in any attempts to control or eradicate this disease in dogs and cats, and is an area which would warrant further investigation.

Within the shelter that was sampled longitudinally, 14 of the 74 cats (19%) that were sampled three or more times tested consistently negative. There are many reasons that might explain this "resistant" cohort including immunity following previous infection with either CPV of FPLV or vaccination either within the shelter or previously (Bohm *et al*, 2004). It is also probable that the biosecurity employed in such shelters allows individual cats to remain unexposed despite the high prevalence of infection in other cats in the shelter.

This study provides strong evidence that cats could act as a reservoir for CPV, with (Mochizuki *et al*, 1996) or without showing any clinical signs typically associated with parvovirus infection. Therefore care must be taken when introducing puppies into an environment where a cat lives, as cats may potentially be able to infect these seronegative puppies. It has also been suggested that as around 80% of dogs are vaccinated (Gaskell *et al*, 2002), herd immunity may well have been achieved in dogs, but the virus is allowed to be propagated in cats, thus allowing its continued survival. This may cause an issue in pups which haven't yet responded to vaccination, and cats may well be as big a danger to infect them as other dogs may be.

It was interesting to note that one of the two amino acid motifs which was suggested to be a substitution which circulates in dogs in the UK only (chapter 3), was again found in cats, that motif being the substitution at position 139 isoleucine to valine. Indeed in some cases this substitution was the only one which differed between the UK sequences and those found elsewhere in the world.

Since all cats are routinely vaccinated when they enter the shelters in this study, one would expect that they would develop some level of immunity to parvovirus infection. Unfortunately no blood was available from the cats or dogs in this study, so antibody levels could not be determined. However, it remains possible that the viruses identified in this study are selected to evade the immunity from such vaccines. Results of preliminary SN tests using polyclonal antibodies from previously vaccinated CPV dogs (authors unpublished observations) also suggest that some of these feline-origin CPV strains may be somewhat antigenically distinct from CPV isolates from dogs. If this is confirmed, it might be worth adding a canine parvoviral component to the multivalent feline vaccines, to prevent or reduce the risk of CPV carriage in the cats, and thus protect in-contact dogs. However, much more work is required to confirm this. Some vaccines have been shown to protect cats from infection by CPV and shedding in cats when vaccinated with a trivalent vaccine (Chalmers *et al*, 1999). There is a lack of information for the protection offered by other vaccines.

Interestingly, all of the 122 dog faecal samples collected in this study were negative for CPV infection. We cannot therefore conclude that infected cats are acting as a reservoir for cross species transmission between the dogs and cats within this shelter. The lack of canine infection suggests that either these viruses identified in cats were not infectious

to dogs, or perhaps more likely, measures to control infection between these two populations including canine vaccination and biosecurity were adequate. Further study will be needed to categorically define the infection status of cats such as these identified in this study, and to clarify their infectious nature to dogs.

Chapter 8

Full genome sequencing of CPV using 454 next generation sequencing technology

Introduction

The single stranded CPV genome is split into 2 open reading frames, with the left [1] 5' ORF encoding the non-structural (NS) proteins, and the right 3' ORF encoding the VP capsid proteins (Reed *et al*, 1988). The 3' end has either one or two palindromic repeats of around 60 nucleotides; the significance of the number of these is unknown (Reed *et al*, 1988, Horiuchi *et al*, 1994). Both termini of the virus are predicted to form GC rich hairpins, with the left termini Y shaped in rodent parvoviruses such as MVM (Astell *et al*, 1979) and the right termini shaped as a club (Bloom et al, 1990). These termini are known to stabilise the genome, and prevent any breaks in it during transport and replication (Bloom *et al*, 1990). Only a limited number of studies have sequenced the entire coding region of the virus, with a very small number successfully managing to sequence the full genome with the termini. Two predicted full genomes have so far been produced. Reed *et al* (1988) produced a full genome by cloning the replicative form DNA from the virus into M13 vectors, and this remains the most commonly referenced full genome, as they mapped on all sites of interest, including splice acceptor sites and protein start and end sites. Controversy has raged about the presence of a 60 base pair repeat at the 5' termini, and this was refuted by Horiuchi *et al* (1994) who produced a chimeric virus, using both CPV and mink enteritis virus. This seems to be because the secondary structure of the termini has proved refractory to standard sequencing technologies

such as PCR and Sanger sequencing, and RACE (rapid amplification of complimentary ends). This lack of knowledge concerning these non-coding regions of parvoviruses clearly represents a major challenge to understanding their full biology; especially as such structures can be critical to viral replication and virulence (Astell *et al*, 1979; Bloom *et al*, 1990).

Recently, new sequencing technologies such as 454 sequencing (Roche) have been developed which allow for rapid and deep sequencing of full genomes of many pathogens, in a very short time (Dong *et al*, 2011). The 454 technology is mainly used to sequence large genomes, such as bacteria (Arnold *et al*, 2011; Crasta *et al*, 2008; Pearson *et al*, 2007) or for within population diversity studies (Lorusso *et al*, 2010; Bimber *et al*, 2010; Solmone *et al*, 2009). That said, it has also been used to revolutionised the genome sequencing field, and has already been used successfully in many studies, including finding one potential cause of the disappearance of honeybees (Cox-Foster *et al*, 2007), uncovering re-arrangements in the human genome (Korbel *et al*, 2007) and sequencing the first genome of a human Neanderthal (Green *et al*, 2006, Noonan *et al*, 2006). There is also potential that this technology may be of use for genomes refractory to sequencing by conventional PCR methods, such as CPV.

Despite the obvious advantages of the new technology such as 454, these techniques do have their drawbacks. Pyrosequencing is known to be unreliable when long runs of the same nucleotide are together (homopolymers), such as several A residues (Dong *et al*, 2011). In addition, as viruses are obligate

intracellular organisms, any sequencing methodology would need to take into account the potential for high levels of host DNA contamination.

As 454 sequencing has never been used for CPV, and the difficulty encountered using conventional PCR to sequence the viral termini, it was of interest to assess the suitability of the 454 next generation technology for sequencing of entire CPV genomes, including the terminal hairpins. If successful, this technology could replace the older methods of PCR amplification and traditional Sanger sequencing and allow huge amounts of genomic information to be produced in a very short period of time, leading to more in depth studies of genomes and evolution.

Methods

Nomenclature

To avoid confusion, throughout this thesis, the genome has been orientated relative to the plus strand, rather than the negative strand which is packaged. Therefore the left terminus is the 5' and the right terminus is the 3'.

Samples

In total, four CPV viruses were used for 454 sequencing, of which three were replicative form (RF) DNA (kindly produced by N. Greenwood, Intervet Schering Plough) and one of which was viral DNA extracted from whole infectious viruses (table 8.1).

Lab ID	DNA type	CPV type	Origin
RL-3	RF DNA	CPV type 2a (flask 36)	
RL-4	RF DNA	CPV type 2 (vaccine)	Nobivac produced by Intervet
RL-5	RF DNA	CPV type 2a (flask 42)	
RL-6.	Viral DNA	CPV Type 2a (PDSA sample 388)	Hospitalised dog with severe gastro enteric disease which presented at the PDSA hospital in Thamesmead hospital in London. (chapter 3)

Table 8.1. *Description of samples used for 454 sequencing.*

Sample preparation

Two methods were used to produce the DNA for on-going sequencing: RF DNA and viral DNA.

Virus RF DNA was prepared by N. Greenwood at Intervet Schering Plough using the protocol of Greenwood et al, 1995. In order to produce RF dsDNA, the virus was inoculated onto A72 dog cells, and allowed to grow until CPE was observed. If no CPE occurred during the first passage, the virus was then passed onto fresh A72 cells and allowed to grow. The DNA was extracted by the Hirt extraction method. Briefly, cells from a 75ml flask were lysed using sodium dodecyl sulphate (SDS) with

EDTA, at pH 7.5. Sodium chloride was then added and the sample mixed. The sample was then centrifuged at 17000g for 30 mins at 4°C to remove the SDS and protein. The viral RF DNA remaining in the supernatant was then banded through caesium chloride, and after harvesting, resuspended in distilled water (McMaster *et al*, 1982, Hirt *et al*, 1967). This DNA was then stored at -80°C prior to sequencing.

To produce viral DNA, viruses were inoculated onto A72 cells or Crandell feline kidney cells (CRFK) and allowed to grow until significant cytopathic effect was observed. Virus from two 75 ml flasks was then extracted by freeze thawing the cells three times at -80°C. This virus and cell mixture was then transferred from flasks to centrifuge tubes, and spun at 55 $x10^3$ g for 2 minutes to pellet any large cellular debris. To further purify the virus, 20 mls of the clarified viral suspension was pelleted through a 20% sucrose cushion on a Sorvall Surespin 630 ultracentrifuge rotor for 16 hours at 167 $x10^3$ g. To obtain maximum DNA, this was done in triplicate, and pooled at the end. The sucrose was removed, and the viral pellet resuspended in 1ml of molecular grade water (Sigma), before storing at 4°C.

Analysis of concentration of DNA

The concentration of the DNA was assessed using Picogreen, which is specific for double stranded DNA, so would not take into account the single stranded DNA packaged by normal infectious virions.

DNA sequencing and sequence analysis

All sequencing and sequence assembly work for CPV was carried out in collaboration with the Centre for Genomics Research, University of Liverpool, using standard techniques of the manufacturer. Sequencing used the Genome Sequencer FLX Titanium Series by 454 life sciences (Roche Diagnostics, UK), which is capable of production of 1,000,000 high quality reads of approximately 500 bases in length per 10 hour sequencing run.

Library preparation and sequencing was carried out according to the manufacturer's instructions. Briefly, purified DNA was fragmented using nitrogen gas and a nebuliser according to standard protocols. Fragments >100bp were purified using DNA AMPure XP (Agencourt, Beckman), and used to prepare a fragment library with the GS FLX Titanium Rapid Library Preparation Kit (Roche 454).

Fragments of these ssDNA libraries were immobilised onto capture beads and amplified using emulsion based clonal amplification. These beads were then inserted into wells of a picotiter plate and sequenced by pryosequencing.

Individual reads were assembled with Newbler (v2.0.01.14: Roche Diagnostics, UK) using default assembly parameters. Contigs (a sequence made up from joining individual small overlapping (contiguous) sequences) over 100bp in length were analysed using Tablet (Plant Bioinformatics Group SCRI), and manually assembled into a "pseudogenome" using Chromas Pro (Technelysium Pty Ltd), and the best described published genome sequence for CPV (CPV-N (Reed *et al*, 1988) Genbank number: M19296). Subsequent analysis of the CPV-N genome, to another published

sequence (chimeric CPV (Horiuchi *et al*, 1994) Genbank number: D26079), as well as those produced here suggested that the CPV-N sequence likely contains artefacts to do with the sequencing methodology employed, particular in relation to a large 255bp repeat predicted at the 5'end of its genome, which also disrupts the 5' hairpin. As a result, subsequent analyses of the genome termini were carried out in relation to the chimeric CPV.

The TRDb database (Benson et al, 1999; http://tandem.bu.edu/trf/trf.html) was used to identify potential repeat sequences, as previously identified by Reed *et al* (1988). To assess for predicted secondary structures in the genomic termini, MFold (Zuker, 2003) was used using default settings, inserting the last 200 nucleotides of the genomes generated here, as well as the previously published genomic termini (Reed *et al*, 1988; Horiuchi *et al*, 1994).

Results

Virus concentrations

As the 454 machine has a requirement for double stranded DNA, the concentrations of the double stranded DNA in each of the four samples as assessed by picogreen were broadly equivalent (table 8.2), and in all cases suitable for ongoing sequencing. This was despite the fact that the DNA for RL-6 was generated by extraction of purified infectious virus, which in theory contains only single stranded DNA (Chapman *et al*, 1995). Double stranded DNA may have been in the

form of RF DNA carry over, or perhaps more likely, annealing of plus and minus single strands extracted from packaged viruses.

