RELEVANCE OF THE VARIABILITY OF THE FELINE IMMUNODEFICIENCY VIRUS IN REGARD TO PATHOGENICITY AND VACCINATION IN NEW ZEALAND

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Abstract

Cats infected with the feline immunodeficiency virus (FIV) show a range of clinical signs. Given the variability of the FIV genome, it is possible that there is variation in certain biological characteristics of FIV, such as pathogenicity. This may also be relevant to vaccination against FIV, as an effective vaccine would have to result in the generation of T cells that recognise a range of different variants in the field. The Fel-O-Vax® FIV vaccine has been available to veterinarians in New Zealand (NZ) for the past 12 years. Despite this, there is a paucity of studies investigating the cross-reactivity of the vaccine-induced immune response against different variants of FIV, and no studies investigating the efficacy of the vaccine in NZ.

The overall aim of the research in this thesis was to determine the relevance of the variability of FIV, in regard to pathogenicity and vaccination in NZ. Firstly, 2 separate assays were designed to assess variation in the ability of different isolates of FIV to induce apoptosis or inhibit mitogen-induced proliferation in lymphoid cells *in vitro*. Results showed that variation in FIV-apoptosis did occur, supporting the argument that FIV variants may also differ in pathogenicity. Secondly, the cross-reactivity of the vaccine-induced immune response was assessed *in vitro* and *in vivo*, by measuring antigen-specific cellular activation and a delayed type hypersensitivity (DTH) response in vaccinated cats following inoculation with NZ field isolates of FIV. Results showed that the response was at least partially cross-reactive, however quantitative differences were detected in the response to each isolate of FIV tested. Finally, efficacy of the Fel-O-Vax® FIV vaccine under NZ conditions was investigated by comparing the prevalence of FIV in vaccinated and unvaccinated cats in the field. Results showed that there was no effect of vaccination on FIV prevalence, suggesting poor efficacy of the Fel-O-Vax® FIV vaccine in NZ.

Results described in this thesis support the argument that there is variation among FIV in NZ, and that this may affect pathogenicity and vaccine efficacy in this country. The evidence presented did not support use of the Fel-O-Vax® FIV vaccine in NZ.

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List of abbreviations

2-ME	2-Mercaptoethanol
7AAD	7 amino actinomycin D
AB	Annexin binding buffer
AICD	Activation induced cell death
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
CD	Cluster of differentiation
cDNA	Complementary DNA
CFGS	Chronic feline gingivostomatitis syndrome
ConA	Concanavalin A
CPM	Counts per minute
CPT	Cell preparation tube
Cq	Quantification cycle
CRD1	First cysteine rich domain of the CD134 molecule
CRD2	Second cysteine rich domain of the CD134 molecule
CRFK	Crandell-Reese Feline Kidney
CTLA-4	Cytotoxic T lymphocyte antigen 4 (CD152)
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
env	Envelope
FBS	Foetal bovine serum
FHV-1	Feline herpesvirus 1
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
FMO	Fluorescence minus one
FSC	Forward scatter
gag	Group-specific antigen
GM	Growth medium
gp100	Surface unit of the FIV envelope glycoprotein
gp35	Transmembrane unit of the FIV envelope glycoprotein
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
MEM	Minimum essential medium
MFI	Mean fluorescence intensity (geometric)
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	Messenger RNA
MUAEC	Massey university animal ethics committee
MUFNU	Massey university feline nutrition unit
MUVTH	Massey university veterinary teaching hopsital
NaCl	Sodium chloride

NEAA	Non-essential amino acids
NK	Natural killer
NZ	New Zealand
OD	Opitcal density
ORF	Open reading frame
p24	Capsid peptide of the FIV gag protein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline (pH 7.2)
PCR	Polymerase chain reaction
PD-1	Programmed death receptor 1
PF	Preventable fraction
PI	Post inoculation
pol	Polymerase
PS	Phosphatidylserine
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute (medium)
rRNA	Ribosomal RNA
RT	Reverse transcriptase
SI	Stimulation index
SSC	Side scatter
SU	Surface unit of the FIV envelope glycoprotein (gp100)
$TCID_{50}$	Median tissue culture infectious dose
TH1/2	T helper 1/2 cells
TM	Transmembrane unit of the FIV envelope glycoprotein (gp35)
TNF	Tumour necrosis factor
UV	Ultraviolet
V1-V9	Hypervariable regions (1-9) of the FIV envelope gene
vif	Viral infectivity factor
VNA	Virus neutralising antibodies

CHAPTER ONE

Review of the literature

1.1. Introduction

The feline immunodeficiency virus (FIV) is a group of genetically related retroviruses that have adapted over time to a range of host species. In addition to domestic cats, at least 19 free-ranging felids as well as one hyena species are confirmed hosts of the virus (Pecon-Slattery *et al.*, 2008b, Troyer *et al.*, 2005). Global seroprevalence studies and phylogenetic analyses of FIV have indicated a likely point of origin in Africa, along with other lentiviruses infecting primates and ungulates (Pecon-Slattery *et al.*, 2008b). The vast genetic diversity of FIV suggests a prolonged period of evolution has occurred, and it is estimated that the virus has been present in felids since the late Pliocene period (from 5.33 - 2.58 million years ago) (Pecon-Slattery *et al.*, 2008b).

FIV is highly specific for its host species, and even within the Felidae family, cross-species transmission is extremely rare (Troyer *et al.*, 2008). FIV has evolved over time to adapt to each host, leading to extensive genetic differences between species (Pecon-Slattery *et al.*, 2008b). Furthermore, phylogenetic analysis of FIV has revealed diversity of the viral genome within particular host species and even within individual hosts, suggesting a continual pattern of evolution of the virus (Pecon-Slattery *et al.*, 2008a, Beczkowski *et al.*, 2015b, Sodora *et al.*, 1994).

Despite being a commonly diagnosed infection, little is understood about the pathogenicity of FIV and the significance of genomic sequence variation. Many factors contribute to the variability of FIV-associated disease, including host, environmental and viral factors. Key determinants of FIV pathogenicity are yet to be identified, but there is evidence to suggest that genetic variation may affect the pathogenicity of individual variants.

The genetic diversity of FIV presents a challenge to vaccine development (Hosie *et al.*, 1995, Elyar *et al.*, 1997). An effective vaccine must induce an immune response that is cross-protective against a range of different FIV variants in the field. A vaccine against FIV was commercially released in 2002 (Fort Dodge Animal Health). Although some studies report good efficacy of the vaccine against laboratory strains of FIV, the vaccine is largely untested in the

field. It is important that veterinarians understand how genomic sequence variation of FIV may affect the efficacy of this vaccine, in order to understand the issues concerning its use.

The aim of this chapter was to review the literature on FIV to gain a thorough understanding of the virus, disease and the host's response to the virus. In addition, it aimed to summarise results from published studies investigating the efficacy of the Fel-O-Vax® FIV vaccine. This information was critically examined and synthesised to highlight pertinent questions surrounding use of the Fel-O-Vax® FIV vaccine in order to generate the hypotheses that are subsequently tested in the experimental chapters of this thesis.

The nomenclature describing FIV can be confusing. The abbreviation "FIV" is used throughout this thesis to refer to the group of FIV that infect domestic cats. Other closely related viruses within the FIV species that infect other feline hosts are named accordingly in the literature (e.g. FIVaju, which infect cheetahs, and FIVppa, which infect leopards) (Troyer *et al.*, 2008). Within the group FIV that infect domestic cats, there is much variation in the genomic sequences of individual viruses. These individual viruses will be referred to as variants. FIV variants are grouped together into subtypes according to phylogenetic relationships, and these are designated subtypes A-F. The term 'clade' is synonymous with 'subtype' in the context of the FIV literature. The terms isolate and strain are used according to the definitions presented in **Table 1-1**.

Table 1-1 Summary of viral nomenclature.
The definition of an FIV subtype refers to the original description of FIV subtypes by Sodora et al, 1994 (Sodora *et al.*, 1994). The differentiation of the terms variant, strain, isolate and quasispecies are discussed by Kuhn and Jahrling 2010 (Kuhn and Jahrling, 2010).

Subtype	A group of variants of FIV that cluster together on a phylogenetic tree (generally <15% pairwise sequence divergence).
Clade	A group of viruses with a common ancestor, clustering together on a phylogenetic tree.
Variant	A term used to differentiate genetically distinct viruses within the FIV species.
Quasispecies	A mixture of related FIV variants existing within an individual animal. These develop over time due to ongoing evolution of the virus within a host.
Strain	A variant of FIV which is well-characterised and recognisable because it possesses a unique, stable phenotype.
Isolate	A virus isolated in cell culture from an individual infected animal at a particular time point.

1.2. Genetic diversity of FIV

1.2.1. The FIV genome

The FIV genome is approximately 9.5 kilobases (kb) in length and contains the major genes that are characteristic of all retroviruses (Talbott *et al.*, 1989). The polymerase (*pol*) gene encodes enzymes necessary for viral replication and assembly (i.e. reverse transcriptase (RT), protease, integrase and deoxyuridine pyrophosphatase) (Elder and Phillips, 1993). The group-specific antigen (*gag*) gene encodes the structural and core components, including the matrix, capsid (p24), nucleocapsid and 'late' domain proteins (O'Connor *et al.*, 1989, Luttge and Freed, 2009). The envelope (*env*) gene encodes the envelope glycoprotein, expressed on the surface of infected cells and virions, and it consists of two subunits, the surface unit (SU, gp100) and the transmembrane unit (TM, gp35)[1] (Pancino *et al.*, 1993b, Talbott *et al.*, 1989). At least three other accessory genes (*vif*, *ORF A* and *rev)* play a role in viral gene expression, infectivity and viral replication (Miyazawa *et al.*, 1994).

FIV is a diploid RNA virus that utilises the enzyme, Reverse Transcriptase (RT), to make a copy of its genome, which is then integrated into the host cell. The error-prone nature of this process, the occurrence of recombination events, and the selection pressures exerted by the host all contribute to the generation of mutations within the FIV genome, thus explaining the vast genetic diversity of this virus (Steinhauer and Holland, 1987). Random point mutations persist most frequently within the *env* gene, where nine hypervariable regions have been identified (V1-V9) (Pancino *et al.*, 1993b). The *gag* and *pol* genes are more conserved than the *env* gene, although some variation within these regions has also been documented (Greene *et al.*, 1993, Hayward and Rodrigo, 2008). Unequal variability across the FIV genome is a reflection of host and environmental selection pressure, as well as the functionality of the corresponding proteins encoded by each gene. The envelope glycoprotein is expressed on the surface of the virion and is involved in cell entry and fusion to the host cell membrane. Mutations within the *env* gene offering

[1] The HIV counterparts are labelled gp120 and gp41 due to their larger size compared to FIV. There is confusion in the literature regarding these terms, and the FIV glycoproteins are often also referred to as gp120 and gp41. In this thesis, they will be referred to as gp100 (surface unit) and gp35 (transmembrane unit).

a selection advantage may include those enhancing viral entry and fusion to host cells, and those altering the structure of exposed epitopes in order to evade the host's adaptive immune response (Pancino *et al.*, 1993a, Pancino *et al.*, 1995). In contrast, there is less opportunity within the *gag* and *pol* gene for mutations to result in a selection advantage. Furthermore, mutations affecting the structural integrity and function of these essential proteins are more likely to be detrimental to viral replication and survival.

In addition to random point mutations, diversity of the FIV genome is further increased by frequent recombination events (Hayward and Rodrigo, 2008, Reggeti and Bienzle, 2004). FIV is a diploid RNA virus, meaning that each virion contains two identical copies of the single stranded RNA genome. For recombination to occur, a cell must be co-infected with two different variants, and hybrid virions must be produced containing one strand of RNA from each virus (Kann *et al.*, 2007a, Sodora *et al.*, 1994, Hu and Temin, 1990). During the process of reverse transcription, the enzyme occasionally switches from one RNA molecule to the other, producing a mosaic provirus that contains genetic information from both strands of RNA. Recombination events are most commonly evident within the *env* gene, but recombination within the *gag* gene has also been reported (Bachmann *et al.*, 1997, Hayward and Rodrigo, 2008, Reggeti and Bienzle, 2004). Recombinant events occurring between genes are less common, but FIV genomes containing *env* and *pol* genes from different subtypes have been identified (Hayward and Rodrigo, 2008).

1.2.2. Subtype classification

FIV is classified into different subtypes, most commonly based on the nucleotide sequence of the *env* gene (**Figure 1-1**) (Sodora *et al.*, 1994, Kakinuma *et al.*, 1995, Pecoraro *et al.*, 1996, Weaver, 2010). Sodora et al (1994) described three discrete clusters of FIV based on the comparison of the variable regions of the *env* gene of 34 viruses (1994). The sequences differed by 17.8 - 26.2% between clusters, with less than 15% difference within clusters. These clusters were thus designated subtypes A, B and C. The accumulation of sequence data over the years and the constant evolution of FIV have resulted in three additional subtypes being defined (subtypes D, E and F) (Kakinuma *et al.*, 1995, Pecoraro *et al.*, 1996, Weaver, 2010). More recent studies

have also identified variants that do not cluster with previously recognised subtypes (designated U for 'unknown') (Weaver *et al.*, 2004, Weaver, 2010, Hayward *et al.*, 2007, Duarte and Tavares, 2006).

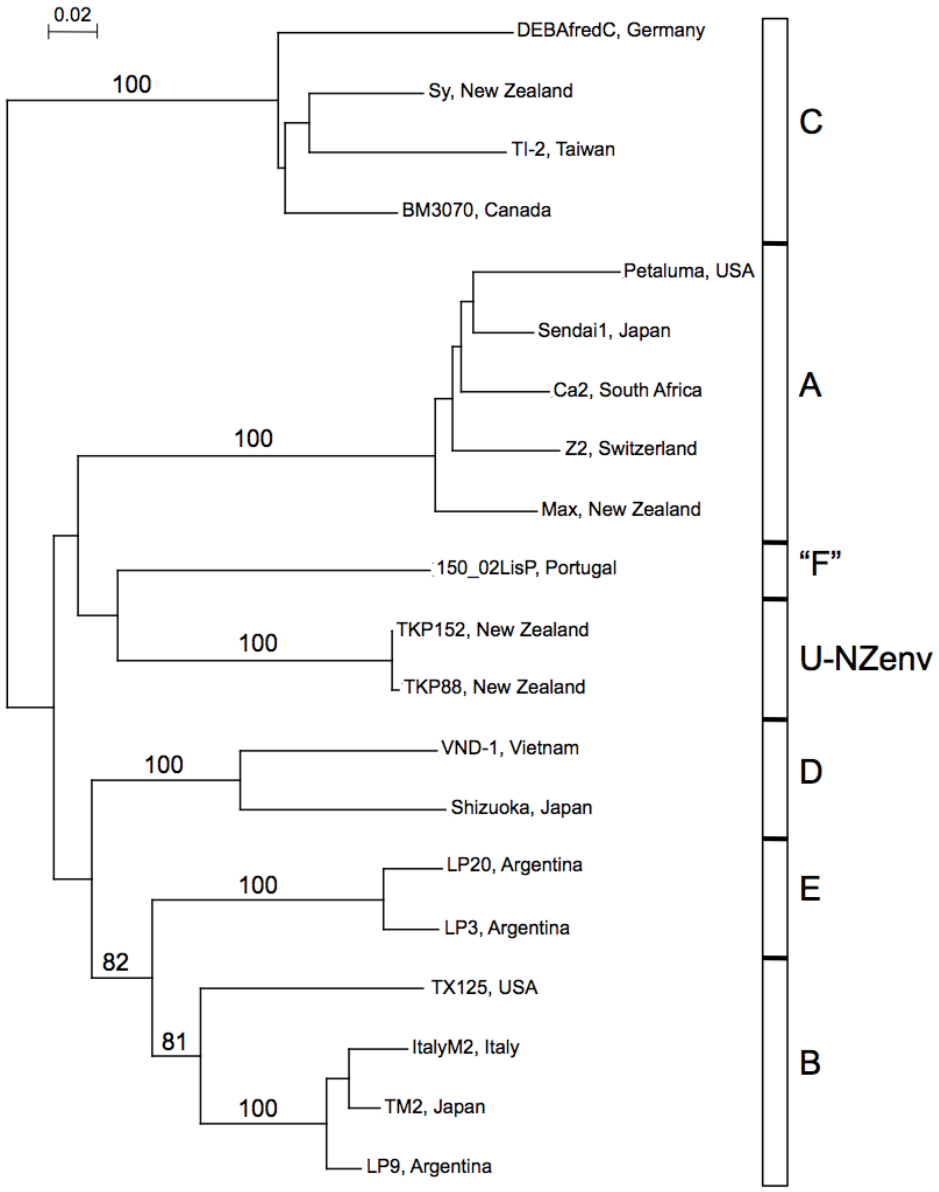


Figure 1-1 Phylogenetic classification of FIV subtypes.
Neighbour joining phylogenetic tree of FIV *env* V3-V5 sequences. Tree was constructed using a general time reversible model incorporating invariant sites (0.4086) and a gamma distribution of mutation rates (shape = 1.6859), as determined by Modeltest3.7 (Posada and Crandall, 1998). Numbers shown are bootstrap values, based on 1000 iterations. Subtypes A to E, "F" (Duarte and Tavares, 2006), and U-NZenv (Hayward and Rodrigo, 2008, Hayward *et al.*, 2007) are shown along the right side of the tree. Strains are DEBAfredC (U57020), Sy (GQ357641), TI-2 (AB016026), BM3070 (AF474246), Petaluma (M25381), Sendai1 (D37813), Ca2 (DQ873714), Z2 (X57001), Max (GQ357642), 150_02LisP (DQ072566), TKP152 (GQ357640), TKP88 (EF153977), VND-1 (AB083502), Shizuoka (D37811), LP20 (D84498), LP3 (D84496), TX125 (AY139094), ItalyM2 (X69501), TM2 (M59418), LP9 (D84497). Reprinted (Hayward and Rodrigo, 2010) with permission from Elsevier.

In some studies, subtype classification has been performed by sequencing the *gag* gene in addition to the *env* gene (Hayward and Rodrigo, 2008, Kakinuma *et al.*, 1995, Steinrigl and Klein, 2003). Perhaps not surprisingly, variants may be classified into different subtypes depending on the region sequenced, and this probably reflects intersubtype recombination or independent evolution at the different locations (Steinrigl and Klein, 2003, Hayward and Rodrigo, 2008).

A clear geographical origin for each FIV subtype is not evident. Subtype A is well distributed globally, being reported in the U.S.A., Canada, Argentina, Nicaragua, Japan, Australia, UK, Germany, Italy, Netherlands, France, Switzerland, South Africa and New Zealand (Nakamura *et al.*, 2003, Kann *et al.*, 2006b, Kann *et al.*, 2006a, Iwata and Holloway, 2008, Pistello *et al.*, 1997, Weaver, 2010, Reggeti and Bienzle, 2004). Subtype B is also widespread – it is present in the U.S.A., Canada, Argentina, Japan, Italy, Australia and Germany (Weaver, 2010, Reggeti and Bienzle, 2004, Kann *et al.*, 2006b). Subtype C was first reported in Canada and has now been detected in New Zealand, Germany, Japan, Taiwan, Vietnam, U.S.A and South Africa (Weaver, 2010, Reggeti and Bienzle, 2004, Nakamura *et al.*, 2003, Kann *et al.*, 2006a). Subtypes D and E are less common, with subtype D found in Japan, Vietnam and Thailand (Keawcharoen *et al.*, 2006, Nakamura *et al.*, 2003, Nishimura *et al.*, 1998). Subtype E has only been reported in Argentina (Pecoraro *et al.*, 1996) and subtype F has only been detected in the U.S.A (Weaver, 2010). This seemingly random geographical distribution of FIV subtypes around the globe may be the result of frequent introductions of the virus into individual countries, or may indicate convergent evolution of the virus over time. More likely however, it simply reflects the random nature of viral mutation and highlights the arbitrary nature of subtype classification.

1.2.3. The clinical relevance of subtype classification

When considering the relevance of FIV subtypes, one must not assume a phenotypic relationship between viruses of the same subtype. There is currently no evidence that subtype classification is predictive of pathogenicity or susceptibility to vaccine-induced immunity. On the contrary, evidence presented later in this chapter highlights that intra-subtype variation does occur

with respect to these characteristics. Subtype classification is too simplistic to explain variations in viral behaviour.

FIV subtype classification has historically been performed via phylogenetic analysis of a very small portion of the *env* gene, usually the V3-V5 region (Sodora *et al.*, 1994). This region encodes part of the surface unit of the envelope glycoprotein, and is one of the regions of greatest genomic sequence diversity. FIV subtyping according to the V3-V5 region provides no information about the diversity of the *gag* or *pol* gene, or even within other regions of the *env* gene (such as the transmembrane portion of the envelope glycoprotein). It is unclear what role the *env* V3-V5 region plays in regards to pathogenicity and susceptibility to the host immune response. It is possible that subtyping based on this region may not be clinically relevant, and variation at other sites of the genome may be more important.

Although subtypes do represent clusters of variants that are genetically similar at the *env* gene, up to 15% diversity of that gene can be observed even within subtypes (Sodora *et al.*, 1994). It is also important to remember that subtype classification is largely based on nucleotide variation rather than amino acid variation. Silent substitutions (i.e. those that do not change the amino acid sequence) increase genetic diversity but cannot affect viral characteristics. Subtyping based on amino acid sequences may be more clinically relevant, as this would at least reflect differences in viral protein composition.

1.3. Biology of FIV

1.3.1. Cell tropism and the virus-host cell interaction

FIV infects host cells using the envelope glycoprotein expressed on the surface of each virion. In the early stages of infection, the virus preferentially replicates in activated CD4+ T cells (Beebe *et al.*, 1994). With chronicity however, the cell tropism of FIV expands to include CD8+ T cells, B cells, macrophages, dendritic cells and astrocytes (Billaud *et al.*, 2000, English *et al.*, 1993, Brown *et al.*, 1991). The alteration in cell tropism over time is explained by evolution of the virus within the host.

Entry of FIV into a host cell *in vivo* is dependent on a two-step process, utilising the primary receptor, cluster of differentiation (CD) 134, as well as a co-receptor molecule, C-X-C chemokine receptor 4 (CXCR4) (Willett *et al.*, 2008, Shimojima *et al.*, 2004, Willett *et al.*, 2006b). The CD134 molecule is a 43 kDa glycoprotein belonging to the tumour necrosis factor (TNF) receptor superfamily. When bound to its natural ligand, CD134 augments T cell function and upregulates cytokine production and anti-apoptotic gene expression on activated T cells (de Parseval *et al.*, 2004a). The CD134 receptor is predominantly expressed on CD4+ T cells, and expression is up-regulated on these cells once activated (Reggeti *et al.*, 2008, de Parseval *et al.*, 2004a). It is not expressed on feline CD8+ cells, regardless of activation state (de Parseval *et al.*, 2004a). CD134 is, however, expressed at a lower concentration on B lymphocytes, dendritic cells and macrophages (Reggeti *et al.*, 2008). The CXCR4 molecule is a chemokine receptor that is expressed on activated (but not resting) T cells, as well as B cells, macrophages and dendritic cells (Willett *et al.*, 2003, Joshi *et al.*, 2005).

The envelope glycoprotein SU binds primarily to the first cysteine rich domain of the CD134 molecule (CRD1) (Willett *et al.*, 2006a). A conformation change ensues, and this exposes the secondary binding site for CXCR4 (**Figure 1-2**) (Willett *et al.*, 1997, Egberink *et al.*, 1999). Viruses isolated from cats in early stages of infection require an additional interaction with the second cysteine rich domain of the CD134 molecule (CRD2) in order to induce the conformation change required for the CXCR4 interaction (Willett *et al.*, 2006a). High concentrations of CD134 are required for this interaction, making CD4+ T cells the primary target in the early stages of infection (due to their high expression of CD134). Over time, viral evolution within the host results in a generation of variants that have a reduced requirement for CRD2 interaction. This was shown by Willet and others (2010) when FIV quasispecies were examined from a cat infected with the subtype A, Glasgow 8 virus (FIV-GL8) six years previously. Comparison of these viruses with the original FIV-GL8 virus showed differences in the amino acid structure of the envelope glycoprotein, enabling CRD2-independent infection of cells. The less stringent binding of CD134 reduces the threshold required to induce the conformational change needed for the CXCR4

interaction. This renders cells with low concentrations of CD134 (such as B cells, dendritic cells and macrophages) permissible to infection.

Certain variants of FIV appear to be able to also infect CD134 negative cells via interaction with the CXCR4 receptor alone. This has been shown to occur in vitro, with laboratory adapted viruses able to infect CD134 negative, CXCR4 positive Crandell-Reese Feline Kidney (CRFK) cells. This phenotype is associated with multiple mutations in the V3 loop of SU (Verschoor *et al.*, 1995, Siebelink *et al.*, 1995c, Richardson *et al.*, 1999). Similarly, FIV has been adapted to infect astrocyte cell lines in vitro, following co-cultivation of uninfected astrocytes with FIV-infected peripheral blood mononuclear cells (PBMC) (Gavrilin *et al.*, 2002). Astrocytes have been shown to express CXCR4, but not CD134 (Koirala *et al.*, 2000, Ishikawa *et al.*, 2008). Evolution of the virus to a CD134-independent phenotype within the brain has been postulated to explain the presence of FIV in astrocytes of infected cats (Meeker, 2007, Fletcher *et al.*, 2011, Macchi *et al.*, 1998). Following establishment of infection within CNS macrophages, cell-to-cell interactions with astrocytes are thought to induce the conformational change necessary to CD134-independent cell entry. Once present in the astrocytes, CD134-independent quasispecies then evolve to maintain a reservoir in astrocytes. For a summary of the cell surface expression of receptors by cells permissible to infection with FIV, refer to **Table 1-2**.

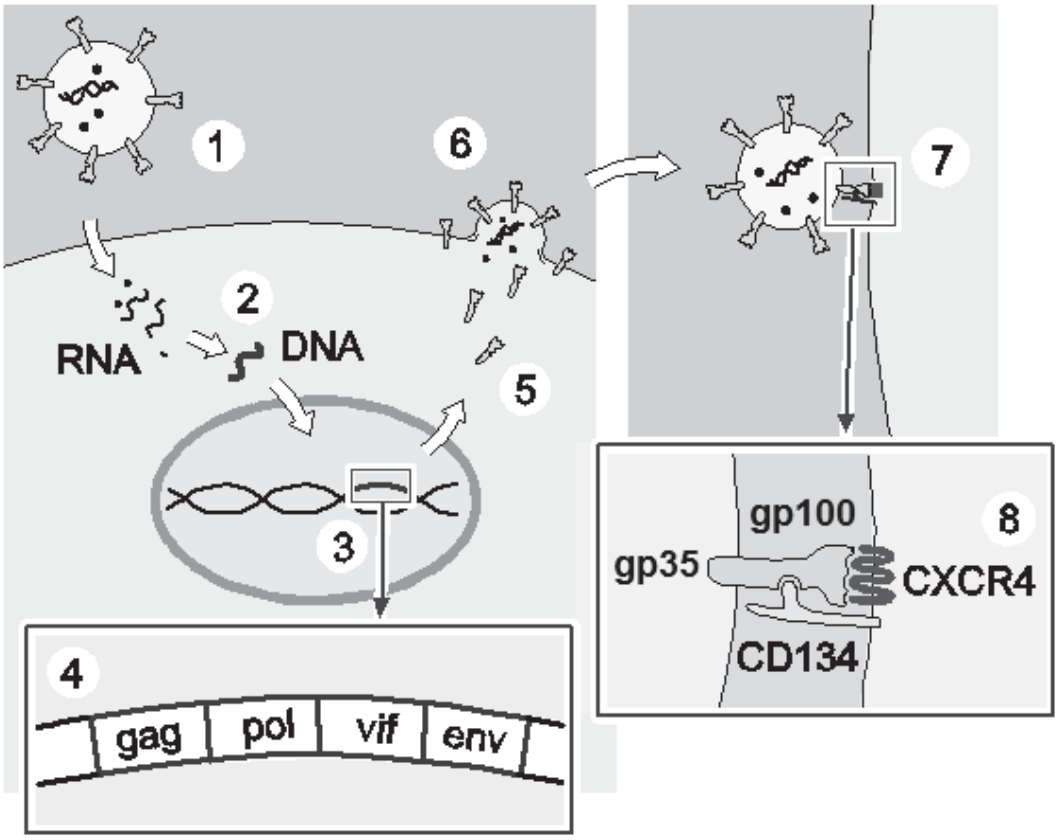


Figure 1-2 The FIV-host interaction.
Following binding, FIV fuses with the host cell membrane and releases viral RNA and proteins, including reverse transcriptase, into the host cell **(1)**. Reverse transcription of viral RNA produces proviral DNA **(2)** which is integrated into the host cell genome **(3)**. The FIV genome is comprised of three major genes (*env*, *gag* and *pol*) as well as accessory genes (*vif* shown) **(4)**. Transcription and translation of viral genes by the host cell produces viral proteins that assemble at the host cell membrane **(5)**. The envelope glycoprotein contains two subunits, gp100 and gp35, which are expressed on the surface of the host cell membrane. Viral budding occurs and a new virion is released into the extracellular space **(6)**. Virions bind to uninfected, susceptible cells via interaction between the envelope glycoprotein and host cell receptors **(7)**. The surface unit of the envelope glycoprotein (gp100) initially binds to CD134 on the host cell. A conformational change occurs, exposing the viral binding site for the host cell co-receptor CXCR4 **(8)**. Binding of CXCR4 allows viral fusion to occur, enabling viral entry into the host cell. Reprinted (Stickney *et al.*, 2013) with permission from BMJ Publishing Group Ltd.

Table 1-2 Cell tropism of FIV.
Cell surface expression of the molecules CD134 and CXCR4 determines the host cell tropism of FIV. Generally, interaction with both molecules is required for cell entry, however certain variants of FIV are able to infect cells that express only CXCR4, or CXCR4 with low concentrations of CD134.

Cell type	Expression of CD134	Expression of CXCR4	Cell permissive to FIV infection
Resting CD4+ T cells	Positive (low)	Negative	No
Activated CD4+ T cells	Positive (high)	Positive	Yes [a]
Resting CD8+ T cells	Negative	Negative	No
Activated CD8+ T cells	Negative	Positive	Yes [c]
B cells	Positive (low)	Positive	Yes [b]
Dendritic cells	Positive (low)	Positive	Yes [b]
Monocytes / macrophages	Positive (low)	Positive	Yes [b]
Astrocytes	Negative	Positive	Yes [c]
CRFK cells	Negative	Positive	Yes [c]

[a] All variants of FIV
[b] CRD2-independent FIV variants only
[c] CD134-independent FIV variants only

1.3.2. Virus assembly

Following transcription of the provirus, the envelope glycoprotein is synthesised within the endoplasmic reticulum and transported through the Golgi apparatus to the cell membrane (**Figure 1-2, section 5**). It is initially produced as a large precursor protein of approximately 150 kDa, and two post-translational cleavage steps yield the SU (gp100) and TM (gp35) (Verschoor *et al.*, 1993). The mature glycoproteins remain associated via non-convalent interactions, and the presence of a cytoplasmic tail on gp35 anchors the complex to the cell membrane (Celma *et al.*, 2007).

The gag proteins are also initially produced as large precursor molecules consisting of the matrix, capsid and nucleocapsid proteins. The gag precursor is directed to the host cell membrane by the interaction with phosphatidylinositol-4,5-bisphosphate, which is concentrated on the inner surface of the host cell membrane (Luttge and Freed, 2009). Multimerisation of the gag precursor forms the shell that houses the viral RNA and enzymes. Following assembly of viral RNA, envelope glycoproteins, and gag precursors at the cell membrane, the virus buds and is released from the cell membrane (Luttge and Freed, 2009) (**Figure 1-2, section 6**). Final viral maturation occurs after budding, when the viral protease cleaves the gag precursor into the capsid,

nucleocapsid and matrix proteins (Luttge and Freed, 2009). The virus particle is then infective to other cells.

1.3.3. Viral transmission

The major route of transmission of FIV is presumed to be via parenteral exposure of saliva or blood from an infected cat (Yamamoto *et al.*, 1989). This most commonly occurs during fighting, where virus enters the wound in saliva from an infected cat. After the acute phase, when the cat enters an asymptomatic phase of infection, the viral concentration in saliva remains relatively low, and this may account for the naturally low transmission rate of FIV (Yamamoto *et al.*, 2007). Infected cats with oral disease are probably at increased risk of transmitting the disease to an uninfected cat, as blood contamination of the wound is more common when oral lesions are present and virus concentration is much higher in blood when compared to saliva (Yamamoto *et al.*, 2007, Matteucci *et al.*, 1993). Vertical transmission of the virus has been reported, occurring either in utero or via milk, and transmission rates are higher when queens become acutely infected whilst pregnant (O'Neil *et al.*, 1995, Yamamoto *et al.*, 1989, Medeiros *et al.*, 2012). Other routes of transmission are reported experimentally, including mucosal transmission via the rectal and vaginal routes, and transmission via intravenous, intramuscular and intraperitoneal inoculation (Burkhard *et al.*, 1997, Yamamoto *et al.*, 1988).

1.3.4. Viral dissemination

In the early stages of infection, dendritic cells are thought to be involved in the process of viral dissemination. This has been shown for HIV infection, whereby virions bind to dendritic cells residing in the skin or at the mucosal surface, via the C-type lectin receptor, CD209 (also known as DC-SIGN). The virion / CD209 complex is then internalised and transported to the local lymph node within the dendritic cell. Virus infectivity is preserved during this process, and the infective particle is presented to $CD4^+$ T cells in the lymphoid tissue (Geijtenbeek *et al.*, 2000). CD209 has also been shown to bind with high affinity to the surface unit of the FIV envelope glycoprotein, and a similar mechanism of dendritic cell presentation of virus to $CD4^+$ T cells is

thought to occur in FIV infection (de Parseval *et al.*, 2004b). In addition, co-culture of feline peripheral blood mononuclear cells (PBMC) with dendritic cells has been shown to enhance FIV replication *in vitro*, suggesting a role for dendritic cells above just presentation of virus to permissible cells (van der Meer *et al.*, 2007, Toyosaki *et al.*, 1993).

Within the lymph node, FIV replicates primarily in CD4+ T cells and then disseminates to other lymphoid and non-lymphoid organs (such as the lung, liver, kidney and brain) (Beebe *et al.*, 1994, Hein *et al.*, 2005, Matteucci *et al.*, 1993). The acute stage of infection is associated with a high plasma viral load for the first 8-10wks, and vague, often mild clinical signs may occur during this period (Diehl *et al.*, 1995a). In some experimental studies, there are reports of severe disease occurring during this period (Diehl *et al.*, 1995b). Suppression of viral replication by the host immune response coincides with an asymptomatic stage of infection of variable duration. In some (but not all) infected cats, the immune response eventually fails to suppress viral replication, and this is associated with a decline in CD4+ T cells, an increase in plasma viral load and the development of an acquired immunodeficiency syndrome (AIDS) (Goto *et al.*, 2000).

1.4. Feline acquired immunodeficiency syndrome

There is enormous variation in the disease course following FIV infection. Many clinical signs have been reported, including lymphadenopathy, fever, weight loss, lymphoma, neurological signs, cytopenias, gingivostomatitis, upper respiratory infections, conjunctivitis, abscessation and diarrhoea (Hutson *et al.*, 1991, Fujino *et al.*, 2009, Yamamoto *et al.*, 1989, Ishida *et al.*, 1989, Hopper *et al.*, 1989, Sparkes *et al.*, 1993, Matsumura *et al.*, 1993, Callanan *et al.*, 1992, Kohmoto *et al.*, 1998, Ishida *et al.*, 1992, Gabor *et al.*, 2001, Maingat *et al.*, 2009). However, there are very few studies comparing the prevalence of disease in FIV infected and uninfected cats (Shaw *et al.*, 1990, Ravi *et al.*, 2010, Liem *et al.*, 2013). One such study that did compare clinical signs in age- and sex-matched FIV infected and uninfected cats was only able to confirm a positive association between FIV and lethargy (OR 3.16), and FIV and oral disease (including stomatitis, gingivitis and periodontal disease, OR 5.7) (Ravi *et al.*, 2010). Another

similar study did not report any statistically significant disease associations, although there was a trend towards an increased prevalence of lymphoid malignancies in the FIV positive group (Liem *et al.*, 2013). Survival analyses of age-matched cats with and without FIV have also failed to show an increased mortality in infected cats (Liem *et al.*, 2013, Ravi *et al.*, 2010). In addition, long-term observations of infected cats have revealed prolonged asymptomatic periods in FIV infected cats (Kohmoto *et al.*, 1998, Addie *et al.*, 2000). In contrast to these studies, there are also reports of natural and experimental infection resulting in the rapid development of AIDS and high mortality rates (Diehl *et al.*, 1995b, Beczkowski *et al.*, 2015c, Ishida *et al.*, 1992).

Although the reasons for such variability in the clinical signs of FIV-infected cats are not clearly understood, multiple factors are likely to be involved. For example, environmental factors may influence disease progression as a result of stress and exposure to pathogens. This was highlighted recently when clinical signs and mortality were compared over time in two cohorts of separately housed FIV infected cats. One group suffered a 63% mortality over a 22 month period, whilst only 6% of cats in the other group died (Beczkowski *et al.*, 2015c). After attempting to exclude other factors (such as viral genetic factors), the authors concluded that the differences in disease progression between the two groups was most likely a result of differences in housing. The cats in the high mortality group were housed together in an overcrowded environment, whereas the cats in the other group were maintained in single-cat households. Crowding causes stress in cats as a result of direct and indirect interactions (via noise, pheromones etc.) with other cats, and this may result in immunosuppression. The immunosuppressive effect of stress in cats is well demonstrated by studies that show reactivation of feline herpes virus 1 (FHV-1) in cats following a period of stress (Gaskell and Povey, 1977). In combination with increased exposure of these cats to opportunistic pathogens, stress was likely to have contributed to the development of feline AIDS in the FIV infected cats housed in the overcrowded environment.

Host factors also contribute to the variability of disease in FIV infected cats. For example, the age at which a cat is infected with FIV seems to be an important factor affecting the severity of disease, with neonatal and aged cats having an increased risk of more severe disease (George

et al., 1993). Likewise, host genotype probably affects disease progression. Non-host factors such as dose of the viral inoculum and route of exposure are important when considering outcomes of experimental infections with FIV. For example, one study demonstrated that cats infected intravenously experienced a greater decline in CD4+ T cells than cats infected with the same viral dose via the vaginal route (Burkhard *et al.*, 2002).

Understanding the role of FIV in causing disease is complicated by the presence of concurrent disease in naturally infected cats. It is well recognised that FIV causes immune dysfunction in infected cats, and in some cases, this may lead to an increased susceptibility to infectious disease. There are many case reports of infectious disease in FIV infected cats, and these have been thoroughly reviewed by Sellon & Hartmann (2006). The presence of disease in an infected cat does not, however, confirm a causal relationship for FIV, and these reports should not be considered as evidence of FIV-induced disease. More robust are those studies comparing the prevalence of infectious disease in FIV-infected versus uninfected cats. Of the organisms studied, only *Toxoplasma gondii*, *Mycoplasma haemofelis*, *Bornavirus* and orthopoxvirus are reported to occur more commonly in FIV-infected cats (Tryland *et al.*, 1998, Huebner *et al.*, 2001, Dorny *et al.*, 2002, Jenkins *et al.*, 2013, Duarte *et al.*, 2015). There is no confirmed association between FIV infection and *Cryptococcus*, *Cryptosporidium*, or *Bartonella* spp. (Kelly *et al.*, 2010, Mtambo *et al.*, 1991, Walker *et al.*, 1994). FIV positive cats co-infected with other pathogens may be at risk of increased severity of disease due to viral-induced immunosuppression. This has been shown previously for *Toxoplasma gondii*, where cats concurrently infected with FIV were predisposed to developing generalised and severe toxoplasmosis, whereas FIV negative cats only experienced transient and mild clinical signs (Davidson *et al.*, 1993).

FIV causes progressive immune dysfunction in infected cats. Previous studies have documented an increased prevalence of oral disease and possibly lymphoid malignancies in infected cats. Despite no confirmed association between FIV and neurological disease, the neurotropic nature of FIV has been well studied as a model for HIV. In order to further appreciate

the clinical impact of FIV, a thorough understanding of the mechanism of FIV-induced immune dysfunction is required.