Library	DsDNA Concentration
RL-3	24.2 ng/ µl
RL-4	5 ng/µl
RL-5	14.7 ng/ µl
RL-6	12 ng/µl

Table 8.2. *Concentration of double stranded DNA in the canine parvovirus isolates as assessed by picogreen. RL-3, -4 and -5 were all purified replicative form (RF) DNA. RL-6 is DNA purified from purified viruses.*

Sequencing

Summary data for the sequence produced for each sample is shown in table 8.3. The proportion of reads mapping to CPV ranged from 23% - 79%. For each of the four samples, genome coverage was predicted to be in excess of 91% in comparison to the published CPV-N sequence (Reed *et al*, 1988). The depth of genome sequencing was lowest for RL-6 (53x), as a result of its having fewer reads and higher cellular DNA contamination than the other samples. Interesting for rl4 despite have the of greatest number reads mapping to CPV they assemble into the most number of CPV contigs (26). In contrast rl6 has the smallest number of reads producing the smallest number of contigs. The reasons for this are unclear but suggest something may be breaking the alignment into individual contigs such as mixed infection.

	RL3	RL4	RL5	RL6
Total no reads	19872	33612	39981	4534
Total no bases	5427166	8997552	10861516	1152620
Ave read length	273.1	267.7	271.7	254.2
Number of reads (%) which map to CPV	8538 (43%)	26500 (79%)	20894 (52%)	1035 (23%)
Total length of CPV pseudogenome	5014	4852	4945	4956
Predicted depth	465X	1462x	1148x	53x
% genome coverage (CPV-N)	94.2	91.2	92.9	93.1
% genome coverage, (chimeric CPV)	98.8	95.6	97.4	97.7
3' terminal gap (CPV-N)	108	271	103	133
3' terminal gap (chimeric)	33	196	28	98
5' terminal gap (CPV-N)	23	24	96	-6
5' terminal gap (chimeric)	23	24	96	-6
Internal gaps (nucleotides)	0	2 (71 and 113)	2 (95 and 21)	0
Total number of contigs	70	112	129	28
Number of contigs mapping to CPV	6	26	16	2
Contigs used to make the pseudogenome	3	11	7	2
Number of contigs mapping to canine genome	56	76	99	16
Number of contigs showing no homology	7	10	13	8
Number of contigs mapping to other	1 (human)	0	1 (mouse)	1 (pig), 1 (cat)

Table 8.3. *Summary of the results obtained from the 454 machine for the four different genomes. It shows the total number of reads, and how many of these were CPV specific as well as the average read length, and the percentage of the genome relative to CPV-N (Reed et al, 1988) and chimeric CPV (Horiuchi et al, 1994) which was sequenced in each genome. Only contigs over 100bp in length were included.*

The resulting pseudogenome produced by RL3 (Flask 36, 2a virus) and RL-6 (PDSA 388 purified from viruses) had no gaps within it, although was not complete (based on Reed *et al*, 1988) with sections missing at the 5' and 3' termini. The 5' terminus

of the RL6 virus was complete, and was 6 nucleotides longer than that predicted by Reed *et al* (1988).

The pseudogenome produced by RL4 (vaccine) and RL-5 (Flask 42 2a virus) had small gaps present in the middle of the genome, so analysis of these was more difficult. As the virus for RL4 had previously had parts of the coding region sequenced for previous studies, these were used to fill in some of the gaps.

Two high quality sequence psuedogenomes were created which had no gaps, RL-3 and RL-6. RL-4 and RL-5 had small gaps within the coding regions.

RL-3- flask 36, CPV-2a RF-DNA

In total, 70 contigs which were over 100 nucleotides in length were obtained from the RL-3 flask of which six corresponded to CPV or the highly related virus feline panleukopenia (FPL) (table 8.4). Three contigs (69, 1 and 3) were needed to produce the genome (pseudogenome) (Figure 8.1). The largest of these was contig 1, which was 3701 nucleotides in length. Contigs 69 (1239 base pairs) and contig 3 (1239 base pairs) were aligned with contig 1 to create the full genome. Contig 3 had an 18 base overlap with contigs 69.

These three contigs produced a final pseudogenome which was 5014 nucleotides in length, 309 nucleotides shorter than that predicted by Reed *et al* (1988), and 61 base pairs shorter than the genome predicted by Horiuchi *et al* (1994). Contig 69 finished 20 nucleotides short of the 3' end of the genome as predicted by Reed *et al* (1988), and the pseudogenome was 278 nucleotides short of the predicted finish

point for the 5' termini. Compared to the genome predicted by Horiuchi *et al* (1994), the genome generated here was again 20 nucleotides short at the 3' end, but was only 12 nucleotides short at the 5' end.

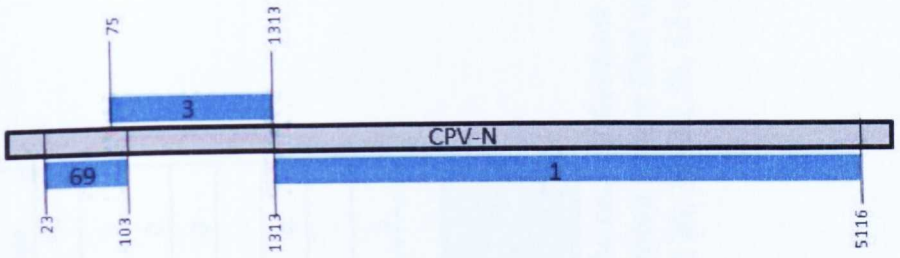


Figure 8.1. *A schematic diagram showing the contigs which make up the RL-3 pseudogenome compared to CPV-N (Reed et al, 1988). The grey bar indicates CPV-N and the blue bars are contigs produced here. The positions of these contigs are shown relative to CPV-N.*

Contig no	Length	Reads	Description	Genbank No	Identities	Gaps
Used in Pseudogenome						
1	3701	6332	CPV	M38245	3690/3702	3
3	1239	2099	CPV	M38245	1239/1239	0
69	103	45	FPL	X55115	103/103	0
Not used in Pseudogenome						
39	377	9	FPL	U22185	123/125	2
41	358	47	CPV	EU659120	358/358	
43	316	6	CPV	GQ857609	315/320	5
Others						
n= 56	Ave 426 (range 100-1200)	Ave 17 (range 2-88)	Dog genome			
33	435	6	Human genome	AC067957		
n=7	Ave 330 (107-526)	ave 4 (range 2-9)	No Homology			

Table 8.4. *Identity of all contigs over 100 nucleotides in length produced from RL-3 (CPV 2a) and the nearest Genbank neighbour of it. Only six contigs correspond to CPV, with the majority of the rest being dog cells. One of the contigs represented human DNA which may have been a contaminant from the growth or extraction procedure at some stage of the purification. Contig 23, 30, 32, 36, 55, 62 and 68 showed no homology to any sequences on Genbank, so the identity / origin of this DNA is unknown.*

A further three contigs (39, 41 and 43) also mapped to CPV, were based on small numbers of reads, and largely contained identical sequences to that found elsewhere within contigs 69,1 and 3, and so were not needed to produce the pseudogenome. Contig 41 mapped to one end of contig one, and was 100% identical. Contig 43 was very similar to an area around nucleotide 2000 of contig one, but differed due to a number of repeats of T nucleotides which causes problems in pyrosequencing. Contig 39 mapped to an area of the genome which was contained within contig 3, and was only different due to a single additional nucleotide found in a run of several A residues (figure 8.1)

Of the remaining 64 contigs, seven corresponded to sequences which did not match anything available on Genbank (table 8.4). One of the contigs matched human cells, which may reflect low level contamination of the sample. The remaining contigs represented dog cell DNA, presumably from the cells which the virus was grown in, suggesting that the purification did not remove all cellular DNA (table 8.4).

RL-6: CPV2a purified from infectious virus

Of the 28 contigs produced from this sequence, two mapped to CPV. Contig one (2735 base pairs) and 2 (2563 base pairs) overlapped by 145 nucleotides.

These two contigs came together to produce a pseudogenome which was 4956 nucleotides in length. This was 367 nucleotides shorter than that predicted by Reed *et al* (1988) and also 119 base pairs shorter than the genome predicted by Horiuchi

et al, 1994. In addition, we predict that the genome is 6 nucleotides longer at the 3' end than predicted by Reed *et al* (1988).

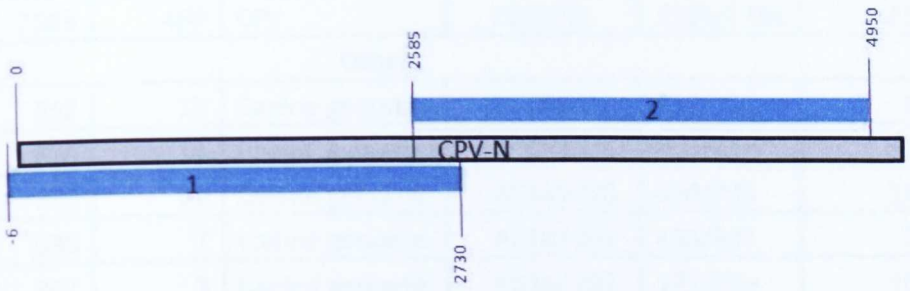


Figure 8.2. *A schematic diagram of the positions of the two large contigs from RL-6. Contig 1 over hangs by six nucleotides at the left hand terminus compared to CPV-N. This is discussed in more detail below. The grey bar indicates CPV-N and the blue bars are contigs produced here. The positions of these contigs are shown relative to CPV-N.*

Contig	Length	Reads	Description	Genbank No	Identities	Gaps
Used in Pseudogenome						
1	2735	546	CPV	M38245	2715/2732	1
2	2563	489	CPV	D26079	2352/2384	25
Others						
3	952	17	Canine genome	AC186058	937/956	5
4	834	14	Canine genome	AC186073	800/844	18
5	733	37	Canine genome	AC189730	690/739	14
6	530	7	Canine genome	AC187001	435/447	3
7	497	3	Canine genome	AB167707	171/199	10
8	475	5	Canine genome	AJ239527	436/476	3
9	411	14	Canine genome	AC188543	405/411	2
10	365	19	Canine genome	AC199142	186/201	5
12	324	3	Canine genome	AC147679	289/327	15
13	310	6	Canine genome	AC183634	307/309	2
14	282	9	Canine genome	AC197829	144/159	6
15	278	29	Canine genome	AC188926	193/199	2
17	215	4	Canine genome	AC203194	182/182	0
19	206	8	Canine genome	AC183632	206/207	1
25	112	4	Canine genome	AC190107	88/98	2
27	109	2	Canine genome	AC186972	104/110	1
26	117	2	Porcine genome	CU326356	31/32	0
28	102	2	Feline genome	AC233697	41/41	0
11	344	6	No homology	Not applicable		
16	245	8	No homology			
18	207	3	No homology			
20	196	3	No homology			
21	173	2	No homology			
22	150	1	No homology			
23	148	1	No homology			
24	129	2	No homology			

Table 8.5. *Identity of all contigs over 100 nucleotides in length produced from RL-6 (DNA isolated from a PDSA sample) and the nearest genbank neighbour of it. Two contigs correspond to CPV, with the majority of the rest being dog cell, or showing no homology to those available on genbank.*

No other contigs were produced which mapped to CPV using this DNA sample. The reason for the small number of CPV specific contigs compared to RL3 is unknown, but may be a reflection of the purification protocol used here.

Of the remaining 26 contigs, 16 were mapped to dog cell DNA, which is unsurprising as the virus was grown on these cells (Table 8.5). Surprisingly, one was similar to pig DNA, which may reflect low level contamination, possibly from trypsin which is sometimes of pig origin. A further eight of the 25 sequences showed no homology to any sequences on Genbank.

RL-4 (CPV-2 vaccine) RF DNA

In total, 112 contigs over 100 nucleotides were obtained from the RL-4 flask, of which twenty six corresponded to CPV or the highly related FPL (table 8.6).

Eleven contigs ranging in size from 1575-101 (average 484), were used to produce the final pseudogenome of 4852 base pairs (figure 8.4). However two small internal gaps (from 300-371 and from 855- 968) still existed towards the left terminus of the genome, between contigs 77 (225 base pairs) and 37 (1915 base pairs), and 37 and 99 (136 base pairs). These two gaps could have been bridged by conventional PCR. However, we were fortunate to already have the appropriate NS gene sequence from a previous study (chapter 5, this thesis), which showed 100% identity where it overlapped contig sequences, and so this was incorporated into the final pseudogenome. Four contigs were manually joined together without any gaps

(contig numbers 96 (146 base pairs), 6 (1078 base pairs), 5 (1575 base pairs) and 73 (239 base pairs) to form the middle part of the virus.

Of the remaining small contigs, contig 77 overlapped at one end of the 109 contig by 31 nucleotides which was a 100% match, but there were seven nucleotide substitutions based on CPV-N, but these were due to long runs of a single nucleotide. Contig 37 is flanked either side by gaps. Contig 96 and 6 show no overlap, but join end to end, as does contig 5 to contig 6, and contig 73 to 5. Contig 73 overlaps with contig 44 by 155 nucleotides which are 100% match. Contig 111 and 40 overlap by 30 nucleotides, again at a 100% match (Figure 8.4).

The pseudogenome was predicted to finish 24 nucleotides short of the 3' end of the genome reported by Reed *et al* (1988), and 146 nucleotides short of the predicted finish point for the 5' termini. Compared to the genome predicted by Horiuchi *et al*, (1994), the genome generated here was again 20 nucleotides short at the 3' end, and was also short at the 5' end.

Of the 15 remaining contigs that mapped to CPV and were not used to produce the final pseudogenome, all were small (range of size, and average) and overlapped larger contigs.

Of the remaining 86 contigs, ten corresponded to sequences which did not match anything available on genbank. The remaining contigs represented dog cell DNA, which was the cells which the virus was grown on, suggesting that the purification did not remove all cellular DNA (table 8.6).

Contig No	Length	Reads	Description	Genbank	Identities	Gaps
Used in pseudogenome						
5	1575	8845	CPV	EU659117	1571/1576	1
6	1078	6877	CPV	EU310373	1078/1078	0
37	485	1915	CPV	EU659120	485/485	0
40	445	1425	CPV	M23255	444/445	0
44	391	3046	CPV	FJ197846	391/391	0
73	239	87	CPV	FJ197846	238/239	1
77	228	188	CPV	D26079	228/228	0
92	155	1527	CPV	GQ414751	155/155	0
109	103	50	FPL	X55115	103/103	0
96	146	1943	CPV	EU659121	146/146	0
111	101	8	CPV	HQ025913	101/101	0
Parvo sequences not used in pseudogenome						
26	580	227	CPV	EU310373	579/580	1
52	336	12	CPV	AB437434	329/336	7
60	297	6	CPV	FJ197846	294/297	3
62	290	7	CPV	FJ197846	282/284	2
64	283	11	CPV	AB437434	275/283	8
65	273	133	CPV	EU659121	179/179	0
66	270	7	CPV	FJ011098	265/270	5
68	265	34	CPV	GQ502248	91/92	1
69	265	9	CPV	AB437434	259/266	6
70	263	7	CPV	FJ197846	262/264	2
87	192	124	CPV	M38245	190/192	1
95	151	11	CPV	GQ421597	150/150	0
97	142	3	CPV	GQ421597	137/139	2
110	103	5	CPV	EU310373	102/103	1
Other sequences not used in pseudogenome						
N= 77	Ave 526 (102-5224)	Ave 16 (range 2-81)	Dog genome			
N= 10	Ave 232 (range 101-513)	Ave 14 (range 2-51)	No homology			

Table 8.6. *Identity of all contigs over 100 nucleotides in length produced from RL-4 (vaccine) and the nearest Genbank neighbour of it. Twenty five contigs correspond to CPV (or the closely related FPL), with the majority of the rest being dog cells, or showing no homology to those available on genbank. For clarity of presentation, the contigs that did not map to CPV are summarised at the bottom of the table.*

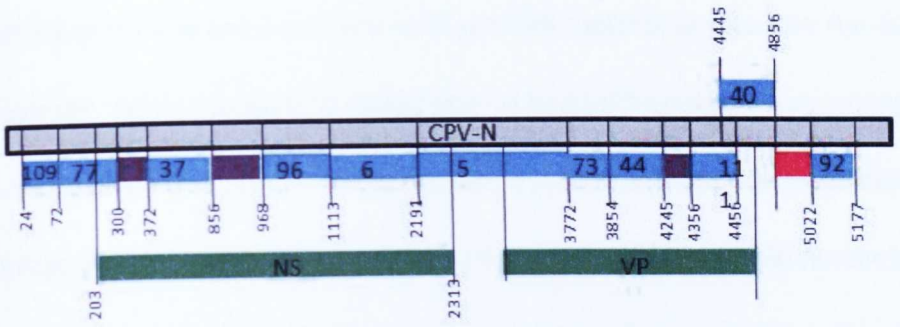


Figure 8.4.*A schematic diagram of the 454 sequencing contigs* used to produce RL4, *and the two amplicons produced by sequencing of PCR amplicons (named NS and VP). This closes the gaps between contigs 77 and 37, and between 37 and 99.The grey bar indicates CPV-N and the blue bars are contigs produced here. The positions of these contigs are shown relative to CPV-N. In addition, this pseudogenome contains some gaps, some of which were closed by PCR amplicons (shown in purple), and some of which remain, shown in red.*

RL-5 CPV type 2a (flask 42) RF DNA

Of the 129 contigs produced form this sequence, 16 mapped to CPV, of which seven ranging in size from 1179-270 (average 732), were used together to form the final pseudogenome of 4945 nucleotides in length.

Two small gaps were still present between contig 5 and 95 and also between 20 and 11. These would have been easily closed using conventional PCR and sequencing but due to time constraints, were joined in the final pseudogenome by N's, the number of which was determined by comparison with CPV-N. This was deemed appropriate as the gaps existed in highly conserved coding regions.

Contig 5 maps to the 3' end of the genome, and is followed by a 95 nucleotide gap before contig 95 starts. Contig 95 maps end to end with contig 9 based on published CPV-N sequence and so it was felt appropriate to join the contigs together. Again, contig 9 mapped end to end with contig 12, as supported by CPV-N and also by two other contigs (85 and 93) which were not assembled into larger contigs due problems with homopolymers. An overlap of 120 nucleotides between contig 12 and the next contig, 84, and another overlap of 150 nucleotides is found between contig 84 and 20. A 21 nucleotide gap is found before contig 11.

In total, the RL-5 pseudogenome which was 378 nucleotides shorter than that predicted by Reed *et al* (1988) and was 130 base pairs shorter than the genome predicted by Horiuchi *et al*, (1994).

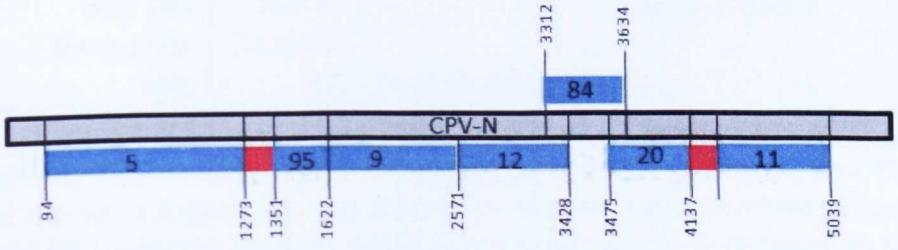


Figure 8.5. *A schematic diagram of the contigs which were used to produce the pseudogenome for RL-5. The grey bar indicates CPV-N and the blue bars are contigs produced here. The positions of these contigs are shown relative to CPV-N. In addition, as this pseudogenome contains some gaps in the sequence, these are indicated with a red bar.*

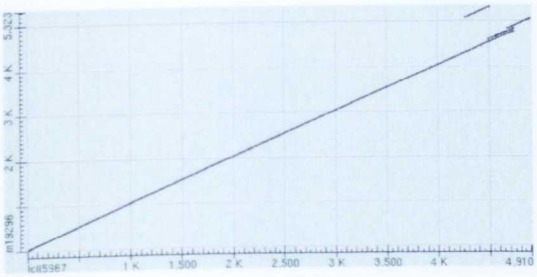
Contig	Length	Reads	Description	Genbank no	Identities	Gaps
Used in pseudogenome						
5	1179	3452	CPV	M38245	1178/1179	0
9	950	4550	CPV	AY742933	949/950	0
11	881	2096	CPV	M38245	878/881	0
12	856	4065	CPV	EU659119	856/857	1
20	661	3907	CPV	FJ197835	661/661	0
84	327	6	CPV	GQ857609	321/329	8
95	270	2033	CPV	EU659120	270/270	0
Parvo sequences not used in pseudogenome						
96	268	408	CPV	EU659120	268/268	0
105	193	121	CPV	AB437434	193/193	0
117	146	61	CPV	EU659121	142/143	1
118	139	85	CPV	EU659121	138/139	1
40	518	48	CPV	HQ025913	517/518	1
78	359	12	CPV	HQ025913	354/359	5
85	322	7	CPV	AB437433	319/322	1
92	297	37	CPV	EU659121	297/297	0
93	286	6	CPV	AB437434	281/291	9
Other sequences not used in pseudogenome						
N=99	Ave 481 (range 103-1796)	Ave 17 (range 2-170)	Dog cells	Not applicable		
N=14	Ave 284 (range110-696)	Ave 13 (range 2-42)	No Homology			
119	135	2	Mouse Cells			

Table 8.7. *Identity of all contigs over 100 nucleotides in length produced from RL-5 (flask 42, 2a virus) and the nearest genbank neighbour of it. Sixteen contigs correspond to CPV (or the closely related FPL), with the majority of the rest being dog cell, or showing no homology to those available on Genbank. A single sample (contig number 119) was found to be most similar to mouse cells, which may represent low level contamination of the sample, either during extraction or sequencing. For clarity of presentation, the contigs that did not map to CPV are summarised at the bottom of the table.*

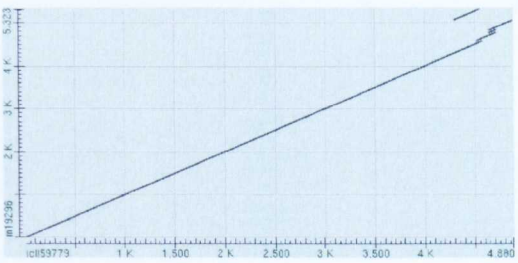
Of the nine that mapped to CPV but were not used in the final pseudogenome, contig 118 and 92 were contained entirely within contig 5. Contig 118 was 100% identical to contig 5 and the reason for its failure to incorporate into contig 5 is uncertain. For contig 92, there was a single C deletion in a homopolymer of seven c residues compare with contig 5. Contig 96 is contained with 100% identity within contig 96, and contig 117 is contained within contig 9, with a single deletion of an A residue in a run of three A residues. Contig 85 and 93 are contained within contig 12, as are contigs 105, 40 and 78.

Comparison of 454 sequences with CPV-N.

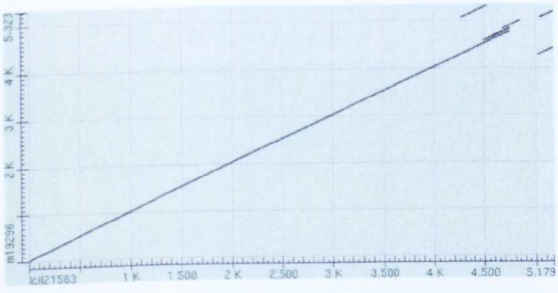
The four pseudogenomes produced here were compared to the previously published CPV-N genome using the similarity dot matrix as available on BLAST (figure 8.6 a-d). High levels of similarity were observed from the start of the genome with all viruses. However the 5' terminus of the genome differs from that reported by Reed *et al* (1988), largely due to the presence of 60 base pair repeats and a predicted 255 base pair duplication at the 5'end of CPV-N. In addition, a gap in RL-5 at around 1200 nucleotides, was a reflection of the missing genetic data in our sequence.