1.4.1. FIV-induced immune dysfunction

FIV-induced immune dysfunction is characterised by a progressive inability of lymphocytes from infected cats to respond to viral and non-specific antigen (Tompkins and Tompkins, 2008). The pathogenesis of the FIV-induced immune dysfunction is multifactorial, in part due to T cell exhaustion and destruction of CD4+ T cells, as well as altered cytokine activity, dendritic cell dysfunction and increased activation of T regulatory cells (Lawrence *et al.*, 1995, Lehman *et al.*, 2010, Miller *et al.*, 2013).

1.4.1.1. Cellular activation and T cell exhaustion

FIV infection is associated with increased activation of B and T lymphocytes in infected cats when compared to uninfected control cats (Tompkins *et al.*, 2002, Paillot *et al.*, 2005, Silvotti *et al.*, 1997, Gebhard *et al.*, 1999). For example, histological examination of the lymph nodes of infected cats reveals follicular and paracortical hyperplasia, indicating B and T cell proliferation respectively (Callanan *et al.*, 1992). Furthermore, when compared to uninfected cats, lymphocytes in the peripheral circulation and lymph nodes of FIV infected cats have increased expression of cellular activation markers (such as CD30, CD152, CD80, CD87 and CD25) (Silvotti *et al.*, 1997, Gebhard *et al.*, 1999, Ohno *et al.*, 1992b, Willerford *et al.*, 1995, Tompkins *et al.*, 2002). Widespread polyclonal activation of B cells probably explains the hypergammaglobulinemia that is often reported in association with FIV infection. The concentration of serum IgG is up to two-fold higher in FIV infected cats when compared to uninfected cats, with elevations occurring early in the disease course and antibodies not specific to viral antigen (Ackley *et al.*, 1990, Flynn *et al.*, 1994, Sparkes *et al.*, 1993). The mechanisms of polyclonal B cell activation in FIV infection are unclear and probably multifactorial, although overproduction of the B-cell stimulatory cytokine, IL-6, may be a contributing factor (Beatty *et al.*, 1998b, Lawrence *et al.*, 1995, Bona, 1996).

One of the earliest effects of FIV on the immune system is a reduction of mitogen-induced lymphocyte proliferation. PBMC from infected cats show an impaired response to mitogens *ex vivo,* as well as a reduced ability to be primed by novel antigen (Hara *et al.*, 1990, Lin *et al.*, 1990, Taniguchi *et al.*, 1990, Torten *et al.*, 1991, Bishop *et al.*, 1992, Lawrence *et al.*, 1992, Barlough *et al.*, 1991). The effect is not confined to a particular lymphocyte subset, with an abnormal response of PBMC to both B and T cell mitogens. Impaired lymphocyte proliferation occurs prior to a reduction in CD4+ T cells, indicating that it is not an effect of reduced cell number of this subset in culture (Bishop *et al.*, 1992). The abnormality is present in asymptomatic cats, but the severity progresses over time, correlating with the clinical stage of infection (Taniguchi *et al.*, 1990, Torten *et al.*, 1991, Barlough *et al.*, 1991, Siebelink *et al.*, 1990). In association with impaired mitogen-induced proliferation, PBMC from FIV infected cats also show decreased production of IL-2 in response to mitogens (Lawrence *et al.*, 1992, Siebelink *et al.*, 1990). In most situations, the addition of exogenous IL-2 to the culture does not abrogate the effect, except perhaps early in infection while cats remain asymptomatic. This suggests that there may be reduced responsiveness of T and B cells to IL-2, as well as reduced production of this cytokine.

T cell exhaustion is associated with a deterioration of T cell function, defined by "poor effector function, sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells" (Wherry, 2011). T cell exhaustion is preceded by a loss of proliferative capacity and IL-2 production, followed by reduced production of other cytokines such as TNF-α and eventual deletion by apoptosis (Kahan *et al.*, 2015). In humans and mice, T cell exhaustion is characterised by dysfunctional lymphocytes that persistently express co-inhibitory molecules, such as CD152 and CD279 (Barber *et al.*, 2006, Khaitan and Unutmaz, 2011). CD152 (also known as cytotoxic T lymphocyte antigen 4, CTLA-4) is normally expressed on T cells following activation. Once bound to its ligands, CD80 or CD86, CD152 suppresses the co-stimulatory signal induced by the CD28 molecule during the activation process, thereby restricting IL-2 production and downregulating the immune response (Walunas *et al.*, 1994). Similarly, CD279 (also known as programmed-death 1, PD-1) is a co-inhibitory molecule

belonging to the CD28 superfamily, that induces a negative signal to B and T cells upon ligation with CD274 (PD-L1) or CD273 (PD-L2). Although both CD152 and CD279 are normally expressed on activated T cells, a persistant and high level of expression is associated with T cell exhaustion (Wherry, 2011). In addition to upregulation of cell surface co-inhibitory molecules, T cell exhaustion is also associated with increased production of inhibitory cytokines such as IL-10 from CD4+ T cells, dendritic cells and possibly monocytes (Wherry, 2011, Ng and Oldstone, 2014). The characteristics have been shown to occur in HIV infection as a result of chronic antigenic stimulation, and this has been reviewed recently by Khaitan & Unutmaz (2011) and Moir & Fauci (2014). The T cell phenotype and dysfunction that occurs in FIV infection is also reminiscent of a state of T cell exhaustion (Lawrence *et al.*, 1992, Barlough *et al.*, 1991, Bishop *et al.*, 1992, Hara *et al.*, 1990, Lin *et al.*, 1990, Taniguchi *et al.*, 1990, Torten *et al.*, 1991, Folkl *et al.*, 2008, Tompkins *et al.*, 2002, Najafi *et al.*, 2014).

1.4.1.2. Loss of CD4+ T cells and apoptosis

Following initial infection with FIV, there is a gradual decline of CD4+ T cells in the peripheral blood and lymph nodes, causing inversion of the CD4+/CD8+ T cell ratio (Novotney *et al.*, 1990, Hoffmann-Fezer *et al.*, 1996, English *et al.*, 1994, Walker *et al.*, 1994, Ackley *et al.*, 1990, Hoffmannfezer *et al.*, 1992, Torten *et al.*, 1991, Tompkins and Tompkins, 2008). In some studies, this has also been associated with an increase in the absolute number of CD8+ T cells (Mihaljevic *et al.*, 2004, Walker *et al.*, 1994, Ackley *et al.*, 1990, Hoffmannfezer *et al.*, 1992). It seems that the absolute number of peripheral $CD4^+$ T cells and the CD4+/$CD8^+$ T cell ratio cannot reliably be used to differentiate between healthy and symptomatic cats infected with FIV (Walker *et al.*, 1994). However, these parameters have been shown to decline in individual cats prior to the development of terminal AIDS (Beczkowski *et al.*, 2015c).

A large proportion of the CD4+ T cells that die during the progression of FIV infection are, in fact, uninfected lymphocytes. There is substantial evidence that these cells are lost as a result of apoptosis within the lymph nodes of FIV positive cats (Garg *et al.*, 2004b, Muro-Cacho *et al.*, 1995, Finkel *et al.*, 1995, Sarli *et al.*, 1998, Tompkins *et al.*, 2002). Spontaneous apoptosis has

been reported in PBMC cultured from infected cats and in certain cell lines infected with FIV (Yamamoto *et al.*, 1997, Sutton *et al.*, 2005, Ohno *et al.*, 1994, Guiot *et al.*, 1993a, Johnson *et al.*, 1996, Garg *et al.*, 2004b, Holznagel *et al.*, 1998, Momoi *et al.*, 1996, Bishop *et al.*, 1993, Guiot *et al.*, 1993b, Guiot *et al.*, 1997). This phenomenon is unlikely to be due to direct infection of cells, as apoptosis occurs *in vitro* prior to active infection of cells (as demonstrated by gag protein expression) (Sutton *et al.*, 2005). In addition, the percentage of apoptotic cells (>50% by 15 days post infection of a feline lymphoblast cell line, MYA-1 cells) greatly exceeds the number of infected cells in culture, which is typically very low (<10%) (Sutton *et al.*, 2005, Finkel *et al.*, 1995).

Apoptosis is an active, genetically programmed mechanism of cell death, characterised by a distinct set of morphological features such as cell shrinkage, membrane blebbing and DNA fragmentation (Kerr *et al.*, 1972). It represents a normal physiological process in embryonic development and healthy tissue turnover, and is also the mechanism by which effector cells are deleted following successful elimination of the pathogen (Akbar and Salmon, 1997, Kerr *et al.*, 1972). In contrast to necrosis, where membrane integrity is lost, apoptosis results in the formation of cellular fragments with an intact cell membrane (apoptotic bodies) that are rapidly phagocytosed without inducing an inflammatory reaction (Wyllie, 1997). In the absence of phagocytosis, apoptotic bodies *in vitro* will eventually swell and lyse, and this has been termed secondary necrosis (Wyllie, 1997). Conventional cell viability assays using cell permeability dyes therefore cannot determine the mechanism of cell death (i.e. apoptosis versus primary necrosis) once membrane integrity is lost.

Apoptosis occurs following activation of one of three different pathways - the intrinsic, extrinsic or perforin / granzyme pathway (**Figure 1-3**) (Elmore, 2007). The intrinsic pathway is triggered when cells are damaged by external factors (such as radiation, free radicals etc.) or following withdrawal of growth factors or hormones. Briefly, these stimuli alter the permeability of the inner mitochondrial membrane, leading to the release pro-apoptotic proteins into the cytosol. Pro-apoptotic proteins activate a series of proteases known as caspases that eventually

activate endonucleases and degrade cytoskeletal proteins. The end result is DNA fragmentation and the formation of apoptotic bodies (Wyllie, 1997). The extrinsic pathway of apoptosis occurs when the caspase cascade is activated by a number of cell surface receptors belonging to the TNF receptor gene superfamily (such as CD95 and CD120) (Elmore, 2007). Binding of ligands such as CD95L and TNF-α to their respective receptors leads to the recruitment of proteins that ultimately trigger activation of the caspase cascade (Pier, 2004). Finally, apoptosis may occur via the perforin / granzyme pathway, which is one mechanism by which cytotoxic T cells may kill tumour cells and virus infected cells. The pore-forming molecule, perforin, is secreted by the cytotoxic cell and this allows entry of the subsequently released proteolytic granules into the target cell. The end result is activation of the caspase cascade and apoptosis of the target cell. A caspase-independent pathway of apoptosis is also initiated by these granules. The mechanisms of apoptosis are complex, with overlap of the different pathways occurring in many situations, and these have been comprehensively reviewed (Elmore, 2007, Wyllie, 1997).

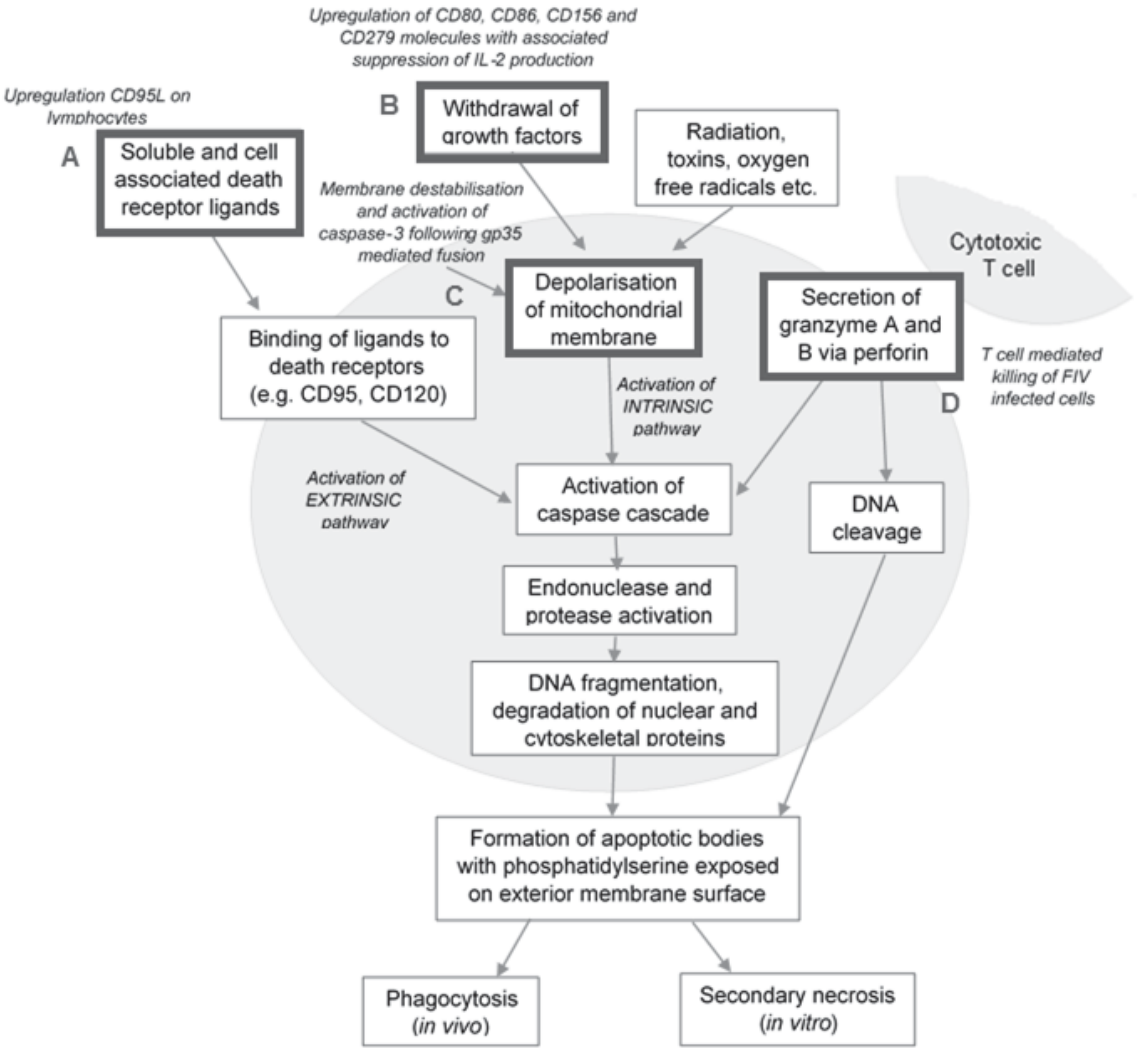


Figure 1-3 Apoptotic pathways and FIV-induced mechanisms of apoptosis.
The diagram depicts the general mechanisms of lymphocyte apoptosis, with suggested virally-induced mechanisms indicated in heavy boxes. FIV infection may cause bystander lymphocyte apoptosis via the extrinsic pathway (A, due to upregulation of CD95L on lymphocytes); intrinsic pathway (via upregulation of co-inhibitory molecules, B; or membrane destabilisation following gp35 mediated viral fusion, C). Apoptosis of infected cells also occurs due to perforin-mediated killing of FIV-specific cytotoxic lymphocytes (D).

Apoptosis is recognised by morphological characteristics (using transmission electron microscopy), DNA fragmentation (observed by DNA electrophoresis) or expression of cell surface markers (detected using flow cytometry) (Bishop *et al.*, 1993, Guiot *et al.*, 1993b, Momoi *et al.*, 1996, Wyllie, 1997). One flow cytometric method involves the concurrent use of the fluorescent DNA binding dye, 7 amino actinomycin D (7AAD), and the phosphatidylserine (PS) binding protein, Annexin V (conjugated to a fluorochrome). 7AAD can only enter cells to bind intracellular DNA once there has been sufficient damage to the cellular membrane to result in increased permeability, such as occurs with necrosis. The cell membrane of viable cells is not permeable to 7AAD and therefore they do not show any dye uptake (Liu *et al.*, 1991). Annexin V is a protein that binds to phospholipids, with a high affinity for PS (Vermes *et al.*, 1995). PS is a phospholipid that is present in abundance in eukaryotic cell membranes (Leventis and Grinstein, 2010). In healthy cells, PS faces the cytosolic surface of the membrane, and is held in place by enzymes that transport phospholipids from the outer to the inner surface of cell membranes (known as flippases) (Clark, 2011). Early in apoptosis, caspases inactivate flippases, allowing PS to freely translocate to the outer surface of the cell membrane (Segawa *et al.*, 2014, Verhoven *et al.*, 1995). It is the presence of PS on the surface of the cell that triggers phagocytosis of the apoptotic bodies (Segawa *et al.*, 2014). As Annexin V binds with high affinity to PS, cells will bind this protein extracellularly once the apoptosis pathway is initiated. Apoptotic cells retain their membrane integrity, and thus do not allow entry of 7AAD to bind intracellular DNA. Dual-colour flow cytometry will therefore identify apoptotic cells as Annexin V positive and 7AAD negative. As already discussed, apoptosis *in vitro* eventually leads to secondary necrosis. These cells become permeable and therefore eventually allow entry of 7AAD, and are detected as double positive cells on flow cytometry. Cells undergoing primary necrosis in culture are permeable from the outset, binding 7AAD intracellularly. These cells will also be positive for Annexin V however, as increased permeability permits access of Annexin V to the PS present at the cytosolic surface of the cell membrane. Viable, non-apoptotic cells will be double negative on flow cytometry, as they are not permeable to either 7AAD or Annexin V and PS is not present on the exterior surface of the membrane of these cells. In summary, a dual-colour, flow cytometric assay

using Annexin V and 7AAD will differentiate between viable cells (Annexin V negative / 7AAD negative), early apoptotic cells (Annexin V positive / 7AAD negative) and dead cells (Annexin V positive / 7AAD positive), but cannot determine whether cell death occurred due to apoptosis or necrosis.

The mechanism of bystander apoptosis in FIV infection is multifactorial, involving activation of both intrinsic and extrinsic pathways. There is evidence that the envelope glycoprotein has a direct pro-apoptotic effect on lymphocytes. This was demonstrated by Garg et al. (2004) when activated PBMC were cultured in the presence of env-expressing CRFK cells (Garg *et al.*, 2004b). The uninfected lymphocytes underwent apoptosis in a manner that was dependent the transmembrane unit, gp35. The authors concluded that apoptosis was occurring as a direct result of interaction of gp35 with the bystander cell membrane, leading to cellular fusion and the formation of a fusion pore and subsequent apoptosis. Similar studies examining the mechanism of HIV-induced apoptosis in bystander lymphocytes have shown that fusion results in activation of caspase-3 at the cell surface, with subsequent mitochondrial membrane depolarisation and activation of the intrinsic pathway of apoptosis (Garg and Blumenthal, 2006).

There is also evidence to suggest that apoptosis of bystander lymphocytes may be the result of activation induced cell death (AICD) via a CD95 / CD95L-mediated interaction. CD95 (also known as Fas) is constitutively expressed on B and T lymphocytes, but expression of its ligand, CD95L (FasL) only occurs following activation of the T cell (Li-Weber and Krammer, 2002, Sieg *et al.*, 1997). The end result of a CD95 / CD95L interaction depends on the activation state of the cell. In naïve T cells, the CD95 / CD95L interaction provides a costimulatory signal that augments the immune response. In contrast, binding of CD95L to CD95 on an activated T cell induces apoptosis via the extrinsic pathway (Lynch *et al.*, 1995). This interaction is one mechanism by which autoreactive T cells are deleted in the periphery (Mogil *et al.*, 1995). In states of chronic antigenic stimulation, repeated exposure to antigen also results in apoptosis of antigen-specific T cells as a result of CD95 / CD95L interactions. FIV infection has also been shown to upregulate expression of the CD95L molecule on infected lymphocytes (as shown by increased mRNA

production by PBMC from infected cats). In addition, cell lines infected with FIV *in vitro* also show increased production of CD95L mRNA (Mizuno *et al.*, 2003). Binding of upregulated CD95L on FIV-infected $CD4^+$ T cells, to CD95 on naïve bystander lymphocytes, may contribute to the apoptosis of uninfected lymphocytes that occurs with FIV infection.

As previously discussed in section 1.4.1.1, inhibitory costimulatory molecules are expressed on activated lymphocytes, and these restrict the immune response and prevent ongoing cell proliferation. For example, CD152 is expressed on T cells 2-3 days after activation, and interaction of this molecule with its ligands (CD80 and CD86) suppresses IL-2 production, resulting in anergy and apoptosis (Walunas *et al.*, 1994). FIV induces widespread cellular activation, and infection is associated with increased expression of the costimulatory molecules CD152, CD80 and CD86 (Tompkins *et al.*, 2002). Interactions of T cells expressing the inhibitory CD152 molecule with bystander lymphocytes may induce apoptosis, thereby contributing to the gradual decline of CD4+ T cells. This is supported by an inverse correlation between CD4+ T cell count and expression of CD152, CD80 and CD86 molecules in FIV infected cats (Tompkins *et al.*, 2002).

1.4.2. FIV-associated neurological disease

The first reported cases of FIV described neurological abnormalities in infected cats (Pedersen *et al.*, 1987). Cats predominantly displayed behavioural changes, with compulsive roaming and abnormal facial movements. Since then, similar reports of neurological signs have been described in both naturally and experimentally infected cats, independent of secondary infections affecting the nervous system. Experimental studies commonly report neurological disease in FIV-infected cats, however cross-sectional studies of naturally infected cats have failed to confirm this association (Liem *et al.*, 2013, Ravi *et al.*, 2010). This may be due to the subtle nature of the signs, as many cats show behavioural changes rather than motor deficits. FIV infected cats with neurological disease may show signs of dementia, loss of social behaviour, aggression, loss of toilet training, altered sleep patterns, and compulsive roaming behaviour (Prosperogarcia *et al.*, 1994, Pedersen *et al.*, 1987, Pedersen *et al.*, 1989, Phillips *et al.*, 1994).

Facial twitching, ataxia, reduced peripheral sensory and motor conduction, seizures and gait abnormalities have also been described however (Pedersen *et al.*, 1987, Power *et al.*, 1997, Power *et al.*, 1998, Steigerwald *et al.*, 1999, Phillips *et al.*, 1996). In addition, microscopic lesions can occur within the brains of asymptomatic cats, and FIV neurotropism appears to be strain dependent (Podell *et al.*, 2000). It is therefore possible that the impact of FIV on the central nervous system is underestimated when studying a naturally infected population of cats (Podell *et al.*, 2000).

As discussed previously in section 1.3.1, FIV is primarily maintained in microglia and other CNS macrophages, and possibly astrocytes within the CNS (Dow *et al.*, 1992, Hein *et al.*, 2005). The virus enters the brain early in the acute stages of infection, via lymphocytes and monocytes trafficking across the blood-brain and blood-CSF barriers (Ryan *et al.*, 2005). The histopathological changes present as a result of FIV infection include lymphocyte-rich perivascular infiltrates within meninges, choroid plexus and spinal cord dura mater; proliferation and activation of microglial cells; and loss of neurons (Ryan *et al.*, 2005, Power *et al.*, 1997, Fletcher *et al.*, 2011, Miller *et al.*, 2011). Neuronal injury occurs progressively over time, beginning in the asymptomatic stages of infection and resulting in the gradual development of behavioural changes and other neurological signs (Podell *et al.*, 2000). The effect of FIV on neurons is thought to be due to an indirect mechanism, as neurons are not susceptible to viral infection (Dow *et al.*, 1990). Co-culture of neurons with FIV infected astrocytes and microglia results in neuronal swelling and cell death, but only in the presence of glutamate in culture (Meeker, 2007, Gruol *et al.*, 1998, Bragg *et al.*, 2002). Furthermore, blockade of the N-methyl-D-aspartate (NMDA) receptor abrogates this effect, suggesting an indirect role of glutamate inducing neuronal injury via the NMDA pathway (Bragg *et al.*, 2002). This may occur as a result of excessive calcium influx and the generation of free radicals within the neurons (Zenger *et al.*, 1997). Glutamate mediated neuronal toxicity can be induced by envelope glycoprotein alone, indicating that this occurs as a direct result of the virus (Bragg *et al.*, 2002, Gruol *et al.*, 1998). Neuronal toxicity is also thought to be exacerbated within the brain by defective uptake of

glutamate by FIV infected astrocytes, thereby increasing the local concentration of glutamate and exposure to neurons (Podell *et al.*, 2000).

1.4.3. FIV-associated neoplasia

Of all the case reports of concurrent neoplasia in FIV-infected cats, the association between FIV and lymphoma is most convincing. Although not statistically significant, there was a trend for an increased risk of lymphoid malignancies in FIV-infected versus uninfected cats in one large Australian cross-sectional study (21.3% versus 13%, p=0.1) (Liem *et al.*, 2013). Another smaller study showed a five-fold increased risk of lymphoma in FIV infected versus uninfected cats, with the risk increasing even further in cats infected with both FIV and FeLV (Shelton *et al.*, 1990). The prevalence of FIV in cats diagnosed with lymphoma is between 16 and 51% (Shelton *et al.*, 1990, Gabor *et al.*, 2001, Court *et al.*, 1997, Zwahlen *et al.*, 1998). Of these studies, the two from Australia showed a prevalence of FIV of 46 and 50% in cats with lymphoma, which is much higher than the expected prevalence in a similar population of cats (14.6%) (Liem *et al.*, 2013, Court *et al.*, 1997, Gabor *et al.*, 2001). Experimentally, FIV is associated with a high prevalence of lymphoma, also supporting a positive association between lymphoma and FIV (Magden *et al.*, 2013, Magden *et al.*, 2010). In contrast to these studies, some authors have reported no association between FIV and neoplasia, although these studies did not specifically examine the risk of lymphoid malignancies in infected cats (Ravi *et al.*, 2010, Shaw *et al.*, 1990). The conflicting results of these studies highlight the difficulty in determining a causative role for FIV in causing disease. Differences in study size and design may account for some of the discordance, but other factors must also be involved. For example, there may be a geographical variation in disease, perhaps due to genetic differences in the viruses circulating in particular regions. Differences in housing, environment and prevalence of other infectious diseases could also contribute to a geographical difference in disease. As there is a wide spectrum of disease that can occur secondary to immunosuppression, very large numbers of cats would be necessary to confirm specific disease associations. Even in a large cross-sectional study, where 1205 cats were tested for FIV, only 66 were actually positive for the disease (including 8 cats that were positive for both

FIV and FeLV) (Ravi *et al.*, 2010). Of the 58 FIV-positive / FeLV-negative cats, and 58 age- and sex-matched FIV-negative cats, neoplasia was diagnosed in only 6 cats. Studies that determine the prevalence of FIV in cats affected with specific diseases may be more robust for this purpose, due to the increased numbers of cases examined (between 50-60 in most cases for lymphoma). Results from these studies do indicate an association between FIV and lymphoma.

The pathogenesis of FIV-associated neoplasia is probably multifactorial. Most studies have failed to detect clonal integration of provirus in lymphoma cells, suggesting an indirect mechanism of lymphomagenesis (Callanan *et al.*, 1996, Terry *et al.*, 1995, Endo *et al.*, 1997, Beatty *et al.*, 1998b). The majority of studies show that FIV-associated lymphomas are of B cell origin, and many are extranodal (Gabor *et al.*, 2001, Callanan *et al.*, 1996, Terry *et al.*, 1995, Endo *et al.*, 1997, Court *et al.*, 1997, Hutson *et al.*, 1991). Activated B cells are at risk of malignant transformation, as a result of replication errors that occur during proliferation. With FIV infection, chronic antigenic stimulation may increase the risk of malignant B cell transformation due to increased activation of cells, as well as impair clearance of defective cells due to defective $CD4^+$, $CD8^+$ and natural killer (NK) cell function (Swann and Smyth, 2007, Simoes *et al.*, 2012).

In contrast to most studies, integrated provirus has been detected in lymphoma cells from a small number of cases (Wang *et al.*, 2001, Beatty *et al.*, 1998a). In one of these cases, further analysis of the site of provirus insertion by Beatty and others (2002) showed integration of the virus at a site that had been previously implicated in several human cancers (Zweemer *et al.*, 2001, Park *et al.*, 2000). The authors suggested a direct role of FIV in oncogenesis, as a result of loss of function of a tumour suppressor gene. In sporadic cases, FIV-induced neoplasia may therefore result from disruption of tumour suppressor genes or activation of oncogenes during insertion of the provirus into the host genome.

1.4.4. FIV-associated gingivostomatitis

Oral lesions are among the most commonly reported clinical sign in naturally and experimentally infected cats (Bandecchi *et al.*, 1992, Hofmann-Lehmann *et al.*, 1995, Knotek *et*

al., 1999, Beczkowski *et al.*, 2015c, Waters *et al.*, 1993, Toi *et al.*, 1994, de Rozieres *et al.*, 2004, Deniz, 2001, English *et al.*, 1994, Gil *et al.*, 2013). The link between FIV and oral disease was confirmed by Ravi and others (2010), when they showed that FIV infected cats in Canada had more than a five-fold increased risk of developing oral disease (which included stomatitis, gingivitis and periodontal disease) when compared to uninfected cats (OR=5.7). Similarly, when a randomly selected group of cats were tested for FIV and examined for the presence of oral disease, FIV infected cats were more likely to have oral lesions, including periodontal disease, gingivitis and/or stomatitis (Tenorio *et al.*, 1991). The association between FIV and gingivostomatitis is also apparent when determining the prevalence of FIV in cats diagnosed with oral disease. Koryna et al. (2014) recently reported an increased risk of FIV seropositivity in Canadian cats with oral inflammatory disease (particularly stomatitis, but also periodontitis). This association was most evident in younger cats. Similarly, Knowles et al. (1989) reported an increased prevalence of FIV in British cats with stomatitis versus unaffected cats (81% seropositivity versus 16% respectively). Interestingly, this study did not detect a difference in FIV seropositivity in stomatitis affected versus unaffected cats from North America. Belgard et al. (2010) reported no association between FIV and gingivostomatitis, with no differences detected in the prevalence of FIV in affected and unaffected cats. Quimby et al. reported similar findings with no significant differences detected. However, in this study, 22.2% (2/9) of cats with stomatitis were positive for FIV versus only 8.3% (3/36) of unaffected cats, but the study was underpowered to detect a difference. Again, these studies highlight the variation in reports of FIV disease associations. Possible bias in the studies may result from differences in the lifestyle of infected versus uninfected cats, potentially affecting the incidence of oral diseases such as periodontitis. However, the studies examining only cats with inflammatory stomatitis also report an increased prevalence of FIV compared to unaffected cats, and this increases the strength of the argument for a true disease association. However, as there are no studies that examine the risk of acquiring FIV infection when stomatitis is present (as opposed to the risk of developing stomatitis as a result of FIV infection), the disease association does not necessary imply a causative role of FIV in cats with stomatitis.

Chronic feline gingivostomatitis (CFGS) is characterised by a mixed inflammatory infiltrate, dominated by IgG plasma cells and CD8+ T cells (Harley *et al.*, 2011). The aetiology of the condition is poorly understood, and is probably multifactorial. Dental hygiene is paramount to successful management of this condition, and the most successful treatment thus far involves tooth extraction (Jennings *et al.*, 2015). Although evidence is lacking, it is commonly accepted that CFGS is caused by an aberrant immune reaction to oral microflora that reside in the plaque and mucosal biofilm. It is possible that viruses may contribute to the aetiology by triggering a loss of tolerance to the microbiome in genetically susceptible individuals. Potential mechanisms could include alteration of microflora and/or the cytokine environment, cross-reactivity of the antiviral immune response to oral bacteria or damage to mucosal surfaces resulting in increased exposure of immune cells to bacteria. Further studies are needed to confirm that CFGS is due to a loss of tolerance to oral microflora, and to ascertain the possible role of viruses in triggering this condition.

In addition to the link between FIV and CFGS, there is an even stronger association between CFGS and feline calicivirus (FCV), supporting the argument that viruses are involved in the aetiopathogenesis of this condition. When compared to healthy cats, cats with CFGS are more likely to be actively shedding FCV (as detected by PCR on oral swabs or tissue biopsy) (Lommer and Verstraete, 2003, Knowles *et al.*, 1989, Belgard *et al.*, 2010, Dowers *et al.*, 2010, Geraldo Junior, 2010). Furthermore, successful treatment resulting in improvement of clinical signs often coincides with cessation of FCV shedding (Addie *et al.*, 2003, Gil *et al.*, 2013). There are numerous case reports and two larger studies investigating the efficacy of feline recombinant interferon-omega (IFN-Ω, Virbagen Omega; Virbac) in the management of CFGS, and these have demonstrated clinical improvement following treatment (Gil *et al.*, 2013, Addie *et al.*, 2003, Leal *et al.*, 2013, Southerden and Gorrel, 2007, Zetner *et al.*, 2010). IFN-Ω is a type I interferon that is normally produced by leucocytes during viral infection. It has indirect antiviral activity against a range of viruses, and has been shown to result in reduced shedding of FCV and clinical improvement of cats with FIV (Gil *et al.*, 2013). In addition to its antiviral effects, IFN-Ω also

has immunomodulatory effects, stimulating natural killer cell activity, enhancing the expression of major histocompatibility complex (MHC) class I, and inhibiting mitogen induced lymphocyte proliferation (Adolf, 1995). It is not clear as to whether the efficacy of IFN-Ω for CFGS is related to its antiviral or immunomodulatory effects, however the association of clinical improvement with a reduction in FCV load in the oral cavity does suggest a role for viruses in this disease (Gil *et al.*, 2013).

Whether or not FIV is involved in triggering an aberrant immune response to oral microflora, the virus may contribute to the pathogenesis of CFGS via other mechanisms. Cats concurrently infected with FIV and FCV tend to show more severe oral lesions, suggesting that FIV may exacerbate the condition (Tenorio *et al.*, 1991, Waters *et al.*, 1993, Knowles *et al.*, 1989, Geraldo Junior, 2010). This could be due to an increased susceptibility to secondary infection. There is some evidence that FIV may influence the oral microflora, as cats with FIV and FCGS have different populations of oral bacteria, when compared to uninfected cats with similar oral lesions (Daniel and Reche Junior, 2005, Weese *et al.*, 2015). Cats with CFGS have been shown to have increased salivary IgG and IgM, but reduced salivary IgA concentrations, and this is most prominent in cats that are also infected with FIV (Harley *et al.*, 2003). Humans infected with HIV also show reduced salivary IgA concentrations, and this is thought to be due to reduced secretion of IgA as well as decreased salivary flow (Sweet *et al.*, 1995). FIV induced reductions in salivary IgA may further exacerbate the condition by predisposing cats to secondary oral infections.

There is much to be learned about the pathogenesis of CFGS and the role of FIV in this disease. Although we can be fairly confident that there is an association between FIV and CFGS, we can only speculate as to the potential mechanisms by which it may be involved. Further studies are required to elucidate the exact nature of the relationship between these two diseases.

1.5. The impact of genetic diversity on the pathogenicity of FIV

The literature presented regarding FIV-associated disease has highlighted the unpredictable clinical course of FIV. As already discussed in section 1.4, host and environmental factors are

likely to contribute to the variability of FIV-associated disease. However, another variable that is yet to be considered, is the influence of viral genetic factors on disease expression.

The literature suggests that some FIV variants are more pathogenic than others as a result of viral genetic factors (Nishimura *et al.*, 1998, Kohmoto *et al.*, 1998, Pedersen *et al.*, 2001). For example, Nishimura and others (1998) reported that cats infected with FIV subtype B variants developed less severe clinical signs than those infected with subtype A variants. Similarly, a long-term prospective study described a fatal immunodeficiency in one cat infected with a subtype A FIV variant and compared this to cats that remained asymptomatic when infected with subtype B FIV variants (Kohmoto *et al.*, 1998). Pederson and others (2001) reported an increased virulence of the FIV-CPGammar strain (subtype C), when compared to the FIV-Petaluma strain (subtype A), with respect to haematological parameters, viral load and histopathological changes in the acute phase of infection. The Glasgow-8 strain (FIV-GL8) has also been described as highly virulent, and infection with this virus is generally associated with a much higher viral load and lower CD4+ T cell count when compared to infection with FIV-Petaluma (Hosie *et al.*, 2000, Hosie *et al.*, 2002). Although these studies describe disease in very small numbers of cats and do not control for the influence of host genetic factors, they do suggest that genomic sequence variation of FIV may be an important factor influencing viral pathogenicity.

Further evidence of a role for sequence variation in pathogenicity is provided by *in vitro* experiments comparing characteristics of different FIV isolates. These studies highlight differences that are the result of genomic sequence variation alone, as they eliminate the effect of host and environmental factors. Properties such as mutation characteristics, replication rate, cell tropism, neurotoxicity and the ability to induce apoptosis vary between different variants (Power *et al.*, 1998, Sodora *et al.*, 1994, Bachmann *et al.*, 1997, Nakamura *et al.*, 2003, Ikeda *et al.*, 2004, Diehl *et al.*, 1995a, de Rozieres *et al.*, 2008, Willett *et al.*, 2006b, Hohdatsu *et al.*, 1996, Sutton *et al.*, 2005, Miller *et al.*, 2011). These properties are likely to influence disease progression *in vivo*, although the exact relationships between the *in vitro* characteristics and *in vivo* behaviour of FIV still need to be elucidated.

1.5.1. Viral evolution

As previously discussed in section 1.2, FIV is continually evolving within the host due to frequent mutations in the genomic sequence of the virus. Some mutations result in amino acid changes, whilst others are silent, and differences have been observed in the frequency of each type of mutation present in FIV variants. A number of studies have demonstrated that FIV variants belonging to FIV subtype B evolve with a higher frequency of silent mutations when compared to FIV variants from subtypes A and C (Nakamura *et al.*, 2003, Bachmann *et al.*, 1997, Sodora *et al.*, 1994, Ikeda *et al.*, 2004). Mutations occur randomly, however sequence variation at the *env* gene may influence survival of new variants due to interactions with the host immune response. FIV variants that escape immunity or fail to elicit a strong immune response are subjected to less selection pressure, which favours the formation of a homogenous population of viruses. Whereas FIV variants that elicit a robust and effective immune response are constantly under pressure and are thus forced to evolve further (Sodora *et al.*, 1994). Variants that are well adapted to their host are likely to be much older viruses, as they exist in a more stable state where amino acid changes no longer offer a survival advantage (Sodora *et al.*, 1994, Bachmann *et al.*, 1997). Previous authors have suggested that FIV variants evolving with a higher proportion of silent mutations (such as FIV-subtype B) are not only well adapted to the host, but are also less pathogenic (Sodora *et al.*, 1994, Nakamura *et al.*, 2003).

1.5.2. Replication rate

Plasma viral load of FIV in infected cats correlates with disease progression and survival (Goto *et al.*, 2002, Diehl *et al.*, 1995a). Cats with rapidly progressive FIV-associated disease have previously been shown to have plasma viral loads 10 to 100 fold higher than that observed in long-term survivors (Diehl *et al.*, 1996). Viral load is determined by the balance between the rate of replication of the virus and the rate of virus elimination by the host immune response. Interestingly, a negative correlation between the plasma viral load and anti-FIV antibody titres has not been demonstrated (Diehl *et al.*, 1996). This suggests that the strength of the humoral response alone is not the main determinant for the ability of the virus to survive *in vivo*. This may

be due to the relative importance of the anti-FIV cellular immune response when compared to the humoral response. Alternatively, viral load in plasma may be determined by the replication rate of the virus, and in support of this is evidence that plasma viral load differs in cats infected with different FIV variants (de Monte *et al.*, 2002). Previous studies *in vitro* have compared the replication rate between FIV variants. *In vivo*, the FIV-CPGammar strain is associated with high viral loads and rapidly progressive disease (Diehl *et al.*, 1995b, Hosie *et al.*, 2002). In a separate study *in vitro*, this virus was shown to replicate much faster than the less pathogenic subtype A strain, FIV-PPR (de Rozieres *et al.*, 2008). Genetic factors influencing replication rate may influence pathogenicity by affecting viral load.

1.5.3. Cell Tropism

As previous discussed in section 1.3.1, the cell tropism of FIV variants differs according to their interaction with host cell receptors. FIV variants requiring a less stringent interaction with the CD134 molecule (CRD2 independent variants) have a broader cell tropism, and are more readily able to infect B cells, CD8+ T cells and monocytes/macrophages (Dean *et al.*, 1999, Phillips *et al.*, 1990, Willett *et al.*, 2006b, English *et al.*, 1993). There is evidence that a broader cell tropism (due to CRD2 independence) is associated with disease progression in infected cats. Beczkowski and others (2014) recently demonstrated this by examining the cell tropism of FIV quasispecies (within individual cats) according to variation at the *env* gene. They found that sick cats and cats that had died during the study had a higher proportion of CRD2 independent viruses (with a broader cell tropism) when compared to healthy cats. Furthermore, the proportion of CRD2 independent quasispecies detected was negatively correlated with the CD4+ T cell count. Together, these findings indicate that cell tropism is related to disease progression, highlighting the influence of *env* sequence variation on pathogenicity.