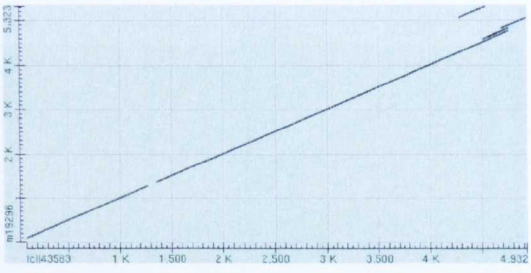
8.6a. RL3 (X axis) against CPV-N (Y axis) (Y axis)



8.6b. RL-6 against CPV-N



8.6c. RL4 (X axis) against CPV- N (Y axis) (Y axis)

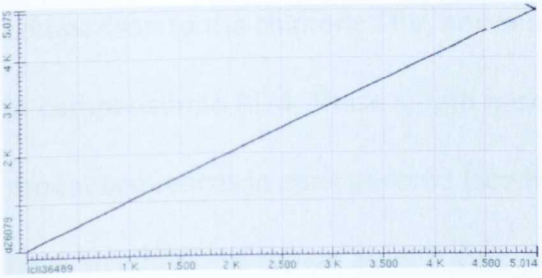


8.6d. RL5 (X axis) against CPV-N

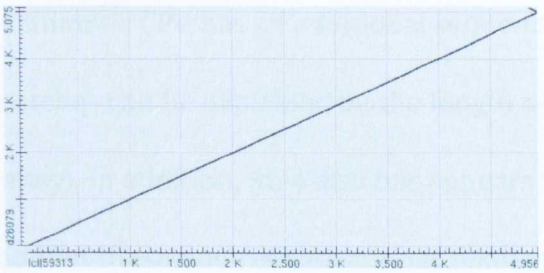
Figure 8.6a-d. *A dot matrix comparison of the genome sequenced using 454 sequencing produced here compared to the previously published genome by Reed et al (1998) (Genbank number M19296).*

Comparison of 454 sequences with chimeric CPV produced by Horiuchi *et al* (1994)(D26079)

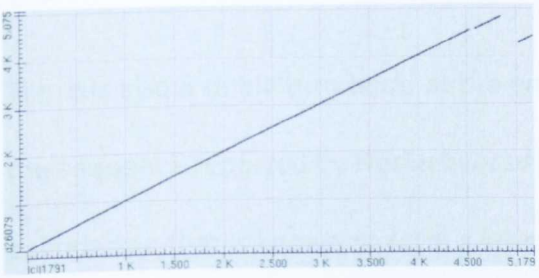
As Horiuchi *et al* (1994) disputed the 255 base pair repeats at the 5' end of the genome, it was also of interest to compare the sequences of the pseudogenomes produced here, to the genome published by Horiuchi *et al* (1994) which was generated to validate the design and manufacture of chimeric viruses.



8.7a. RL3 (X axis) compared to D26079 (Y axis)



8.7b. RL6 (X axis) compared to D26079



8.7c. RL4 (X axis) compared to D26079 (Y axis)

8.7d. RL5 (X axis) compared to D26079 (Y axis)

Figure 8.7a-d. *A dot matrix comparison of the genome sequenced using 454 sequencing produced here compared to the previously published genome by Horiuchi et al (1994) (Genbank number D26079).*

The data here suggest that there is no 255 base pair repeat found in our pseudogenomes, and as CPV-N is the only genome where a 255 base pair repeat has been reported, it appears that this may be due to the sequencing protocol used by Reed *et al* (1988).

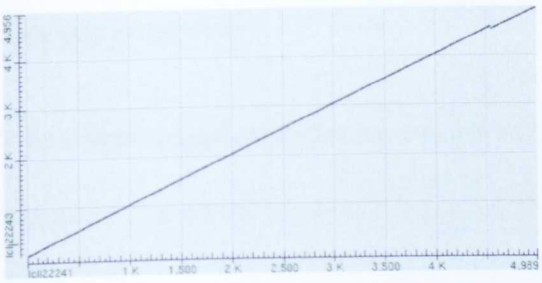
When our sequences were compared to the sequence reported by Horiuchi *et al* (1994), it suggests a gap in the co-linearity of the genomes at around 4500 base pairs in each genome. The direction of displacement of the distal genome segment suggests that RL-3, RL-4 and RL-5 have a short region of additional sequence in

comparison to the chimeric CPV, and that chimeric CPV has an additional sequence in comparison to RL-4. These length mismatches can be explained by the length of repeat sequences in each genome (see below). In addition, RL-4 also has appears to have an additional repeat at its 3' end, and that these two repeats are not tandem, but are separate from each other. The latter repeat is likely to be an artefact of the large number of contigs (11) used to produce this pseudogenome, and how it was originally assembled using the CPV-N sequence.

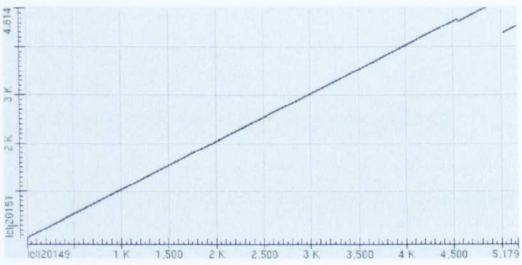
There is also a small 'turn back' at the end of RL-3, RL-5 and RL-6 when compared to the sequence reported by Horiuchi *et al* (1994), and this is indicative of a palindrome that is known to form a stem loop in chimeric CPV (see below).

Comparison of the 454 sequences with the RL-6 sequence

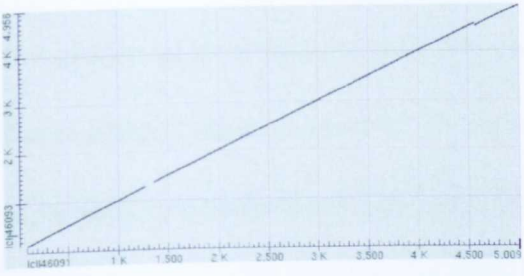
As the 5' termini of the viruses are controversial, with two differing opinions (discussed above) it was of interest to ascertain if the newer viruses had different termini. Therefore the sequences produced by RL3, 4 and 5 were compared to RL6, which was the pseudogenome produced using only two contigs, and gave the best genome for comparison.



8.8a. RL3 (X axis) compared to RL6 (Y axis)



8.8b. RL4 (X axis) compared to RL6 (Y axis)



8.8c. RL5 (X axis) compared to RL6 (Y axis)

Figure 8.8a-d. *A dot matrix comparison of the genomes sequenced using 454 sequencing produced here compared to RL6, again produced here. 8.9a = RL3, 8.9b= RL4 (with PCR products used to fill some gaps in coding regions), 8.9c= RL5.*

These analyses confirm that RL6 is missing sequence around position 4500 when compared to the pseudogenomes of RL3, RL4 and RL5, probably due to the presence of repeat sequences in these three genomes (see also below). In addition, RL-4 has an additional non-tandem repeat at its 3' end.

Analysis of termini

It has been suggested that canine parvovirus genomes form complex terminal hairpins. Therefore the termini of published CPV genomes, and the psudogenomes produced here, were analysed for their ability to form these structures.

Analysis of the 5' left end termini of the viruses

The predicted structure of the 5' left end terminus of RL-6 is shown in figure 8.10. The predicted structure is of a "Y"-shaped terminus, containing two long stems separated by a small bubble of single stranded sequence, and distally, two small stem loops (3 and 4), which have been referred to by others as "rabbit ears".

The sequences of the viruses (for CPV-N, chimeric CPV, RL-3, RL-4, RL-5 and RL-6) were analysed to ascertain if there were sufficient differences to alter this predicted terminal structure (figure 8.9).

In general only minor differences were apparent between sequences, and these were restricted to loops, and suggest that the primary sequence as well as the secondary structure is important for viral function.

There were differences in the length of the termini. RL-6, CPV-N and chimeric CPV had identical termini, apart from the fact that RL-6 is six nucleotides longer. There is also a substitution at nucleotide 49, a G in chimeric CPN, but a C in CPV-N and RL-6 (numbered according to CPV-N) which affects the bubble at the base of the ears. This additional 6 nucleotides base-pair 100% and allow the stem of the terminus to be extended by six nucleotides.

RL3 and RL-4 are shorter, with the entire bubble observed in RL-4 not being formed due to the lack of the joining bases at the base of the bubble to the stem, and this may be formed in vitro. The bubble is present in RL-3 but is only joined to the stem by a single nucleotide, likely to make it relatively unstable.

RL-6 is 2 base pairs shorter in loop (ear) 3 to a 2 base pair gap, and this suggests that the loop in CPV-N may be bigger than that observed in RL-6. It also suggests that insertions and deletions may be accepted in the genome termini, providing that they occur in the bubbles, or the tops of the loops.

Despite minor sequence changes in the left termini, the overall structure of the viral genome is still predicted to highly conserved (figure 8.9), suggesting that in the left termini, the structure rather than the sequence is conserved. In some cases, there appear to be differences in the termini, but these are likely due to the different start points of the virus, with some genetic data missing in some of our sequences.

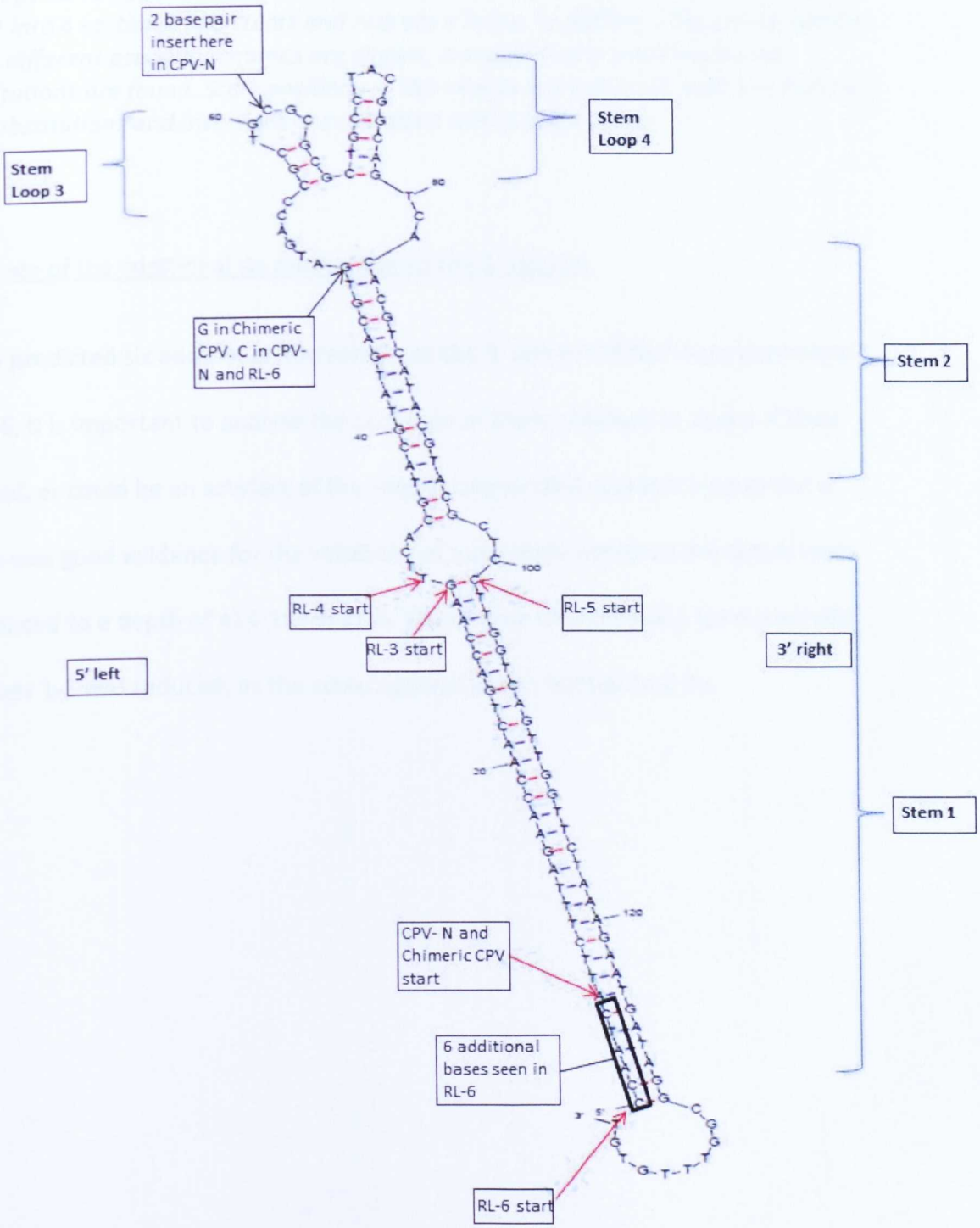

2 base pair insert here in CPV-N
Stem Loop 3
Stem Loop 4
G in Chimeric CPV. C in CPV-N and RL-6
Stem 2
RL-4 start
RL-3 start
RL-5 start
5' left
3' right
Stem 1
CPV- N and Chimeric CPV start
6 additional bases seen in RL-6
RL-6 start