1.5.4. Neurotoxicity

The occurrence of neurological disease in some cats but not others reflects the neurotropism and neurotoxic potential of individual FIV variants. In support of this, experimental studies have

shown that certain variants (such as subtype A FIV-PPR and subtype B FIV-MD) are more consistently associated with neurological disease than others (such as subtype C FIV-CPGammar) (Bragg *et al.*, 1999, Phillips *et al.*, 1994, Phillips *et al.*, 1996, Miller *et al.*, 2011, Power *et al.*, 1998, Meeker *et al.*, 1997). As already discussed in section 1.3.1, neurotropism is strain dependent. Neurotropic viruses must be able to infect macrophages readily, allowing them to become established within the CNS macrophage reservoir. In addition, viruses that have evolved to a CD134-independent state (or have increased propensity to evolve towards this state *in vivo*), are also infective to astrocytes, thereby increasing their neurovirulence. In support of this, FIV-PPR, which has been shown to cause neurological disease in experimentally infected cats, does not require interaction with the CD134 molecule for cell entry. In contrast, FIV-CPGammar can only infect cells that express the CD134 receptor, and this virus is uncommonly associated with neurological disease in infected cats (Willett and Hosie, 2013).

In vitro studies comparing neurotoxicity between different FIV variants have shown that envelope glycoprotein from FIV-PPR is more neurotoxic than envelope glycoprotein from FIV-Petaluma (Bragg *et al.*, 1999). Similarly, infection with FIV-MD *in vitro* leads to more profound impairment of glutamate uptake by astrocytes when compared to cells infected with FIV-Petaluma (Zenger *et al.*, 1997). These studies indicate that genetic variation at the *env* gene may account for some of the differences in neurovirulence.

1.5.5. Lymphocyte apoptosis

As previously discussed in section 1.4.1.2, FIV causes apoptosis of uninfected 'bystander' lymphocytes within lymph nodes, largely contributing to the loss of CD4+ T cells. The mechanism is multifactorial, but there does appear to be a direct effect of the envelope glycoprotein in inducing apoptosis (Garg *et al.*, 2004b). Mutations of the *env* gene may therefore influence pathogenicity if they alter the pro-apoptotic potential of the envelope glycoprotein. Experimentally, mutations affecting the short cytoplasmic domain of gp35 have been shown to interfere with fusion capacity (Celma *et al.*, 2007). As fusion is a pre-requisite for envelope glycoprotein-mediated apoptosis, it is possible that such mutations may affect the ability of the

virus to induce apoptosis in bystander lymphocytes. Such FIV variants would therefore cause a less profound decline in the CD4+ T cell count. In support of this, Hosie and others (2002) demonstrated that the GL8 strain of FIV induced a more rapid decline of CD4+ T cells in infected cats when compared the Petaluma strain. *In vitro* studies are required to determine whether this is related to an increased ability of the GL8 strain to induce apoptosis via an envelope glycoprotein-mediated mechanism. There is one such study comparing the apoptotic capacity of FIV variants from different felid species (FIV-PPR from the domestic cat and FIV-Oma from a wild Pallas cat), using a flow-cytometric assay on MYA-1 cells (Sutton *et al.*, 2005). The authors demonstrated that more cells underwent apoptosis following exposure to FIV-PPR compared to FIV-Oma. Similar studies comparing apoptosis induced by different domestic cat variants of FIV are warranted, in order to determine the impact of this factor on the variability in disease progression.

1.6. Natural immunity against FIV

Soon after initial infection with FIV, the host generates an immune response against the virus that involves both cell-mediated and humoral components. Although unable to eliminate infection completely, the host immune response is capable of suppressing viral replication and clearing some infected cells and viral particles. The immune response therefore maintains a low viral load, allowing the infected cat to remain asymptomatic for a variable period of time. Over time, probably due to progressive immune dysfunction, the immune response becomes less effective and viral replication increases. This corresponds to progression of infection into the clinical stages of the disease. The time course of FIV infection therefore depends largely on the ability of the host to maintain a low viral load.

1.6.1. Intrinsic anti-FIV immunity

Cellular restriction factors are proteins, such as APOBEC3 (apolipoprotein B mRNA-editing catalytic polypeptide 3), Trim5α (tripartite motif protein), and tetherin, that are constitutively expressed in host cells (Muenk *et al.*, 2010). Following infection with FIV, these

restriction factors are packaged into virions and inhibit virus replication, thus limiting dissemination of the virus within the host. APOBEC3, for example, impairs viral replication by deaminating cytosine residues in RNA and single-stranded DNA, resulting in mutations in the newly synthesised nucleic acid (Taniwaki *et al.*, 2013). FIV has evolved to evade this defence mechanism, by degrading APOBEC3 via the accessory protein, viral infectivity factor (Vif), encoded by the *vif* gene. This prevents the packaging of APOBEC3 proteins into virions. Recently, FIV was also shown to be able to degrade APOBEC3 released in virions via the viral Protease (Yoshikawa *et al.*, 2017). There appears to be variation in the effectiveness of the APOBEC3 mechanism, with one variant of APOBEC3 reported to be able to overcome degradation by Vif (Yoshikawa *et al.*, 2016). In addition, the anti-APOBEC3 activity of Vif in one strain of FIV (subtype B, TM2) has been shown to be attenuated compared to 3 other isolates of subtypes A, C and D (Yoshikawa *et al.*, 2017).

1.6.2. Innate anti-FIV immunity

Within one week post-infection, potent antiviral activity can be detected from CD8+ T cells of infected cats (Flynn *et al.*, 2002). The rapidity of this response, as well as its non-MHC restricted nature, suggest that it forms part of the host's innate defence mechanism (Hohdatsu *et al.*, 1998). Numerous studies have confirmed that the factors involved in this early response are generated from non-cytolytic CD8+ T cells, and that it is mediated by a soluble factor, in a dose-dependent fashion. Hohdatsu and others (1998) showed that FIV replication *in vitro* was enhanced when PBMC from infected cats were depleted of CD8+ T cells prior to culture – an effect that was abrogated following the re-introduction of the CD8+ T cells. Likewise, Flynn and others (1999) demonstrated suppression of FIV replication in MYA-1 cells *in vitro*, following co-culture with PBMC, but not CD8+ depleted PBMC, from FIV infected cats. In both these studies, infected cells were not destroyed, and the antiviral effect was not dependent on cell-cell contact (suggesting a soluble factor was involved). Choi and others (2000) showed that the soluble antiviral factor could be generated from CD8+ T cells from uninfected cats, following exposure to FIV antigen *in vitro*. Since then, other authors have demonstrated that stimulation of CD8+ T

cells with FIV antigen presenting cells are required to elicit the antiviral response (Phadke *et al.*, 2004, Li *et al.*, 2005).

Suppression of viral replication by soluble factors is thought to occur at the level of FIV mRNA synthesis. In the presence of the anti-FIV soluble factor, proviral DNA continues to be integrated into the host cell, but viral particles are not released by the cell (as shown by reduced viral RNA in cell culture supernatants) (Hohdatsu *et al.*, 2002). The exact nature of the soluble antiviral factor is currently unknown. There is evidence that multiple factors are actually involved, and that these act together to produce the antiviral effect (Li *et al.*, 2005). Previous studies attempting to correlate antiviral activity with interferon production have excluded both IFN-α and IFN-γ as causative factors (Li *et al.*, 2005). Based on molecular size of the antiviral factors, α and β chemokines are also unlikely to be involved (Li *et al.*, 2005). The innate antiviral response declines over time, and this is associated with an increase in proviral load and plasma viraemia, as well as a decrease in CD4+ T cell counts (Hohdatsu *et al.*, 2005, Hohdatsu *et al.*, 2003b). It is likely that the non-cytolytic T cell response is at least partly responsible for controlling viral replication in the asymptomatic stage of FIV.

1.6.3. Acquired anti-FIV immunity

1.6.3.1. Cell-mediated response

FIV-specific, MHC class-I restricted cytotoxic T lymphocytes (CTL) are detected in the blood of FIV infected cats within 2-7 weeks post infection (Flynn *et al.*, 2002, Beatty *et al.*, 1996, Song *et al.*, 1992). The presence of effector cells in circulation declines as infection progresses, with effector cells limited to the lymph nodes in the chronic stages (Beatty *et al.*, 1996). *In vitro* studies using env- and gag-expressing target cells demonstrate that MHC I molecules containing gag derived peptides elicit the strongest CTL response (Flynn *et al.*, 2002, Beatty *et al.*, 1996). In particular, conserved gag capsid epitopes are targeted by cytotoxic lymphocytes, and this may confer broad specificity to the response (Song *et al.*, 1995).

1.6.3.2. Humoral response

Antibodies against FIV are detected in serum within 4-6 weeks post-infection (de Ronde *et al.*, 1994, English *et al.*, 1994). Multiple linear B-cell epitopes have been identified within the gag protein and the surface and transmembrane regions of the envelope glycoprotein, however the majority of these epitopes do not induce neutralising antibodies (Lombardi *et al.*, 1993, Richardson *et al.*, 1996, Pancino *et al.*, 1993a, Lombardi *et al.*, 1994). The presence of virus-neutralising antibodies (VNA) is variable in infected cats (Hosie *et al.*, 2011, Inoshima *et al.*, 1996). Early studies showed rapid development of broadly neutralising antibodies post-infection, and authors emphasized the importance of an epitope within the V3 loop of gp100 (which corresponds to the CXCR4 binding site of the surface component of the *env* gene product) (Richardson *et al.*, 1996, Lombardi *et al.*, 1993, Sundstrom *et al.*, 2008). These studies utilised CRFK cell-based systems for detection of VNA, therefore requiring the use of laboratory adapted CD134-independent strains of FIV (de Parseval *et al.*, 2006, Lombardi *et al.*, 1993, de Ronde *et al.*, 1994). The CXCR4 binding site (as well as epitopes in this region) is persistently exposed on CD134-independent strains, allowing antibodies directed against the V3 region to bind and neutralise the virus *in vitro* (Richardson *et al.*, 1996). In contrast, the V3 region of the surface unit is generally hidden on field viruses prior to binding of the CD134 receptor. This explains the ineffectiveness of env-directed VNA against viruses in naturally infected cats, as the majority of field viruses are resistant to these antibodies (Hosie *et al.*, 2011).

Experiments demonstrating VNA using a T-cell based assay (rather than CRFK cells) are more applicable to natural infection, as these allow the use of viruses that are representative of field variants (as opposed to CRFK cell-based systems that require the use of laboratory adapted strains). Using T-cell based assays, epitopes located within the highly variable V4 and V5 regions of gp100 have been shown to be more important in virus neutralisation (Siebelink *et al.*, 1995b, Hosie *et al.*, 2011). However, the majority of cats show a response that is narrow in specificity and the presence of broad, cross-subtype neutralising sera is rare (0.9% of cats) (Hosie *et al.*, 2011, Siebelink *et al.*, 1993, Beczkowski *et al.*, 2015a). In addition, the emergence of VNA

resistant variants has been shown to occur over time with virus evolution *in vivo*, and this phenotype is associated with mutations in the V5 loop of gp100 (Willett *et al.*, 2010). Escape from the humoral immune response may occur with chronicity of infection, contributing to disease progression.

Non-neutralising antibodies may also contribute to anti-FIV immunity. Complement mediated lysis of viral particles has been demonstrated by incubating FIV with complement and serum from infected cats (Fevereiro *et al.*, 1993). Incubation with heat-inactivated serum or complement alone fails to induce virolysis (as shown by an RT release assay in cell-free supernatant), suggesting an antibody-dependent mechanism of complement activation. Non-neutralising antibodies may also inhibit essential viral enzymes, preventing viral replication. For example, antibodies from cats infected with FIV for more than 1-2 years have been shown to specifically block FIV reverse transcriptase, inhibiting its activity *in vitro* by up to 78% (Fevereiro *et al.*, 1991). Further study is warranted to investigate the role of non-neutralising antibodies in anti-FIV immunity.

Autoantibodies directed against the CD134 receptor have been described in FIV infected cats. In a study of FIV infected cats, 63% had detectable anti-CD134 antibodies, and their presence was not detected in FIV negative cats that were healthy or infected with other viruses (Grant *et al.*, 2009). These antibodies were able to prevent FIV infection *in vitro* by displacing the virus prior to cell entry, but only after binding of FIV to CD134 induced a conformational change on CD134 that exposed the anti-CD134 antibody epitope (Grant *et al.*, 2009). The significance of anti-CD134 antibodies remains unclear, but their presence does seem to correlate with reduced viral load and better overall health status (Grant *et al.*, 2009). It is likely that they play a role in controlling infection for prolonged periods during the asymptomatic stage.

1.7. FIV vaccination

Since the discovery of FIV, researchers have been attempting to develop a safe and effective vaccine to protect cats against infection with FIV. Numerous vaccine candidates have

been trialled over the years, but few have shown acceptable efficacy against challenge with FIV. Subunit vaccines have been largely unsuccessful, whilst recombinant vector vaccines, live attenuated virus vaccines and DNA vaccines have shown only limited efficacy against challenge with vaccine strains, and this has been previously reviewed by Uhl *et al.* (2002). The most success has been achieved using whole inactivated virus. In particular, a dual-subtype vaccine has afforded the most protection, and this has led to the development and commercialisation of the Fel-O-Vax® FIV vaccine (Fort Dodge Animal Health, Fort Dodge, IA). For the remainder of this section, the discussion will focus on the Fel-O-Vax® FIV vaccine and its prototypes. Studies of the other vaccine candidates have been reviewed by Leocollinet & Richardson (2008).

1.7.1. Evidence for efficacy of the Fel-O-Vax® FIV vaccine

The dual-subtype, Fel-O-Vax® FIV vaccine was first released in 2002 in the USA, and has since been registered for use in Canada, Australia, New Zealand and Japan (Yamamoto *et al.*, 2010). It is manufactured using paraformaldehyde inactivated whole virus and killed FIV-infected cells (Uhl *et al.*, 2002). Two strains of FIV are currently included in the vaccine, one from subtype A (the Petaluma strain, FIV-Pet) and one from subtype D (the Shizuoka strain, FIV-Shi) (Yamamoto, 2002). The use of a dual-subtype vaccine was hoped to broaden immunity, eliciting humoral and cell-mediated responses that are cross-reactive against FIV variants from different subtypes (Omori *et al.*, 2004, Kusuhara *et al.*, 2005, Yamamoto *et al.*, 2007, Pu *et al.*, 2001, Pu *et al.*, 2005, Hohdatsu *et al.*, 1997b, Huang *et al.*, 2004). A summary of the dual-subtype vaccine efficacy studies to date (including Fel-O-Vax® FIV and its prototypes) are presented in **Table 1-3**.

Table 1-3 Summary of the dual-subtype FIV vaccine efficacy studies.
Studies are listed according to the challenge virus in order to highlight differences in vaccine efficacy against different FIV variants. Viruses included in the vaccine are named homologous viruses, whereas all other challenge viruses are named heterologous, as they are genetically different to the vaccine virus. The subtype and country of origin are listed for each challenge virus, and details of the studies are included for comparison. The preventable fraction is listed for each study and an overall mean preventable fraction is calculated as the average value for each challenge virus. There are clearly differences in vaccine efficacy according to the challenge virus.

Type of challenge[a]	Virus	Sub-type	Country of origin	Ref.[b]	Vaccine[c]	Route[d]	Method of detection[e] (sample[f])	Time (wk)[g]	Dose (CID_{50})[h]	Vacc. infected (%)	Controls infected (%)	PF[i]	Mean PF[j]
Homologous	Petaluma (FIV-Pet)	A	U.S.A.	1	Prototype (KIC + A-MDP)	IP	VI (PBMC)	2	10	0/4 (0)	4/4 (100)	100	100
				2	Prototype (IWV + FD-1)	IV	VI (PBMC, LN, BM), PCR (PBMC, LN, BM)	3	20	0/6 (0)	9/9 (100)	100	
									50	0/4 (0)	5/5 (100)	100	
				3	Fel-O-Vax® FIV	IV	VI (PBMC), PCR (PBMC)	4	20, 50	0/12 (0)	16/16 (100)	100	
				4	Prototype (IWV)	?	VI (PBMC, LN, BM), PCR (PBMC, LN, BM)	?	25	0/3 (0)	4/4 (100)	100	
									50	0/4 (0)	4/4 (100)	100	
				10	Prototype (IMV + FD-1)	IV	VI (PBMC), PCR (PBMC)	3-4	?	0/6 (0)	6/6 (100)	100	
	Shizuoka (FIV-Shi)	D	U.S.A.	1	Prototype (KIC+A-MDP)	IP	VI (PBMC)	2	10	0/4 (0)	4/4 (100)	100	100
Heterologous	FIV-FD/US	A	U.S.A.	3	Fel-O-Vax® FIV	IM	PCR (PBMC)	52	1.47	9/27 (33)	25/34 (74)	54.7	68.4
				5	Fel-O-Vax® FIV	IM	VI (PBMC), PCR (PBMC)	52	11	4/25 (16)	17/19 (89)	82.1	

	FIV-FD/DutA	A	Nether-lands	3	Fel-O-Vax® FIV	IM	?	52	1.73	3/24 (13)	13/15 (87)	85.6	85.6
	Glasgow 8 (GL8)	A	U.K.	3	Prototype (IWV)	IV	VI (PBMC), PCR (PBMC)	4	10	9/15 (60)	15/15 (100)	40	26.7
				6	Fel-O-Vax® FIV	IM	VI (PBMC), RT-PCR (plasma)	4	10	6/6 (100)	5/5 (100)	0	
				10	Prototype (IMV + FD-1)	IV	VI (PBMC), PCR (PBMC)	3-4	?	12/20 (60)	20/20 (100)	40	
	FIV-FC1	B	U.S.A.	3	Fel-O-Vax® FIV	IV	VI (PBMC), PCR (PBMC)	4	15	2/21 (10)	16/16 (100)	90.5	87.3
				7	Fel-O-Vax® FIV	IV	VI (PBMC, LN, BM),	3	15	0/8 (0)	9/9 (100)	100	
				8	Fel-O-Vax® FIV	IV	PCR (PBMC, thymus, spleen, LN, BM)	52	1000 PBMC[k]	4/14 (29)	5/5 (100)	71	
				10	Prototype (IMV + FD-1)	IV	VI (PBMC), PCR (PBMC)	3-4	?	1/4 (25)	10/10 (100)	75	
					Fel-O-Vax® FIV	IV	VI (PBMC), PCR (PBMC)	3-4	?	0/8 (0)	10/10 (100)	100	
	Amori-2 (FIV-Ao2)	B	Japan	9	Fel-O-Vax® FIV	Co	PCR (PBMC)	2 – 82[l]	N/A	0/6 (0)	4/8 (50)	100	100
	Bangston (FIV-Bang)	A/B recom.	U.S.A.	2	Prototype (IWV + FD-1)	IV	VI (PBMC, LN, BM), PCR (PBMC, LN, BM)	3	10	1/5 (20)	4/4 (100)	80	55.3
									100	3/5 (60)	5/5 (100)	40	

				3	Fel-O-Vax® FIV	IV	VI (PBMC), PCR (PBMC)	4	10, 25, 100	11/28 (39)	23/23 (100)	60.7	
				10	Prototype (IMV + FD-1)	IV	VI (PBMC), PCR (PBMC)	3-4	?	4/14 (29)	10/10 (100)	71	
					Fel-O-Vax® FIV	IV	VI (PBMC), PCR (PBMC)	3-4	?	3/4 (75)	4/4 (100)	25	
	FIV-NZ1	F'/C recom.	New Zealand	3	Fel-O-Vax® FIV	IV	VI (PBMC), PCR (PBMC)	4	50	5/9 (56)	6/6 (100)	44.4	53.1
				10	Prototype (IMV + FD-1)	IV	VI (PBMC), PCR (PBMC)	3-4	?	2/8 (25)	10/10 (100)	75	
					Fel-O-Vax® FIV	IV	VI (PBMC), PCR (PBMC)	3-4	?	3/5 (60)	10/10 (100)	40	

[a] Challenge type – homologous (vaccine virus), heterologous (non-vaccine virus)
[b] References – 1 (Hohdatsu *et al.*, 1997b), 2 (Pu *et al.*, 2001), 3 Unpublished studies from the University of Florida, referenced by Yamamoto and others (2010), 4 (Omori *et al.*, 2004), 5 (Huang *et al.*, 2004), 6 (Dunham *et al.*, 2006b), 7 (Pu *et al.*, 2005), 8 (Huang *et al.*, 2010), 9 (Kusuhara *et al.*, 2005), 10 (Coleman *et al.*, 2014).
[c] Vaccine type – Killed FIV-infected cells (KIC), inactivated whole virus (IWV), adenyl-muramyl dipeptide adjuvant (A-MDP), Fort-Dodge proprietary adjuvant (FD-1), combination of KIC and IMV with FD-1 (Fel-O-Vax® FIV).
[d] Route of challenge – Intraperitoneal (IP), intravenous (IV), intramuscular (IM), contact with infected cats only (Co).
[e] Method of virus detection – Virus isolation (VI), polymerase chain reaction to detect provirus (PCR), polymerase chain reaction to detect viral RNA (RT-PCR).
[f] Sample – peripheral blood mononuclear cells (PBMC), lymph node biopsy (LN), bone marrow biopsy (BM).
[g] Time – The number of weeks between vaccination and viral challenge.
[h] Challenge dose – Number of median cat infectious doses (CID_{50}).
[i] Preventable fraction % (PF) = (% controls infected - % vaccinates infected) / % controls infected x 100.
[j] Mean preventable fraction (%) for each variant of FIV.
[k] 1000 PBMC from an infected cat used as inoculum.
[l] Vaccinated and control cats were housed together with FIV-infected cats for a period of 1.5yrs, beginning 2wks after final vaccination.

All of the published efficacy studies on the dual-subtype (prototype and Fel-O-Vax® FIV) vaccine report a 100% preventable fraction (PF) against the vaccine strains, FIV-Pet and FIV-Shi (Hohdatsu *et al.*, 1997b, Pu *et al.*, 2001, Yamamoto *et al.*, 2010, Omori *et al.*, 2004). The PF of the vaccine is defined as the percentage of vaccinated versus unvaccinated cats that are protected from FIV infection following challenge with the virus. So in this case, 100% of vaccinated cats were protected against infection with homologous virus when compared to the unvaccinated control population. However, efficacy against heterologous challenge (using variants not included in the vaccine) is highly variable, with the reported PF ranging from 0% to 100% (Pu *et al.*, 2001, Yamamoto *et al.*, 2010, Huang *et al.*, 2004, Dunham *et al.*, 2006b, Pu *et al.*, 2005, Huang *et al.*, 2010, Kusuhara *et al.*, 2005). Even the two trials performed to obtain United States Department of Agriculture (USDA) approval for the vaccine reported variable efficacy, with one study of 44 cats demonstrating a PF of 82.1%, but the other reporting only 54.7% efficacy against the same heterologous subtype A variant of FIV (FIV-US/FD) (Huang *et al.*, 2004). It is unclear what factors may have influenced these differing results, especially as the study reporting a 54.7% efficacy remains unpublished, with only one reference to it in the literature (Yamamoto *et al.*, 2010). Some studies report good cross-protective immunity against heterologous virus of subtypes A and B. For example, one study reported a PF of 85.6% against a Dutch, subtype A virus (FIV-FD/DutA) (Yamamoto *et al.*, 2010). Similarly, the studies that challenged vaccinated cats with a subtype B virus from the U.S.A. (FIV-FC1) showed a PF ranging from 71 to 100% (Yamamoto *et al.*, 2010, Pu *et al.*, 2005, Huang *et al.*, 2010, Coleman *et al.*, 2014). Kusuhara and others (2005) designed a study that mimicked the natural transmission of FIV, by housing vaccinated and unvaccinated cats with cats infected with the subtype B virus, Amori-2 (FIV-Ao2). They also reported a PF of 100%. In contrast to these studies demonstrating promising efficacy of the Fel-O-Vax® FIV vaccine, other trials have demonstrated poor efficacy against some variants of FIV. On average, when vaccinated cats were challenged with a subtype A/B recombinant Bangston virus (FIV-Bang), only 55.3% of cats were protected (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014). Similarly, challenge of Fel-O-Vax® FIV vaccinated cats with a New

Zealand F'/C recombinant virus[2] (FIV-NZ1) resulted in an average PF of only 42.2% (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014). Perhaps more concerning however, was the complete lack of protection (PF of 0%) and apparent enhancement of infection seen when Fel-O-Vax® FIV vaccinated cats were challenged with the subtype A isolate, Glasgow 8 (FIV-GL8) (Dunham *et al.*, 2006b). When compared to the unvaccinated cats, the vaccinated cats experienced increased viral loads following infection with this virus – a phenomenon has been reported previously, following vaccination and homologous challenge with other FIV vaccines (Hosie *et al.*, 1992, Siebelink *et al.*, 1995d). Two studies examining the efficacy of the dual-subtype prototype vaccine (containing whole inactivated virus only) also reported poor efficacy against the FIV-GL8 virus (PF 40%), suggesting that there may be certain properties associated with this virus that allow escape from vaccine-induced immunity (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014).

Differences in the design of efficacy studies may explain some of the discordance in the literature regarding efficacy of the Fel-O-Vax® FIV vaccine. For example, the route of virus inoculation (i.e. intramuscular versus intravenous) would affect antigen presentation, possibly influencing susceptibility of the virus to vaccine-induced immunity. Also, the viral titre of the challenge inoculum is probably important, as efficacy of the vaccine appears to be reduced with high-dose challenge. This was demonstrated by Pu and others (2001) when vaccinated cats were challenged with 10 and 100 median cat infectious doses (CID_{50}) of a heterologous virus (FIV-Bang). Increasing the challenge dose by a factor of 10 decreased the PF from 80% to only 40%. The infectious dose received during natural transmission has been suggested to be closer to 0.8 CID_{50} (Yamamoto *et al.*, 2010), so the clinical significance of high-dose challenge studies is questionable.

Aside from these factors, there are apparent differences in vaccine efficacy against genetically distinct variants of FIV (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014). Although there is some evidence of cross-protective immunity against heterologous variants, there is just as much

[2] FIV-NZ1 is a recombinant virus, with the *env* gene clustering with subtype C, and the *pol* and *gag* genes belonging to an unknown subtype (designated F')

evidence to the contrary, casting doubt on the true efficacy of the vaccine in the field. The apparent poor efficacy against the subtype A variant, FIV-GL8, is interesting, as this virus was isolated from an asymptomatic cat in the early stages of infection. In contrast, many of the other challenge viruses were isolated from sick cats, late in infection (Yamamoto *et al.*, 2010, Hosie and Beatty, 2007). Previous authors have suggested that variants isolated from cats early in infection are more representative of the types of viruses transmitted in the field, as asymptomatic cats are more likely to be roaming and fighting with other cats (Hosie and Beatty, 2007). It is therefore important that the commercial vaccine is able to protect against such field viruses.

1.7.2. Vaccine-induced immunity

The question of efficacy of the Fel-O-Vax® FIV vaccine is complicated by a lack of knowledge regarding vaccine-induced immunity. It is not imperative that the mechanism of immunity is understood in order to recommend a vaccine, however, for the Fel-O-Vax® FIV vaccine, this information may help to predict the likely cross-reactivity of the vaccine-induced immune response because of the diversity of the virus in the field. The inclusion of whole virus and infected cells in the vaccine hinders our ability to determine the mechanisms important for protection, and protective epitopes of FIV have not yet been elucidated. In addition, there are no natural correlates of sterilising immunity with which to compare vaccine-induced immunity, as cats do not recover from infection. The information gathered from investigations thus far suggests that both humoral and cell-mediated components are important for protective vaccine-induced immunity.

It is clear from numerous studies that FIV vaccination induces FIV-specific antibodies. What is not clear however, is their relative importance in protecting against challenge with FIV. Antibodies against p24, gp100 and gp35 are detected using ELISA soon after vaccination with Fel-O-Vax® FIV (Yamamoto *et al.*, 1993, Hohdatsu *et al.*, 1997b). Previous authors have shown that passive transfer of partially purified antibodies from Fel-O-Vax® FIV vaccinated cats protected unvaccinated cats against challenge with homologous FIV-Pet (PF 87.5 – 100%). In contrast, challenge of similar unvaccinated, transfused cats with the heterologous

subtype B virus, FIV-FC1, resulted in no protection (PF 0%) (Uhl *et al.*, 2008, Coleman *et al.*, 2014). Protection against homologous challenge correlated with the presence of VNA to FIV-Pet but not FIV-FC1. As shown previously in **Table 1-3**, the Fel-O-Vax® FIV does in fact protect some cats against challenge with FIV-FC1 (PF 71 – 100%) (Yamamoto *et al.*, 2010, Pu *et al.*, 2005, Huang *et al.*, 2010). These results suggest that the humoral response is responsible for vaccine-induced protective immunity against some, but not all variants of FIV.

In support of this, studies measuring the VNA titre in Fel-O-Vax® FIV vaccinated cats demonstrate the presence of VNA to homologous, but not heterologous viruses. For example, Coleman and others (2014) reported positive VNA titres (1/>10) to FIV-Pet and FIV-Shi in cats vaccinated with the Fel-O-Vax® FIV vaccine. In contrast, minimal to no neutralising titres were present against the heterologous viruses, FIV-GL8, FIV-FC1 and FIV-NZ1. These results did not correlate with protection against FIV-FC1 challenge in this same study however, as all vaccinated cats were protected against infection despite the absence of VNA against this virus. Furthermore, Pu and others (2001) showed that the presence or absence of VNA did not correlate with protection from homologous (FIV-Pet) or heterologous (FIV-Bang) challenge. Although induced by the Fel-O-Vax® FIV vaccine, VNA display narrow specificity and are therefore unlikely to cross-protect against a broad range of FIV variants. The importance of non-neutralising antibodies induced by the Fel-O-Vax® FIV vaccine is currently unknown. Further studies are required to determine if other mechanisms of humoral immunity (such as antibody dependent cell-mediated cytotoxicity) contribute to cross-protective immunity against heterologous challenge.

There is evidence that vaccination with the Fel-O-Vax® FIV vaccine also elicits a cell-mediated response, and that this response may have broader specificity than the humoral response. This was suggested by Pu and others (2006) when they challenged unvaccinated cats with FIV-Pet, following adoptive transfer of purified PBMC from fully or partially MHC-matched, Fel-O-Vax® FIV vaccinated cats. Three out of four cats receiving purified CD4+ and CD8+ T cells, two of out three cats receiving purified CD4+ T cells and two out of three cats receiving purified CD8+ T cells were protected from infection. In contrast, none of cats receiving purified B cells

from vaccinated cats, or CD4+ and CD8+ T cells from unvaccinated cats were protected (Pu *et al.*, 2006). In a similar study, unvaccinated cats were challenged with the heterologous, subtype A virus, FIV-FC1. Three out of five cats receiving purified CD4+ and CD8+ T cells from MHC-matched, Fel-O-Vax® FIV vaccinated cats were protected from infection, whereas none of the cats receiving CD4+ and CD8+ T cells from vaccinated, unmatched cats or unvaccinated cats were protected (Yamamoto J.K., 2009). These studies provided evidence for an MHC-restricted, FIV-specific immunity which was at least partially protective against heterologous challenge. Other authors have demonstrated increased production of IFN-γ, IL-2 and perforin from T cells post-vaccination, following stimulation with homologous virus (FIV-Pet or FIV-Shi) *in vitro* (when compared to media stimulated cells) (Pu *et al.*, 2001, Omori *et al.*, 2004). These studies suggest the presence of an FIV-specific, TH1 and cytotoxic response induced by the Fel-O-Vax® FIV vaccine.

From this information, we can see that the Fel-O-Vax® FIV vaccine induces both a humoral and cell-mediated immune response. What remains to be determined is the relative importance of each component in protecting against a broad range of FIV variants. It seems likely that the humoral and cellular immune responses are synergistic in protecting vaccinated cats against infection. Antibodies may be responsible for clearance of virus from the circulation, whilst cellular components +/- antibodies are probably necessary for elimination of FIV infected cells.

1.7.3. The impact of genetic diversity of FIV on the efficacy of the Fel-O-Vax® FIV vaccine

As already discussed in section 1.7.1, the efficacy of the Fel-O-Vax® FIV is not equal against all variants of FIV. The identity of those epitopes important for protective immunity following vaccination has not yet been established. However, if such epitopes are encoded by non-conserved regions of the genome (such as hypervariable regions of the *env* gene), then poor cross-reactivity of the vaccine-induced immune response against different variants may explain the variability in efficacy.

Section 1.6.3.2 discussed the emergence of VNA-resistant variants of FIV as a result of mutation of the *env* gene (Siebelink *et al.*, 1993, Siebelink *et al.*, 1995a, Willett *et al.*, 2010). Similar mutations may also confer resistance against vaccine-induced VNA. Evolution of the *env* gene could affect viral susceptibility to a cell-mediated response in vaccinated cats, as previous authors have demonstrated that protective immunity against homologous challenge was correlated with the presence of *env*-specific cytotoxic T lymphocytes (Hosie and Flynn, 1996, Flynn *et al.*, 1996). In that study, cats were vaccinated cats with a single subtype vaccine, however further work is underway to map the T cell epitopes important in protective immunity induced by the dual-subtype vaccine. So far, stimulation of T cells from dual-subtype vaccinated cats with RT or a combination of RT and p24 induced T cell proliferation, indicative of recognition of T cell epitopes on these peptides. Furthermore, vaccination of cats with an RT/p24 peptide combination protected cats against low dose, heterologous challenge with a subtype B virus (Yamamoto *et al.*, 2010). From these studies, we can conclude that important epitopes for the dual-subtype vaccine-induced immune response are located on some *pol* and *gag* proteins. Other important epitopes are probably also present on the envelope glycoprotein, as demonstrated in the single-subtype vaccine studies described above. Although cross-reactivity of the vaccine-induced response is more likely with epitopes located on the *gag* and *pol* proteins, this cannot be assumed, as genomic sequence variation occurs even at these locations (Hayward and Rodrigo, 2008).

Given the inconsistent results of previous challenge studies, and the uncertain importance of epitopes encoded by highly variable regions of the genome, further studies are required to confirm the efficacy of the Fel-O-Vax® FIV vaccine against different variants of FIV. Subtype classification does not appear to be predictive of protection [as shown by the resistance of subtype A FIV-GL8 to immunity induced by the subtype A vaccine strain (Dunham *et al.*, 2006b), and infection of vaccinated cats with subtype A virus (Westman *et al.*, 2016a)], so further studies challenging vaccinated cats with different subtypes of FIV are unlikely to provide definitive answers. Experiments that further analyse the mechanisms of vaccine-induced immunity, identify

the exact location of important epitopes and assess the cross-reactivity of the vaccine-induced response would provide more useful information in determining vaccine efficacy.

1.7.3.1. Assays to assess cross-reactivity of vaccine-induced immune responses

In vitro assays using different isolates of FIV may enable assessment of cross-reactivity of the Fel-O-Vax® FIV vaccine-induced immunity against a wider range of variants than *in vivo* studies. Previous studies have assessed the cross-reactivity of VNA as previously discussed in section 1.7.2 (Pu *et al.*, 2001, Hohdatsu *et al.*, 1997b). Determining the cross-reactivity of the cell-mediated response is more difficult, and to the author's knowledge, this has not yet been performed. Chromium-51 release cytotoxic assays are laborious and require the use of a radioactive isotope. Other assays measuring cytokines and cytotoxic mediators provide only indirect information about the cell-mediated response. An assay measuring cross-reactivity of the cell-mediated response must be simple and direct, in order to screen a number of isolates of FIV.

Flow cytometric measurement of cell-surface marker expression has been used to demonstrate antigen-specific T cell activation in humans (Hanevik *et al.*, 2011, Kahi *et al.*, 1998, Caruso *et al.*, 1997). Expression of the activation antigen, CD25, in response to *in vitro* antigen exposure parallels results obtained in cell proliferation assays (Caruso *et al.*, 1997). High sensitivity and specificity has been reported for use of this assay to predict previous exposure to pathogens eliciting a cell-mediated immune response (e.g. *Toxoplasma gondii*) (Kahi *et al.*, 1998). In addition, increased expression of CD25 on CD4+ T lymphocytes has been observed in atopic dogs following exposure to allergens (Simpson *et al.*, 2009). In peripheral blood, CD25 (the alpha chain of the interleukin-2 receptor) is expressed on activated T and B cells, T regulatory cells, monocytes, NK cells and a subset of dendritic cells (Kindt T.J., 2007). It is not expressed on resting T and B cells (Smith, 1988). Resting T cells express a low-affinity IL-2 receptor, comprised of beta and gamma chains. Following cellular activation, the alpha chain (CD25) is co-expressed, increasing the affinity of the receptor for IL-2 (Willerford *et al.*, 1995). Assays measuring antigen-induced expression of CD25 have been used previously to determine the cross-reactivity of the vaccine-induced cell-mediated response in pigs vaccinated against swine

influenza virus. In one such study, a T cell memory response against heterologous virus (as shown by CD25 expression) was demonstrated following stimulation of vaccinated PBMC with wild type virus (Kappes *et al.*, 2012). A similar assay may be useful to determine cross-reactivity of the Fel-O-Vax® FIV vaccine-induced immune response.

The concern with *in vitro* assays is that results may not correlate with immunity *in vivo*. For this reason, challenge studies are usually performed as a final step in determining vaccine efficacy. Although numerous challenge studies have been performed for FIV vaccination, these experiments have an ethical cost and are expensive and laborious. In addition, large numbers of viruses and cats would be required to draw any useful conclusions regarding the specificity of the Fel-O-Vax® FIV vaccine-induced immune response. The use of a delayed-type hypersensitivity (DTH) response to viral antigen may be a viable alternative for this purpose.

The DTH response is characterised by a firm cutaneous swelling, developing 24-48 hours after intradermal injection of antigen into a sensitised individual (Crowle, 1975). During the induction phase of this reaction, memory CD4+ T cells (elicited by vaccination) recognise antigen presented on MHC molecules of antigen presenting cells. The memory cells become activated, generating TH1 effector cells that accumulate around the antigen deposit. Release of cytokines (such as IFN-γ and IL-2) and chemokines result in recruitment of inflammatory cells and activation of macrophages that phagocytose antigen. The resultant mononuclear infiltrate is detected on the surface of the skin as a discrete swelling, and this can be measured to quantify the response. The DTH reaction occurs only with prior antigen exposure, following development of an antigen-specific population of memory T cells. Non-immune animals will not generate a detectable swelling, enabling assessment of efficacy of vaccine-induced cell-mediated immunity. For a positive response to occur, antigen processing, presentation, cytokine production and effector responses must be functional. Therefore, the DTH response evaluates all components of the T-cell response (Tizard, 2009, Pier, 2004).

The DTH response to injected antigen has been used previously for diagnosis of infections that induce a strong cell-mediated immune response. For example, the tuberculin skin test has been extensively used for over 100 years to diagnose tuberculosis in humans and cattle (Anders, 1900, Delepine, 1900). The DTH response has also been used to evaluate vaccine efficacy against diseases such as tuberculosis, cryptococcosis and others (Lee *et al.*, 2011, Nichols *et al.*, 2002). Cats have been shown to develop a DTH response to feline herpesvirus, *Yersinia pseudotuberculosis* and mixed antigen derived from vaccines (Fargeaud *et al.*, 1984, Otto *et al.*, 1993, Cave *et al.*, 2007). It has also been used to assess the efficacy of a vaccine candidate against FIP (Hohdatsu *et al.*, 2003a). To the author's knowledge, this test has not been used to assess vaccine efficacy against FIV. The simplicity of this test, together with its relevance to immunity *in vivo*, makes the DTH response a promising option to assess cross-reactivity of the cell-mediated response against different variants of FIV.

1.8. FIV in New Zealand

The overall aim of the research in this thesis was to investigate the relevance of the variability of FIV in regard to pathogenicity and vaccination, specifically in NZ. In order to do this, an understanding of the epidemiology and genetic characteristics of FIV in NZ was required.

Results from prevalence studies of FIV in NZ are similar to those from other countries with large populations of stray and feral cats. In 1988, pet cats of variable health status had an overall FIV prevalence of 14.4% (Swinney *et al.*, 1989). When health status was taken into account, FIV occurred in 6.8% of healthy pet cats, versus 27.3% of sick pet cats (Swinney *et al.*, 1989). As is shown in other studies, feral cats in NZ have a higher prevalence of FIV when compared to pet cats, ranging from 11 to 36%, depending on the region sampled (Hayward *et al.*, 2007). Recent phylogenetic analyses based on the *env* gene has shown a predominance of subtype C virus in NZ (up to 48% in one study), whilst the remaining samples were designated subtype A, or were unable to be classified (Hayward *et al.*, 2007, Kann *et al.*, 2007b). Recombinant viruses are common and co-infection has also been shown to occur (Hayward *et al.*, 2007, Kann *et al.*, 2007b). It is clear

from these studies that there are considerable differences in the genomic sequence of FIV in NZ compared to countries such as Australia or the U.S.A (where subtypes A and B predominate) (Kann *et al.*, 2006b). The relevance of overseas studies must therefore be carefully considered, as there may be differences between countries in the pathogenicity of FIV and its susceptibility to vaccine-induced immunity.

1.8.1. Current considerations for use of the Fel-O-Vax® FIV vaccine in NZ

As discussed previously in section 1.7.1, the efficacy of the Fel-O-Vax® FIV vaccine appears to vary according to the challenge virus. There are no studies investigating the efficacy of the vaccine in NZ, and the literature to date casts doubt on the likely efficacy of the vaccine in this country. Two previous studies from the U.S.A. challenged Fel-O-Vax® FIV vaccinated cats with a NZ- derived recombinant virus (FIV-NZ1). In both studies, the PF was reported as only 40-44% (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014). The authors of these studies suggest that for whatever reason, vaccine efficacy appears to be reduced against recombinant viruses – an observation that is concerning for veterinarians in NZ, given the high incidence of recombination detected in FIV from this country (Hayward and Rodrigo, 2008).

The current World Small Animal Veterinary Association (WSAVA) vaccine guidelines (2016) have classified the Fel-O-Vax® FIV vaccine as a "non-core" vaccine, which is defined as "a vaccine that is required by only those animals whose geographical location, local environment or lifestyle places them at risk of contracting specific infections" (Day *et al.*, 2016). Other organisations, such as the American Association of Feline Practitioners (2006) recommend its use as a non-core vaccine, to be administered only to cats at high risk of acquiring the infection. There are no clear guidelines on use of the vaccine in cats in NZ. Despite the paucity of evidence confirming efficacy of the Fel-O-Vax® FIV vaccine against FIV variants in NZ, the vaccine has been registered in this country for the past 12 years, and is now recommended by many veterinarians. In fact, a recent study showed that 35.7% of NZ veterinarians recommend this vaccine "often or always" (Cave *et al.*, 2016). Interestingly, Boehringer-Ingelheim have recently announced that the Fel-O-Vax® FIV vaccine will no longer be available in the U.S.A. once the

current stocks have been sold.[3] This is likely to have implications on vaccine availability elsewhere in the world.

Given the widespread use of the vaccine in a country where the efficacy is questionable, large-scale follow-up testing of vaccinated pet cats would provide useful information regarding vaccine efficacy in this country. One such study has been performed in Australia, whereby 301 cats (including 89 cats previously vaccinated with the Fel-O-Vax® FIV vaccine and 212 FIV-unvaccinated cats) were tested for FIV using PCR on whole blood (Westman *et al.*, 2016a). Five vaccinated cats tested positive for FIV (5.6%), compared to 25 out of 212 unvaccinated cats (11.8%) – resulting in a protective rate of only 56% for the Fel-O-Vax® FIV vaccine under field conditions. Similar studies are desperately needed in NZ in order to make recommendations for use of the vaccine in this country.

There are differing opinions of professionals within the veterinary industry as to the importance of proving adequate efficacy of a vaccine prior to recommending its use. The comment is often made that "some protection is better than none", suggesting that veterinarians should be using the Fel-O-Vax® FIV vaccine even if it provides only partial efficacy against FIV in the field. Alternatively, one may consider use of this vaccine to provide non-sterilising immunity, however there are no studies investigating the efficacy of the Fel-O-Vax® FIV vaccine in reducing disease severity in vaccinated cats that become infected. If the vaccine is to be recommended despite questionable efficacy, then one must consider the potential for negative consequences. The possibility of vaccine enhancement of infection has already been demonstrated as a result of vaccination with the Fel-O-Vax® FIV vaccine (Dunham *et al.*, 2006b). Other factors to consider include the possibility of injection site sarcomas, the unnecessary euthanasia of misdiagnosed stray cats, and the risk of non-specific vaccine reactions. Another factor that is rarely considered is the potential for selective expansion of vaccine resistant variants, especially in a closed population such as NZ. The clinical importance of creating such a selection pressure

[3] https://mvma.memberclicks.net/assets/docs/Seminar/2016summerseminarproceedingswolf.pdf

would depend on whether there are differences in pathogenicity between variants of FIV, some evidence for which has already been presented in section 1.5. More studies are needed to determine the significance of genomic sequence variation and its relevance to FIV vaccination.

1.9. Conclusion

The ongoing evolution of FIV has resulted in the existence of a diverse continuum of viruses. Clinical disease in FIV-infected cats is unpredictable and genomic sequence variation of FIV is likely to be responsible for at least some of the variation observed. The genetic diversity of lentiviruses also presents an obstacle to effective vaccine development, and this is reflected in the numerous previous unsuccessful attempts to develop an effective vaccine to prevent infection with HIV. A dual-subtype vaccine against FIV is now available, however efficacy of the Fel-O-Vax® FIV vaccine in the field is yet to be proven. Further studies are required to determine whether the vaccine generates broad spectrum immunity against heterologous virus, and *in vitro* assays assessing cross-reactivity of the cell-mediated response may prove useful for this purpose. Ultimately, follow-up testing of vaccinated cats for FIV is desirable in order to assess efficacy of the vaccine in the field. Consideration should be given to the impact of vaccine-mediated selection pressure on viral evolution, especially if vaccine efficacy is poor. Further studies are needed to determine the role of genomic sequence variation on the pathogenicity of FIV. These are the questions that need to be answered before we can confidently make recommendations regarding use of the Fel-O-Vax® FIV vaccine in NZ.

The overall aim of the research in this thesis was to determine the relevance of the variability of FIV, in regard to pathogenicity and vaccination in NZ. As this aim related specifically to FIV in NZ, preliminary work was necessary to isolate viruses from the NZ field and produce virus stock of a known titre for use in subsequent assays. Chapter 2 describes the methods used for this process and provides genetic information about the viruses isolated. The aim of the work in chapter 3 was to test the hypothesis that FIV variants differ in pathogenicity. Experiments were designed to compare *in vitro* properties that served as markers for viral

pathogenicity, including FIV-induced apoptosis and FIV-induced inhibition of mitogen proliferation. The efficacy of the Fel-O-Vax® FIV vaccine against NZ field viruses was then investigated using a series of *in vitro* and *in vivo* methods. Chapter 4 describes an *in vitro* assay to measure antigen-specific cellular activation, as well as the use of a DTH response to determine whether T cells generated following vaccination with the Fel-O-Vax® FIV vaccine recognise antigens from field isolates of FIV. The research presented in this chapter tested the hypothesis that there would be no difference in the magnitude of the DTH response generated, and in the expression of CD25 in response to stimulation with the NZ field isolates of FIV prepared in chapter 2. Finally, the work in chapter 5 aimed to investigate the efficacy of the Fel-O-Vax® FIV vaccine under NZ conditions. The hypothesis was that the prevalence of FIV in vaccinated pet cats would be lower than the prevalence in unvaccinated pet cats, indicating at least some protection of Fel-O-Vax® FIV vaccinated cats against FIV in the field. The evidence presented in this thesis is summarised in chapter 6, and conclusions are made about the variability of FIV in NZ. In particular, the relevance of FIV variation in regards to vaccination is discussed, and comments are made regarding the likely efficacy of the Fel-O-Vax® FIV vaccine in this country.

CHAPTER TWO

Preparation of FIV stock

2.1. Introduction

The overall aim of the research presented in this thesis was to determine the relevance of the variability of FIV, in regard to pathogenicity and vaccination in NZ. In subsequent chapters, *in vitro* experiments are described in which NZ isolates of FIV are used in order to gain more specific information about the virus and vaccine efficacy in this country. For these experiments, preparatory work was required to identify, isolate and prepare titrated stock of a number of NZ field virus isolates. In addition, an FIV strain included in the Fel-O-Vax® FIV vaccine was prepared for use as a positive control in subsequent experiments.

FIV has previously been isolated from plasma, saliva and peripheral blood mononuclear cells (PBMC) from infected cats (Matteucci *et al.*, 1993, Dunham *et al.*, 2006a, Matteucci *et al.*, 1995, Bandecchi *et al.*, 1992, Meers *et al.*, 1992, Miyazawa *et al.*, 1989b, Yamamoto *et al.*, 1988). PBMC were the sample of choice for isolation of the virus from FIV antibody-positive cats (81% sensitivity) when compared to plasma and saliva (14% and 18% sensitivity, respectively) (Matteucci *et al.*, 1993). This may be due to low concentrations of cell-free virus in plasma or saliva, and/or due to the presence of inhibitory factors in these samples (Matteucci *et al.*, 1993). PBMC separation from whole blood is relatively straight-forward, and since viral isolation from this sample is most sensitive, it was the preferred sample for isolation of FIV in this study.

Numerous variants of FIV have been isolated overseas and are available from previous authors for use in experimental work. Some of these viruses are well characterised strains that have been studied both in the laboratory and *in vivo* (de Rozieres *et al.*, 2004, Ikeda *et al.*, 2004, Kohmoto *et al.*, 1994, Kohmoto *et al.*, 1998, Maki *et al.*, 1992, Miyazawa *et al.*, 1989a, Pedersen *et al.*, 2001). However, laboratory strains may not fully represent all FIV circulating in the field, as repeated passage in culture is thought to result in adaptation of the virus to laboratory conditions, which may affect certain viral characteristics (such as cell tropism and susceptibility to virus neutralising antibodies) (Willett *et al.*, 2006b, Bendinelli *et al.*, 2001, Richardson *et al.*, 1996). To ensure that the viruses used in subsequent studies described in this thesis were

representative of field FIV, viruses were isolated from naturally infected cats, and the number of passages in culture was minimised.

FIV variants in NZ are genetically distinct compared to FIV elsewhere in the world. Approximately 70% of NZ viruses belong to either subtype C, or are of an unknown subtype not found elsewhere in the world. In contrast, the majority of FIV variants found in the U.S.A. and Australia (where many FIV studies originate from) belong to subtypes A or B (Pistello *et al.*, 1997, Nakamura *et al.*, 2003, Reggeti and Bienzle, 2004, Kann *et al.*, 2006a, Kann *et al.*, 2006b, Iwata and Holloway, 2008, Weaver, 2010, Hayward *et al.*, 2007, Hayward and Rodrigo, 2008, Kann *et al.*, 2007b). The presence of genetically distinct viruses in NZ is particularly relevant when considering the efficacy of the Fel-O-Vax® FIV vaccine in this country, highlighting the importance of using NZ specific isolates of FIV for work presented in subsequent chapters of this thesis.

The aim of the work described in this chapter was to prepare and titrate stock of FIV isolated from naturally infected cats in NZ. In addition, FIV stock of the vaccine strain, Petaluma (FIV-Pet), was to be prepared and titrated for use in subsequent experiments described in this thesis.

2.2. Materials and methods

2.2.1. Recruitment of FIV positive cats

FIV positive cats were recruited from the Massey University Veterinary Teaching Hospital (MUVTH) and local participating veterinary clinics. Veterinarians from various regions of the North island of NZ were contacted via mail and asked to recruit cats that had been previously diagnosed as FIV-positive on serology. A blood sample (0.5 mL whole blood in EDTA) for PCR and sequencing of the virus was collected from all cats meeting the inclusion criteria. A second blood sample (8 mL heparinised blood) was subsequently collected for virus isolation from a subset of cats within this group. The veterinarians were asked to complete a questionnaire for each cat, detailing any clinical signs that may have been present at the time of venepuncture. A

sample of the submission form is included in **Appendix 1**. The study protocol was approved by the Massey University Animal Ethics Committee (MUAEC, protocol #10/48).

2.2.2. Conventional PCR

2.2.2.1. DNA extraction

DNA was required for amplification of FIV provirus. DNA was extracted from 200 µL of whole blood using the High Pure PCR Template Preparation Kit (Roche), according to the manufacturer's directions. Briefly, 200 µL of binding buffer and 40 µL of proteinase K were added to whole blood, and the mixture was incubated at 70 ºC for 10 minutes. Isopropanol (100 µL) was added, and the solution centrifuged at 8,000 x g for 1 minute in high pure spin filter tubes. The sample was treated with 500 µL of inhibitor removal buffer, washed twice, and then eluted in 200 µL of pre-warmed elution buffer. The quantity and quality of extracted DNA was assessed using spectrophotometry. DNA was stored at -20 ºC for subsequent amplification by PCR.

2.2.2.2. Amplification of the envelope gene by PCR

A conserved region of the envelope (*env*) gene was amplified from proviral DNA using a nested PCR assay. The outer (primary) primer sequences had previously been designed to amplify a 1,230 bp fragment of the V3-V6 region of the *env* gene, based on the genome of the subtype B strain, TM2 (Nishimura *et al.*, 1996). The inner (nested) primer sequences were designed to amplify an 859 bp region of the primary fragment. Sequences for primary and nested primers are shown in **Table 2-1**.

Following optimisation, the PCR reaction was performed in a total volume of 10 µL, consisting of 5.0 µL Faststart Master Mix (Roche), 0.2 µM each of forward and reverse primers, 1.0 µL of template DNA and 3.8 µL of H_20. For the nested reaction, 1.0 µL of the primary PCR product was used as template DNA. Amplification conditions for both primary and nested reactions consisted of initial denaturation at 95 ºC for 10 minutes, followed by 35 cycles of template denaturation (15 seconds at 95 ºC), primer annealing (15 seconds at 50 ºC), and

elongation (1 minute at 72 ºC). PCR products were identified by gel electrophoresis (100 V for 40 minutes), using a 1% agarose gel stained with ethidium bromide. A molecular weight marker (DNA Molecular Weight Marker XIV, Roche) was included for estimation of the product's size.

Table 2-1 PCR primers used for amplification of the *env* gene in conventional PCR.
The expected PCR products of the primary and nested PCR were 1,230 bp and 859 bp long, respectively. Primer sequences were previously identified by Nishimura and others (1996) from the subtype B, TM2 variant of FIV.

Reaction	Primer	Location on FIV genome	Product size	Sequence (5' to 3')
Primary	Forward (VE1S) Reverse (VE1R)	7134 – 7154 8345 – 8364	1230bp	GAGTAGATACWTGGTTRCAAG CATCCTAATTCTTGCATAGC
Nested	Forward (VE2S) Reverse (VE2R)	7326 – 7345 8165 – 8184	859bp	CAAAATGTGGATGGTGGAAY ACCATTCCWATAGCAGTRGC

2.2.3. Sequencing of the PCR product

PCR products were excised from agarose gel under ultraviolet (UV) light and transferred to a gel extraction column (Bio-Rad Laboratories, Inc.). The gel was frozen in liquid nitrogen for 1 minute, then thawed and centrifuged at 13,000 x g for 3 minutes. Extracted DNA was harvested into collection tubes and assessed using spectrophotometry. The DNA was submitted for sequencing by the Massey Genome Service (Massey University, Palmerston Nth, NZ) in a total volume of 15 µL H_20, containing 3.2 pmol of one primer and approximately 18 ng of the PCR product. Sequences were determined by capillary electrophoresis on the ABI3730 DNA Analyzer.

2.2.4. Phylogenetic analysis

Sequence reads were aligned and trimmed using Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ), and a consensus sequence of the PCR product was constructed for each virus. Consensus sequences were then aligned and individual pairwise comparison performed to determine the percentage similarity between the sequences. Reference FIV sequences from each subtype were downloaded from GenBank® and included in the alignment. An unrooted phylogenetic tree was constructed using a Jukes-Cantor matrix and Neighbour-Joining algorithm. Viruses not clustering with any of the reference subtype sequences were tested for recombination using Recombinant Identification Program 3.0 (R.I.P.,

http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html), with a 200 bp sliding window and a 90% threshold for significance. Reference sequences were also included in the R.I.P. analysis.

2.2.5. Reverse transcriptase quantitative PCR

2.2.5.1. Viral nucleic acid extraction

For optimisation of the reverse transcriptase qPCR, a positive control was generated by extracting RNA from the Fel-O-Vax® FIV vaccine using the RNeasy Mini Kit (Qiagen), according to the manufacturer's directions. Where RNA was not specifically required (i.e. for virus isolation and detection in cell culture supernatant), the Viral Nucleic Acid Extraction Kit II (Geneaid) was used to extract DNA and RNA, according to the manufacturer's directions. Briefly, 200 µL of undiluted vaccine was mixed with 400 µL lysis buffer and incubated at room temperature for 10 minutes. AD buffer (450 µL) was added prior to centrifugation at 16,000 x g for 1 minute. The sample was washed twice and the purified nucleic acid eluted into 50 µL RNase-free water.

2.2.5.2. One-step reverse transcriptase quantitative PCR (FIV qRT-PCR)

The first 164 bp of the *gag* gene was amplified from extracted nucleic acid using previously published primer sequences (**Table 2-2**) (Wang *et al.*, 2010). The reaction was performed in a total volume of 10 µL, consisting of 5.0 µL of the SYTO 9 reagent, Accumelt HRM Supermix (Quanta), 0.5 µL Transcriptor High Fidelity Reverse Transcriptase (Roche), forward and reverse primers (each at a concentration of 0.3 µM), 2.0 µL of template nucleic acid and 2.2 µL of H_2O. Initial amplification conditions consisted of reverse transcription at 50 ºC for 10 minutes, initial denaturation at 95 ºC for 5 minutes, then 50 cycles of template denaturation (95 ºC for 5 seconds), primer annealing (54 ºC for 10 seconds), and elongation (70 ºC for 8 seconds).

The optimisation process included use of an alternative enzyme and detector system (One-Step SYBR Green RT-PCR Kit, Quanta qScript), and adjustment of the primer concentrations and cycle conditions as required to achieve amplification of the desired product. Gel electrophoresis of the PCR products was performed as previously described in section 2.2.2.2,

when sequence confirmation of specific amplicons was required. Primers were examined for their propensity to anneal to each other using commercially available software (Oligo 7 Primer Analysis Software, 2006, Molecular Biology Insights).

Table 2-2 Real-time PCR primers for amplification of the *gag* gene.
Primer sequences amplified the first 164 bp of the *gag* gene as designed by Wang and others (2010).

Primer	Product size	Sequence (5' to 3')
Forward (FIVf) Reverse (FIVr)	164bp	ATGGGGAAYGGACAGGGGCGAGA TCTGGTATRTCACCAGGTTCTCGTCCTGTA

2.2.5.3. Two-step reverse transcriptase quantitative PCR (FIV RT-qPCR)

The qRT-PCR described in section 2.2.5.2 was modified to a two-step process, by producing complementary DNA (cDNA) from RNA in a separate reaction. The cDNA was produced in a total volume of 20 µL, consisting of 16 µL of template nucleic acid and 4 µL of qScript cDNA Supermix (Quanta). The reaction proceeded over 5 minutes at 25 ºC, 30 minutes at 42 ºC and 5 minutes at 85ºC. Quantitative PCR was then performed on cDNA as in section 2.2.5.2, with the exception that RT was not included in the reaction, and the initial reverse transcription step was omitted. A positive control (cDNA from the Fel-O-Vax® FIV vaccine) was included in all reactions. The identity of the product obtained with the positive control template was confirmed by sequencing of the band (section 2.2.3) following gel electrophoresis (section 2.2.2.2).

The FIV RT-qPCR was optimised by using different detector systems (SYTO 9 versus SYBR green) and adjusting primer concentrations and cycling conditions as required to achieve a target qPCR efficiency of 90 - 100% ($-3.6 \geq$ slope ≥ -3.3) and an R^2 value >0.99. Once optimised, repeatability and accuracy of the assay was confirmed by generating three standard curves with 10-fold serial dilutions of the positive control (Fel-O-Vax® FIV vaccine cDNA). Where semi-quantification of different samples was required (e.g. to assess a rise in viral RNA in culture over time) all samples were tested in a single batch, and comparisons were made between the quantification cycle (Cq) for each sample.

2.2.5.4. Determining sensitivity of the FIV RT-qPCR

The sensitivity of the FIV RT-qPCR assay was subsequently assessed to determine if the assay was suitable for diagnosis of FIV in the field. Additional primers (FIV-long_F and FIV_long_R) were designed using Geneious Pro 4.8.5 software to amplify an 874 bp region of the *gag* gene that included the 164 bp region targeted by the FIVf and FIVr primers described in section 2.2.5.2 (**Table 2-3**). The long PCR product was amplified from vaccine cDNA using conventional PCR in a total volume of 20 µL, consisting of 4.0 µL HOT FIREpol® DNA polymerase mix (Solis BioDyne), forward and reverse primers (each at a concentration of 0.2 µM), 2.0 µL of template nucleic acid (cDNA produced from the Fel-O-Vax® FIV vaccine, as described in section 2.2.5.3) and 13.2 µL of H_2O. Amplification conditions consisted of initial denaturation at 95 ºC for 15 minutes, 35 cycles of template denaturation (95 ºC for 10 seconds), primer annealing (60 ºC for 30 seconds), and elongation (72 ºC for 2 minutes), and a final elongation period of 7 minutes at 72 ºC. The PCR band was then cut out of the gel, snap frozen in liquid nitrogen and centrifuged at maximum speed for 5 minutes. The supernatant was then used as template for a second round of PCR as described above. The PCR band was then extracted from the second gel, and the DNA concentration (in ng/µL) was measured on a Qubit™ 3.0 Fluorometer (Invitrogen™) using the dsDNA Broad Range Assay Kit (Invitrogen™). The copy number was calculated from the measured DNA concentration using a copy number and dilution calculator (Thermo Fisher Scientific), and a stock solution of 10^8 copies/µl was produced. Serial ten-fold dilutions (containing 10^0 – 10^8 copies/µL) of the stock solution were then used as standards in the FIV RT-qPCR assay described in section 2.2.5.3. The sensitivity of the assay was determined by the lower limit of detection of the standards.

Table 2-3 Additional primers used to determine sensitivity of the FIV RT-qPCR.
Primers were designed to amplify an 874 bp region of the *gag* gene, that included the smaller, 164bp product targeted by the FIV RT-qPCR (section 2.2.5.2). Primer sequences were designed using Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ).

Primer	Product size	Sequence (5' to 3')
Forward (FIV_long_F)	874 bp	GCAGTTGGCGCCCGAACAGG
Reverse (FIV_long_R)		TTATCTGCAGCGCACCCTGGT

2.2.6. Virus isolation

2.2.6.1. PBMC separation

Seven FIV-positive cats were chosen for isolation of FIV from PBMC. Cats were selected based on phylogenetic analysis of their virus, in order to isolate a range of genetically distinct FIV that were representative of the population sampled. One additional uninfected cat was used as a PBMC donor for virus isolation. Heparinised whole blood (8 mL) was diluted in an equal volume of saline (0.9% sodium chloride, NaCl) and layered over density medium solution (Ficoll-Paque, GE Healthcare Life Sciences) at a ratio of 2 mL diluted blood : 1 mL density medium solution. Tubes were centrifuged at 450 x g for 45 minutes, the PBMC were collected from the plasma / medium interface and transferred to a separate tube containing 10 mL of phosphate buffered saline, pH 7.2 (PBS). Cells were centrifuged at 350 x g for 10 mins and resuspended in another 10 mL of PBS. A final low speed centrifugation for 10 mins at 250 x g was performed to reduce platelet contamination.

2.2.6.2. Virus isolation using donor PBMC

PBMC from the donor cat were cultured at 37 °C in 5% CO_2, in RPMI media (GIBCO®) supplemented with 10% foetal bovine serum (FBS, GIBCO®), 1% penicillin / streptomycin (GIBCO®), 1% glutamax (GIBCO®) and 5 µg/mL of Concanavalin A (ConA, Sigma-Aldrich®), at a concentration of $1.5x10^6$ cells/mL. PBMC from infected cats were separated and added to the donor cultures after 3 days, at a ratio of 1:1, and cells were cultured for a further 27 days. Every 3 days, half of the media was replaced with fresh media. Samples of cell culture supernatant were collected at each media change and frozen at -80 ºC for subsequent detection of viral nucleic acid using the FIV RT-qPCR as described in section 2.2.5.3.

2.2.6.3. Virus isolation using donor MYA-1 cells

Optimisation of virus isolation was performed by co-culturing PBMC from infected cats with an IL-2 dependent, feline lymphoblastoid cell line (MYA-1 cells, CRL-2417, ATCC®). These cells had been characterised by previous authors as free of retrovirus but highly permissible

to FIV infection (Miyazawa *et al.*, 1989b). Initial optimisation of media composition was required to sustain the cells in culture, as they failed to proliferate under certain conditions (e.g. when newborn calf serum was used instead of FBS). MYA-1 cells were therefore maintained in growth medium (GM) that consisted of ATCC® modified RPMI, (containing 2 mL L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate, GIBCO®), supplemented with 0.05 mM 2-mercaptoethanol (2-ME, Sigma-Aldrich®), 100 units/mL recombinant human IL-2 (BD Biosciences) and 10% FBS. Half of the media was replaced every 2-3 days, with the addition of 100 units/mL (of total volume in flask) of IL-2 each time. Cells were split as required to maintain a density of 1-2x10^5 cells/mL. This was performed by counting a sample of cells, adding fresh media to dilute the cells to the required volume and aliquotting the additional volume of media into different flasks.

PBMC from infected cats (3-4x10^6 cells) were co-cultured with 1x10^6 MYA-1 cells in 5 mL of GM and cultured for 20 days. To determine the optimal time to harvest the virus, supernatant was initially collected daily for quantification of viral nucleic acid using the FIV RT-qPCR described in section 2.2.5.3. Cell-free supernatant containing virus was then harvested from all subsequent cultures at the determined time for peak viral output, and stored at -80 ºC.

2.2.6.4. Passage of FIV isolates

To confirm successful isolation of live FIV, each virus isolated was further passaged in MYA-1 cells. Uninfected MYA-1 cells were centrifuged and resuspended in 1 mL of FIV-positive cell culture supernatant from the original virus isolation (passage 0), and incubated for 2 hrs at 37 ºC. Cells were then cultured for 20 days in a total volume of 5 ml, with half media changes performed every 5 days as described in section 2.2.6.3 (passage 1). FIV RT-qPCR was performed on a sample of supernatant collected at each media change to monitor viral load in supernatant. A rising concentration of FIV nucleic acid over time (as shown by a decrease in Cq values) was considered indicative of successful passage of the virus in MYA-1 cells.

2.2.7. Production of virus from infectious molecular clones

Infectious molecular clones of the subtype A strains, Petaluma (PetF14) and Glasgow-8 (GL8) were obtained.[4] The Petaluma clone (34TF10) had been produced using the bacteriophage λ vector, EMBL-4 (Talbott *et al.*, 1989). The FIV GL8 clone (414) had been produced from an E.coli plasmid, pBR328 (Hosie *et al.*, 2002). The Petaluma strain had been chosen for this study as it is one of the viruses contained in the Fel-O-Vax® FIV vaccine. The UK strain, GL8, had previously been shown to readily infect cats vaccinated with the Fel-O-Vax® FIV vaccine, suggesting that it can escape vaccine-induced immunity (Dunham *et al.*, 2006b).

2.2.7.1. Transfection of CRFK cells with the PetF14 clone

CRFK cells were seeded onto 6-well plates at a density of $1x10^5$ cells/ml in Advanced Minimum Essential Media (MEM, GIBCO®) supplemented with 2% FBS and 1% glutamax and cultured at 37 ºC in 5% CO_2 for 24 hrs. CRFK cells were chemically transfected using a non-liposomal lipid-based reagent (Xtreme Gene 9 DNA Transfection Reagent, Roche). DNA from the PetF14 clone (1 µg) was mixed with 100 µL of diluted transfection reagent (3/100 in Opti-MEM® reduced serum media, GIBCO®) and incubated at room temperature for 15 mins. CRFK cells were then cultured in the presence of the DNA mixture at 37 ºC in 5% CO_2 for 3 days. Aliquots of supernatant were collected every day and frozen at -80 °C for quantification of viral RNA (to observe a rising viral concentration, as shown by a decrease in Cq value) using the FIV RT-qPCR described previously in section 2.2.5.3. On day 3, all of the supernatant was collected, and cells were transferred to a 25 cm^3 flask with fresh media. Half media changes were performed every 3-4 days, with supernatant stored for subsequent detection of viral RNA.

2.2.7.2. Infection of MYA-1 cells with FIV rescued from transfected CRFK cultures

In subsequent experiments, MYA-1 cells were used as donor cells for infection with FIV released from transfected CRFK cells. CRFK cells were transfected with the PetF14 and GL8 clones as described in section 2.2.7.1, with the exception that the CRFK supernatant was replaced

[4] Generously gifted by Dr. Margaret Hosie, Institute of Infection, Immunity and Inflammation, University of Glasgow, UK.

after 18 hrs with fresh RPMI (supplemented as described in section 2.2.6.3) containing $1x10^6$ MYA-1 cells. Cells were co-cultured for 24hrs, after which time the MYA-1 cells were transferred to a separate 25 cm^3 flask and maintained in culture as described in section 2.2.6.3. Samples of MYA-1 supernatant were collected daily for the first 4 days, then every 3 days until day 17. All supernatants were stored at -80 ºC, with aliquots from each day collected for subsequent detection of virus by the FIV RT-qPCR.

2.2.7.3. Transfection of 293T cells with the GL8 clone

An alternative cell line to CRFK cells was also transfected with the GL8 clone. The human embryonic kidney cell line (293T, ATCC®) is a highly transfectable adherent cell line, used previously to produce high titres of retroviruses (Pear *et al.*, 1993). These cells were maintained in Advanced Dulbecco's Modified Eagle Medium (DMEM, GIBCO®), supplemented with 2% FBS, and 1% glutamax and transfected as described in section 2.2.7.1.

2.2.7.4. Viral RNA extraction and detection using RT-qPCR

Measurement of FIV RNA was used to confirm successful production of FIV in CRFK cells following transfection (as extraction of total nucleic acid would have included clonal DNA). RNA was extracted from CRFK cell culture supernatant using the TRIzol® LS Reagent (Invitrogen™) according to the manufacturer's directions. Briefly, 750 µL TRIzol® was added to 250 µL of CRFK cell culture supernatant. The mixture was incubated for 5 mins at 25 ºC prior to adding 200 µL chloroform. The mixture was shaken, incubated for a further 10 mins at 25 ºC and centrifuged at 12,000 x g for 15 mins at 4 ºC. The aqueous phase (containing RNA) was aspirated and transferred to a new microcentrifuge tube. Isopropanol (500 µL) was added and the mixture incubated for 10 minutes at 25 ºC prior to centrifugation at 12,000 x g for 10 minutes at 4 ºC. The supernatant was aspirated and the RNA precipitate washed in 1 mL of 75% ethanol and pelleted by centrifugation at 7,500 x g for 5 mins at 4 ºC. Finally, the RNA pellet was air-dried for 10 mins and resuspended in 50 µL of RNase-free water at 60 ºC for 10 mins. A rise in virus concentration

(as shown by a decrease in Cq value) was then detected using the FIV RT-qPCR described in section 2.2.5.3.

To confirm stable integration of FIV DNA into CRFK DNA following transfection, genomic DNA was extracted from trypsinised CRFK cells at day 15 after the initial transfection procedure, using the High Pure PCR Template Preparation Kit (Roche). Viral DNA was then amplified using the FIV qPCR as described in section 2.2.5.3 (without the reverse transcription step).

2.2.8. Concentration of virus stock

Cell culture supernatants collected from each FIV-infected culture at various time points were pooled and centrifuged at 1,500 x g for 5 minutes to remove cellular debris. The virus containing supernatant was then concentrated from 15 mL to 200 µL by ultrafiltration at 4,000 x g for 30 minutes using Amicon® Ultra-15 100 kDa centrifugal filter units (Millipore®). The virus stock was stored at -80 ºC in 100 µL aliquots.

2.2.9. Quantification of virus stock

2.2.9.1. Semi-quantification of viral nucleic acid by FIV RT-qPCR

Nucleic acid from each virus stock were extracted according to the manufacturer's directions using the Viral Nucleic Acid Extraction Kit II (Geneaid). The FIV RT-qPCR was performed on each sample in duplicate as described in section 2.2.5.3, with a reference standard curve of Fel-O-Vax® FIV vaccine cDNA included in the assay. Semi-quantification was performed by comparing the Cq values of the amplification curve for each virus stock.

2.2.9.2. Endpoint dilution assay

2.2.9.2.i. Comparison of DNA extraction kits for detection of proviral DNA in culture

Detection of FIV proviral DNA by qPCR was used for identification of FIV infected cultures in an endpoint dilution assay. The assay was based on detection of FIV provirus (DNA) rather than infectious virus (RNA) due to the associated reduced costs and labour. Due to the

expected low copy number of provirus in culture, two different methods of DNA extraction were tested to maximise sensitivity of the assay. MYA-1 cells from FIV-infected cultures were centrifuged, counted and resuspended at four different concentrations (10^6, 10^5, 10^4 and 10^3 cells/mL). DNA was then extracted from each sample using the Genomic DNA Mini Kit (Geneaid) and the High Pure PCR Template Preparation Kit (Roche). FIV provirus was amplified using the FIV qPCR described in section 2.2.5.3.

2.2.9.2.ii. Inactivation of FIV for use as a baseline control

A baseline control containing cells incubated with inactivated virus was included in the endpoint dilution assay to differentiate between infected cells (with integrated provirus) and uninfected cells (with only original FIV stock DNA present in supernatant). FIV (MUVTH003) was thermally inactivated at 56 ºC for 30 minutes. Passage of the inactivated virus in MYA-1 cells was then attempted as described in section 2.2.6.4. Passage of untreated (live) virus stock of MUVTH003 in MYA-1 cells was performed as a positive control. DNA was extracted from all cultures at day 15 and tested for FIV provirus using the FIV qPCR described in section 2.2.5.3. The Cq values of the amplification curves were compared to ensure that there was no difference in virus concentration over time in the inactivated virus cultures (indicating no replication of the inactivated virus).

2.2.9.2.iii. Virus stock titration

MYA-1 cells were seeded onto 96-well plates at a density of $2x10^5$ cells/mL in 100 µL per well. Virus stocks were thawed rapidly at 37 ºC and 10-fold serial dilutions were produced in GM. A 50 µL aliquot of each dilution was then added to MYA-1 cells in duplicate. Additional virus stock was inactivated thermally at 56 ºC for 30 minutes and added to separate wells with MYA-1 cells to serve as a baseline control for virus replication. Plates were incubated for 15 days at 37 ºC in 5% CO_2.

Following culture, DNA was extracted from the entire volume of each well using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's directions. The FIV

qPCR (section 2.2.5.3) was performed to amplify the FIV *gag* gene of the proviral DNA integrated into infected MYA-1 cells, as well as any FIV DNA added with the original virus stock. FIV provirus was detected rather than the infectious form of the virus, due to reduced costs and labour associated with the qPCR, compared to RT-qPCR. Wells were considered infected if the Cq value at a given dilution was lower than that obtained from the corresponding well containing inactivated virus. The titre of the virus was expressed as $TCID_{50}$/mL, which was calculated using the Reed & Muench method (Reed and Muench, 1938).

2.2.9.3. P24 ELISA

A quantitative ELISA measuring the concentration of FIV p24 antigen was performed on virus stock diluted 10-fold and 100-fold in unsupplemented RPMI media, using the commercial QuickTiter™ FIV Lentivirus Quantitation Kit (Cell Biolabs Inc.). To generate a standard curve for quantification, 2-fold dilutions of recombinant p24 antigen in assay diluent were prepared (1.56 ng/mL – 100 ng/mL) according to the manufacturer's directions. All samples were treated with Triton X-100 for 30 minutes prior to performing the ELISA. Test samples, FIV p24 standard solutions and negative controls (assay diluent and RPMI media) were all tested in duplicate according to the manufacturer's directions. Each microwell was measured by spectrophotometry at 450 nm. Regression analysis was performed on the linear aspect of the standard curve, using the normalised absorbance values for each concentration of the p24 standard [calculated by subtracting the mean optical density (OD) of the negative controls from the OD measured for each concentration of p24]. The p24 concentration was then calculated for each virus stock sample using the regression equation generated. When the normalised absorbance of a sample was below the linear range of the standard curve, the ELISA was repeated using a more concentrated sample (1/3 and 1/6 dilutions or 1/5 and 1/10 dilutions, depending on results from the initial analysis). The final concentration of p24 in the undiluted stock solutions was then calculated by multiplying the p24 concentration by the dilution factor and averaging the two values.

2.3. Results

2.3.1. Recruitment of FIV positive cats

A total of 22 FIV seropositive cats were recruited from 6 participating veterinary clinics in the North island of NZ. Samples were named according to the clinic of origin in order of acquisition. The majority of cats (15/22) were clinically normal and had been routinely tested for FIV prior to administration of the Fel-O-Vax® FIV vaccine. The remaining cats had been diagnosed with a variety of diseases, including lymphoma (1/22), chronic kidney disease (1/22), cellulitis (1/22), chronic rhinitis (1/22), and a nasopharyngeal polyp (1/22). Two cats had also been euthanised without a diagnosis due to non-specific and worsening clinical signs. A summary of the results are included in **Appendix 2**. Clinical data from the NZ field viruses isolated and used for subsequent chapters in this thesis is provided in

Table 2-4 Clinical characteristics of cats infected with selected NZ field isolates.

Virus	Age	Sex	Breed	Disease status
MUVTH002	10 yrs	Male desexed	Domestic shorthair	Sick (chronic kidney disease)
MUVH003	8 mths	Male desexed	Domestic shorthair	Sick (nasopharyngeal polyp)
CVK001	5 yrs	Female desexed	Domestic shorthair	Asymptomatic
RVC009	3 yrs	Male desexed	Domestic shorthair	Asymptomatic

2.3.2. Conventional PCR

The selected region of the *env* gene was successfully amplified from whole blood from 20 of the 22 serologically FIV-positive cats. The remaining 2 samples were consistently negative on PCR (**Figure 2-1**).

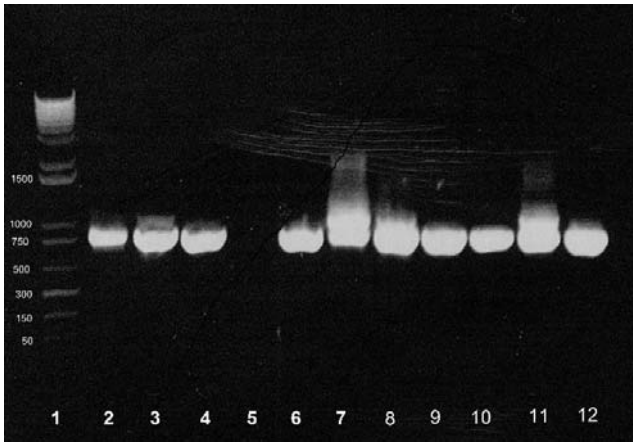


Figure 2-1 Gel electrophoresis showing conventional FIV PCR results.
Gel electrophoresis of PCR products amplified from whole blood from a group of cats positive for FIV on serology (lane 1 – DNA ladder; 2 – MUVTH002; 3 – CVK001; 4 – CVK002; 5 – RVC003; 6 – RVC002; 7 – RVC004; 8 – RVC005; 9 – RVC001; 10 – CSV001; 11 – SRV003; 12 – SRV004; 13 – No template control). Results show the presence of a band at the expected site (859 bp) in 10/11 samples. The RVC003 sample (lane 5) was consistently negative on PCR, as indicated by the absence of a band at the expected site. **Phylogenetic analysis**

Sequencing of the PCR product from 13 reactions was successful. The majority of sequences (10/13) clustered with the subtype C reference sequences, a further 2 sequences clustered with the subtype A reference sequences, and the remaining sequence did not cluster with any of the reference sequences (**Table 2-5** and **Figure 2-2**). Pairwise comparison of the viruses used in subsequent chapters of this thesis showed 78 – 90.2% similarity over approximately 520 bp of the sequenced region of the *env* gene (**Table 2-6**).

Table 2-5 Pairwise comparison of NZ FIV isolates to reference sequences.
Figures indicate nucleotide similarity (%) of the 13 successfully sequenced NZ FIV (left column) with representative reference sequences from each subtype (labelled as subtype identity). The reference sequence that is most similar to each NZ sample is highlighted, indicating subtype of the isolate. The RVC001 sequence was not clearly similar to any one reference sequence. Reference sequences were downloaded from GenBank® and pairwise comparisons were performed using Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ) to align sequences.