Figure 8.9. *The structure of the 3' terminus of RL6, which is representative of all the viruses, and is also the longest. The six additional bases observed in the RL-6 pseudogenome appear not to alter the structure of the virus terminus. The terminus is split into 4 sections, two stems and two stem loops. In addition, the start positions of the different psedudogenomes are shown, along with any positions where substitutions are found. Start positions of the viruses are indicated with a red arrow, and substitutions and insertions are indicated with a black arrow.*

Coverage of the additional six nucleotides at the 3' termini

As we predicted six additional nucleotides at the 3' termini of the virus sequenced in RL-6, it is important to analyse the coverage of these residues to assess if they are real, or could be an artefact of the sequencing or DNA extraction procedure. There was good evidence for the reliability of nucleotide positions 4-6 which were sequenced to a depth of 414-15x in RL-6. The degree of confidence for nucleotide residues 1-3 was reduced, as the coverage was lower, limited to 2-4x.

CPV-N nucleotide number								1	2	3	4	5	6	7	8	9	10	11	12	13	14
Virus	CPV-N							A	T	T	C	T	T	T	A	G	A	A	C	C	A
Virus	RL-6	C	C	T	A	T	C	A	T	T	C	T	T	T	A	G	A	A	C	C	A
RL-6 nucleotide number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Coverage		2	2	4	14	14	15	19	19	19	19	19	20	20	20	21	23	25	25	25	26

Table 8.8. *The coverage of the 3' termini of RL-3 compared to that of CPV-N. For clarity this is shown with CPV-N (Reed et al, 1988), and the six additional bases indicated in bold. The number of times (coverage) that each nucleotide was sequenced in RL-6 is also shown at the bottom.*

Analysis of the right hand end termini of the viruses

The right hand termini of parvoviruses are predicted to form a hairpin (Bloom *et al*, 1990). However analysis of the terminal nucleotides of the first published genome, CPV-N (Reed *et al*, 1988), were not predicted to form a hairpin (data not presented). The genome obtained by Horiuchi *et al* (1994) was predicted to form a "tight" hairpin containing almost 162 bases of double stranded stem, and two small single stranded loops each of three bases (figure 8.10).

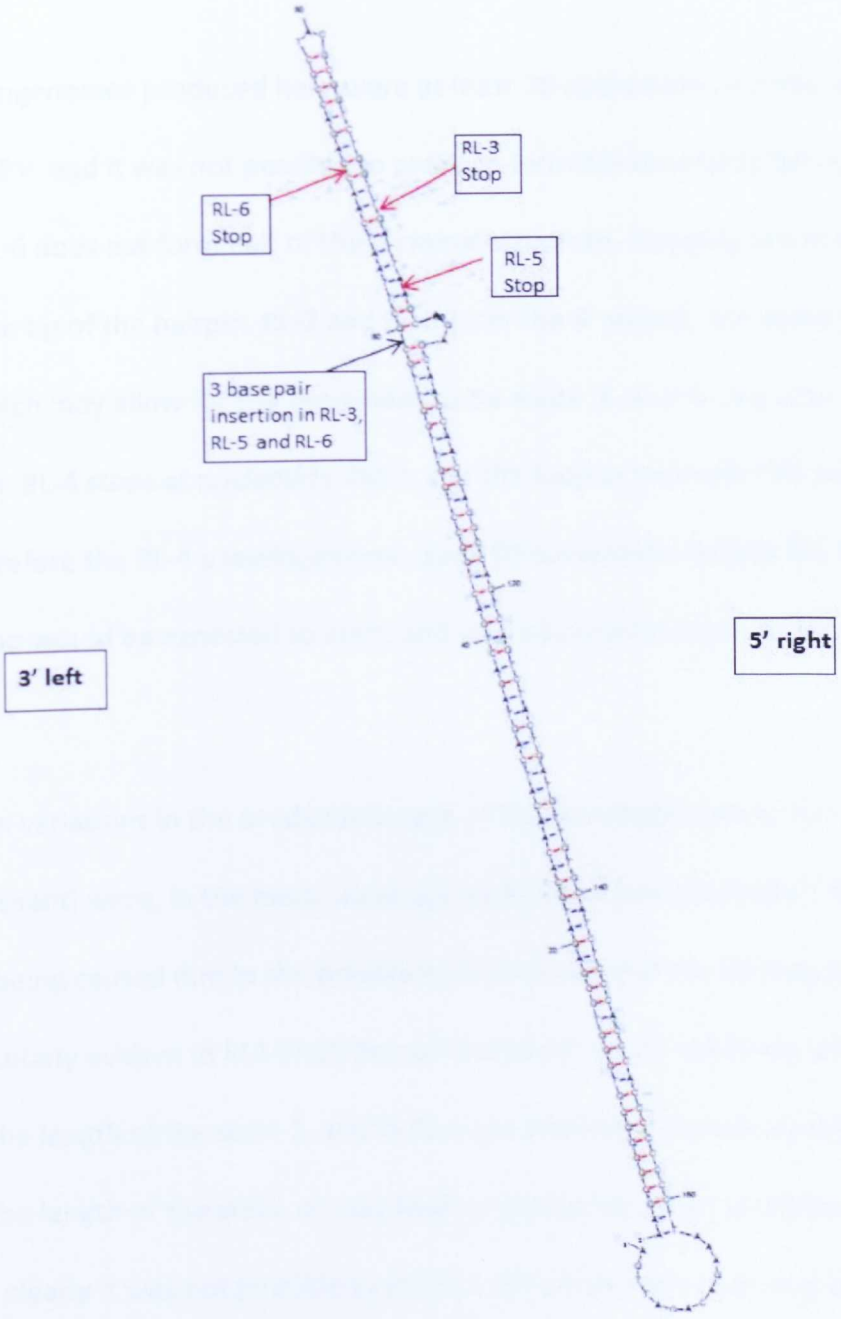

RL-3
Stop
RL-6
Stop
RL-5
Stop
3 base pair
insertion in RL-3,
RL-5 and RL-6
3' left
5' right

Figure 8.10. *Structure of the right end terminus of the chimeric CPV (Horiuchi et al, 1994). The ending positions of the different psedudogenomes are shown, along with any positions where substitutions are. Start positions of the viruses are indicated with a red arrow, and substitutions and insertions are indicated with a black arrow.*

The pseudogenomes produced here were at least 78 nucleotides shorter than chimeric CPV, and it was not possible to produce terminal structures for all of these viruses. RL-6 does not form half of the terminal structure, stopping ten nucleotides short of the tip of the hairpin. RL-3 and RL-5 form the 3' strand, and some of the 5' strand, which may allow for the remainder to be made *in vivo* during viral replication. RL-4 stops at nucleotide 4827, and the loop in chimeric CPV starts at 4901. Therefore the RL-4 pseudogenome stops 80 nucleotides before the terminal hairpin loop would be expected to start, and so is not predicted to form a terminal hairpin.

Apart from variations in the predicted length of the pseudogenomes, the sequences (where present) were, in the main, identical, with only a few insertions / deletions probably being caused due to the presence of homopolymers in the sequence. This was particularly evident in RL3 (insertion of two additional G residues), which may increase the length of the stem 1, and in RL-6 (an additional T residue), which may increase the length of the stem, or may lead to production of an additional bubble. However, clearly it was not possible to check if the other stem had substituted to match the substitutions observed here.

In addition to these potential homopolymer problems, there was also a three base insertion in RL-3, Rl-5 and RL-6 (RL-4 was not of sufficient length) of TCT, which was

not observed in chimeric CPV (figure 8.10). This occupies an area at the small bubble observed in the structure of chimeric CPV and may have an effect in increasing the size of the bubble in the pseudogenomes sequenced here.

With the exceptions of the inserts due to homopolymers and the three base inserts, there were no substitutions within the virus, suggesting again that in general, the termini again were well conserved.

Analysis of repeats within the CPV genomes

The published sequence of CPV-N has been predicted to have a 200 base pair and a 60 base pair repeat at the 5' end of the genome (Reed *et al*, 1988). It was therefore of interest to find out if there were similar repeat sequences in the genomes presented here. Apart from RL6, all the genomes were predicted to contain at least one tandem repeat, with CPV-N containing two. The length of the repeats varied from 19 in chimeric CPV to 62 in CPV-N.

Virus	Repeat length	No repeats	Position
Chimeric CPV	19	2	4533--4571
CPV-N	62	2	4513--4636
	61	3	4701--4892
RL3	60	2	4488--4608
RL4	60	2	4495--4615
RL5	60	2	4509--4629
RL6	None		

Table 8.9. *Analysis of repeats in the genomes of CPV N (M19296, Reed et al, 1988), chimeric CPV (D26079, Horiuchi et al, 1994) and the four pseudogenomes sequenced here. The repeat length, number of times these repeats are found within*

the genome, and the relative position of these repeats within their respective genomes are also indicated.

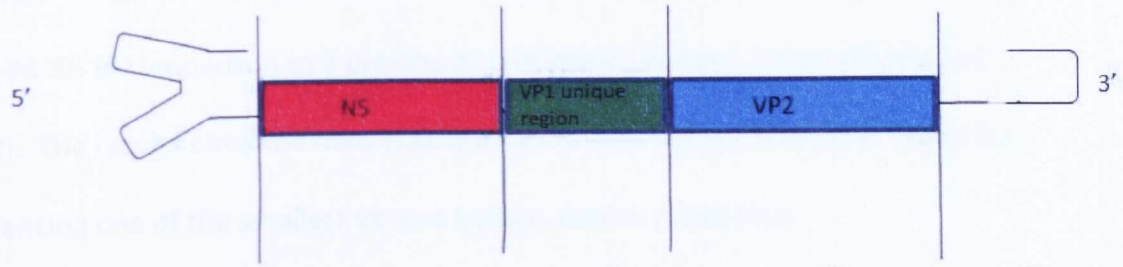
Comparison of the substitutions observed within these and published genomes

As there are few published sequences of CPV full genomes, it was of interest to ascertain the levels of substitution within both the coding regions, and the terminal non-coding regions of the viruses. Therefore sequence from the two previously sequenced genomes (CPV-N: Genbank accession number M19296, (Reed *et al*, 1988) and chimeric CPV (Genbank accession number D26079 (Horiuchi *et al*, 1994), were compared to the four sequences generated here.

Between the six genomes there were only 286 (approximately 5.3%, Figure 8.11) nucleotides different (where data was present). In total, few substitutions were observed in the 5' and within the coding region, with most substitutions being observed after the coding region for the capsid genes at the 3' end.