Nucleotide similarity (%)							
NZ field viruses	**FIV reference sequence (with GenBank® accession number)**						
	A_Petaluma (M25381)	**B_FC1 (AY621093)**	**C_NZ1 (GQ406243)**	**D_Shizuoka (D37811)**	**E_LP3 (D84496)**	**F_150_02LisP (DQ072566)**	**U_TKP88 (EF153977)**
RVC001	84.8	81.6	86	78.1	80.5	85.9	79.8
RVC002	78.5	81.6	93.5	77.9	79.6	80.7	80.4
RVC004	78.6	80.6	92.6	77.3	78.4	79.4	79.8
RVC005	89.3	80.3	78.8	77.6	80.3	85	79.1
RVC007	77.9	79.7	93.2	77.3	78.9	78.4	80.1
RVC008	76.9	79.3	91.4	76.8	78.7	78.8	79.5
RVC009	90.7	80.1	79.2	78.5	81.3	88.3	78.4
CVK001	78.8	80	91.6	75.6	78	78.7	79.4
CVK003	78.4	80.8	92.3	77.5	80.4	80.2	80.5
MUVTH001	77.3	80.5	91.6	77.8	78.6	79.7	78.3
MUVTH002	78.1	80.5	92	76.9	78.7	79.1	78.6
MUVTH003	78.6	80.3	91.9	77.7	78.9	78.7	79.9
SRV001	77.6	80.6	92.6	76.8	78.3	79.3	79.3

Table 2-6 Pairwise comparison of selected NZ FIV isolates.
Pairwise comparison of the 4 NZ sequences that were selected for used in subsequent chapters of this thesis with the reference subtype A Petaluma strain. The figures represent the percentage similarity for each NZ isolate when compared to the other NZ isolates. RVC009 and Petaluma are closely related, both belonging to subtype A. MUVTH002, MUVTH003 and CVK001 are closely related, all belonging to subtype C. Pairwise comparisons were performed using Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ) to align sequences.

Nucleotide pairwise identity (%)					
	PetF14	**MUVTH002**	**MUVTH003**	**CVK001**	**RVC009**
Petaluma		78.0	78.6	78.8	90.7
MUVTH002	78.0		92.2	92.0	
MUVTH003	78.6	92.2		91.7	78.2
CVK001	78.8	92.0	91.7		78.9
RVC009	90.7	78.9	78.2	78.9	

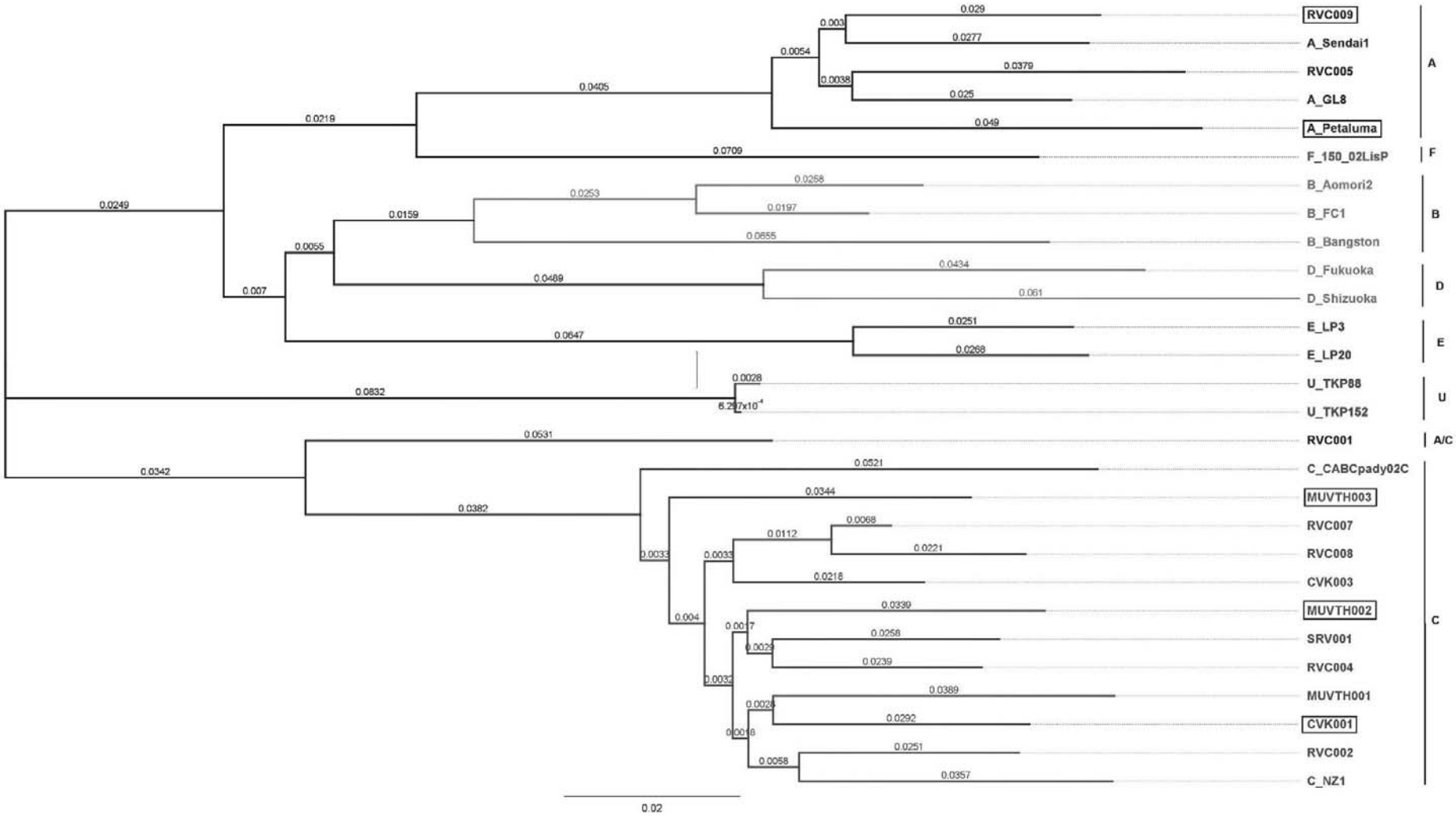

RVC009
A_Sendai1
RVC005
A_GL8
A_Petaluma
F_150_02LisP
B_Aomori2
B_FC1
B_Bangston
D_Fukuoka
D_Shizuoka
E_LP3
E_LP20
U_TKP88
U_TKP152
RVC001
C_CABCpady02C
MUVTH003
RVC007
RVC008
CVK003
MUVTH002
SRV001
RVC004
MUVTH001
CVK001
RVC002
C_NZ1
A
F
B
D
E
U
A/C
C
0.02

Figure 2-2 Phylogenetic analysis of NZ FIV isolates.
(Page 79) Unrooted neighbour-joining tree showing subtype classification of NZ isolates with reference sequences from each subtype downloaded from GenBank® and included for comparison. Subtype classification is included alongside the sequence names. Subtype A is represented in by the reference sequences Petaluma (M25381, U.S.A.), Sendai1 (D37813, Japan) and GL8 (X69496, U.K.). Subtype B is represented by the reference sequences Aomori2 (D37817, Japan), FC1 (AY621093, U.S.A.) and Bangston (AY620002, U.S.A.). Subtype C is represented by the reference sequences CABCpady02C (U02392, Canada) and NZ1 (GQ406243, NZ). Subtype D is represented by the reference sequences Fukuoka (D37815, Japan) and Shizuoka (D37811, Japan). Subtype E is represented by the reference sequences LP3 (D84496, Argentina) and LP20 (D84498, Argentina). Subtype F is represented by the reference sequence 150_02LisP (DQ072566, U.S.A.). The reference sequences TKP152 (GQ357640) and TKP88 (EF153977) represent NZ sequences that do not group with a known subtype (designated "U" for unknown).

The majority of the NZ viruses sequenced in this study cluster with subtype C reference sequences (MUVTH003, RVC007, RVC008, CVK003, MUVTH002, SRV001, RVC004, MUVTH001, CVK001 and RVC002). The RVC005 and RVC009 sequences cluster with subtype A reference sequences. The RVC001 sequence does not cluster with any of the known subtypes and was subsequently shown to be an A/C recombinant isolate. The isolates selected for culture and used in subsequent chapters of this thesis are highlighted in boxes.

```
             1        10        20        30        40        50        60
             |        |         |         |         |         |         |
A_Petaluma   CGTCATGGAAACAAAGAAATAGATGGGAGTGGAGACCAGATTTTGAAAGTAAAAAGGTGA
    CVK001   CGTCATGGAGGCAAAGGAATAGGTGGGAATGGAGACCAGATTTTGAGAGTGAAAAGGTAA
  MUVTH002   CATCATGGAGGTCTAGGAATAGGTGGGAATGGAGACCAGATTTTGAAAGTGAAAAGGTAA
  MUVTH003   CGTCATGGAAGCAACGGAATAGGTGGGAATGGAGACCAGACTTTGAAAGTGAAAAGGTAA
    RVC009   CATCATGGAGACAAAGGAATAGATGGGAATGGAGACCAGATTTTGAAAGTGAAAAAGTGA

A_Petaluma   AAATATCTCTACAGTGCAATAGCACAAAAAACCTAACCTTTGCAATGAGAAGTTCAGGAG
    CVK001   AAATATCATTACAATGTAACAGTACAAAAACCTTAACTTTTGCAATGAGAAGTACAGCTG
  MUVTH002   AAATATCATTACAATGTAACAGTACAAAAGACTTAACTTTTGCAATGAGAAGTGCCGCTG
  MUVTH003   AAATAGCATTACAATGTAACAGTACAAAAAACTTAACTTTTGCAATGAGAAGTGCGGCTG
    RVC009   AAGTATCTCTACAGTGTAATAGCACAAAAAACCTAACTTTTGCAATGAGAAGTTCAGGAG

A_Petaluma   ATTATGGAGAAGTAACGGGAGCTTGGATAGAGTTTGGATGTCATAGAAATAAATCAAAAC
    CVK001   ATGTTGGTGATGTTGTGGGAGCATGGATTGAATTTGGATGTCATAGAAACCGAACAYGGC
  MUVTH002   ATTTTGGTGATGTTGTAGGAGCATGGATAGAATTTGGATGTCATAGAAACCGATCAAAAA
  MUVTH003   ATTTTGGTGATGTTGTAGGAGCATGGATAGAATTTGGATGTCATAGAAACAAATCACGAA
    RVC009   ATTATGGAGAAGTAACGGGAGCTTGGATAGAGTTTGGATGTCATAGACAGAAATCAMAAT

A_Petaluma   TTCATGCTGAAGCAAGGTTTAGAATTAGATGTAGATGGAATGTAGGGAGTAATACCTCGC
    CVK001   AGCATACAGASGCAAGATTTAGAATAAGATGTAGATGGAATGTTGGCTCTAACACTTCTC
  MUVTH002   CGCATACAGAKRCAAGATTTAGAATAAGATGTAGATGGAATGTTGGCTCTAACACCTCTC
  MUVTH003   CTCATTCAGATGCAAGATTTAGAATAAGATGTAGATGGAATGTTGGCTCTAACACTTCTC
    RVC009   CTCATACTGAAACAAGGTTTAGAATTAGATGTAGATGGAATATAGGGGATAATACCTCAC

A_Petaluma   TCATTGATACATGTGGAAACACTCAAAAAGTTTCAGGTGCGAATCCTGTAGATTGTACCA
    CVK001   TAATTGAYACATGTGGAACAGACCTAAATATCTCAGGAGCTAATCCTGTAAATTGTACTA
  MUVTH002   TAATTGACACATGTGGAAATGACACAAATATCTCAGGAGCTAATCCTGTAAATTGTACTA
  MUVTH003   TAATTGATACATGTGGAAAAGACAAAAATATCTCAAGCGCTAATCCTGTAAACTGTACTA
    RVC009   TCATTGATACATGTGGAGAAGCTCAAAATGTTACAGGTGCAAATCCTGTAGATTGTACCA

A_Petaluma   TGTATTCAAATAAAATGTACAATTGTTCTTTACAAAACGGGTTTACTATGAAGGTAGATG
    CVK001   TGGAAGCAAAGACTCTGTACAATTGCTCATTACAGGAGGGATTTACTATGAAAATAGAAG
  MUVTH002   TGAAAGCAAAGACTCTGTACAATTGCTCATTACAAGAAGGATTTACTATGAAAATAGAAG
  MUVTH003   TGATAGCAAAGACTTTGTACAATTGCTCATTACAAGAGGGATTTACTATGAAAATAGAAG
    RVC009   TGTATGCAAATAGAATGTATAACTGTTCCTTACAAAACGGGTTTACTATGAAGGTAGATG

A_Petaluma   ACCTTATTATGCATTTCAATATGAAAAAGGCTGTAGAAATGTATAATATTGCTGGAAATT
    CVK001   ATCTTATAATGCATTTTAACATGACAAAGGCTGTAGAAATGTATAATATTGCTGGAAATT
  MUVTH002   ATCTTATAATGCATTTTAATATGACAAAGGCTGTAGAAATGTATGACATTGCTGGAAATT
  MUVTH003   ATCTTATAATGCATTTTAACATGACAAAGGCTGTGGAAATGTATAATATTGCTGGAAATT
    RVC009   ACCTTATTATGCATTTCAATATGACAAAAGCTGTAGAAATGTATAACATTGCTGGAAATT

A_Petaluma   GGTCTTGTACATCTGACTTGCCATCGTCATGGGGGTATATGAATTGTAATTGTACAAATA
    CVK001   GGTCATGTAAATCTGATTTACCTAAAGACTGGGGTTATATGAAATGTAATTGTACAAGTA
  MUVTH002   GGTCATGTAAATCTGATTTACCTACGGACTGGGGTTACATGAAATGTAATTGTACTAAT-
  MUVTH003   GGTCATGTAAATCTGATTTACCTAGAGACTGGGGTTACATGAAGTGTAATTGTACAAGT-
    RVC009   GGTCTTGTACATCTGACTTGCCATCAACATGGGGGTATATGAATTGTAATTGTACAAATA

A_Petaluma   GTAGTAGTAGTTATAGTGGTACTAAAATGGCATGTCCTAGCAATCGA-GGCATCTTAAGG
    CVK001   CAAGT-GAAAATGTATCGGGA--AAGATGAAATGTCCT-GCAGGGGATGGGATATTAAGA
  MUVTH002   ------GRAAMTGTAGAGGGA--AAGATGAAATGTCCT-GGAAAGGATGGGATATTAAGA
  MUVTH003   ------GAAGAGGCATCG--CATAAGATGAAATGTCCT-GCAAAGAATGGGATATTAAGA
    RVC009   ---GTAGTARCGAT---GTTAACAAAATGGCATGTCCTAG-AAGCCAAGGCATCTTAAGA

A_Petaluma   AATTGGTATAACCCAGTGGCAGGATTACGACAATCCTTAGA-ACAGTATCAAGTTGTAAA
    CVK001   AATTGGTATAATCCAGTAGCAGGATTAAGACAAGCTTTAGATA-AGTATCAAGTGGTAAA
  MUVTH002   AATTGGTATAATCCAGTAGCAGGATTAAGACAAGCTTTAGATA-AATATCAAGTGGTAAA
  MUVTH003   AATTGGTATAATCCAGTAGCAGGATTAAGACAAGCTTTAGATA-AATATCAAGTGGTAAA
    RVC009   AATTGGTATAACCCAGTAGCAGGATTACGACAATCCTTAGACAAAGTATCAAGTTGTAAA

A_Petaluma   ACAACCAGATTACTTA
    CVK001   ACAGCCAGATTATATA
  MUVTH002   ACAGCCAGATTATATA
  MUVTH003   ACAGCCAGATTATATA
    RVC009   GCAACCAGATTATTTA
```

Figure 2-3 Nucleotide alignment of the selected FIV isolates used in subsequent chapters of this thesis.
A 859 bp region of the *env* gene was amplified and sequenced. Sequence reads were trimmed and aligned using Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ). The corresponding region of the *env* gene from a reference sequence (Petaluma) was downloaded from GenBank® and included for comparison (M25381).

Further analysis was performed to investigate the RVC001 sequence for recombination. R.I.P. analysis was used to determine the reference sequence with the highest similarity to RVC001 at each point along the sequence (**Figure 2-4**). The output revealed a clear cross-over point, where the reference sequence sharing the most similarity to RVC001 changed from a subtype C virus (CABCpady02C) to a subtype A virus (GL8). A second cross-over point was evident, where the most similar reference sequence became another subtype C virus (NZ1). These results suggested two intragenic recombination events between subtype A and subtype C viruses.

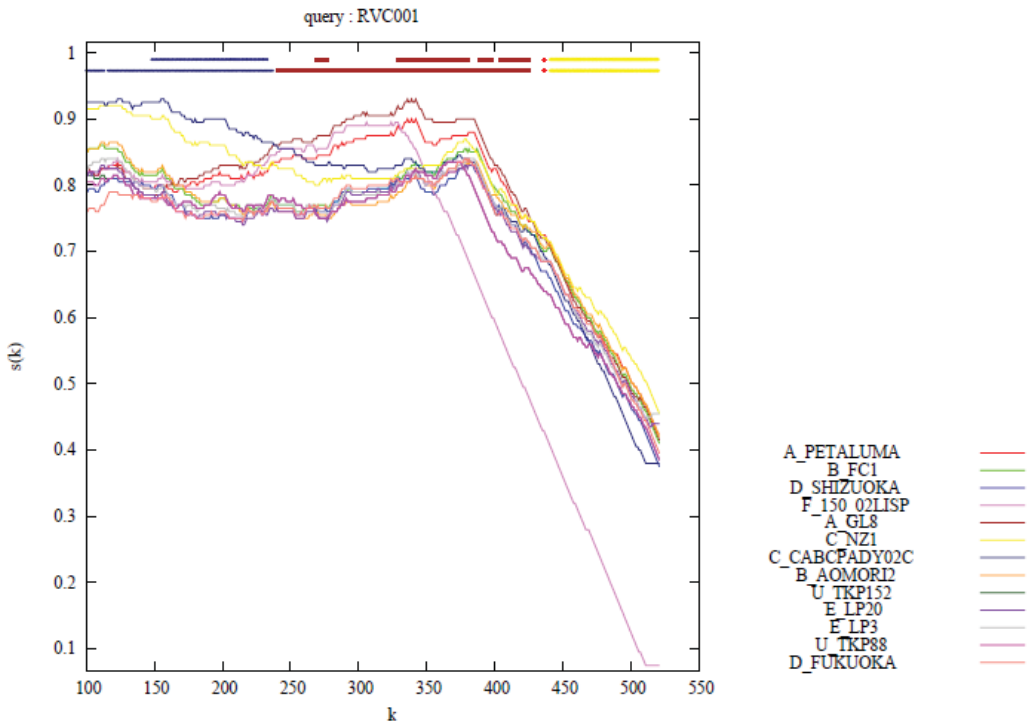


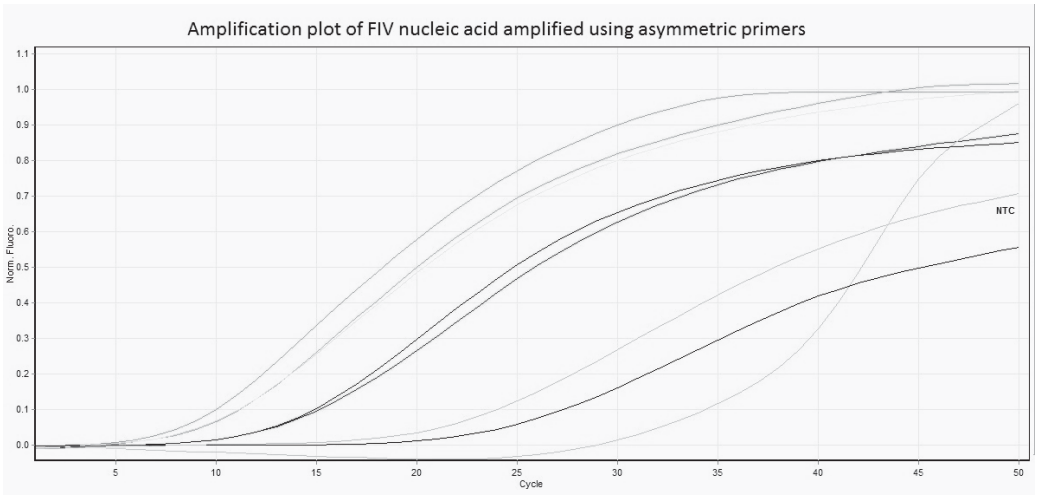
Figure 2-4 Recombinant analysis of NZ RVC001 isolate.
R.I.P. output showing the similarity of the RVC001 sequence to each of the reference sequences included in the phylogenetic analysis. The x-axis represents the position of the RVC001 sequence at the centre of the moving window (k). The y-axis represents the similarity, s (k), of each reference sequence to the RVC001 sequence at each window. The lower bar and text across the top of the graph shows the reference sequence with the most similarity to RVC001 (the "best match"). The upper bar shows the regions where the "best match" is significantly more similar to RVC001 compared to all other sequences. Analysis was performed using the Recombinant Identification Program 3.0 (R.I.P., http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html), with a sliding window of 200 bp and the significance set at 90%.

2.3.4. Reverse transcriptase quantitative PCR

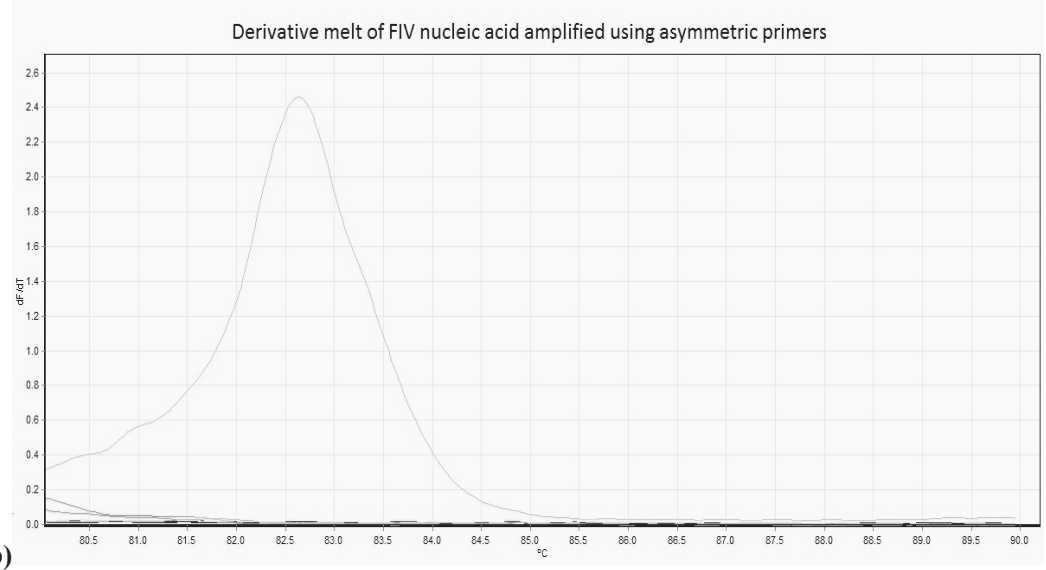
2.3.4.1. FIV qRT-PCR

The FIV qRT-PCR initially resulted in no amplification of the positive control (RNA from the Fel-O-Vax® FIV vaccine). To optimise the reaction, various enzyme systems, primer concentrations and cycling conditions were used. Firstly, the concentration of reverse transcriptase (RT) in the reaction was reduced, but no amplification occurred regardless of RT concentration. Increasing the template concentration did not result in successful amplification either. Adjustment of primer concentrations to an asymmetric combination (FIVf 4: FIVr 1) resulted in amplification of the target, but significant non-specific amplification also occurred in all samples including no-template controls (**Figure 2-5**). Non-specific amplification could not be reduced by adjustment of cycling conditions (prolongation of the RT step, decreased annealing and extension times, increased extension temperature or increased temperature for reverse transcription) or by the addition of random hexamers to the mixture. SYBR green was also replaced by a SYTO 9 dye, but this also failed to result in specific amplification of the desired product.

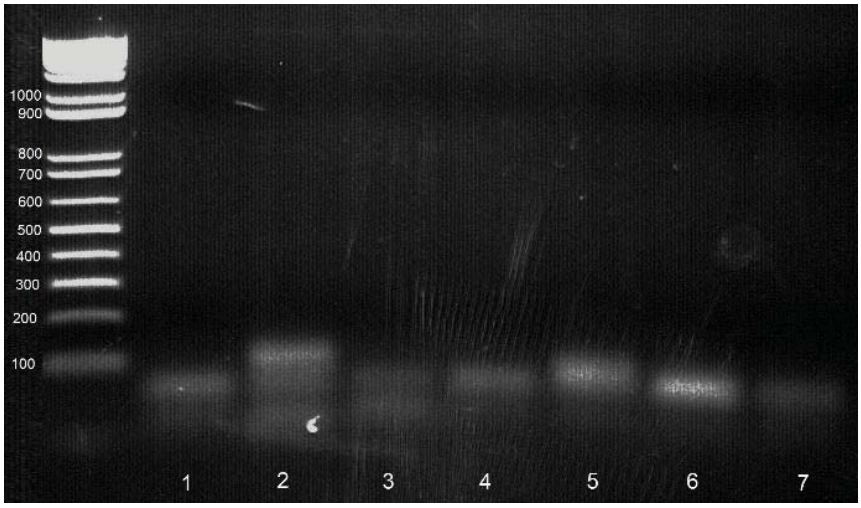
Examination of RT-PCR products by gel electrophoresis indicated non-specific amplification of small products (<100 bp), consistent with the formation of primer-dimers (**Figure 2-6**). The primers were therefore examined for their propensity to anneal to each other using commercially available software (Oligo 7 Primer Analysis Software, 2006, Molecular Biology Insights). Results showed that strong duplex formation was possible at the 3' end of both forward and reverse primers (ΔG = - 5.2 kcal and -2.2 kcal respectively) as well as a relatively strong overall dimer formation (ΔG = - 6.0 kcal). Selected output from Oligo 7 analysis of primers is displayed in below (**Figure 2-7**).


Amplification plot of FIV nucleic acid amplified using asymmetric primers
Norm. Fluoro.
Cycle
NTC

a)


Derivative melt of FIV nucleic acid amplified using asymmetric primers
-dF/dT
°C

b)


1000
900
800
700
600
500
400
300
200
100
1
2
3
4
5
6
7

c)

Figure 2-5 Optimisation of the FIV qRT-PCR.
(Page 83) Results are a representative sample from the optimisation process, using nucleic acid extracted from the Fel-O-Vax® FIV vaccine. The reaction was performed with a range of asymmetric primer concentrations (0.8 forward : 0.2 reverse, 0.4 : 0.1, 0.2 : 0.1, 0.1 : 0.2, 0.1 : 0.4, 0.2 : 0.4, 0.2 : 0.8). **a)** Amplification plot showing amplification in all samples including no template control (indicated as NTC). The quantification cycle (Cq) is represented along the x-axis, and the y-axis shows the fluorescence of the reporter dye minus the baseline fluorescence (ΔR).
b) The derivative melt curve demonstrating which products consist of amplified target sequences versus non-specific amplification. The target sequence was successfully amplified in only one sample, as shown by the specific product with a melting temperature of 82.5 ºC. This product was amplified using an asymmetric primer combination of 400µM forward : 100µM reverse.
c) Gel electrophoresis of RT-PCR products show an indiscrete product of approximately 164 bp in lane 2, representing late amplification of the target sequence. All other lanes (1, 3-7), including the no template control in lane 7 contain smaller products consistent with primer-dimers.

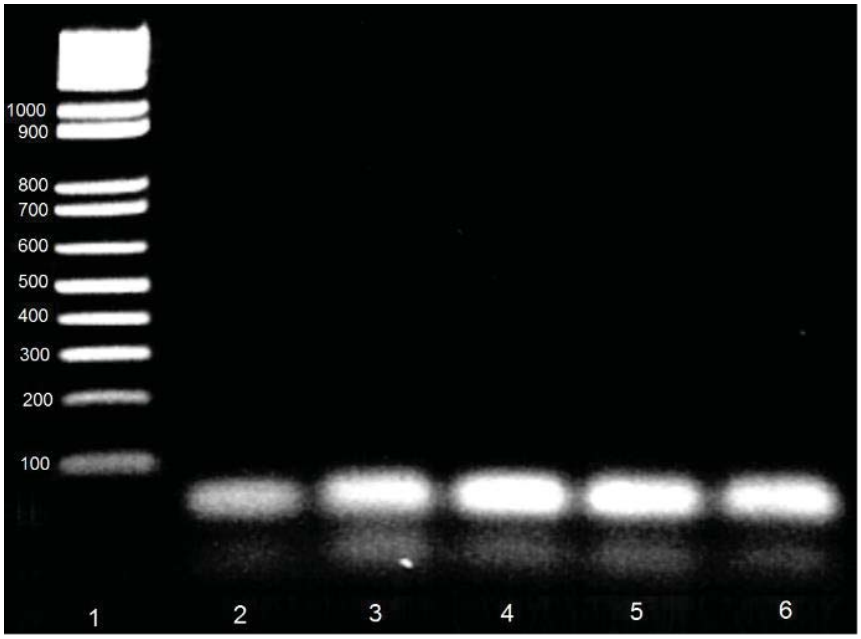


Figure 2-6 Gel electrophoresis showing primer-dimer formation with the FIV qRT-PCR.
Representative sample from the optimisation of the FIV qRT-PCR. All lanes, including the no-template control (lane 6), contain products <100 bp in size, consistent with non-specific amplication (probably primer-dimers).

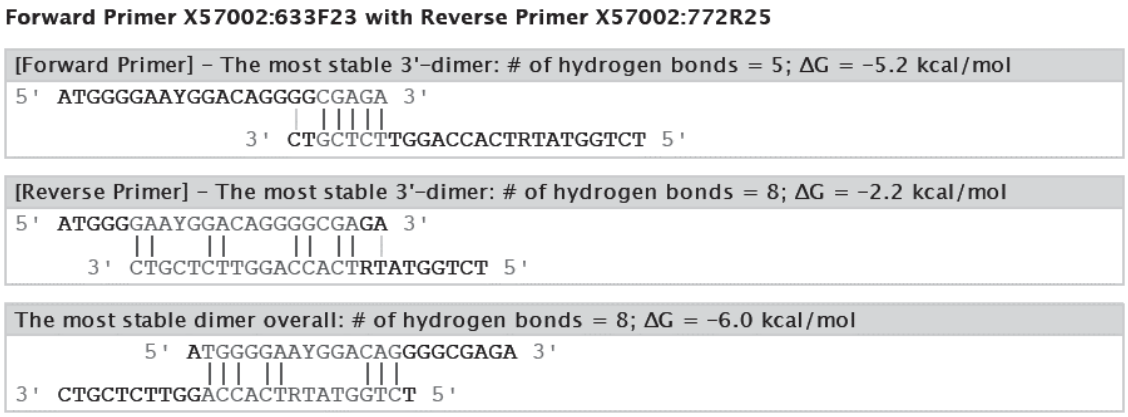


Figure 2-7 Oligo 7 output showing potential for duplex formation between primers.
The top window shows the most stable 3' terminal duplex of the forward primer (shown as light grey). The middle window shows the most stable 3' terminal duplex of the reverse primer. The bottom window shows the most stable dimer overall. The ΔG value represents the energy required to dissociate the most stable duplex, with values ≤ - 2.0 kcal indicating strong duplex formation. These results show that the primers used in this study were prone to primer-dimer formation due to potentially strong duplex formation at the 3' end of each primer, as well as strong overall dimer formation.

2.3.4.2. FIV RT-qPCR

Successful amplification of the desired product was achieved from the positive control (RNA from the Fel-O-Vax® FIV vaccine) using a two-step process (**Figure 2-8**). Sequencing and alignment of the vaccine PCR product to reference sequences of the FIV strains contained in the Fel-O-Vax® FIV vaccine revealed that a 164 bp product of the *gag* gene had been amplified as predicted. The amplified product was 98.1% homologous to the corresponding region of the *gag* gene from the vaccine strain, Shizuoka (**Figure 2-9**).

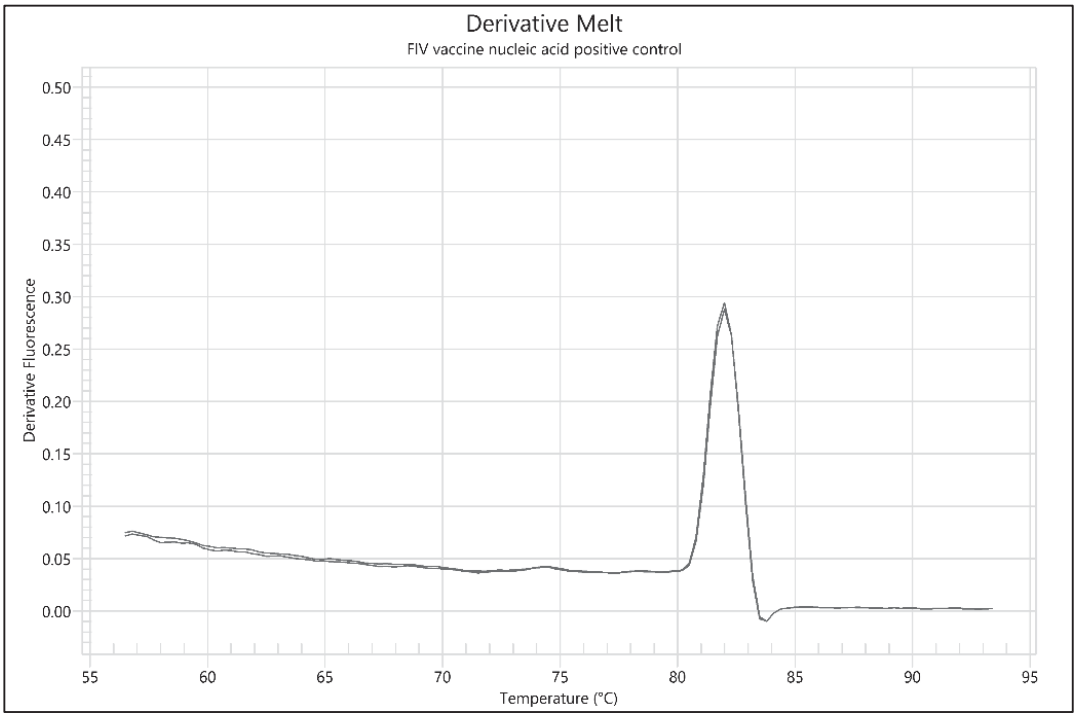


Figure 2-8 Melt curve results from the FIV RT-qPCR.
The FIV R-qPCR was used to amplify the *gag* product from nucleic acid extracted from the Fel-O-Vax® FIV vaccine. In contrast to results from the qRT-PCR, this assay reveals amplification of the target product as shown by the specific melt curve. The melting temperature is represented along the x-axis, and the relative fluorescence is shown on the y-axis.

```
                1        10        20        30        40        50        60
                |        |         |         |         |         |         |
      Petaluma  ATGGGGAATGGACAGGGGCGAGATTGGAAAATGGCCATTAAGAGATGTAGTAATGTTGCT
       Shizuoka ATGGGGAATGGACAGGGGCGAGATTGGAAAACGGCCATAAAGAGATGTAGTAATGTTGCT
Vaccine product ATGGGGAATGGACAGGGGSGAGATTGGAAAACGGCCATAAAGAGATGTAGTA-TGTTGCT

      Petaluma  GTAGGAGTAGGGGGGAA-GA-GTAAAAAATTTGGAGAAGGGAATTTCAGATGGGCCATTA
       Shizuoka GTAG--GTACGGGACAACGAAGTAAGAAGTTCGGGGAAGGAAATTTTAGATGGGCCTTGA
Vaccine product GTAG--GTACGGGACAACGAAGTAAGAAGTTCGGGGAAGGAAATTTTAGATGGGCCTTGA

      Petaluma  GAATGGCTAATGTATCTACAGGACGAGAACCTGGTGATATACCA
       Shizuoka GAATGGCCAATGTAACTACAGGACGTGAACCTGGTGATATACCA
Vaccine product GAATGGCCAATGTAACTACAGGACGAGAACCTGGTGAYATACCA
```

Figure 2-9 Nucleotide alignment of the sequenced FIV RT-qPCR product.
The first 164 bp of the *gag* gene from the vaccine strains, FIV-Petaluma and FIV-Shizuoka, are aligned with the RT-qPCR product sequenced following amplification of vaccine nucleic acid. Pairwise comparison shows 98.1% similarity of the vaccine PCR product to the Shizuoka sequence, and 86.1% similarity to the Petaluma strain, indicating amplification of the Shizuoka nucleic acid contained within the vaccine. Alignment was generated using the Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ). Reference sequences of the *gag* gene were downloaded from GenBank® (Petaluma – M25381, Shizuoka – AY679785).

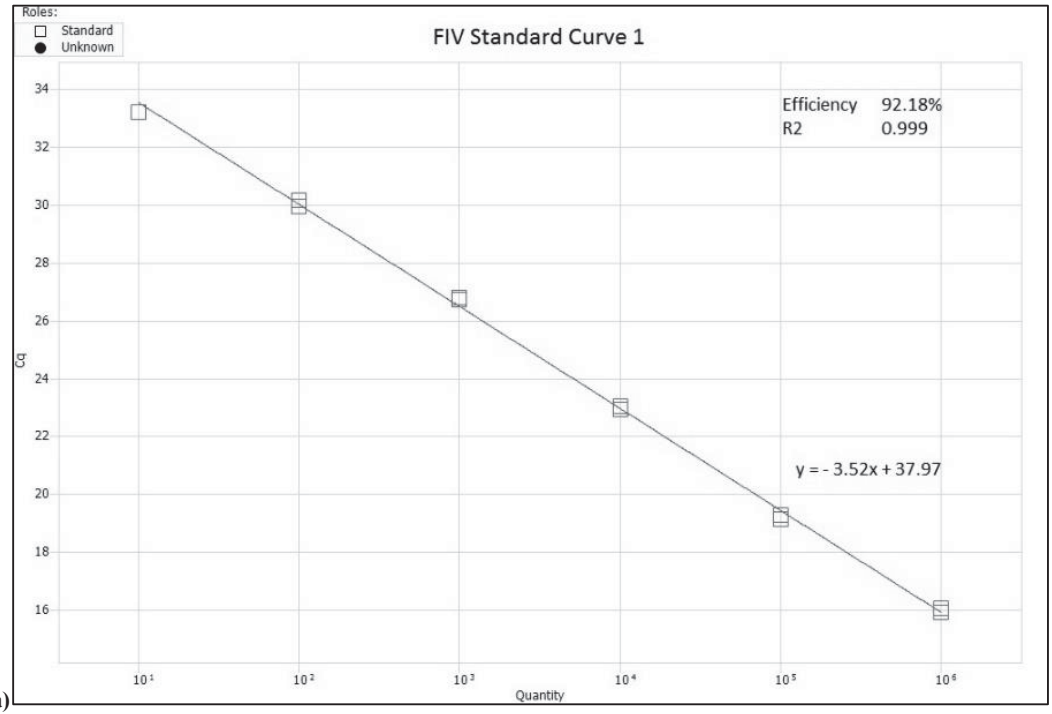
The FIV RT-qPCR was optimised with a SYTO 9 dye (Accumelt HRM Supermix, Quanta) and an asymmetric primer ratio of 1 FIVf : 5 FIVr. The number of cycles was also reduced to 35

to eliminate non-specific amplification. Efficiency was increased by incrementally extending the primer annealing time. The final conditions used in all subsequent experiments are detailed below (**Table 2-7**). An average efficiency of 90.5% was achieved using this assay, with an R^2 value consistently exceeding 0.99 (**Figure 2-10**). A summary of the optimisation process for the FIV RT-qPCR is included in **Appendix 3**.

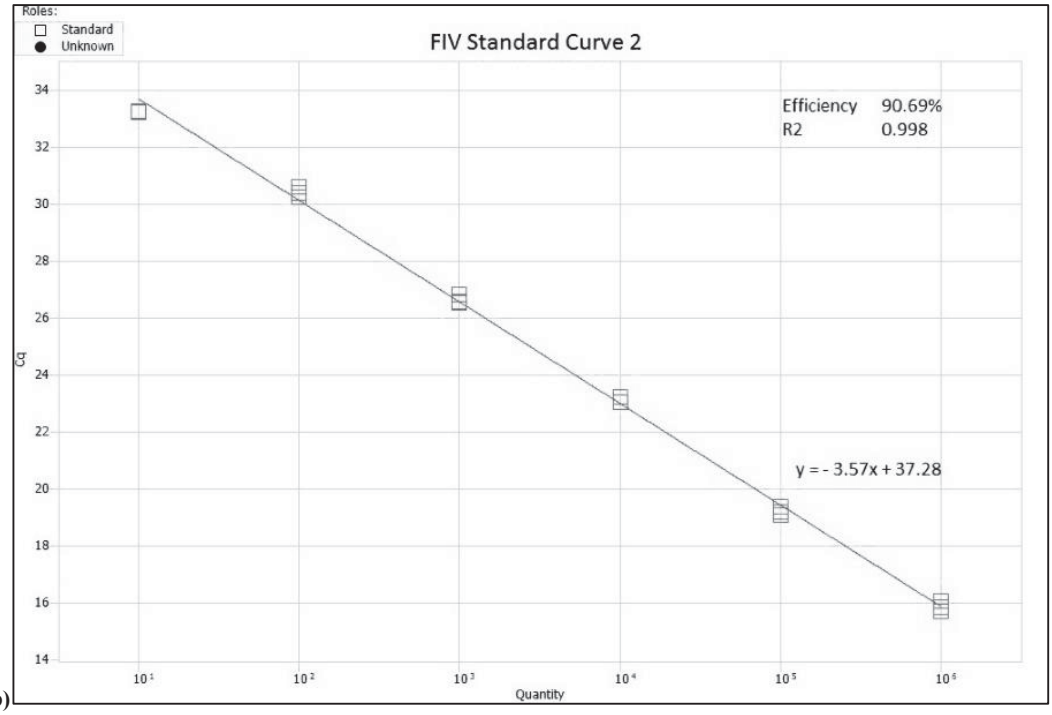
Table 2-7 Final amplification conditions for the optimised FIV RT-qPCR.

Reagents	SYTO 9 supermix (Accumelt HRM, Quanta)	5.0 µL
	Forward primer (FIVf)	0.1 µL (0.2 µM)
	Reverse primer (FIVr)	0.5 µL (1.0 µM)
	Template (Viral cDNA)	2.0 µL
	H20	2.4 µL
	Total volume	10 µL
Cycling conditions	Initial denaturation	95 °C for 5 min
	Denaturation	95 °C for 5 sec
	Annealing	58 °C for 16 sec
	Elongation	72 °C for 15 sec
	Number of cycles	35
	Melting curve	55 °C to 95 °C

a)


Roles:
Standard
Unknown
FIV Standard Curve 1
Efficiency 92.18%
R2 0.999
y = - 3.52x + 37.97
Cq
Quantity

b)


Roles:
Standard
Unknown
FIV Standard Curve 2
Efficiency 90.69%
R2 0.998
y = - 3.57x + 37.28
Cq
Quantity

c)

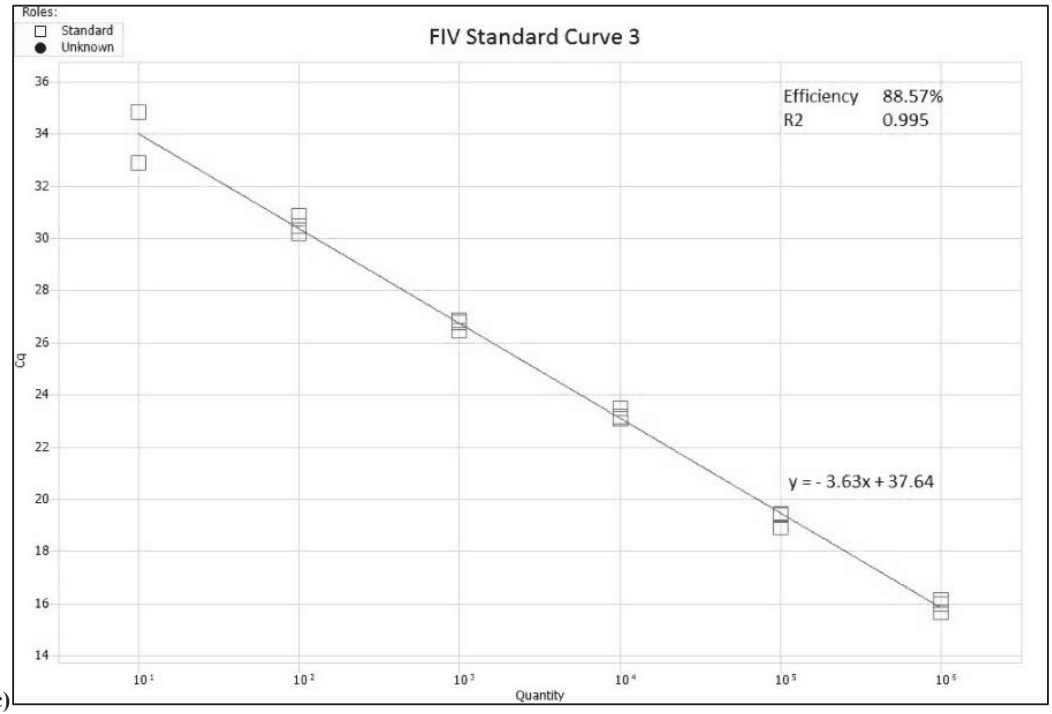


Figure 2-10 Standard curves generated using the optimised FIV RT-qPCR.
The reactions were performed under the conditions detailed in Table 2-7. The x-axis represents the relative copy number of the target DNA, and the y-axis represents the quantitation cycle (Cq). Standards comprised cDNA from the Fel-O-Vax® FIV vaccine. The efficiency and R^2 values are shown in the upper right corner of each graph, with values consistently >90% and >0.99 respectively.

2.3.4.3. Determining sensitivity of the FIV RT-qPCR

The 874 bp region of the *gag* gene was successfully amplified from cDNA produced from the Fel-O-Vax® FIV vaccine nucleic acid, and a stock solution of this product was produced at a concentration of 10^8 copies/µL. Serial dilutions (10^2 to 1 copy/ µL) of the stock solution were used as standards for the FIV RT-qPCR. The specific 164 bp product was amplified from all dilutions of the standards, including one of the replicate of the highest dilution (**Figure 2-11**). The assay was therefore able to detect as little as 1 – 10 copies/µL of target FIV DNA, confirming excellent sensitivity.

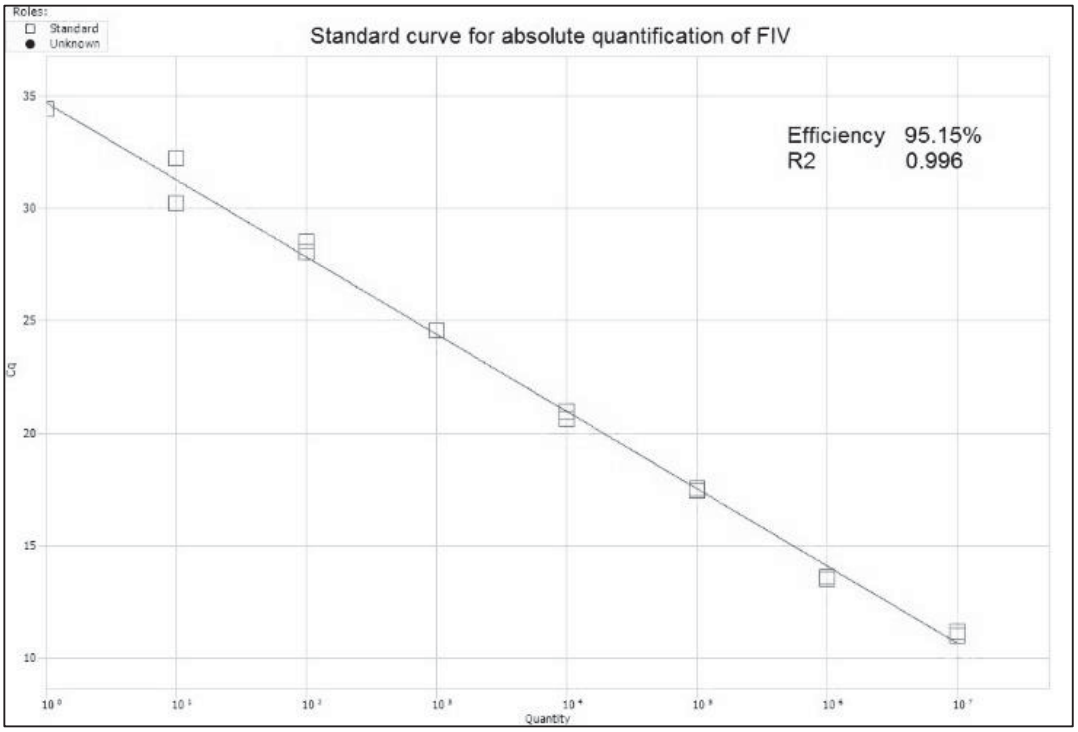


Figure 2-11 Standard curve generated for absolute quantification of FIV.
Standards comprised an 874 bp PCR product of the *gag* gene that included the target 164 bp sequence. The 164 bp product was successfully amplified from all standards including one replicate of the highest dilution (1 copy/µL), indicating that the assay was able to detect as little as 1-10 copies of FIV target DNA.

2.3.5. Virus isolation

2.3.5.1. Virus isolation using donor PBMC

Seven FIV infected cats (one infected with a subtype A virus, and the other six infected with subtype C viruses) were selected based on phylogenetic analysis to obtain a range of viruses representative of the population sampled. PBMC from each cat were co-cultivated with activated donor PBMC from uninfected cats. Quantification of viral nucleic acid in cell culture supernatant over a period of 27 days showed minimal to no production of FIV in four cultures (as shown by no difference in the Cq value over time) (**Figure 2-12**). Although a transient rise in FIV nucleic acid was detected in three of the cultures (as shown by a decrease in Cq value), the concentration subsequently declined, indicating that viral replication was not sustained.

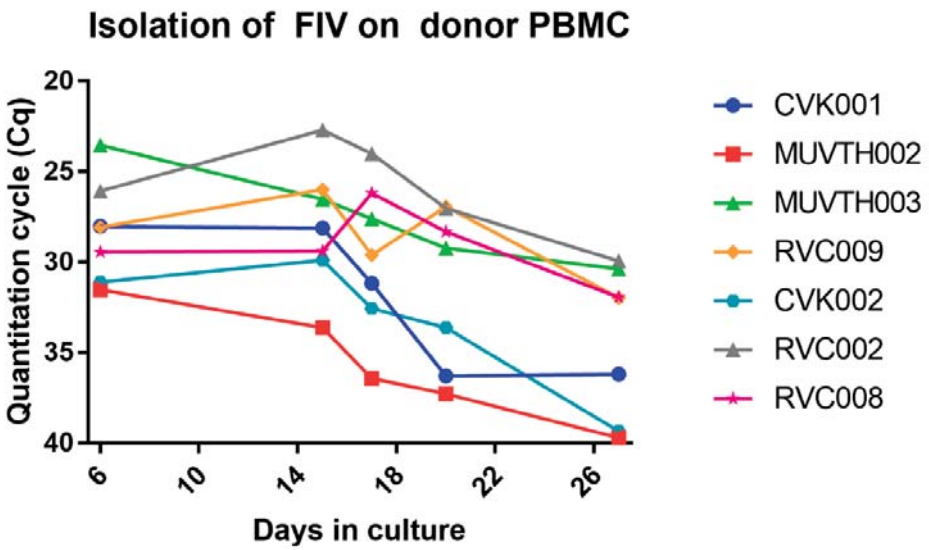


Figure 2-12 Virus isolation from FIV-infected PBMC co-cultured with donor PBMC.
Viral nucleic acid (cDNA from viral RNA and proviral DNA from infected cells) was detected in cell culture supernatant using the FIV RT-qPCR described in section 2.2.5.3. The y-axis represents the quantity of nucleic acid as expressed by the quantification cycle (Cq) at each time point (x-axis). The quantity of nucleic acid declined over time in four cultures, suggesting unsuccessful infection of donor cells. A transient rise in viral load was detected in three cultures, but subsequent decline of the nucleic acid in culture indicated that FIV replication was not sustained.

2.3.5.2. Virus isolation using donor MYA-1 cells

Co-cultivation of PBMC from an FIV infected cat (MUVTH002) with MYA-1 cells resulted in a progressive increase in the quantity of viral nucleic acid in cell culture supernatant over time (as shown by a decrease in Cq value) (**Figure 2-13**). This confirmed active replication of FIV in culture. Based on daily measurement of viral nucleic acid concentrations in this experiment, the optimal time for harvest of the virus was day 9. Three more field viruses were subsequently isolated using this method (MUVTH003, RVC009 and CVK001).

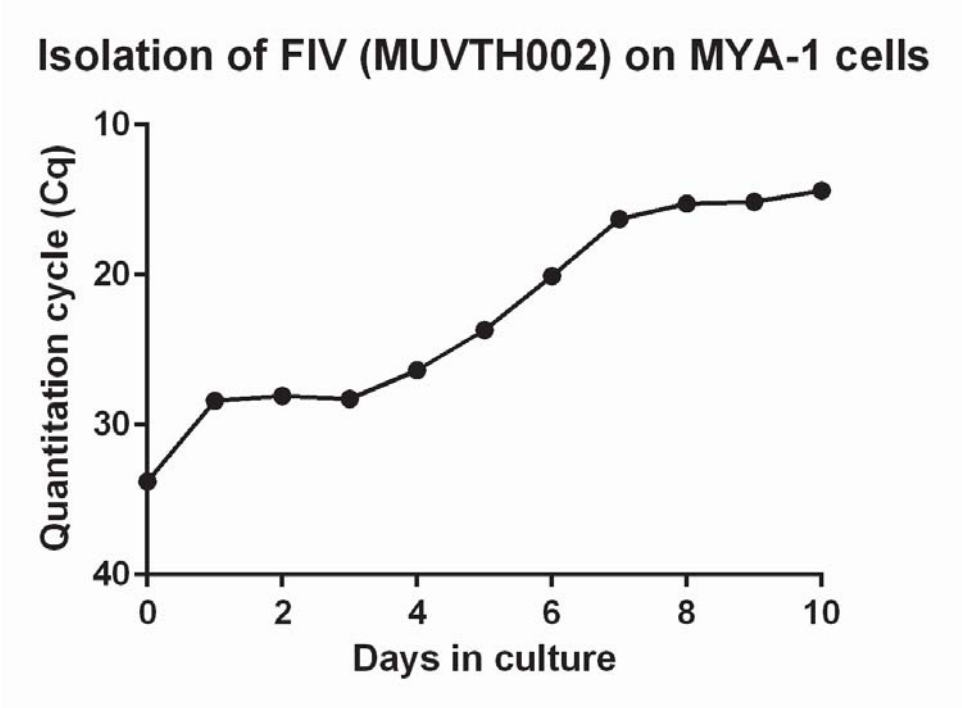


Figure 2-13 Virus isolation from FIV-infected PBMC co-cultured with MYA-1 cells.
Viral nucleic acid was detected in cell culture supernatant using the FIV RT-qPCR described in section 2.2.5.3. The y-axis represents the quantity of nucleic acid as expressed by the quantification cycle (Cq) at each time point (x-axis). The quantity of nucleic acid increased over time, suggesting replication of the virus in culture.

2.3.5.3. Passage of FIV isolates

To ensure that live FIV had been isolated, the isolates from cultures infected with MUVTH002, MUVTH003, CVK001 and RVC009 were passaged one more time in MYA-1 cells. The viral nucleic acids increased (as shown by a decrease in Cq value) in MYA-1 cultures after passaging of all three viruses, indicating that live virus had been obtained from the original virus isolation experiments. Cell cultures infected with MUVTH003 showed a late rise in viral nucleic acid following an initial decrease, presumably due to late replication of the virus (**Figure 2-14**).

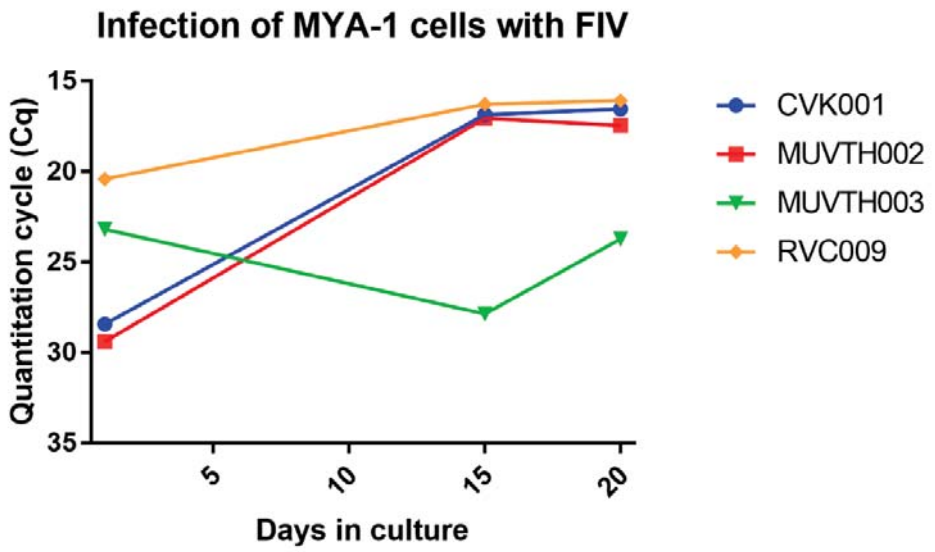


Figure 2-14 Quantification of viral RNA in culture of MYA-1 cells following passage of FIV isolates.
Viral nucleic acid was detected in cell culture supernatant using the FIV RT-qPCR described in section 2.2.5.3. The y-axis represents the relative quantity of viral nucleic acid as shown by the quantification cycle (Cq) at each time point (x-axis). The rising quantity of viral nucleic acids over time indicates active replication of the virus in MYA-1 cells and successful passage of all 4 NZ isolates.

2.3.6. Production of virus from infectious molecular clones

2.3.6.1. Transfection of CRFK cells with the PetF14 clone

Transfection of CRFK cells with the PetF14 clone resulted in successful virus production, as shown by the presence of viral RNA in the CRFK supernatant. Viral RNA concentrations peaked on day 1 (as shown by a decrease in Cq value) and gradually declined over time (**Figure 2-15**). Proviral DNA was also detected in CRFK cells on day 14, confirming successful transfection of cells (**Figure 2-16**). These results indicate successful transfection and release of PetF14 virus from CRFK cells, but no ongoing production of virus beyond day 1.

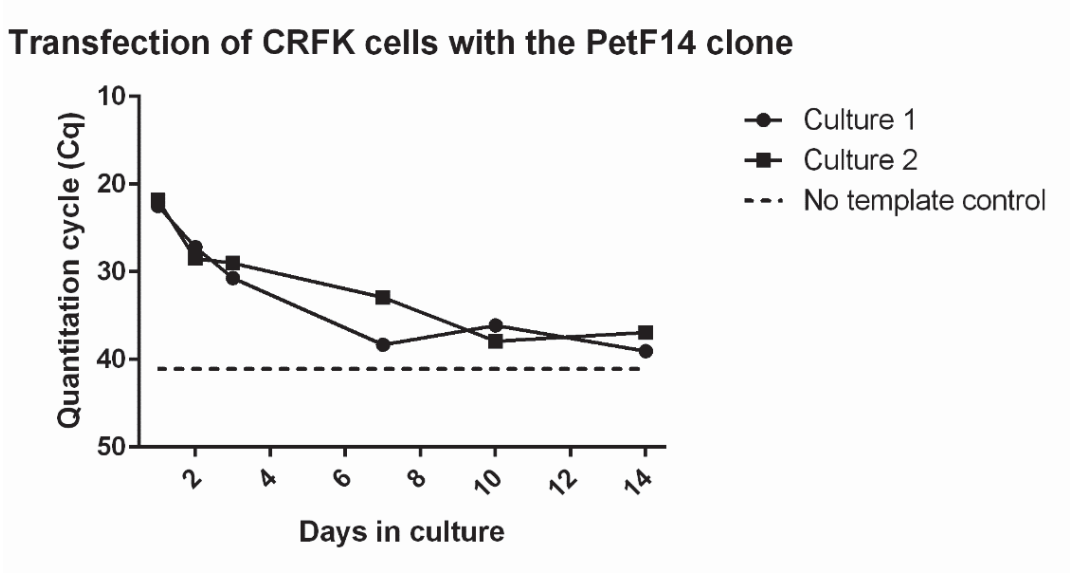


Figure 2-15 Viral replication following transfection of CRFK cells with PetF14 clones.
CRFK cells were transfected with the PetF14 clone in two separate cultures and viral RNA was measured using the FIV RT-qPCR assay as described in section 2.2.5.3. The y-axis represents the quantity of viral RNA as shown by the quantification cycle (Cq) at each time point (x-axis). The hashed line represents the Cq value of the negative (no template) control. Viral RNA is present at day 1, indicating release of virus from transfected cells. The declining quantity of viral RNA over time suggests that the FIV infection was not maintained in culture.

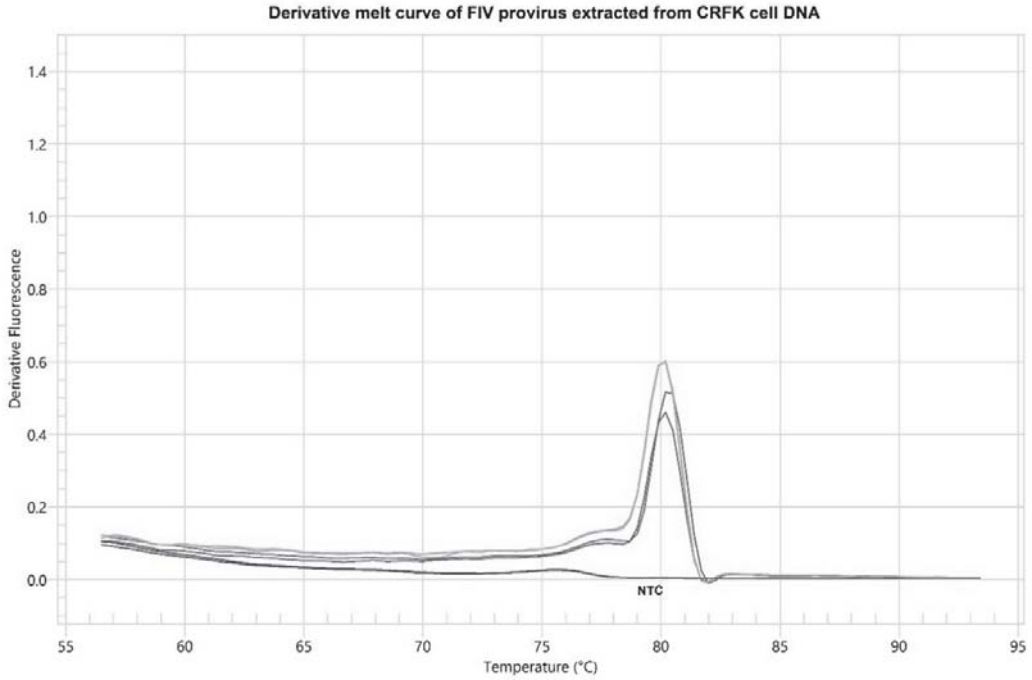


Figure 2-16 Melt curve analysis of FIV DNA from transfected CRFK cells.
DNA was extracted from trypsonised CRFK cells, 14 days post-transfection with the PetF14 clone. CRFK cell DNA from each of the two cultures were tested in duplicate using the FIV qPCR described in section 2.2.5.3. The no template (negative) control is labelled as NTC. The presence of viral DNA in the CRFK samples indicates successful transfection of cells with the FIV clone.

2.3.6.2. Infection of MYA-1 cells with FIV rescued from transfected CRFK cultures

Donor MYA-1 cells became infected with FIV-PetF14 following co-culture with transfected CRFK cells. The quantity of viral RNA was increased at day 7 compared to days 1-4 post-transfection (as shown by a decrease in Cq value). This indicated that active replication of FIV in donor cells occurred between days 4 and 7. Subsequent to day 7, the viral RNA concentration declined over time, suggesting poor ongoing replication of the virus in MYA-1 cells. There was no active production of viral RNA from the MYA-1 cells co-cultured with the GL8 transfected CRFK cells (**Figure 2-17**).

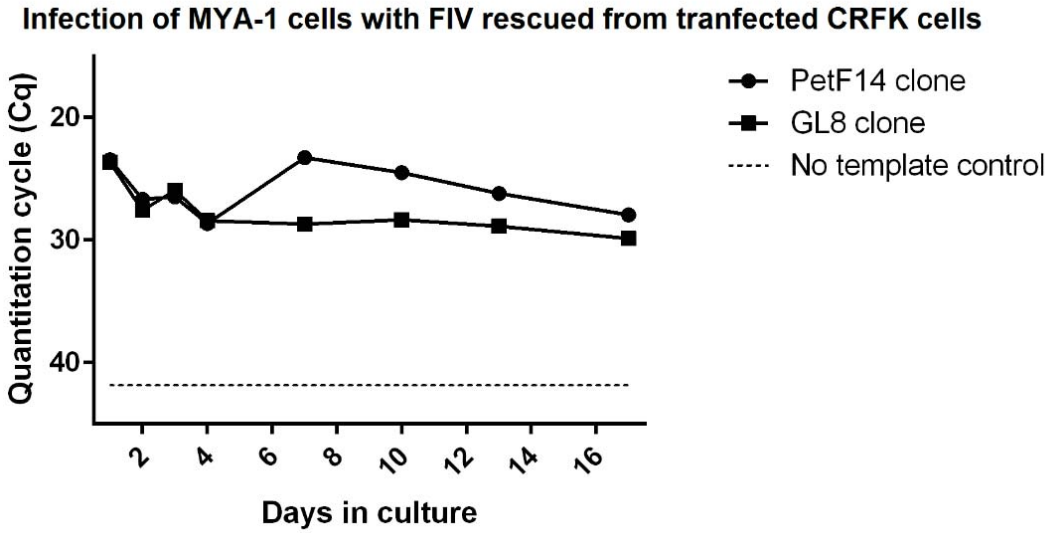


Figure 2-17 Viral RNA sampled from transfected CRFK cells and infected MYA-1 cells.
Supernatant was sampled from PetF14 or GL8 transfected CRFK cells on day 1, CRFK/MYA-1 cell co-cultures on day 2, and MYA-1 cell cultures on days 3, 4, 7, 10, 13 and 17. The y-axis represents the quantity of viral RNA as shown by the quantification cycle (Cq) over time (x-axis). The hashed line represents the Cq value of the negative (no template) control. Viral RNA was present at day 1 in CRFK supernatant, indicating release of virus from cells transfected with both viruses. Peak viral production from MYA-1 cells infected with the supernatant from PetF14 transfected CRFK cells occurred at day 7, indicating successful infection of MYA-1 cells and release of virus. There was no active production of FIV RNA from MYA-1 cells co-cultured with the GL8 transfected CRFK cells.

2.3.6.3. Transfection of 293T cells with the GL8 clone

Transfection of 293T cells with the GL8 clone, followed by co-culture of transfected cells with MYA-1 cells, also resulted in viral RNA production from 293T cells, but no sustained replication of FIV-GL8 in MYA-1 cells. As propagation of the GL8 virus in MYA-1 cells was consistently unsuccessful, this was not available for use in subsequent experiments in this thesis.

2.3.7. Quantification of virus stock

2.3.7.1. Semi-quantification of viral nucleic acid by FIV RT-qPCR

The FIV RT-qPCR was used to semi-quantity FIV nucleic acid contained in each virus stock solution, by comparison of Cq values. The virus stock consisted of pooled supernatant from MYA-1 infected cells, concentrated to include only proteins larger than 100 kDa (as described in section 2.2.8). The concentration of FIV nucleic acid in the MUVH003 stock was considerably lower than that of the other stock solutions (**Figure 2-18**).

Melt analysis revealed differences in the melting point of the amplified region of the *gag* gene from each virus. In particular, the product amplified from the subtype A isolate, RVC009, had a lower melting temperature (80.5°C) when compared to the products amplified from the other stock solutions (82.3 – 82.9°C) (**Figure 2-19**). Differences in melting temperature between products occur due to variation in nucleic acid composition of the sequences. These results therefore suggest that there was some sequence variation between isolates at the amplified region of the *gag* gene in addition to the *env* gene variation described in section 2.3.3.

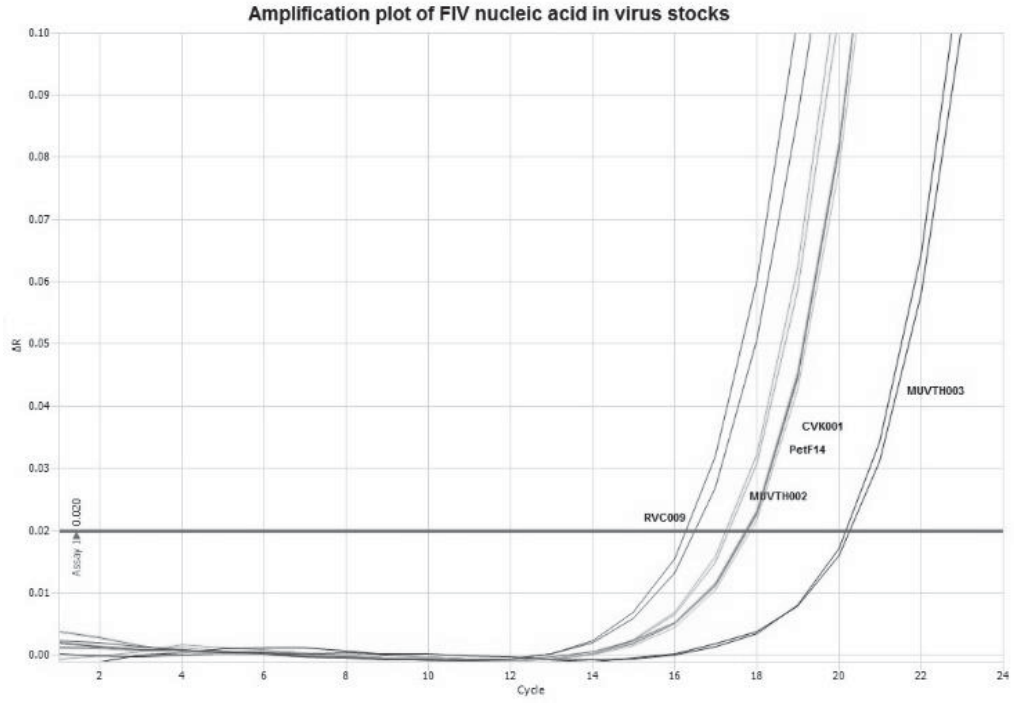


Figure 2-18 Amplification plots of FIV DNA and cDNA in virus stocks.

The Cq values were compared to semi-quantify the amount of viral nucleic acid contained in each of the virus stocks following amplification of FIV nucleic acid using the FIV RT-qPCR described in section 2.2.5.3. The RVC009 stock contained the highest amount (HIGH) of viral nucleic acid (average Cq = 16.38), the MUVTH002, PetF14 and CVK001 stocks contained a similar amount (MEDIUM) of viral nucleic acid (average Cq = 17.29, 17.75 and 17.78 respectively) and the MUVTH003 stock contained the lowest amount (LOW) of viral nucleic acid (average Cq = 20.22).

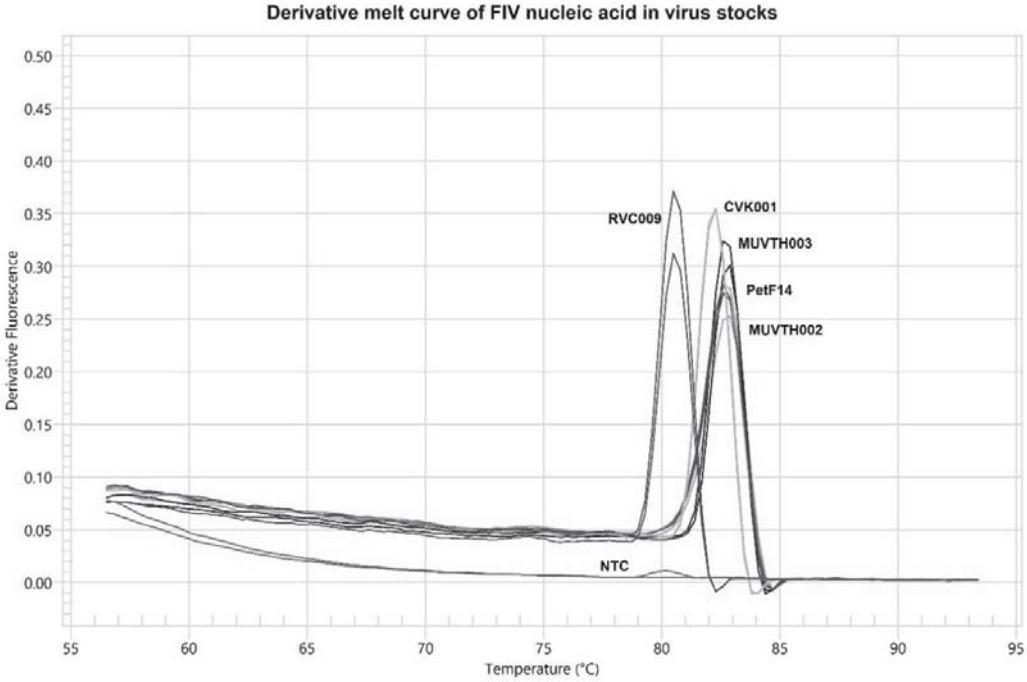


Figure 2-19 Melt curve analysis following FIV RT-qPCR on virus stock.
The *gag* FIV product was amplified from each virus stock solution using the FIV RT-qPCR described in section 2.2.5.3. The product amplified from the RVC009 stock has a lower melting temperature (80.5 °C) when compared to that amplified from the other stock (82.3 – 82.9°C). The no template controlled is labelled as NTC.

2.3.7.2. Endpoint dilution assay

2.3.7.2.i. Comparison of DNA extraction kits for detection of proviral DNA in culture

DNA was extracted from serial dilutions of MUVTH002-infected MYA-1 cells, using two different DNA extraction kits. Use of the High Pure PCR Template Preparation Kit (Roche) resulted in detection of FIV provirus from all cell concentrations tested using the FIV qPCR described in section 2.2.5.3. In contrast, FIV provirus was only detected in the sample containing the highest concentration of MYA-1 cells following extraction of DNA using the Genomic DNA Mini Kit (Geneaid) (**Figure 2-20**). This suggested that the Roche kit was more suitable than the Geneaid kit for extracting very small quantities of DNA. As the quantity of FIV provirus was expected to be low in the cell cultures prepared for the endpoint dilution assay, the Roche kit was used for all subsequent DNA extraction procedures.

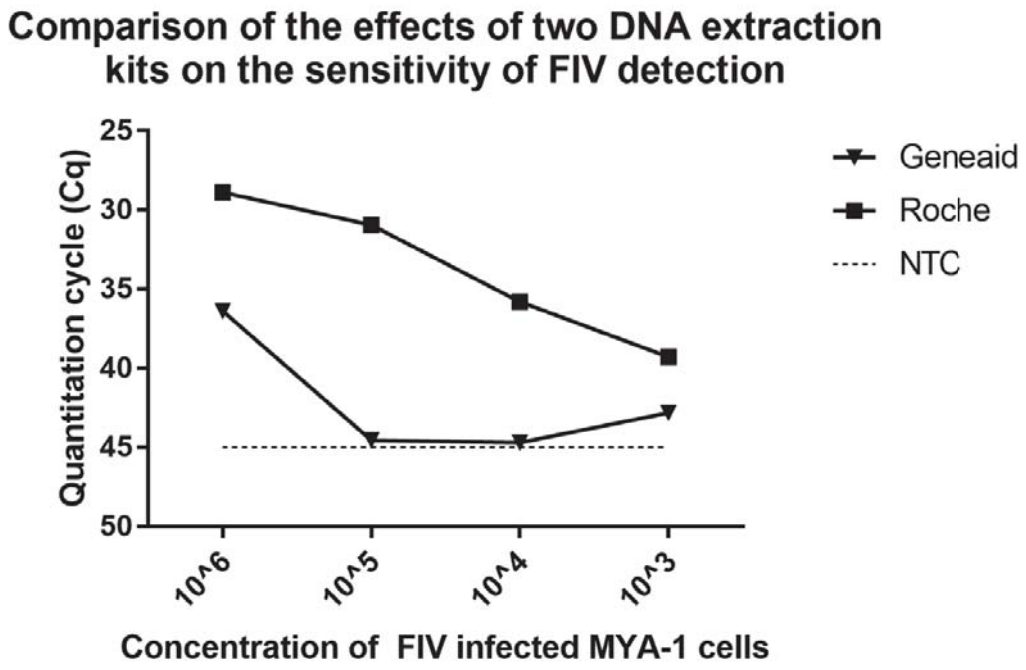


Figure 2-20 Comparison of DNA extraction methods.
Ten-fold serial dilutions of MYA-1 cells from FIV infected cultures were prepared and DNA was extracted using either the High Pure PCR Template Preparation Kit (Roche) or the Genomic DNA Mini Kit (Geneaid). FIV DNA was then amplified using the FIV qPCR described in section 2.2.5.3. The quantity of DNA present in the samples following DNA extraction is shown on the y-axis as the quantification cycle (Cq) at each time point (x-axis). The hashed line represents the negative (no template) control (NTC). Use of the Geneaid kit did not result in extraction of sufficient DNA for detection by PCR at lower concentrations of provirus, whereas use of the Roche kit enabled detection of viral DNA at all cell concentrations tested.

2.3.7.2.ii. Virus stock titration

The quantity of FIV provirus in MYA-1 cells was compared after 15 days in cultures that had been infected with live virus and with thermally inactivated virus. As was expected, the quantity of FIV DNA (shown by the Cq value) amplified from the cultures containing inactivated virus was much lower than that amplified from the cultures infected with live FIV (Cq 33.3 versus 20.3) (**Figure 2-21**). The presence of DNA in the cultures containing inactivated virus was not unexpected, given that virus stock was produced from cell culture supernatant (and therefore may have contained FIV provirus released from necrotic cells).

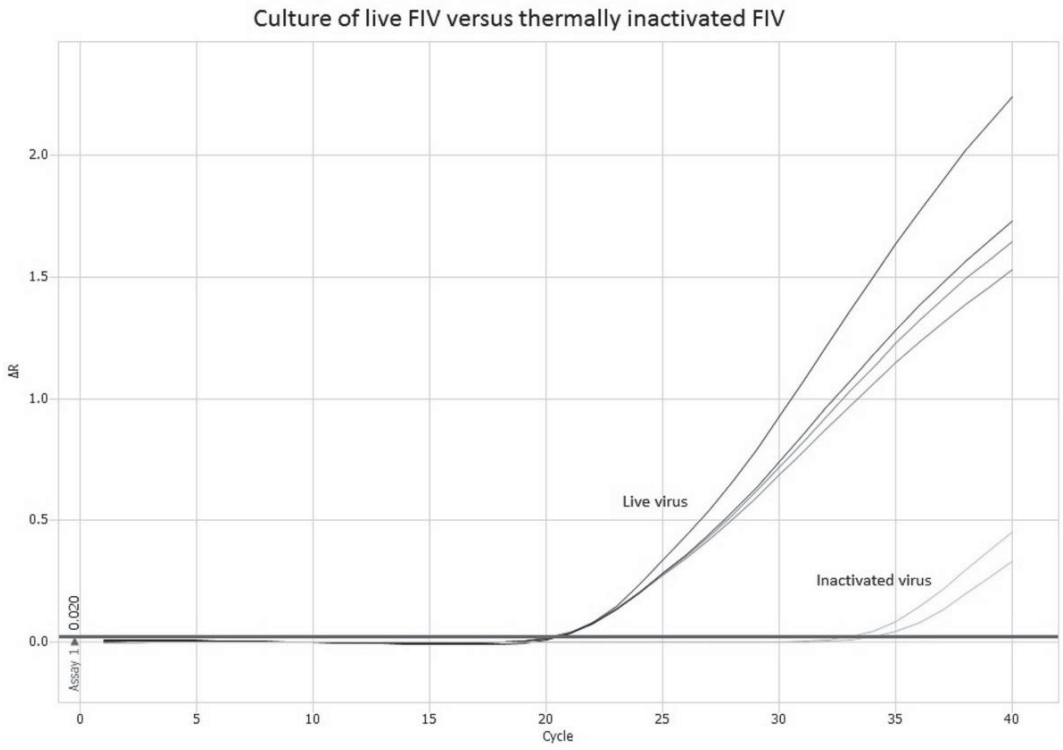


Figure 2-21 Amplification of live versus inactivated FIV.
Amplification plots of FIV provirus tested in duplicate from two separate MYA-1 cultures, 15 days after infection with live FIV (MUVH003) or thermally inactivated virus. The Cq value is shown along the x-axis, and the y-axis shows the fluorescence of the reporter dye minus the baseline fluorescence (ΔR). Results showed amplification of provirus from cultures to which inactivated virus was added, but at a much lower level compared to the live virus, suggesting detection of inactivated virus with no subsequent replication in culture.