Prior to the start of the NS gene, only a two base insertion in the chimeric CPV genome, and pseudogenome 4 and 5 was observed, although these may be due to homopolymers (figure 8.9). Only 40 substitutions (14%) were observed within the coding regions. Nineteen substitutions were found within the coding regions for the NS gene, as well as a four base pair insertion which is present in CPV-N and RL-6, which may be due to homopolymers. Of these, nine amino acid substitutions were observed. In addition, nineteen substitutions were observed within the coding region for VP2, which lead to fifteen amino acid susbtitutions. Two nucleotide substitutions, leading to one amino acid substitution were observed between the

NS gene and the VP2 gene, an area which encodes the VP1 unique region. After the termination of the VP2 gene coding region, 246 substitttions (86%) were present (Figure 8.11), although some of these substitutions may be due to the 200 base pair repeat found in CPV-N (Reed *et al*, 1988) but not in the other sequences. Additionally, the presence of 60 base pair repeats (table 8.9) also increases the number of substitutions.



Insertions	2	5	0	0	N/A
DNA mutations	0	19	2	19	246
Coding AA mutations	-	9	1	15	-

Figure 8.11. *A cartoon of the genome, spilt into the three coding regions and the two termini. The number of insertions, DNA substitutions and coding amino acid substitutions found in a comparison of the six genomes (four pseudogenomes produced here, CPV-N and chimeric CPV) are also shown below it.*

Discussion

Traditional sequencing technologies have found it difficult to produce full parvovirus genomes, mainly due to the fact that the termini are very difficult to amplify by PCR (Ishikawa *et al*, 2003). This is likely due to the extensive hydrogen bonding which occurs in the termini allowing for the formation of the hairpins (Astell *et al*, 1979, Bloom *et al*, 1990). This reason, along with parvovirologists use

of single amino acid substitution to type of viruses using the VP2 gene, has meant that there is currently only one full genome obtained from a virus (Reed *et al*, 1988), and another which was produced in a chimeric virus (Horiuchi *et al*, 1994). Here we have used relatively simple DNA extraction procedures both on purified viral DNA and replicative form DNA, followed by Roche 454 sequencing, to determine the sequence of four CPV genomes. The resulting pseudogenomes ranged in length from 4852 – 5014 bases, representing a sequencing coverage from 95.6-98.8% in comparison to a previously published genome, CPV-N (Reed *et al*, 1988). The results obtained here clearly indicate that this technology is useful for sequencing one of the smallest viruses known, canine parvovirus.

As the termini of CPV have rarely been sequenced, it was of interest to analyse these, and the structures which they may form. Using two previously sequenced genomes (CPV-N and chimeric CPV) as well as the four pseudogenomes produced here, allowed potential analysis of twelve termini.

CPV-N (Reed *et al*, 1988) produced a terminal hairpin at the 5' end, which was of 130 bases in length and displayed a predicted structure of a Y shaped terminus. In contrast, it was not predicted to form a hairpin structure at the 3' end, probably due to the presence of a 255 base pair repeat which may affect the folding of the genome.

Chimeric CPV (Horiuchi *et al*, 1994) produced a Y shaped termini at the 5' end similar to that observed in CPV-N, but also formed a large 162 base pair, club shaped termini at the 3' end.

Of the four pseudogenomes produced by 454 sequencing, the termini fell into three categories: 1) those which produced a terminus larger than predicted, 2) those which produced a terminus where part of the structure was missing, but where sufficient remained to prime replication, and 3) those which were deficient in terminal sequence and were not predicted to form a hairpin.

1) Larger stem loops. Of the eight termini analysed from the pseudogenomes, only RL-6 produced a terminus which was six bases longer at the 5' end, than the genomes previously predicted. The sequence coverage of the termini lends support to the extended termini size, with high level coverage of at least three of the six additional nucleotides. In addition, the fact that it is an exact match to the longer arm of the genome, allowing a larger hairpin to form, is further confirmation that the six nucleotide extension is likely to be true. If this larger hairpin is genuine, it may be more stable (Bloom *et al*, 1990), allowing an increased stability of the virus. Further analysis may increase the size of this terminus even further.

2) Shorter stem loops. Five of the termini (3' terminus of RL-3 and RL-5 and the 5' terminus of RL-3 and RL-4) were predicted to produce some of the terminal structure, with sufficient genetic data present to allow for formation of the entire proximal stem, but only sufficient to produce part of the distal stem. These termini are likely to have sufficient genetic data present to allow for priming of the production of the distal strand which can occur *in vivo*.

3) Absent stem loops. Two of the termini (3' end RL-4 and 5' end RL-5) were not predicted to form hairpins, forming only part of the proximal stem. This would not

allow for priming to allow production of the distal strand *in vivo*, and therefore suggests that there is some genetic data missing.

Taken together, it appears that the viruses conserve the structure of the termini, with a Y shaped terminus at the 5' end, and a club shaped terminus at the 3' end. Additionally, the sequence of the virus termini is highly conserved, suggesting that the sequence as well as the structure is important. That said, small substitutions were identified within the stem, and larger substitutions and insertions within the bubble structures. It will be interesting to determine the effect of such substitutions on the replication and pathogenicity of this virus.

Between the six genomes (four produced here, CPV-N and chimeric CPV) there were only 286 (approximately 6%) nucleotides different (where data was present), further suggesting the high level of conservation within parvoviruses. It was of interest that the majority of the substitutions were outside the coding regions, forming a hyper-variable region localised towards the ends of the genome, these are mainly due to the presence of repeats. A two base pair insertion was contained within the 3' end, prior to the start position for the NS gene (nucleotide 270, Reed et al, 1988). By comparison, 246 substitutions (246/286, 86%) were found after the coding region for VP2 ends (residue 4540). Some of the substitutions at the 5' end, may be due to the 200 base pair repeat, which is only present in CPV-N and is likely to be an artefact of the sequencing and genome preparation procedures used for this virus (Reed *et al*, 1988). This suggests that the ends of the virus (i.e. between the hairpin structures and the start or end of the coding region) are more fluid and can substitute relatively freely compared to the coding regions as the former are

not required to form protein, without affecting the virus too much. It also suggests that the viruses can tolerate substitutions within the termini allowing for the formation of 'bubble' structures, some of which are utilised by the virus, such as for the nicking of the DNA by NS1 (Nuesch *et al*, 1995). In the coding region, only 39 substitutions (40/286, 11.2%) were observed within the coding regions. nineteen substitutions were observed in the NS gene, as well as a four base insertion observed in CPV-N and RL-6. Nineteen were observed in the coding region for the VP2 gene with the remaining two found in the VP1 unique region. Of these substitutions, fifteen (15/19; 79%) lead to amino acid substitutions in the VP2 gene, whereas nine (9/19: 47%) caused amino acid substitution in the NS gene. In addition, one of the two substitutions in the VP1 unique region lead to a coding change.

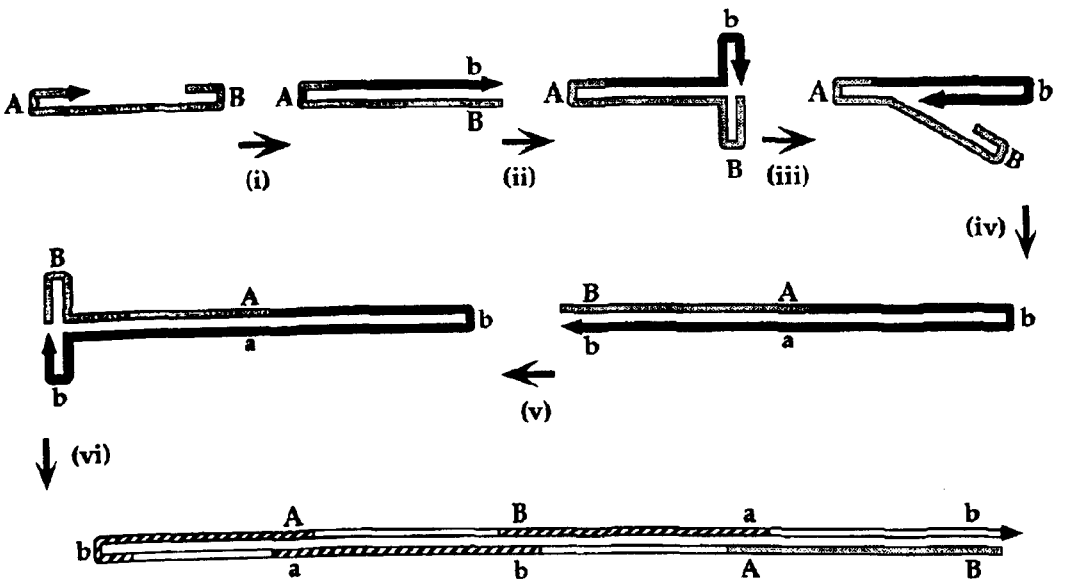


Figure 8.12. *A summary of the replication strategy thought to be used by canine parvovirus. The light grey is the parental strand (indicated by the A and B), and the newly synthesised strand is shown in black (indicated by a and b) with the arrow at the 3' end. Steps (i) to (v) show the virus replicating to form a genome tetramer shown in (vi), in which there are three progeny genomes in addition to the parent strand. As shown in (vi) the genomes overlap on opposite strands and these are then*

excised to form the new genomes which are inserted into the preformed capsids. The hairpins snap together rapidly to form the new genomes. (Cotmore et al, 1995).

In this study, our starting material was DNA purified from two different sources. Three of the four samples were from replicative form dsDNA, a reflection of the need for double stranded template DNA for Roche 454 library preparation. Although this DNA is harder to purify, it also allows for the potential to view some of the genetic intermediates predicted to form following rolling hairpin replication (figure 8.12). In particular, this model of parvoviral replication predicts the formation of concatamers of genomes joined end to end in opposite polarities. Interestingly, when sequencing RF DNA, we found no evidence for such structures in the contigs generated possibly suggesting these parvoviral genomes are replicated by a different strategy not relying on concatamerisation / rolling circle. However, and perhaps more likely, an additional explanation is that such concatamers do indeed exist, but remain unresolved due the complexity of the informatics. In support of this is the lack of intact termini for these three pseudogenomes despite the high sequence depth (≥465x) for the rest of the genome. This is an area that warrants future studies, because an ability to observe these structures would give added insight into the replication of these viruses.

The fourth virus sequence (RL-6) generated here was based on DNA purified from viral particles. This sample arguably gave the best sequence of the four as only two contigs were needed to assemble the pseudogenome, which covered 93.1% of the CPV-N genome (second highest coverage). This result was perhaps surprising because parvoviruses are generally regarded as single stranded viruses (Chapman

et al, 1995), whereas 454 sequencing has an absolute requirement for double stranded DNA template (Meyer *et al*, 2008). Why this sequence worked so well is uncertain but options include some alternate packaging of plus and minus strands by individual virus particles, such that the purified DNA was a mixture of the two that were able to reanneal. The virus is known to package mainly negative strand DNA, but can package a small (around 5%) amount of plus strand DNA (Chapman *et al*, 1995), which may be responsible for the high double stranded DNA concentration. Alternatively, some RF-DNA may have survived the purification process. Whatever the reason, this purification method is simpler than RF-DNA and provides an option to look more closely at the termini of the genome (top left figure 8.12). In addition, it may be possible to generate near-whole genome sequences directly from high-titre clinical samples, removing the need to grow the virus in cell culture, with the associated substitutions that can occur due to viral adaptation to *in vitro* growth such as has been reported for vaccines (Hoare *et al* 1997; O'Brien *et al*, 1986).