The $TCID_{50}$/mL was calculated following detection of FIV DNA in cultures infected with 10-fold serial dilutions of each virus. At low dilutions ($>10^{-3}$), viral DNA was detected in the wells containing inactive virus (representing DNA contained in the stock solution added to the cultures). However, the quantity of FIV DNA in the cultures infected with inactive virus was consistently lower than the quantity present in the cultures infected with live viruses. Therefore, a culture was considered to be infected with FIV if the Cq value was less than that measured in the well containing a corresponding dilution of the inactivated virus. The tissue culture infective dose for each virus stock was calculated to be between $10^{4.8}$/mL and $10^{5.8}$/mL. A sample of the end point dilution assay results are provided in **Table 2-8**, and results from all viruses are included in **Appendix 4**.

Table 2-8 Representative results from endpoint dilution assay.
Wells infected with live virus were considered positive when the Cq value was less than that from the well containing the corresponding dilution of inactivated virus. Positive wells are labelled '+'. Where FIV DNA was detected in a well infected with inactivated virus, it is labelled '(+)', so long as the quantity of FIV DNA was lower than the corresponding well infected with live virus. If FIV DNA was not detected in a given well, it is labelled '-'. The proportion of infected wells positive at each dilution is given as a percentage. The proportionate distance, 50% endpoint dilution and final titre (in $TCID_{50}$/mL) was calculated using the Reed & Muench method (Reed and Muench, 1938).

PetF14		**10**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**	**10^{-11}**	**10^{-12}**
Presence of FIV DNA	Live virus	+	+	+	+	+	-	-	-	-	-	-	-	-
		+	+	+	+	-	-	-	-	-	-	-	-	-
	Inact. virus	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
		(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
% infected		100	100	100	100	50	0	0	0	0	0	0	0	0

Proportionate distance	0
50% endpoint dilution	10^{-4}
$TCID_{50}$/mL	$10^{5.3}$

2.3.7.3. P24 ELISA

A standard curve was generated using serial dilutions of recombinant p24 protein from the commercially available ELISA. The curve was linear in the concentration range of 10-100 ng/mL p24. The range of p24 concentrations calculated for the undiluted viral stock was 48-71 ng/mL (**Figure 2-22** and **Table 2-9**). For one viral stock solution, RVC009, the p24 concentration calculated for the 1/5 dilution did not correlate with that calculated for the 1/10 dilution. As the absorbance of the 1/10 dilution fell below the linear range of the standard curve, this was likely to be the source of the inaccuracy, so the final concentration was calculated from the 1/5 dilution only. The absorbance of both diluted samples of MUVTH003 fell below the linear range of the standard curve despite repeating the assay with more concentrated samples. Due to the limited availability of reagents, this assay was not repeated a third time, so the MUVTH003 virus was not quantified according to p24 concentration.

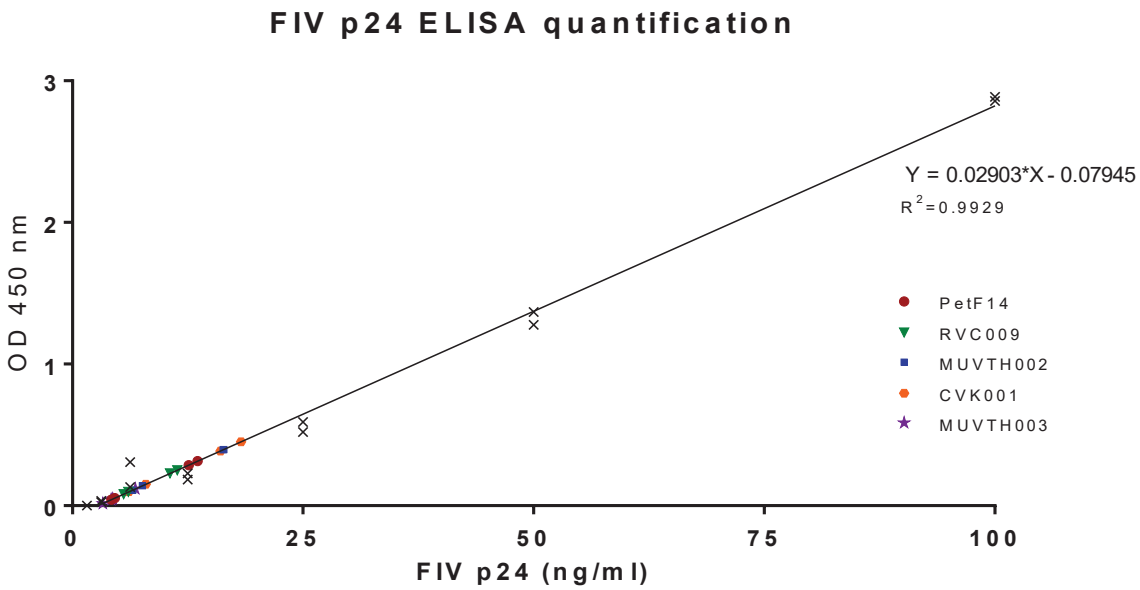


Figure 2-22 Standard curve used to calculate the p24 concentration of virus stocks.
An ELISA was used as described in section 2.2.9.3 to generate a standard curve with serial dilutions of recombinant p24 protein. The normalised optical density (OD) measured at 450 nm for each standard dilution is shown on the y-axis, and values are plotted against the known concentration of the p24 solution on the x-axis (and marked as 'x' in duplicate). A regression equation was generated and used to create the standard curve from which the p24 concentrations in the virus stocks dilutions was calculated.

Table 2-9 The p24 concentration of each virus stock solution.
The final concentration was obtained by multiplying the p24 concentration of each dilution (as calculated from the ELISA standard curve) by the dilution factor, then averaging the two values. The concentration of MUVTH003 could not be calculated, as the OD value measured for each dilution was below the linear range of the standard curve.

Viral stock	Dilution factor	P24 concentration (ng/mL)	Concentration of p24 in undiluted stock solution (ng/mL)
PetF14	3	16.22	48.30
PetF14	6	7.99	
MUVTH002	3	19.34	60.53
MUVTH002	6	10.50	
MUVTH003	3	8.61	<10
MUVTH003	6	7.87	
CVK001	3	20.10	61.61
CVK001	6	10.49	
RVC009	5	14.21	71.07
RVC009	10	9.31	

Results from each method of quantification were compared to ensure consistency in determination of virus concentration (**Table 2-10**). The stock solution of MUVTH003 contained a comparatively low concentration of the virus, as determined by low concentrations of FIV nucleic acid, p24 and low titre. The RVC009 stock solution contained a high concentration of virus, as indicated by all 3 methods.

Table 2-10 Summary of quantification results for each virus using 3 different methods.
The amount of viral nucleic acid (cDNA and DNA) was semi-quantitated, based on comparison of Cq values

Virus stock	Amount of FIV cDNA/DNA.	Titre ($TCID_{50}$/mL)	P24 concentration (ng/mL)
PetF14	MEDIUM	$10^{5.3}$	48.30
MUVTH002	MEDIUM	$10^{5.8}$	60.53
MUVTH003	LOW	$10^{4.8}$	<10
CVK001	MEDIUM	$10^{5.8}$	61.61
RVC009	HIGH	$10^{5.8}$	71.07

2.4. Discussion

The aim of the work presented in this chapter was to prepare stock of a known titre of several NZ FIV isolates, for use in subsequent work described in this thesis. In addition, virus stock of the vaccine strain, Petaluma, was also prepared as a positive control for subsequent assays investigating the cross-reactivity of the vaccine-induced immune response.

Phylogenetic analysis comparing the 4 isolated NZ variants of FIV revealed differences in the sequence of the V3-V6 region of the *env* gene. Pairwise comparison confirmed heterogeneity at this location, showing that the nucleotide sequences differed by 8-22% between viruses. Based on sequencing of the *env* gene, three of the NZ viruses were classified as subtype C (MUVTH002, MUVH003 and CVK001), and the other was classified as subtype A (RVC009). These 4 viruses represented FIV types most commonly identified in this study, which was in agreement with previous studies on FIV subtypes circulating in NZ (Kann *et al.*, 2007b, Hayward *et al.*, 2007). Of the 13 successfully sequenced viruses, 10 belonged to subtype C, 2 belonged to subtype A, and 1 virus was found to be an A/C recombinant. Although the prevalence of subtype C in this

study was higher than previously reported (77% versus 10-48%), this should be interpreted with caution as this study was not designed to determine the subtype distribution of FIV in NZ. Sampling only a very small number of cats from a limited geographical location may have introduced bias into the results, especially if viruses sampled from the same location shared a common ancestor. The discovery of a recombinant isolate of FIV is not surprising given the nature of the virus. Intersubtype recombination may occur when a cell is co-infected with two different subtypes of FIV. Hybrid virions are produced, leading to a recombination event during reverse transcription of the virus (Hu and Temin, 1990). Both co-infection and recombination of FIV have been reported previously, in NZ and overseas (Kann *et al.*, 2007a, Hayward and Rodrigo, 2008, Reggeti and Bienzle, 2004, Bachmann *et al.*, 1997).

In addition to differences identified in the *env* gene between the 5 isolates prepared for this study, there was also evidence of sequence variation at the *gag* gene. Melt analysis revealed a difference in the melting temperature of the qPCR products obtained from each virus. The melting temperature is the point at which the 2 strands of DNA separate, resulting in dissociation of the dye and a rapid drop in fluorescence. The shape and location of melting curves are dependent on nucleotide (G and C) content and distribution, length and sequence of the product (Ririe *et al.*, 1997). In this study, the melting temperature of the RVC009 product was approximately 2 °C lower than that of the 4 other products, suggesting a difference in the sequence of the amplified region of the *gag* gene from this virus compared to the others. Although the RVC009 and Petaluma viruses both belonged to subtype A according to phylogenetic analysis of the *env* gene, differences between their *gag* genes may have resulted from intergenic recombination of one of the viruses, or may simply reflect intrasubtype variation at this location (Hayward *et al.*, 2007). Amplification and sequencing of a larger region of the *gag* gene by conventional PCR would be required to investigate these differences further. Sequence variation at the *env* gene was considered to be most relevant to this study, as it is the region of highest variation and encodes the envelope glycoprotein that is expressed on the exterior surface of virions and infected cells

(and therefore most likely to be involved in the characteristics described in subsequent chapters of this thesis).

The NZ field isolates and Petaluma strain of FIV were quantified using different methods. This was to enable standardisation of the virus stock in subsequent experiments, according to either infectious dose ($TCID_{50}$) or protein concentration (p24). The amount of viral nucleic acid was also semi-quantified (based on Cq values following RT-qPCR). Some variation was expected using these different methods to measure the concentration of virus. In support of this, studies comparing methods of quantification of HIV have shown poor correlation between infectious dose and p24 or RNA concentrations (Marozsan *et al.*, 2004). This is because nucleic acid and protein content do not reflect infectivity, as these components may be present regardless of the viability of the virus. Similar results from each method used in this study suggest that the virus stock produced contained mostly viable virus.

From the 22 FIV antibody-positive cats initially recruited, only 20 tested positive for FIV provirus by PCR in this study. The samples that tested negative by PCR may have been true negatives, with a false positive result obtained by serology. The specificity of in-house serology tests commonly used for diagnosis of FIV in NZ ranges from 98.2 – 100% (when compared to Western blot or PCR in a population with a low prevalence of FIV) (Sand *et al.*, 2010). False positive results are possible however, as a result of maternally derived antibodies in young kittens, cross-reactivity of the assay and previous vaccination (Barr, 1996). Alternatively, the PCR results for these two cats may have been falsely negative. The sensitivity of the conventional PCR assay developed in this study was not determined, but the sensitivity of commercially available PCR ranges from 41 – 93%, with false negatives occurring most commonly due to sequence variation of the virus (Crawford *et al.*, 2005). The PCR used in this study was a nested assay, with primers previously shown to amplify the sequences commonly found in NZ. It was based on the highly variable *env* gene, so that this region could be sequenced and compared between isolates. Therefore, the possibility that the 2 serologically positive cats were in fact infected with FIV cannot be excluded, either with a provirus load below the level of detection by the assay, or

harbouring a virus that differed in sequence to such an extent as to prevent primer binding. An assay targeting the more conserved regions of the *gag* or *pol* gene may have been able to detect FIV provirus in these cats.

Medical records from each of the FIV positive cats were reviewed. The majority (15/22) of the recruited cats had been routinely tested for FIV prior to vaccination with the Fel-O-Vax® FIV vaccine, and as would be expected, these cats were generally clinically normal. A small group of cats were tested for FIV following presentation with a variety of clinical signs. Two cats had non-specific clinical signs and were euthanased, and the remaining five cats had been diagnosed with either lymphoma, renal disease, nasal disease, a nasopharyngeal polyp or cellulitis. The variety of clinical signs in cats recruited for this study is in accordance with the literature on FIV infection (Hutson *et al.*, 1991, Fujino *et al.*, 2009, Yamamoto *et al.*, 1989, Ishida *et al.*, 1989, Hopper *et al.*, 1989, Sparkes *et al.*, 1993, Matsumura *et al.*, 1993, Callanan *et al.*, 1992, Kohmoto *et al.*, 1998, Ishida *et al.*, 1992, Gabor *et al.*, 2001, Maingat *et al.*, 2009). No conclusions can be made from the clinical presentation of the FIV infected cats recruited in this study, as the clinical information provided data at a single time point only, and no information was available regarding the time course of infection to clinical signs in the sick cats. However, the findings do highlight the frequent occurrence of FIV positive cats that are clinically normal, which reflects the prolonged asymptomatic period, characteristic of lentiviral infections.

To isolate the virus from infected cats, a technique was required to detect viral replication in culture. Methods previously reported include ELISA to measure viral protein production (e.g. p24), functional assays to detect RT activity, and qPCR to detect viral DNA and/or RNA. There are advantages and disadvantages of each method, and these are more thoroughly reviewed by Ammersbach & Bienzle (2011). Quantitative RT-PCR was chosen in this study due to its high sensitivity and ease of use following establishment of the assay. Use of a one-step RT-PCR was desirable, as this reduces the time required and the risk of inaccuracy associated with multiple steps. Despite extensive troubleshooting, development of the qRT-PCR was unsuccessful using the selected primers. Visualisation of products by gel electrophoresis and melt curve analysis

revealed non-specific amplification in all samples, including no-template controls. The products were small and consistent with primer-dimer formation. Primer-dimers are formed when primers anneal to themselves or to each other, rather than to the template. Amplification of the hybridised primers then occurs, consuming qPCR reagents and inhibiting specific amplification of the template DNA. Primer-dimer formation is more common with certain primer combinations, depending on the number and location of the complementary bases they contain. The primer sequences selected for this study had been designed by previous authors for use in qPCR, however their assay was designed to amplify viral DNA rather than RNA (Wang *et al.*, 2010). Amplification of RNA requires the addition of RT to the reaction in order to produce cDNA from the RNA template. The presence of RT in the reaction increases the probability of primer-dimer formation, as the enzyme can add nucleotides to the 3' end of annealed primers, further stabilising the primer-dimer product (Chumakov, 1994). Analysis of primer duplex formation using computer software in this study confirmed the propensity for primer-dimer formation with the selected primers. The energy required to dissociate these primers at the 3' ends exceeded the recommended value ($\Delta G \geq$ -2.0kcal recommended, ΔG = -5.2kcal and -2.2kcal for selected forward and reverse primers in this study) (Science, 2008). In addition there was risk of relatively strong overall dimer formation (ΔG = -6.0kcal, recommended $\geq$-6.0kcal). As attempts to optimise the reaction and reduce primer-dimer formation had been unsuccessful, a two-step reaction was developed in preference to designing a new set of primers, with the reverse transcription step separate to qPCR amplification.

Optimisation of the FIV RT-qPCR was required to increase the specificity of the amplification, as low level primer-dimer formation still occurred in this reaction. An asymmetric primer concentration of 1 forward : 5 reverse successfully decreased primer-dimer formation, by reducing the amount of excess primer available for mixed duplex formation. Optimisation was also performed to improve the efficiency of the reaction. The efficiency of a qPCR reaction is a measure of the yield of product after each cycle, and an efficiency of 100% means that the amount of DNA doubles with each cycle. Efficiency greater than 100% implies some degree of non-

specific amplification. To improve efficiency in this reaction, the detector dye SYBR green was replaced with a SYTO 9 dye. Both of these dyes are fluorescent and bind selectively to double stranded DNA, however SYTO dyes have proven superior to SYBR green, resulting in improved efficiency (Eischeid, 2011, Monis *et al.*, 2005). Efficiency was further improved by incrementally increasing both the elongation and annealing times, until an efficiency >90% was achieved. The resultant assay was reproducible and accurate, with an acceptable efficiency (average 90.5%) and an R^2 value exceeding 0.99.

Virus isolation was initially attempted by co-culturing PBMC from infected cats with activated donor PBMC. This method has been described by previous authors and was one of the methods used in the original papers describing the isolation of FIV from infected cats (Matteucci *et al.*, 1993, Pedersen *et al.*, 1987, Friend *et al.*, 1990). Attempts to cultivate FIV in activated donor PBMC were largely unsuccessful in this study. The quantity of viral nucleic acid declined over time in 4/7 cultures, suggesting either a failure to infect donor cells or very low level replication in culture (with media changes obscuring a rise in the levels of FIV nucleic acid). In three of the cultures, a transient increase in FIV nucleic acid concentration did occur, but this was not sustained over time. FIV may have been transiently produced in these cultures, with the infection unable to be maintained by the donor cells in culture. Alternatively, release of proviral DNA from dying cells may have accounted for the small increase observed in nucleic acid concentration. Previous authors have supplemented activated PBMC cultures with human recombinant IL-2 (Matteucci *et al.*, 1993, Pedersen *et al.*, 1987, Friend *et al.*, 1990). IL-2 is required by activated T cells for survival as well as proliferation (Vella *et al.*, 1998). Although stimulation with ConA does result in production of IL-2 from activated cells, autologous production may not be sustained *in vitro* for more than a few days (Katial *et al.*, 1998, Farrar *et al.*, 1980). Failure to supplement cultures with IL-2 in this study may have resulted in apoptosis of activated T cells, thereby reducing the availability of donor cells to maintain FIV replication in culture.

Lymphoid cell lines, such as the MYA-1 cells used in this study, are often preferred to donor PBMC for isolation of FIV (Dunham *et al.*, 2006a, Crawford *et al.*, 2005, Giannecchini *et al.*, 1996, Matteucci *et al.*, 1995, Meers *et al.*, 1992). MYA-1 cells are of T cell origin and express CD4, MHC class II and CD25 (Miyazawa *et al.*, 1992). They are highly permissible to FIV infection and high quantities of virus are produced upon co-culture with FIV infected PBMC (Miyazawa *et al.*, 1989b). The percentage of FIV positive MYA-1 cells in a given culture generally exceeds that of infected donor PBMC, suggesting that they are more permissive to FIV infection than PBMC (Miyazawa *et al.*, 1989b). In addition, as they are readily maintained in culture indefinitely, there is increased availability of cells for the virus to infect. MYA-1 cells grow rapidly in culture, as long as their media requirements are strictly adhered to. In this study, the use of new born calf serum instead of FBS resulted in poor growth and eventual cell death of MYA-1 cells. They also have a requirement for supplementation with IL-2, which can be a limiting factor depending on availability and cost. However, the use of MYA-1 cells eliminated the need for collecting PBMC from donor cats, making them an excellent alternative to PBMC for FIV isolation.

Infectious molecular clones were used to produce stocks of 2 well characterised FIV strains (FIV-Petaluma and FIV-Glasgow-8). Transfection of an adherent cell line with an infectious molecular clone enables large quantities of a live virus to be produced from proviral DNA. Production of live Petaluma virus was successful, as shown by the presence of viral RNA in the supernatant of transfected cells and subsequent infection of MYA-1 cells with FIV from this supernatant. In contrast, live infectious virus was not produced from the GL8 clone. Following transfection, viral RNA was present in media from transfected CRFK cells, but the supernatant was not infectious for MYA-1 cells. The reason for this is unclear, but it is possible that the transfection procedure resulted in the production of replication-deficient virus. When virus is produced from transfected cells, virions contain host proteins from the transfected cells. As CRFK and 293T cells are not natural hosts for the GL8 strain of FIV (as opposed to the Petaluma strain which can replicate in CRFK cells), the presence of host restriction factors in virions may have

resulted in impaired replication. Alternatively, the cloning or transfection process may have resulted in a lethal mutation of the virus. Repeated attempts to obtain live virus by transfection were not successful, so the GL8 strain of FIV was not available for use in subsequent experiments.

The overall aim to produce stocks of FIV for use in future experiments was achieved, however there were several limitations of the various methods used. In particular, many of the experiments in this chapter were performed only once, so the repeatability to these results cannot be confirmed. Multiple replicates were used where appropriate to improve validity of the data, however day to day variability of the assays was not assessed. Given that the majority of the experiments in this chapter were designed for the purposes of learning laboratory techniques and optimisation of methods, time and limited resources were not used to repeat experiments that would not change the outcome of the research.

As previously discussed, only a small region of the *env* gene from each virus was sequenced. Sequence variation at this region is not necessarily representative of variation across other regions of the genome. In subsequent chapters of this thesis, work is described that compares characteristics of the NZ FIV isolates as a result of genomic variation of the virus (with all other factors controlled). As extensive sequencing was not performed in this study, we will not be able to determine whether any biological differences found between the isolates are a result of variation at the *env* gene, versus variation elsewhere in the genome.

Although attempts were made to quantify the virus stock accurately and according to a method relevant for subsequent assays, there were limitations of the methods used. For example, one limitation of the endpoint dilution assay was the use of qPCR to identify infected cultures. This method may have resulted in underestimation of the infectious titre of the virus stock, due to the insensitivity associated with trying to detect very small quantities of provirus. However, as subsequent experiments in this thesis required a standardised quantity of each virus relative to each other, rather than an accurate absolute concentration, the assay was deemed suitable for the purposes of this study.

Quantification according to viral protein concentration was performed in addition to calculation of the infectious titre. The main limitation of this assay was the range of concentrations that could be accurately extrapolated from the standard curve. As virus stock contained a low concentration of p24 protein, measurement of diluted samples was difficult, as the absorbance often measured below the linear region of the standard curve. Measurement of undiluted samples of virus stock would have been preferable, however the amount of virus available for this assay was limited. In order to minimise this factor as a source of inaccuracy, the ELISA was used to calculate the p24 concentration at two different concentrations of virus stock. The calculated values correlated well with the dilution factor for 3/5 virus stocks. Calculation of the p24 concentration of the RVC009 virus stock was based on the absorbance of a single dilution, as the absorbance of the second dilution measured below the linear range of the standard curve. Therefore, inaccuracy of the p24 concentration in the RVC009 virus stock is possible. In addition, the p24 concentration of the MUVTH003 virus stock was too low to enable accurate quantification at the dilutions tested. The use of this virus for subsequent assays requiring standardisation of virus stock by protein concentration was therefore not possible, limiting the number of viruses available for such testing.

2.5. Conclusion

Five genetically distinct isolates of FIV were produced and quantified for use in subsequent work presented in this thesis. The subtype classification of the 4 NZ isolates (3 subtype C, 1 subtype A) was typical of the subtype distribution in the sampled population. In addition, virus stock of 1 of the strains included in the Fel-O-Vax® FIV vaccine (FIV-Petaluma) was produced from an infectious molecular clone, enabling use of this virus as a positive control in future assays comparing vaccine cross-reactivity.

Quantification of these viruses according to the different methods used in this chapter enabled virus stocks to be standardised in a manner appropriate to each subsequent assay in this thesis, in order to make direct comparisons between the viruses.

CHAPTER THREE

Comparison of the *in vitro* pathogenicity of NZ isolates of FIV

3.1. Introduction

Many clinical signs have been reported in association with FIV infection, but there have also been frequent reports of cats remaining asymptomatic for many years (Hutson *et al.*, 1991, Fujino *et al.*, 2009, Yamamoto *et al.*, 1989, Ishida *et al.*, 1989, Hopper *et al.*, 1989, Sparkes *et al.*, 1993, Matsumura *et al.*, 1993, Callanan *et al.*, 1992, Kohmoto *et al.*, 1998, Ishida *et al.*, 1992, Gabor *et al.*, 2001, Maingat *et al.*, 2009, Ravi *et al.*, 2010, Liem *et al.*, 2013). Multiple factors probably contribute to the variability of FIV-associated disease in natural and experimental infections, including environmental stress, exposure to pathogens, the cat's age at the time of infection, the dose of inoculum and the route of exposure. The effect of viral factors on pathogenicity is poorly understood, but it is likely that sequence variation of the FIV genome is also responsible for at least some of the differences observed.

FIV mutates frequently during replication. Consequently, there is a high degree of genetic diversity among viruses. FIV is continually evolving and will therefore respond to various selection pressures exerted. As discussed in section 1.2.1, the FIV genomic sequence may differ by as much as 38% at the *env* gene alone, and this has led to phylogenetic identification of at least 6 different subtypes of FIV (A-F) (Hayward and Rodrigo, 2008, Sodora *et al.*, 1994, Duarte and Tavares, 2006, Kakinuma *et al.*, 1995, Pecoraro *et al.*, 1996, Weaver *et al.*, 2004, Weaver, 2010). The clinical significance of the genetic diversity of FIV is unclear, but experimental studies suggest that there may be an effect on pathogenicity. For example, at least 2 different studies have described less severe clinical signs in cats infected with a virus from subtype B compared to those infected with a subtype A virus (Nishimura *et al.*, 1998, Kohmoto *et al.*, 1998). There are also multiple reports describing rapidly progressive and fatal disease in cats infected with a certain subtype C virus (FIV-CPGammar) (Pedersen *et al.*, 2001, Diehl *et al.*, 1995b, Obert and Hoover, 2000). Although this suggests that FIV variants differ in pathogenicity, *in vivo* studies such as these are confounded by various host and environmental factors that may influence disease expression. Experimental studies comparing viral characteristics *in vitro* enable a more direct comparison between viruses, as the impact of host and environmental factors is eliminated.

Previous experiments have shown that FIV variants differ in mutation rate over time, replication rate, cell tropism, and their potential for indirect neurotoxicity (Power *et al.*, 1998, Sodora *et al.*, 1994, Bachmann *et al.*, 1997, Nakamura *et al.*, 2003, Ikeda *et al.*, 2004, Diehl *et al.*, 1995a, de Rozieres *et al.*, 2008, Willett *et al.*, 2006b, Hohdatsu *et al.*, 1996, Sutton *et al.*, 2005, Miller *et al.*, 2011). These studies provide convincing evidence that FIV variants do differ in their *in vitro* characteristics. The question that remains is whether these differences equate to differences in pathogenicity within the host.

Previous authors have designed *in vitro* studies based on the observation that some variants (e.g. FIV-PPR) are more consistently associated with neurological disease than others (e.g. FIV-CPGammar) (Phillips *et al.*, 1996, Power *et al.*, 1998, Miller *et al.*, 2011, Diehl *et al.*, 1995b). *In vitro*, the former variants were shown to cause more indirect neurotoxicity when compared to variants of FIV not typically associated with neurological disease (Bragg *et al.*, 1999, Phillips *et al.*, 1994, Phillips *et al.*, 1996, Miller *et al.*, 2011, Power *et al.*, 1998, Meeker *et al.*, 1997). Similar studies would be useful to investigate other *in vitro* properties that may be linked to the mechanisms of FIV-induced immune dysfunction within the host.

The immunopathogenesis of FIV is multifactorial. As discussed in 1.4.1.2, loss of $CD4^+$ T cells contributes to FIV-induced immune dysfunction. Within the lymph nodes of infected cats, there is widespread apoptosis of $CD4^+$ T cells, and this also occurs experimentally when cultures of PBMC or lymphoblasts are infected with FIV *in vitro* (Yamamoto *et al.*, 1997, Sutton *et al.*, 2005, Ohno *et al.*, 1994, Guiot *et al.*, 1993a, Johnson *et al.*, 1996, Garg *et al.*, 2004b, Holznagel *et al.*, 1998, Momoi *et al.*, 1996, Bishop *et al.*, 1993, Guiot *et al.*, 1993b, Guiot *et al.*, 1997, Muro-Cacho *et al.*, 1995, Finkel *et al.*, 1995, Sarli *et al.*, 1998, Tompkins *et al.*, 2002). The apoptotic lymphocytes are presumed to be uninfected bystander cells, as the percentage of cells undergoing apoptosis in culture (up to 50%) greatly exceeds the percentage of cells typically infected with the virus (<10%) (Finkel *et al.*, 1995). In addition, apoptosis has been shown to occur prior to infection of lymphocytes, as demonstrated by the absence of the gag protein within affected cells (Sutton *et al.*, 2005). One mechanism of FIV-induced apoptosis *in vitro* involves interaction of

uninfected cells with gp35 (Garg *et al.*, 2004b). Apoptosis occurs when gp35 expressed on the exterior of infected cells interacts with FIV cellular receptors on uninfected lymphocytes, resulting in the generation of a fusion pore. Membrane destabilisation follows, and the intrinsic pathway of apoptosis is activated. Apoptosis is an active mechanism of cell death that is triggered following various intrinsic or extrinsic stimuli to the cell. In contrast to necrosis, cell membrane integrity is not lost when a cell undergoes apoptosis, and cellular components are contained within apoptotic bodies that are removed via phagocytosis. *In vitro*, in the absences of phagocytes, apoptotic bodies ultimately undergo secondary necrosis, and in this situation, membrane integrity is eventually lost (Wyllie, 1997).

In addition to inducing apoptosis, FIV infection also results in suppression of lymphocyte proliferative responses. *In vitro*, the proliferative indices of mitogen-stimulated PBMC from FIV infected cats are reduced when compared to those of PBMC from uninfected cats (Hara *et al.*, 1990, Lin *et al.*, 1990, Taniguchi *et al.*, 1990, Torten *et al.*, 1991, Bishop *et al.*, 1992, Lawrence *et al.*, 1992, Barlough *et al.*, 1991). As discussed in 1.4.1.1, lymphocytes from FIV infected cats show characteristics of immune exhaustion as a result of chronic antigenic stimulation, with reduced proliferative responses, impaired production of IL-2 and persistent expression of co-inhibitory molecules such as CD152 and CD279 (Lawrence *et al.*, 1992, Siebelink *et al.*, 1990, Folkl *et al.*, 2008, Tompkins *et al.*, 2002). In addition, there may be a direct inhibitory effect of retroviral proteins on lymphocyte proliferation. This was shown previously by authors culturing PBMC with UV-inactivated HIV (Amadori *et al.*, 1988). Mitogen-stimulated T cells (but not B cells) exposed to HIV showed reduced proliferative indices in a dose-dependent manner, when compared to T cells not exposed to the virus. Affected cell cultures also produced less IL-2, but addition of IL-2 to the culture did not restore normal proliferative activity. The authors suggested that the mechanism of HIV-mediated inhibition of lymphocyte proliferation *in vitro* may involve downregulation of the expression and/or the affinity of the receptor for IL-2. Other authors have demonstrated molecular mimicry between the receptor binding sites of the IL-2 molecule and the transmembrane portion of the HIV and FIV envelope glycoproteins (Serres, 2000). The

significance of this is unclear, but competitive interaction of viral proteins with the IL-2 receptor may be involved in the suppression of lymphocyte proliferation by HIV, and possibly also FIV. If such an effect also occurs *in vivo*, then this mechanism may contribute to FIV-induced immune dysfunction within the host.

The aim of the work presented in this chapter was to test the hypothesis that FIV variants differ in pathogenicity. In order to eliminate the effect of host and environmental factors on pathogenicity, *in vitro* assays were designed for this purpose. FIV-induced apoptosis and viral inhibition of mitogen-induced lymphocyte proliferation were chosen as *in vitro* markers of pathogenicity, due to the potential immunosuppressive effect of these properties *in vivo*. These two assays were used to compare the effect of previously prepared NZ isolates of FIV on cells in culture. If the pathogenicity of these FIV isolates differs *in vitro*, then they may also differ in their biological properties *in vivo*.

3.2. Materials and methods

3.2.1. Development and optimisation of a flow-cytometric apoptosis assay

3.2.1.1. PBMC separation

Whole blood was collected from healthy donor cats housed at the Massey University Feline Nutrition Unit (MUFNU, Palmerston North, NZ) and the protocol was approved by the MUAEC (protocol # 14/22). Following venepuncture, two methods of PBMC separation were trialled. The first method involved collection of 8 mL of whole blood into Cell Preparation Tubes (CPT) with sodium citrate (BD Vacutainer CPT™, BD Biosciences). PBMC were then isolated by gradient centrifugation (at 1,500 x g for 20 mins), washed in 10 mL PBS and pelleted by centrifugation at 300 x g for 10 minutes according to the manufacturer's directions. The second method involved separation of PBMC using a density gradient medium (Ficoll-Paque, GE Healthcare Life Sciences) as described in section 2.2.6.1.

PBMC were prepared for flow cytometry immediately after separation from whole blood, by resuspending the cell pellet in 500 µL of Annexin Binding Buffer (BD Biosciences) diluted

1:4 in PBS (AB). Cells were analysed immediately by flow cytometry using FACScalibur (BD Biosciences), and forward and side scatter (FSC/SSC) profiles were examined to assess the quality of the PBMC separation.

3.2.1.2. Establishment of a positive control for the apoptosis assay using feline PBMC

A dual-colour, flow-cytometric assay was established to measure apoptosis in feline PBMC, using the PS binding compound, Annexin-V and the DNA binding dye, 7AAD. A positive control for the assay was developed using camptothecin (Sigma-Aldrich), which is a DNA topoisomerase I inhibitor that induces apoptosis via activation of caspase 3 (Ferraro *et al.*, 2000). PBMC were separated using density gradient medium, as described in section 2.2.6.1. Cells were seeded in a 24-well plate, at a density of $1.5x10^6$ cells/mL, in a total volume of 1 mL RPMI media (GIBCO®), supplemented with 10% FBS (GIBCO®), 1% glutamax (GIBCO®) and 1% Penicillin/Streptomycin (GIBCO®) and incubated at 37 °C in 5% CO_2. Camptothecin was added to cultures immediately at a concentration of 6 μM or 20 μM [3 replicates per concentration of camptothecin (one for each time point), with an additional 3 replicates treated with 20 μM and cultured for use as controls for flow cytometry]. Cells were harvested in duplicate at different time points (1, 2, and 3 hrs after addition of camptothecin) and prepared for flow cytometry to assess the degree of apoptosis. Cells were transferred to microcentrifuge tubes and pelleted by centrifugation at 300 x g for 10 minutes. Supernatant was discarded, and cells were resuspended in 1 mL of PBS, pelleted again (300 x g for 10 minutes) and resuspended in 100 μL of AB. Cells were then incubated with 1 μL Annexin-V, conjugated to Alexa-fluor 647 (Invitrogen) and 1 μL 7AAD (Invitrogen) at a dilution of 1/100 each for 15 minutes at room temperature. Unstained and single stained controls (7AAD or Annexin-V only) were also prepared from additional PBMC treated with 20 μM of camptothecin. AB was added to each sample immediately prior to flow cytometric analysis using FACScalibur (BD Biosciences). Cells were gated based on FSC/SSC graphs to include only single lymphocytes, and the percentage of lymphocytes undergoing apoptosis, necrosis or cellular activation was analysed using FlowJo® software (TreeStar, Inc., Ashland, OR).

Viable cells were defined as double negative cells (Annexin-V negative / 7AAD negative), apoptotic cells as single positive cells (Annexin-V positive / 7AAD negative) and necrotic cells as double positive cells (Annexin-V positive / 7AAD positive). Therefore, it was possible to differentiate between viable cells, early apoptotic cells and necrotic cells, but not between cells that underwent necrosis as a primary event or secondary to apoptosis.

3.2.1.3. Optimisation of cell culture conditions

3.2.1.3.i. The effects of media composition on PBMC viability

The effect of cell culture media on cell viability was assessed by comparing apoptosis in PBMC cultured using five different media formulations (**Table 3-1**). PBMC were separated using density gradient medium as described in section 2.2.6.1. Cells were then cultured in a total volume of 1 mL of each media formulation (3 replicates per media type), at a density of $1.5x10^6$ cells/mL at 37 °C in 5% CO_2. Cells cultured with each media formulation were harvested at days 1, 2 and 3 (1 replicate per time point) and prepared for flow cytometry as described in section 3.2.1.2. The percentage of cells undergoing apoptosis and necrosis were compared for each media formulation tested.

Table 3-1 Media formulations used for optimisation of PBMC culture.
Five different modifications of growth media were compared in the optimisation process. All formulations consisted of a base media of RPMI, and were supplemented with various combinations of serum and additives as listed below. Media are labelled 1-5 and are referred to as such in the text.

	Media[a]	Serum[b] (%)	L-glutamine[c] (%)	Antibiotics[d] (%)	NEAA[e] (%)	HEPES[f] (%)	Na pyruvate[g] (%)	IL-2[h] (U/mL)	ConA[i] (µg/mL)
1	RPMI	10	1	1	-	-	-	-	-
2	RPMI	8.5	0.5	0.5	1	1	1	-	-
3	RPMI	10	1	1	-	-	-	10	-
4	RPMI & condit. media [j]	10	1	1	-	-	-	-	-
5	RPMI	10	1	1	-	-	-	-	5

[a] RPMI media (GIBCO®)

[b] Foetal bovine serum (FBS, GIBCO®)

[c] L-Glutamine (GIBCO®)

[d] Penicillin / Streptomycin (GIBCO®)

[e] MEM non-essential amino acids (NEAA) 100x (GIBCO®)

f N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid buffer 1M (GIBCO®)

[g] Sodium pyruvate solution (GIBCO®)

[h] Recombinant human interleukin-2 (BD Biosciences)

[i] Concanavalin A (ConA, Sigma-Aldrich®)

[j] Conditioned media consisted of supernatant collected from uninfected PBMC cultured in RPMI media supplemented with 10% FBS, 1% glutamax and 1% penicillin/streptomycin for a period of 3 days. 1mL of conditioned media was added to 9mL of fresh RPMI prior to supplementation with FBS, glutamax and antibiotics.

3.2.1.3.ii. The effects of ConA concentration on cell viability

The effect of ConA concentration on cell viability was assessed by culturing cells for 1 day in the presence of increasing concentrations of ConA (0, 1, 3 and 5 µg/mL). PBMC were collected and cultured as described in section 3.2.1.2. Cells were prepared for flow cytometry using Annexin-V and 7AAD in order to assess the percentage of cells undergoing apoptosis and necrosis. In addition, the percentage of cells becoming activated in culture was also assessed. Prior to incubation with Annexin-V and 7AAD, cells were incubated for 30 minutes in the dark, in a total volume of 50 µL PBS with 5 µL of cell culture supernatant containing a FITC conjugated antibody against the alpha chain of the IL-2 receptor (CD25)[5]. PBS (1 mL) was then added, and cells were pelleted by centrifugation (300 x g for 10 minutes). The supernatant was discarded and cells were incubated with Annexin-V and 7AAD in AB as described in section 3.2.1.2. The

[5] Antibody generously gifted by Dr. Mary Tompkins, Department of Microbiology, Pathology and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC.

optimal concentration of ConA was defined as the amount that caused maximum cell activation without compromising cell viability.

3.2.2. Demonstration of FIV-induced apoptosis

3.2.2.1. FIV-induced apoptosis in ConA stimulated feline PBMC

Feline PBMC were separated and cultured as described in section 3.2.1.2, in the presence of ConA (1 μg/mL) and a non-titrated FIV isolate [100 μL supernatant collected from MYA-1 cultures infected with the MUVTH002 isolate (9 days post infection) as described in section 2.2.6.3]. Controls consisted of cultures treated with ConA or fresh media only. Cells were harvested at days 2 and 7, and prepared for flow cytometric assessment of Annexin-V and 7AAD fluorescence as described in section 3.2.1.2.