Chapter 9

Final discussion and future work

Canine parvovirus (CPV) is a ubiquitous gastrointestinal pathogen of both domestic and wild canids (Carmichael *et al*, 1981) which, despite widespread vaccination, still infects young dogs and occasionally vaccinated dogs (Decaro *et al*, 2009). Additional, recent reports suggest that cats can be infected with CPV and shed virus (Truyen *et al*, 1996; Ikeda *et al*, 2002; Gamoh *et al*, 2003; Mochizuki *et al*, 1996). This is in addition to feline panleukopenia virus (FPL), the common parvovirus of cats (Goto *et al*, 1983; Cave *et al*, 2002; Decaro *et al*, 2008). CPV emerged in 1978 (McCandlish *et al*, 1979; Johnson *et al*, 1979; Osterhaus *et al*, 1980), but the original CPV type 2 has since evolved into three new antigenic types known as 2a, 2b and 2c (Truyen *et al*, 1998; Parrish *et al*, 1988b; Martella *et al*, 2006). The differences between the types relate to an altered reactivity to a panel of monoclonal antibodies but do not lead to different clinical syndromes and do not allow the viruses to group phylogenetically together in all cases (chapter three and six). Indeed few differences (115 nucleotide substitutions leading to 40 amino acid substitutions) within the VP2 gene exist between FPLV (the hypothetical ancestral virus) and CPV (Martyn *et al*, 1990).

Nearly all previous studies on viral diversity and evolution have targeted the most common capsid gene (VP2), to type the viruses, using methods such as real time PCR (RT-PCR), sequencing of small sections of the VP2 gene, monoclonal antibodies

and other methods which allow for rapid virus typing (Decaro *et al*, 2005b; Davies, 2008; De Ybanez *et al*, 1995). Most of these comprise region-specific typing methods, the significance of which are unknown (Yilmaz *et al*, 2005; Costa *et al*, 2005; Kapil *et al*, 2007). In this thesis, we obtained full VP2 gene sequences from a considerable number of viruses which could be interrogated for phylogeny, evolution and viral transmission.

To investigate the evolution of the virus, it was important to develop a relatively sensitive and specific conventional PCR assay (chapter 2). This was produced targeting the VP2 gene, which is known to be the most variable area of the virus, targeted by others in evolution studies (Yilmaz *et al*, 2005; Costa *et al*, 2005; Kapil *et al*, 2007 and many others). Although the assay developed here was sensitive particularly compared to haemagglutination (PCR detecting 117 more positives than HA), it would be beneficial to assess the assay with known titres of virus, or known number of DNA copies to ascertain how low a titre can be detected by the assay.

In addition to using a sensitive assay which gave full VP sequence, we used strategic sampling of three different dog populations; healthy dogs from a kennel, a case control study of animals which were showing signs of gastroenteritis (which in the main, did not require hospital treatment) (chapter 2), and a case defined study for animals hospitalised with diarrhoea (chapter 3). Generally, previous studies have used viruses from a single outbreak or from a single hospital, and / or are limited to using only partial VP2 gene sequence analysis or typing by key amino acid substitutions where it is difficult to draw meaningful conclusions about virus

evolution, spread and the diversity of viruses within a country (Costa *et al*, 2005; Davies 2008; Decaro *et al*, 2005; de Ybanez *et al*, 1995; Kapil *et al*, 2007; Yilmaz *et al*, 2005). Taken together, the strategic sampling, the sensitive assay, and the in depth sequencing and analysis has lead to some interesting insights, furthering our knowledge of parvovirus infections of cats and dogs, both within the UK, and worldwide.

We confirm previous studies that CPV remains a severe gastrointestinal virus in puppies and unvaccinated dogs (Yesilbag *et al,* 2007; Hammond *et al*, 1983; Nandi *et al*, 2010; Hirasawa *et al*, 1996; Castro *et al,* 2007). However, the advice is simple, and has remained so since the virus has emerged;- 'If you have a pet dog (or for that matter, a pet cat), then get it vaccinated, as it will almost certainly prevent it from getting parvovirus!'. When we analysed viruses isolated from hospitalised animals (chapter 3), with signs indicative of CPV, a high prevalence (58%) was found.

Within chapter 3, where we sequenced 150 viruses, 50 different DNA sequence types were identified, and apart from one case, all appeared unique to the UK. Phylogenetic analysis provided clear evidence for spatial clustering at the international level, and for the first time also at the national level, with the geographical range of some sequence types appearing to be highly restricted within the UK. Evolution of the VP2 gene in this dataset was associated with a lack of positive selection. In addition, the majority of predicted amino acid sequences were identical to those found elsewhere in the world, suggesting CPV VP2 has evolved a highly fit conformation. Based on typing systems using key amino acid

substitutions, 43% of viruses were CPV 2a, 57% CPV 2b, with no type 2 or 2c found. However phylogenetic analysis suggested complex antigenic evolution of this virus, with both type 2a and 2b viruses appearing polyphyletic, such that typing based on specific amino acid substitutions may not reflect the true epidemiology of this virus.

This led us to further support Hoelzer *et al*'s (2008) suggestion of decreased movement of the new CPV types rather than the rapid worldwide spread which was found with the original CPV-2 spread in naive dogs in the late 1970s. We show here, that viruses do cluster by country (where sufficient sample numbers are available) and suggest that in future, viruses may diverge differently within each country, and it is possible that a country specific vaccine may be required in the future to protect against its own specific strain in that country. We further these studies, and suggest that in addition to clustering by country, CPV also clusters within countries. As we mapped two substitutions within the VP2 gene which are only found within the UK (Val-139-Iso and Arg-274-Lys), this clustering may mean that in the future it will be possible to pinpoint exactly where an animal was infected with the virus.

Despite a high prevalence of CPV in hospitalised animals (chapter 3 and 4), low levels of variation were observed in the viruses. There was large variation with the 65 type 2a and 85 2b viruses, with neither type being monophyletic. This also suggests that the virus can also be imported into the country, bringing in new types which supplement the viruses already within the UK.

The majority of studies on CPV analysed to date were from clinical cases of dogs with severe diarrhoea indicative of CPV infection, or historically from myocarditis cases. However, studies involving clinically normal animals or mildly ill animals are

extremely rare. It is possible that these asymptomatic animals may potentially carry the virus as has been observed with cats infected with CPV (Chapter 7) and FPLV (Csiza *et al*, 1971). In order to ascertain if there was a carrier state of CPV in dogs, (i.e. healthy dogs carrying CPV), we analysed healthy animals in a kennel, non-hospitalised animals in a case control study, and hospitalised animals (discussed above). In chapter 4, clinically healthy animals showed no signs of CPV shedding in faeces, whereas the prevalence of CPV increases with the severity of disease, to a maximum (58%) in hospitalised animals. This suggests that there is not a carrier state of CPV in dogs. As real time PCR has been shown to be more sensitive than conventional PCR used here (Desario *et al*, 2005), the high prevalence reported here may still represent an under estimate.

Unlike most other studies of CPV, we have analysed the NS-1 gene as well as the VP2 gene of the same viruses. Although the evolution of CPV is complex, it appears from analysis of the NS-1 genes that recombination occurs within parvoviruses, with the same NS-1 gene being observed in a 2a and 2b virus (chapter 6). Unfortunately, the PCR products derived for the VP2 gene and the NS-1 gene did not overlap, so it was not possible to technically confirm this, but this would be an additional piece of work to do in the future. This high level of recombination meant it was not possible to type CPV by the NS-1 gene, as is normally done with the VP2 gene. Such has been the low level of interest in the sequencing of the NS-1 gene that only 41 (11 full CPV NS-1, 27 from full genomes (17 CPV, 10 FPLV), 3 FPLV NS-1) were available on genbank, compared to 740 (384 full, 356 partial) VP2 genes available. For this reason it was not possible to carry out the same level of analysis

for the NS-1 genes as was done for the VP2 genes. Therefore it was not possible to assign specific substitutions to CPV NS-1 genes from the UK, but it was possible to assign four substitutions (247 H, 248 T, 545 Q and 595 H) which differentiated CPV NS-1 genes from those of FPL viruses. Further studies should concentrate on increasing the knowledge of the CPV NS-1 genes to ascertain the role which this gene plays in CPV infection, and how it influences the clinical outcome of the infection.

In chapter 5, analysis of the NS-1 genes showed that many of the sequences predicted to form regulatory elements in other parvoviruses (mainly in minute virus of mice, MVM) were also conserved within UK CPV isolates. This suggests that the evolution of the NS-1 gene will be more restricted to conserve these important motifs than the VP2 gene. Further work is required to investigate the significance of variation, the residues which are phosphorylated, and residues known to offer significant functions in other viruses such as MVM, such as ATPase and helicase.

Recent reports have shown that cats can become infected with CPV (Ikeda *et al*, 2002; Mochizuki *et al*, 1996; Gamoh *et al*, 2003) and it was of interest to try and investigate the potential risk which cats pose to dogs. To investigate prevalence and shedding of CPV by clinically healthy cats, a longitudinal study was undertaken, using both cat and dog faecal samples at the same high throughput rescue shelter. It was found that approximately 33% of clinically healthy cats carried CPV, and shed the virus for long periods, up to six weeks in some cases. This is comparison to none detected in asymptomatic dogs in our studies, and a maximum of 22 days shedding reported for experimental dogs (Carmen *et al*, 1980). Analysis of the VP2 gene of

these viruses indicated that 55% of these viruses are identical to those from clinically ill dogs in chapter 3, suggesting that clinically healthy cats can be shedding potentially deadly CPV silently, allowing for infection of dogs. This is further substantiated by the fact that cats carry virus where the specific substitutions discussed previously are also found, increasing this potential for cross species transmission. Our results dispute the findings of Battilani *et al* (2011) where quasispecies of viruses were found in an infected cat. Due to the high level and relatively long shedding period, it is of great interest to analyse further cats to ascertain the risk they may pose. It will also be beneficial to increase levels of vaccination from the current estimated 30% (Gaskell *et al*, 2002) as the cross protection from FPLV vaccines may help prevent CPV infection and shedding (Chalmers *et al*, 1999). It appears from this study that cats may be playing a significant role in the current epidemiology of CPV infection, as CPV now appears to be the most common parvovirus infection in cats, especially when coupled with the rare diagnosis of FPL in cats in diagnostic laboratories (author's unpublished observations).

Of particular interest was that the shelter cats were vaccinated in the shelter with a trivalent cat vaccine can still become infected and shed virus, suggesting it may be beneficial to investigate the potential to include a CPV component in current cat vaccines. Preliminary results suggested that some of the CPV isolates from cats were not neutralised by FPLV sera (chapter 7). Future work should focus on the ability of CPV to circumvent FPLV vaccine induced immunity, in order to prevent viral shedding and possible disease. Unfortunately, it was not possible, due to

ethical restraints, to do more invasive testing of the cats, including blood sampling for antibodies to CPV and FPLV to ascertain the effect CPV is having on the feline immune system and also to do immunofluorescence testing to ascertain tissue distribution of the virus within the CPV-infected cats. Further studies are required to investigate the role and importance of cross species transmission, and also the protection offered by FPLV vaccines to protect cats from CPV infection, as has been carried out by Chalmers *et al* (1999).

Although several studies have shown that clinically affected cats may be infected with CPV (Ikeda *et al*, 2002; Mochizuki *et al*, 1996; Gamoh *et al*, 2003), it would be of interest to obtain samples of cat faeces from households, from which a dog presents with clinical CPV, to ascertain whether cats may be subclinically infected with the virus being shed by the dog. Additionally, some cats within the shelter (19 %) despite being exposed to high levels of virus, remained negative, suggesting a potential resistance to infection, likely due to previous vaccination with FPLV vaccines, or previous FPLV or CPV exposure. This could be further explored by blood sampling animals, which can be used to ascertain antibody levels, and the major histocompatibility complex (MHC) type of the cats. Interestingly, the resistance to infection appears to occur in cats of varying ages, further suggesting that immunity or MHC type may be influencing infection. It may also be that cats which are resistant to infection have a slightly different transferrin receptor (the receptor for CPV entry (Palermo *et al*, 2003) which may be alternatively glycosylated to prevent virus infection. It will be of great interest to ascertain the differences within

infections of FPLV and CPV infections in cats, such as the receptors used, the difference in canine and feline receptors and the infection process.

The invention of new technologies, such as "next generation" sequencing has allowed us, and others, to perform more in depth analysis of full genomes of organisms at an ever decreasing cost. We used Roche 454 sequencing to analyse four CPV genomes, with coverage of over 90% compared to previously sequenced viruses. Additionally, it appears that the previously un-analysed termini of the viruses are relatively stable, and well conserved, with no defined end point to the genome, as evidenced by the addition of six nucleotides to the 5' end of the genome. As 454 technology allows for good, high quality, full genome sequencing of CPV, it can be used in further studies to analyse the termini of viruses, and evaluate evolution using these full genomes. However, for this to occur, a greater understanding of the viral replication strategy, specifically that used by CPV is required.

As CPV emerged relatively recently, it is one of the few organisms for which we are able to analyse a true evolutionary history, from the first recorded cases to the present cases, to ascertain the evolution of this virus. In a study of 252 historic viruses, we showed that variation and divergence of the virus from its FPLV ancestor increases over time, whilst still showing a high level of conservation, despite selection pressures from the immune system.

The main aim of this thesis was to analyse CPV evolution, and our studies indicated that the mechanisms by which CPV evolves appear to be complex and rapid. There also appears to be purifying selection of some residues of VP2, evidence that the

immune system plays a role in the evolution of the viruses, as well as other more random substitutions. Unfortunately the significance of these substitutions to either the structure or the immunogenicity of the virus is unknown, but they can clearly still cause disease as they were isolated from clinically ill animals. As a wealth of information now exists regarding differences and substitutions within CPV VP2 genes, it is important to begin to assess the significance of such substitutions, for immunogenicity, structure and ability to be neutralised by vaccines. This can be done using epitope mapping experiments with peptide libraries and polyclonal antisera from both dogs and cats infected with CPV to analyse if there is a difference in feline and canine parvovirus linear epitopes. However many epitopes within parvoviruses are thought to be conformational (Kauffmann *et al*, 2007; Corcoran *et al*, 2004) Unfortunately, due to ethical and home office licensing constraints, it was not possible to investigate this within this thesis, as experimental infections are likely required for such samples to be obtained.

As we had significant information on viruses isolated within one country over a 30 year period (chapter 6), we were able to calculate an evolution rate of 6.6×10^{-4} nucleotide substitutions per site per year (95% confidence 4.8-8.4). The calculated evolution rate was higher than previously reported, but this may be due to the inclusion of FPLV in the calculations. Additionally, the historic ancestor was calculated to be around 1973, which fits with the emergence of the virus in 1978 (approximately 32 years ago). As the virus is thought to have emerged first in Europe, (Koptopoulos *et al*, 1986), we may be predicting an earlier ancestor than

that reported in studies from the USA (Shackelton *et al*, 2005) or Brazil (Pereira *et al*, 2007). In addition, Chapter 6 also suggests multiple viral incursions into the UK, which may be due to animals entering the UK from many different countries. The entry of animals with disease seems unlikely as six months quarantine was required before the animal was allowed to enter the country prior to the introduction of the pets travel scheme in 2001. This suggests that virus introduction on fomites, such as peoples' shoes is common.

The specific viruses reported within countries, and also shown here within cities (chapter 3) raises issues for sampling regimes used in other studies where only samples from a single, or small number of locations, or from a single outbreak are used to predict a virus type circulating around that country. Therefore, it is important that more structured sampling across an entire country is used to provide an accurate picture of the viruses circulating, to prevent a biased picture of virus types and diversity being reported, which may not be truly representative of the disease epidemiology.

Current modified live vaccines are reported not to be shed in faeces and so it is unlikely that these vaccines are influencing the evolution of CPV. However, vaccine virus and field virus shedding has been confirmed using techniques such as haemagglutination, and virus isolation which are known to be less sensitive that newer methods such as PCR (Desario *et al*, 2005). Consequently this is an area which would be relatively easy to test using PCR, and should be addressed to confirm that virus is not shed in faeces post vaccination. Our studies suggests that CPV is maintained within the population, not through evolution to evade current

high efficacy and high duration of immunity modified live CPV vaccines which provide sterile immunity, but through an unwillingness of some members of the public to correctly vaccinate their dogs, suggesting that the message of the importance of the CPV vaccination procedure in dogs is not reaching all dog owners.

The full gene (VP2 or NS-1), or in some cases, the full genomes, generated using the new sequencing technology which were used and analysed throughout this thesis rather than the typing of viruses by real time PCR (Decaro *et al*, 2005b, 2006, 2006b, 2006c) or by sequencing small sections of the gene (Decaro *et al*, 2005; Davies, 2008; De Ybanez *et al*, 1995) such has been used in previous studies, allows us to generate high quality information with significant strength in the conclusions drawn. Within this thesis, and following discussions with parvovirologists, pharmaceutical companies and veterinarians questions are beginning to be raised about the significance and values of the current typing system used for CPV. Information generated within this thesis, including the polyphyletic nature of the viruses discussed in chapter 3 and 5, the lack of a distinct clinical phenotype associated with each type, and the recombination observed within the NS-1 gene suggest that although the typing system represents an altered monoclonal antibody panel reactivity, it is not supported clinically, or phylogenetically. There are also sufficient differences phylogenetically, as well as the host differences between the original CPV type 2 and the newer variants to suggest these viruses may be different. However types 2a and 2b are not monophyletic, show variation within each type, cause similar symptoms in dogs, and are very similar, as exemplified by

the single amino acid substitution (Asp/ Asn- 426-Glu) which identifies type 2c (Decaro *et al*, 2005).

Therefore it was of interest to try to suggest a new, more accurate typing system (figure 9.1). This new typing system suggests that feline panleukopenia virus (FPLV), mink enteritis virus (MEV), raccoon parvovirus (RPV) and blue fox parvovirus (BFPV) which are all very similar viruses and groups them together to be carnivora parvovirus one (CPV-1). CPV 2 which no longer circulates in the field (Parrish *et al*, 1988b; Truyen *et al*, 1998), but is still observed in some modified live virus vaccines (Pratelli *et al*, 2001; Decaro *et al*, 2009), is now grouped into a new group, carnivora parvovirus two. Carnivore parvovirus-3 now contains the new variants of canine parvovirus, which includes viruses isolated from cats and dogs, removing the taxonomic problems with canine parvovirus of feline origin, which has received some impractical names, including feline- canine parvovirus or CPV of feline origin. Any new variants of CPV which may emerge should be placed into carnivora parvovirus three group, unless there is a significant difference in the virus, i.e. its genome is significantly longer or shorter due to insertions or deletions, the proteins of the virus are altered due to reading frame shifts, it begins to infect and be transmitted amongst other non-carnivore hosts, such as humans, or it no longer reacts antigenically with the CPV antisera produced by vaccination. This new typing system appears better from a veterinary and biological point of view, removing the need for typing and often expensive experimental testing by vaccine companies when new types appear.

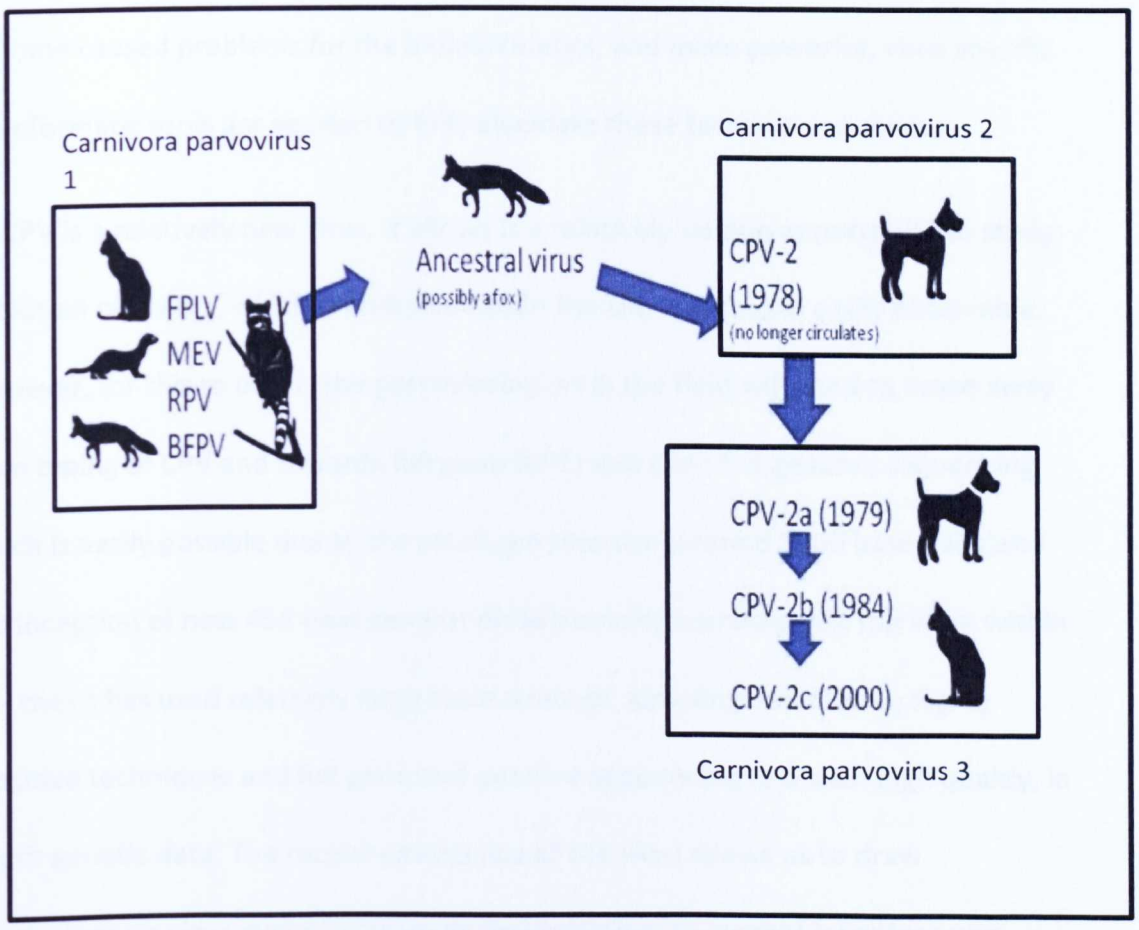


Figure 9.1.*Proposed new classification system for parvoviruses which may help to try and alleviate some of the problems with the current system raised in this thesis. Feline panleukopenia (FPLV), mink enteritis virus (MEV), raccoon parvovirus (RPV) and blue fox parvovirus (BFPV) are all grouped together into carnivore parvovirus group one, the original CPV-2 is grouped into carnivore parvovirus group two (and is now not found in field infections) and the new variants of CPV (2a, 2b and 2c) are grouped into carnivore parvovirus group 3. Reasons for a new carnivora parvovirus group 4 to be created are discussed above.*

As discussed previously, this thesis has used relatively large data sets and gene sequences of around 2000 base pairs to produce some high quality data with some complex analysis. Problems were encountered during the analysis stage, mainly due to lack of computer processing capacity. Therefore if we are to undertake further more complex studies in the future then it will be of great value to obtain a more powerful computer, rather than using online clusters, such as the one at the

Scottish Crop Association. Additionally, the complex nature of the termini of the genome caused problems for the bioinformatics, and more powerful, virus specific bioinformatic tools are needed to fully elucidate these terminal sequences.

As CPV is a relatively new virus, it allows is a relatively unique opportunity to study evolution of a virus, which is endemic within the UK, and thus is easily obtainable. However, for this to occur, the parvovirologists in the field will need to move away from typing of CPV and towards full gene (VP2) and even full genome sequencing, which is easily possible due to the small genome size (around 5000 base pairs)and the inception of new 454 next generation sequencing technologies.The work within this thesis has used relatively large scale strategic sampling techniques, highly sensitive techniques and full gene and genome sequencing to obtain high quality, in depth genetic data. The recent emergence of the virus allows us to draw conclusions on virus evolution from its first infection, to current infections and beyond, and apply these results to the evolution of pathogens and organisms in general. It is also important to remain vigilant, with continued pharmacovigilance.

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