3.2.2.2. FIV-induced apoptosis in unstimulated feline PBMC

The experiment described in section 3.2.2.1 was repeated using unstimulated PBMC. Cells were incubated for 2 days in the presence of the FIV isolate, with controls consisting of cells cultured in fresh media alone.

3.2.2.3. FIV-induced apoptosis in MYA-1 cells

3.2.2.3.i. Establishment of a positive control for the apoptosis assay using MYA-1 cells

MYA-1 cells were cultured as described in section 2.2.6.3, in 96-well plates (100 μL per well). Camptothecin was added to cultures at a concentration of 20 μM, and cells were subsequently cultured for a period of 2 hrs, 4 hrs and overnight (18 hrs). Cells were collected and prepared for flow cytometry as described in section 3.2.1.2. The optimal time period for treatment with camptothecin was established by comparing the percentage of apoptotic and necrotic cells at each time point.

3.2.2.3.ii. FIV-induced apoptosis in MYA-1 cells

MYA-1 cells were cultured as described in section 2.2.6.3, in 96-well plates (100 μL per well). Cells were infected with 2 different isolates of FIV (50 μL supernatant collected from

MYA-1 cultures infected with the MUVTH002 or the RVC009 isolates as described in section 2.2.6.3) in duplicate, and incubated for 1 day at 37 °C, 5% CO_2. Controls consisted of cells cultured in fresh media alone. Cells were prepared for flow cytometry as described in section 3.2.1.2.

3.2.3. Comparison of apoptosis and necrosis of MYA-1 cells, induced by different isolates of FIV

Apoptosis of MYA-1 cells was assessed over time, following incubation with the 5 FIV isolates prepared in chapter 3. These consisted of 3 NZ isolates belonging to subtype C (MUVTH002, MUVTH003, CVK001), 1 NZ isolate belonging to subtype A (RVC009) and 1 subtype A, laboratory adapted strain obtained from an infectious molecular clone of the Petaluma strain (PetF14). Virus stocks were standardised to $10^{4.8}$ $TCID_{50}$/ml. MYA-1 cells were cultured as described in section 2.2.6.3, at a density of $1.5x10^5$ cells/mL in 96-well plates (200 µL per well). Wells were inoculated with 20 µL of each virus in triplicate (multiplicity of infection 0.03)[6]. To ensure that apoptosis was not induced by factors other than FIV, negative controls consisted of the following:

1. MYA-1 cells inoculated with 20 µL of fresh media.
2. MYA-1 cells inoculated with 20 µL of conditioned media (cell culture supernatant collected from uninfected MYA-1 cells cultured for 3 days).
3. MYA-1 cells inoculated with filtrate obtained following ultrafiltration of supernatant from virally infected MYA-1 cell cultures, using Amicon® Ultra-15 100 kDa centrifugal filter units (Millipore®) as described in section 2.2.8 (containing viral and non-viral proteins <100kDa).

[6] The multiplicity of infection (MOI) was calculated using the following equation:
MOI = ($TCID_{50}$ x 0.69) / Number of cells in each well

A positive control consisted of MYA-1 cells incubated with camptothecin (20 μM) for 4 hrs. Cells were harvested for analysis at 1, 3, 8 and 10 days after inoculation, and prepared for flow cytometry as described in section 3.2.1.2.

3.2.4. Effect of FIV infection on MYA-1 cell concentration in culture

A crude assessment of cell concentration over time was made retrospectively by calculating cell flow rate (the number of events acquired per second) during acquisition of data for flow cytometry from the apoptosis study described in section 3.2.3. As all cells from each well were counted, cell flow rate was representative of cell density in culture.

3.2.5. Inhibition of mitogen-induced lymphocyte proliferation by different isolates of FIV

Heparinised whole blood (1 mL) was collected from 6 healthy, FIV-negative cats housed at the MUFNU. Blood was diluted 1 : 4 in RPMI, supplemented with 10% FBS, 1% glutamax, 1% Penicillin/Streptomycin and 0.05 mM 2-ME and transferred to 96-well plates (100 μL per well). Cells were stimulated with ConA (5 μg/mL) and wells were immediately inoculated in quadruplicate with 20 μL of each of the 5 viruses described in section 3.2.3. Control wells consisted of unstimulated cells (without ConA) and ConA stimulated cells without the virus. Cells were cultured for 2 days at 37 °C in 5% CO_2 following inoculation with the viruses. Radioactive [^{3}H] thymidine (0.5 μCi per well) was then added to each well, and cells were cultured for an additional 18 hrs. Distilled water (50 μL) was added to each well to lyse red blood cells, and cells were harvested directly from the plate onto a filter mat and washed manually 10 times. Mats were sealed in plastic with scintillation fluid and the radioactivity was measured using a scintillation counter, expressed as counts per minute (cpm) per well.

3.2.6. Statistical analysis

The normality of the data was confirmed using an Anderson Darling test. To assess the effect of FIV on the proportion of cells undergoing apoptosis and necrosis in sections 3.2.1 - 3.2.3, a general linear model of ANOVA was used. The following statistics were assessed: geometric mean fluorescence intensity (MFI) of Annexin-V, MFI of 7AAD, percentage of viable (double

negative) cells, percentage of apoptotic (single positive) cells, and the percentage of necrotic (double positive) cells. Model factors included 'time' (day) and 'treatment'.

Firstly, the 3 different control conditions were assessed for any differences in each parameter over time (fresh media, conditioned media, virus filtrate). Secondly, the overall effect of FIV infection (all isolates combined) compared to a pooled control was assessed for each parameter. This was assessed over time, as well as at the individual time points. Finally, differences among FIV isolates were examined over time, and at individual time points (treatments – pooled results from all three control conditions, MUVTH002, MUVTH003, CVK001, RVC009 and PetF14). Where the effect of treatment reached significance, a Bonferroni pairwise comparison was used to determine which means were different.

Differences in the cell concentration were assessed using one-way ANOVA at each time point to compare the mean cell flow rate (cells/sec) for each treatment. Tukey's pairwise comparison was used to determine which means were significantly different.

The effect of virus on mitogen-induced cell proliferation was assessed using a general linear model of ANOVA, with the model factors of 'treatment' and the random variable 'cat'. Within each treatment group, the mean absolute count per minute (cpm) for each cat was normalised by subtracting the mean cpm of unstimulated wells from the cpm of stimulated wells. The stimulation index (SI) was calculated for each treatment as the ratio of the cpm of the stimulated wells and the mean cpm of unstimulated wells for each cat. The normalised cpm and the SI were assessed separately as responses in the model.

All statistical analyses were performed using Minitab® Statistical Software (Version 17, Minitab Inc., Pennsylvania, U.S.A) A *p*-value of less than 0.05 was considered significant.

3.3. Results

3.3.1. Development and optimisation of a flow-cytometric apoptosis assay

3.3.1.1. PBMC separation

Attempts to separate feline PBMC from whole blood using the CPT™ vacutainers were unsuccessful, with granulocyte contamination consistently occurring (as shown by a population of small, granular cells on the FSC/SSC profiles) (**Figure 3-1**). In contrast, separation of PBMC using Ficoll-Paque density gradient medium resulted in a clear population of PBMC with minimal to no granulocyte contamination. Consequently, Ficoll-Paque was used for PBMC separation in all subsequent experiments.

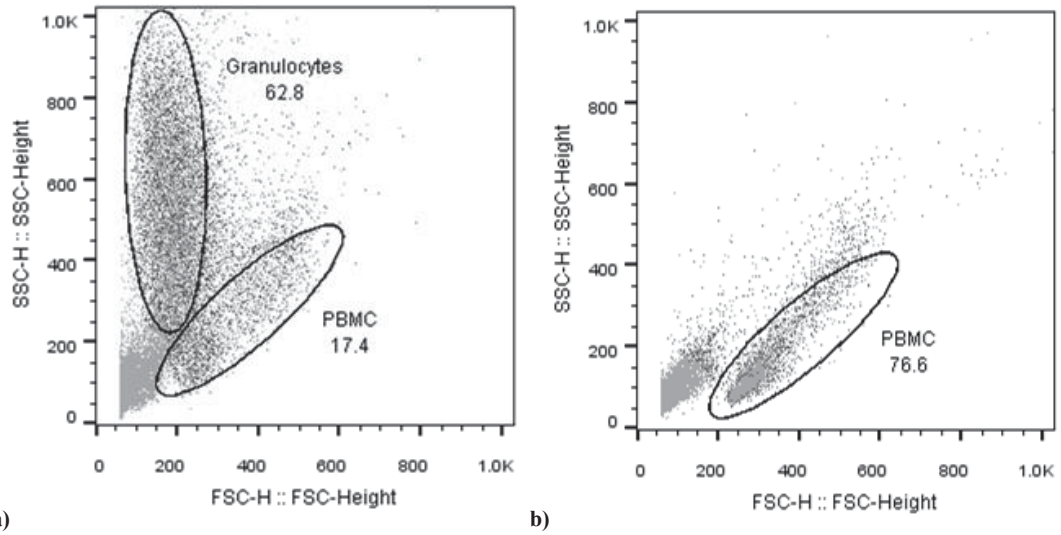


Figure 3-1 Flow cytometry graph of PBMC separation.
FSC/SSC plots demonstrate PBMC separated from a single blood sample using two different methods **a)** CPT™ vacutainers and **b)** density gradient medium (Ficoll-Paque, GE Healthcare Life Sciences). The x-axis represents forward light scatter (FSC), and is proportional to cell size. Side light scatter (SSC) is represented on the y-axis and is a measure of internal complexity (i.e. granularity). Distinct populations of granulocytes and PBMC are circled separately and labelled. The numbers represent the percentage of all cells that belong to each population. Samples prepared using the CPT™ vacutainers were contaminated by granulocytes (62.8% of all cells), whereas samples prepared using the Ficoll-Paque density gradient medium showed minimal to no contamination with granulocytes. Using this latter method, 76.6% of cells isolated were PBMC, including populations of lymphocytes and monocytes.

3.3.1.2. Establishment of a positive control for the apoptosis assay using feline PBMC

Treatment of PBMC with camptothecin resulted in apoptosis, but there was no difference in the percentage of cells undergoing apoptosis following treatment with the 2 different concentrations of camptothecin (p=0.681). There was also no significant difference over time

(within the 3 hour period measured) in the percentage of apoptotic cells (p=0.413) (**Figure 3-2** and **Figure 3-3**).

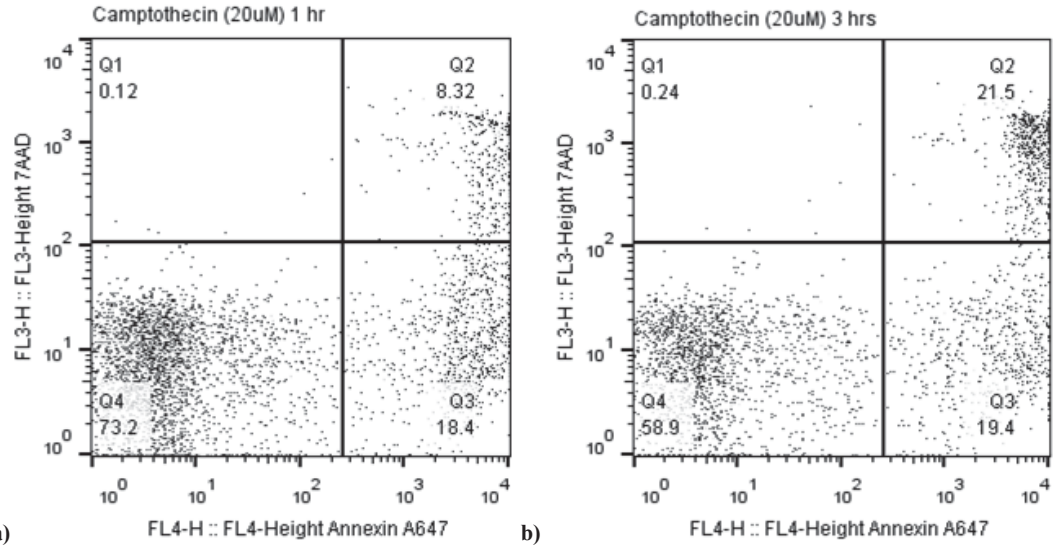


Figure 3-2 Flow cytometry graphs demonstrating camptothecin-induced apoptosis.
Graphs show Annexin-V fluorescence (FL4, x-axis) and 7AAD fluorescence (FL3, y-axis) of PBMC treated with 20 µM camptothecin for **a)** 1 hr and **b)** 3 hrs. Each dot represents a cell that is either viable (located in quadrant 4 – annexin-V negative / 7AAD negative), apoptotic (quadrant 3 – annexin-V positive / 7AAD negative) or necrotic (quadrant 2 – annexin-V positive / 7AAD positive). Numbers represent the percentage of total cells located within each quadrant. There were no significant differences between the two time points in either the percentage of apoptotic cells (p=0.413) or necrotic cells (p=0.545).

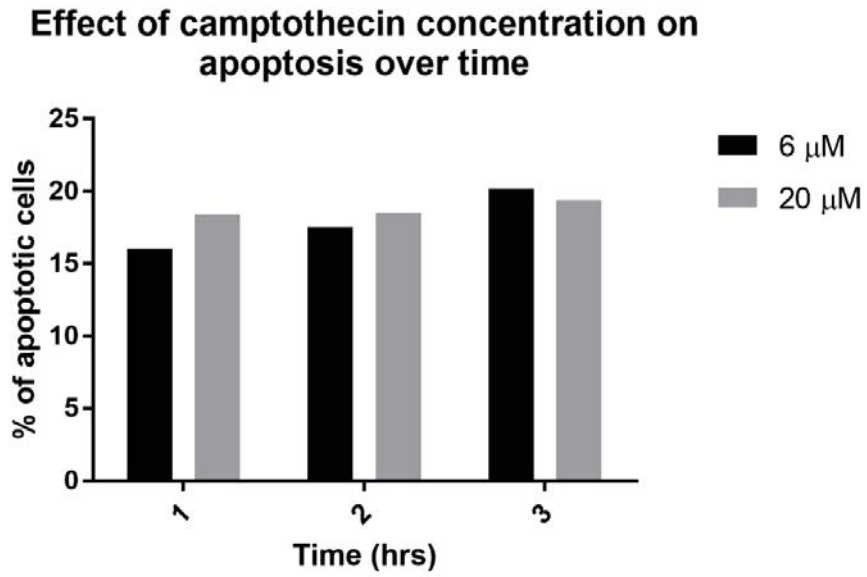


Figure 3-3 The effect of time and concentration on camptothecin-induced apoptosis.
Feline PBMC were cultured in triplicate, in the presence of 2 different concentrations of camptothecin (6 and 20 μM) and harvested at 3 different time points (1, 2, 3 hrs). The percentage of cells undergoing apoptosis (annexin-V positive / 7AAD negative cells) was measured using a dual-colour flow cytometric assay. There was no difference in the percentage of apoptotic cells in cultures treated with 6 μM versus 20μM camptothecin (p=0.681).

3.3.1.3. Optimisation of cell culture conditions

3.3.1.3.i. The effects of media composition on PBMC viability

The effect of media composition on cell viability and apoptosis at different time points (days 1, 2 and 3) was assessed using the dual-colour flow cytometric apoptosis assay (**Figure 3-4**). All cultures except those treated with ConA showed >80% viability of PBMC on day 1. In all cultures, cell viability decreased by day 3, as shown by the reduced proportion of double negative cells in cultures on days 2 and 3. There was no effect of using an alternative media containing less serum, supplementing media with IL-2 or adding conditioned media to the cultures.

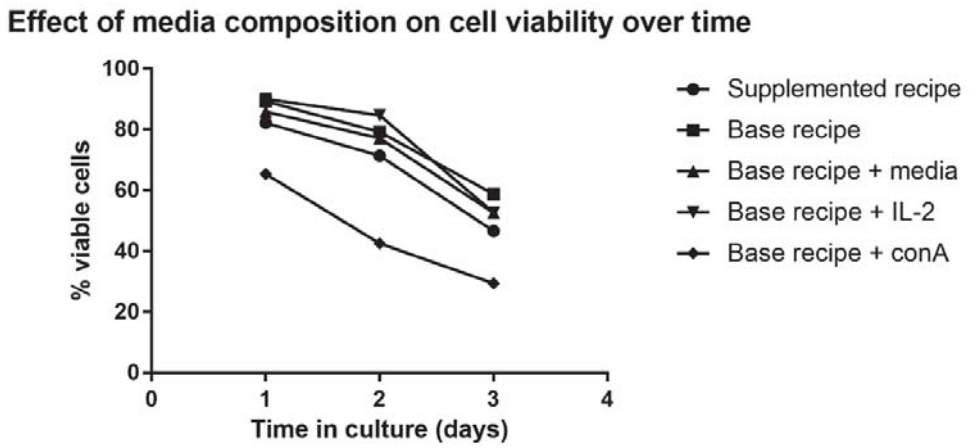


Figure 3-4 The effect of media composition on cell viability over time.
Feline PBMC were cultured in triplicate using 5 different media formulations (1-5) for a period of 3 days (with 1 culture harvested at each time point). Dual-colour flow cytometry was performed as described in section 3.2.1.2 to assess cell viability (defined as Annexin-V negative / 7AAD negative cells). The percentage of viable cells declined over time. Cultures containing media 5 had reduced cell viability compared to other media formulations. Media formulations consisted of: 1. RPMI with 10% FBS, 1% glutamine and 1% penicillin/streptomycin; 2. RPMI with 8.5% FBS, 0.5% glutamine, 0.5% penicillin/streptomycin, and 1% each of additional NEAA, HEPES and Na Pyruvate; 3. Formulation 1 + 10 units/mL of IL-2; 4. Formulation 1 + conditioned media (1mL added to 9mL of media 1); 5. Formulation 1 + 5 µg/mL of ConA (see **Table 3-1** for details).

3.3.1.3.ii. The effects of ConA on cell viability

The effect of ConA on cell viability was inversely related to concentration, with cell viability decreased to <40% in media with >3 µg/mL of ConA (**Figure 3-5**). Cellular activation increased with the concentration of ConA, with 65.8% of cells expressing CD25 at a concentration of 5 µg/mL of ConA, versus 51.8% at a concentration of 1 µg/mL of ConA. Treatment of cells with ConA at a concentration of 1 µg/mL for 1 day was determined to be optimal for activation of lymphocytes with the least effect on cell viability.

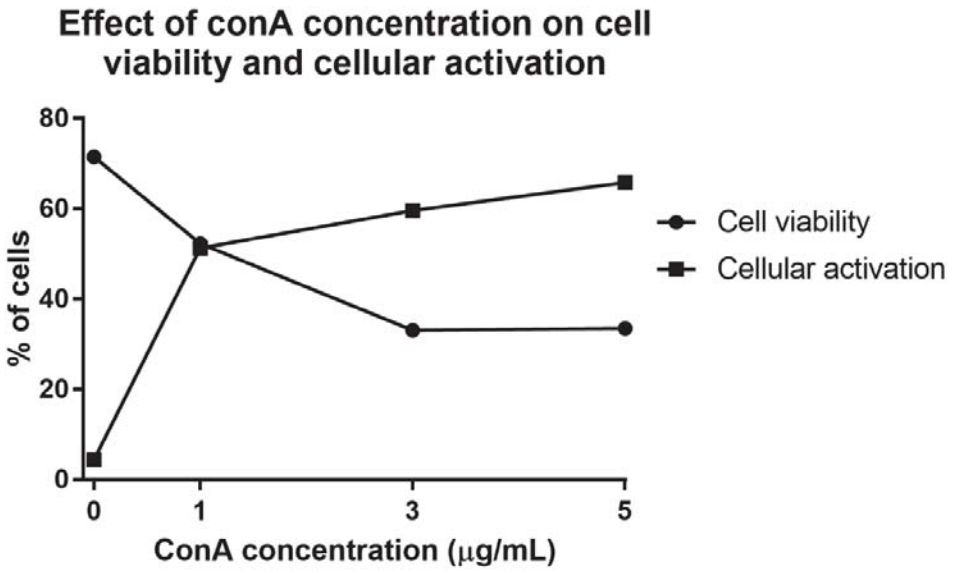
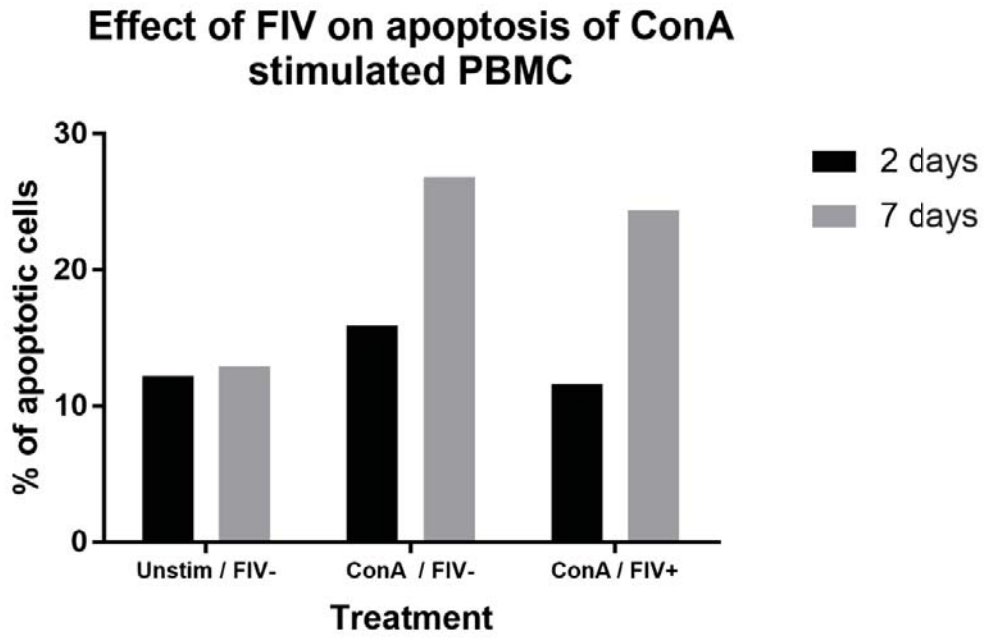


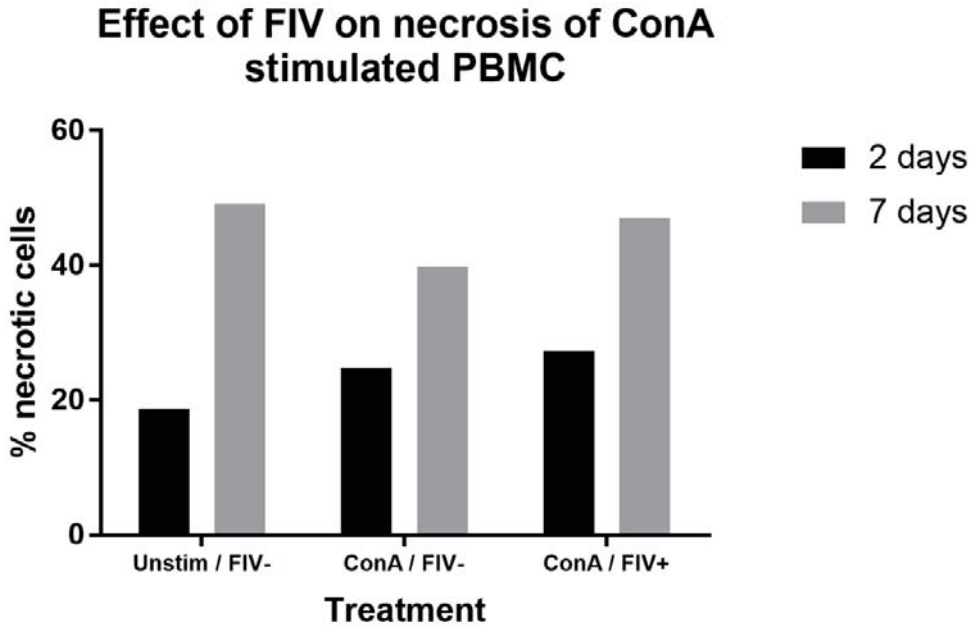
Figure 3-5 The effect of ConA on cell viability and cellular activation.
Feline PBMC were cultured for a period of 1 day in the presence of 3 different concentrations of ConA (1, 3 and 5 μg/mL). The negative control consisted of PBMC cultured in the absence of ConA (0 μg/mL). Dual-colour flow cytometry was performed as described in section 3.2.1.2 to assess cell viability (represented by the percentage of Annexin-V negative / 7AAD negative cells) and cellular activation (represented by the percentage of CD25 positive cells). Each square indicates the percentage of cells that became activated in the presence of each concentration of ConA. Each cross represents the percentage of viable cells at each concentration of ConA. The intercept of the two lines represents the optimal concentration of ConA required to activate cells yet maintain viability (1.0 μg/mL).

3.3.1.4. FIV-induced apoptosis in ConA stimulated feline PBMC

Stimulation of feline PBMC with ConA (1 μg/mL) resulted in increased apoptosis at day 7 when compared to unstimulated cells (26.8% versus 12.9%). Infection of ConA stimulated cells with FIV did not increase the percentage of apoptotic cells at day 7 (24.4% in FIV infected cultures versus 26.8% in uninfected cultures). At day 2, ConA stimulated cells infected with FIV showed the highest proportion of necrosis (27.3%), but this was only slightly higher than cultures of uninfected, ConA stimulated cells (24.8%). By day 7, all cells had undergone extensive necrosis (>40%), including those in the untreated cultures (**Figure 3-6**).



a)



b)

Figure 3-6 The effect of ConA and FIV infection on apoptosis and necrosis of feline PBMC.
Cells were cultured in triplicate, and stimulated with ConA (1.0 µg/mL) and infected with an FIV isolate (MUVTH002). Controls consisted of unstimulated / uninfected PBMC and ConA stimulated / uninfected PBMC. Dual-colour flow cytometry was performed as described in section 3.2.1.2 to assess **a)** apoptosis (represented by the percentage of Annexin-V positive / 7AAD negative cells and shown along the y-axis) at days 2 and 7, and **b)** necrosis (represented by the percentage of Annexin-V positive / 7AAD positive cells and shown along the y-axis) at days 2 and 7. There was no effect of FIV infection on the percentage of cells undergoing apoptosis or necrosis when compared to ConA stimulated / uninfected PBMC.

3.3.1.5. FIV-induced apoptosis in unstimulated feline PBMC

Infection of unstimulated PBMC with FIV did not result in increased apoptosis or necrosis after 2 days when compared to uninfected, unstimulated cells (13.2% versus 12.6%).

3.3.1.6. FIV-induced apoptosis in MYA-1 cells

3.3.1.6.i. Establishment of a positive control for the apoptosis assay using MYA-1 cells

Treatment of MYA-1 cells with 20 μM camptothecin resulted in increased apoptosis at all time points when compared to untreated cells. The percentage of apoptotic cells increased over time from 2 to 4 hrs, but there was no additional increase in apoptosis when cells were treated for 18 hrs (untreated 11.2%, 2 hrs camptothecin 18.3%, 4 hrs camptothecin 20.2%, 18 hrs camptothecin 20.5% apoptosis) (**Figure 3-7** and **Figure 3-8**).

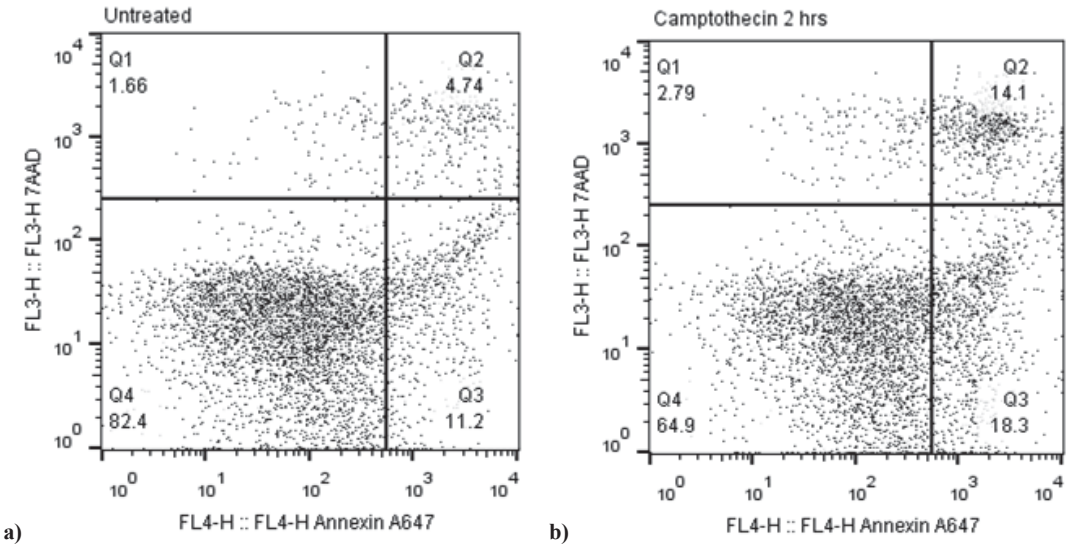


Figure 3-7 Flow cytometry graphs demonstrating camptothecin-induced apoptosis in MYA-1 cells.
Representative graphs showing Annexin-V fluorescence (FL4, x-axis) and 7AAD fluorescence (FL3, y-axis) on **a)** untreated MYA-1 cells and **b)** MYA-1 cells treated with 20 μM camptothecin for 2 hrs. Quadrant 4 represents viable cells, quadrant 3 represents apoptotic cells and quadrant 2 represents necrotic cells and figures indicate the percentage of cells in each quadrant. Treatment with camptothecin resulted in increased apoptosis and necrosis when compared to untreated cells.

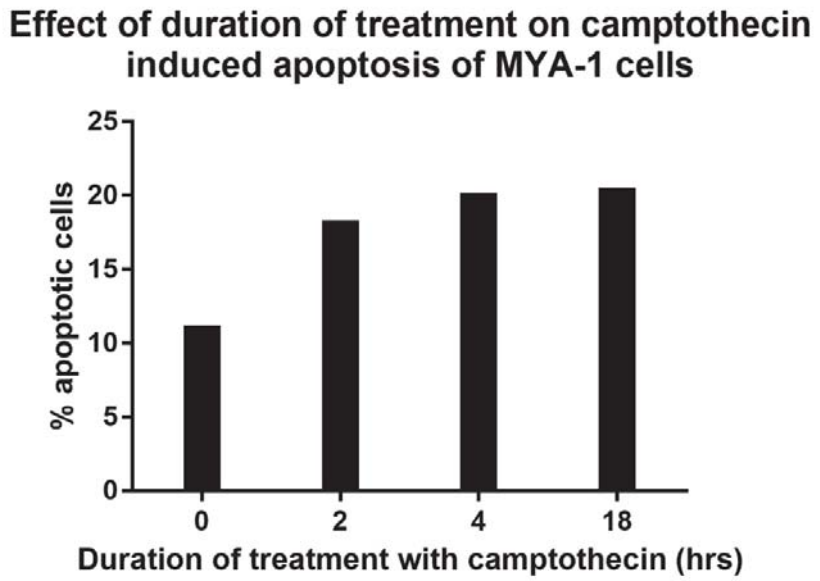


Figure 3-8 The effect of time on camptothecin-induced apoptosis of MYA-1 cells.
Cells were incubated with 20 μM camptothecin for a period of 2 hrs, 4 hrs and overnight (18 hrs). Untreated cells were included as negative controls (0 hrs). Dual-colour flow cytometry was performed as described in section 3.2.1.2 to assess apoptosis in MYA-1 cells, and the percentage of Annexin-V positive / 7AAD negative cells (single positive, "sgl pos" are shown along the y-axis. Apoptosis was increased at 4 hrs versus 2 hrs of treatment, but there was no benefit of prolonged treatment beyond 4 hrs in this study.

3.3.1.6.ii. FIV-induced apoptosis in MYA-1 cells

In the initial pilot study using unquantified virus (supernatant from MYA-1 infected cells), FIV infection did not result in increased apoptosis and necrosis on day 1, when compared to uninfected controls (mean 13.2% versus 11.2%, p=0.245, **Figure 3-9**).

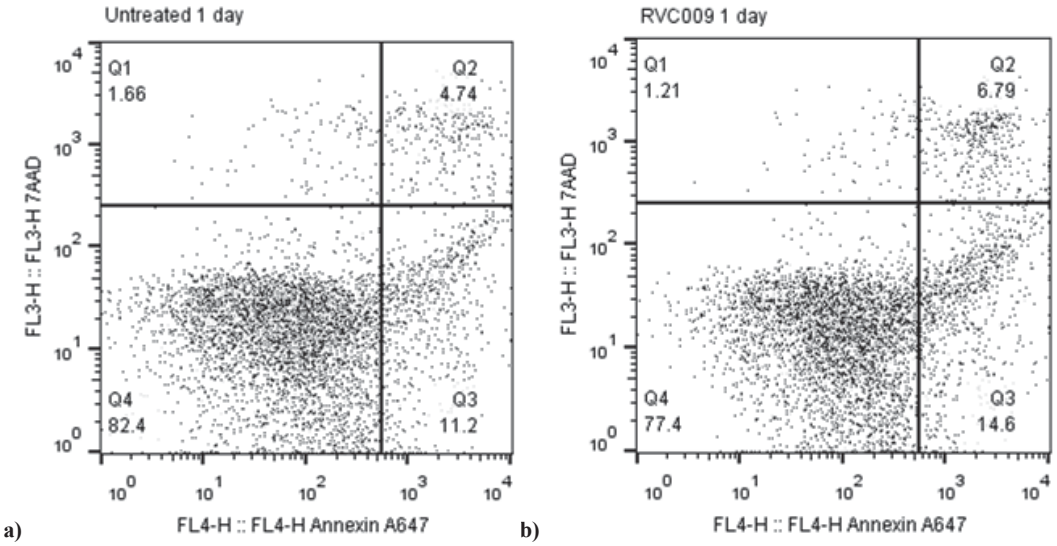


Figure 3-9 Flow cytometry graphs demonstrating the effect of FIV infection on apoptosis in MYA-1 cells. Representative graphs showing Annexin-V fluorescence (FL4, x-axis) and 7AAD fluorescence (FL3, y-axis) on **a)** uninfected MYA-1 cells and **b)** FIV (RVC009) infected MYA-1 cells after 1 day. Quadrant 4 represents viable cells, quadrant 3 represents apoptotic cells and quadrant 2 represents necrotic cells and figures indicate the percentage of cells in each quadrant. Infection with FIV did not result in increased apoptosis and necrosis when compared to uninfected controls (p=0.245).

3.3.2. Comparison of apoptosis and necrosis of MYA-1 cells induced by different isolates of FIV

There was no significant difference observed among control conditions in the percentage of apoptotic cells (p=0.919), necrotic cells (p=0.507), MFI Annexin-V (p=0.623) or MFI 7AAD (p=0.604) over time (**Figure 3-10**). Therefore, results from all 3 control conditions were pooled for subsequent analysis of the data.

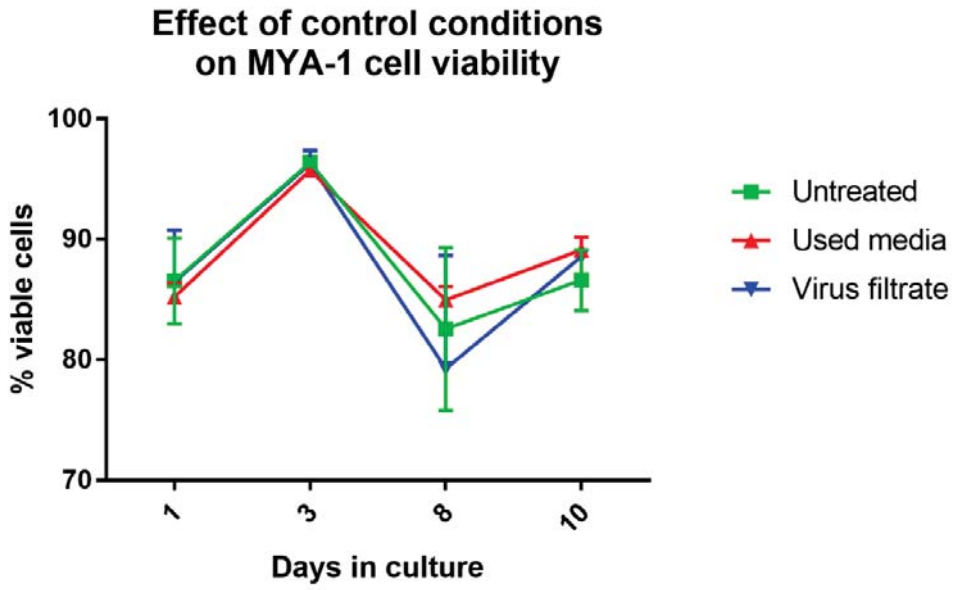


Figure 3-10 The effect of control conditions on MYA-1 cell viability.
Cells were cultured in triplicate and treated with 3 different control conditions – fresh media alone ("untreated"), media obtained from uninfected MYA-1 cell cultures after 3 days of culture ("used media"), and filtrate obtained following ultrafiltration of virus stocks, as described in section 2.2.8 ("virus filtrate"). Dual-colour flow cytometry was performed as described in section 3.2.1.2 to assess the percentage of viable MYA-1 cells (Annexin-V negative / 7AAD negative). Each data point represents the mean percentage of viable cells at each time point, with error bars depicting the standard error of the data. There was no difference over time between each control condition in the percentage of viable cells in culture (p=0.761). Error bars represent the 95% CI for the mean.

Overall, MYA-1 cell cultures infected with all isolates of FIV showed decreased cell viability over time when compared to control cultures. This was shown by a decrease in the percentage of viable (Annexin-V negative / 7AAD negative) cells over time in FIV infected cultures compared to uninfected cultures (mean 75.0% versus 88.1%, p=0.00). When individual time points were considered, this effect was evident at days 1, 8 and 10, but not at day 3 post-infection. **Figure 3-11** shows a representative flow cytometry graph comparing the percentage of apoptotic and necrotic cells in one FIV infected culture versus and infected culture at one timepoint. **Figure 3-12** is a histogram comparing the MFI of Annexin-V in the same two cultures showing increased apoptosis in the FIV infected culture.

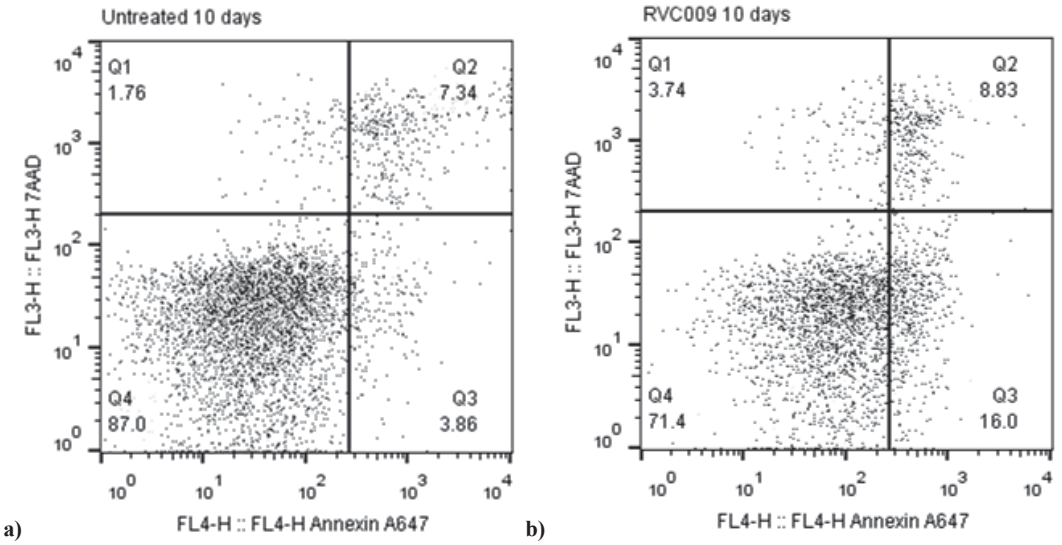


Figure 3-11 Flow cytometry graphs demonstrating the effect of FIV infection on MYA-1 cell apoptosis at day 10. Representative graphs demonstrate Annexin-V fluorescence (FL4, x-axis) and 7AAD fluorescence (FL3, y-axis) at day 10 in **a)** uninfected MYA-1 cells and **b)** RVC009 infected MYA-1 cells. Quadrant 4 represents viable cells, quadrant 3 represents apoptotic cells and quadrant 2 represents necrotic cells and figures indicate the percentage of cells in each quadrant. The FIV infected culture showed an increased percentage of apoptotic and necrotic cells when compared to the uninfected culture.

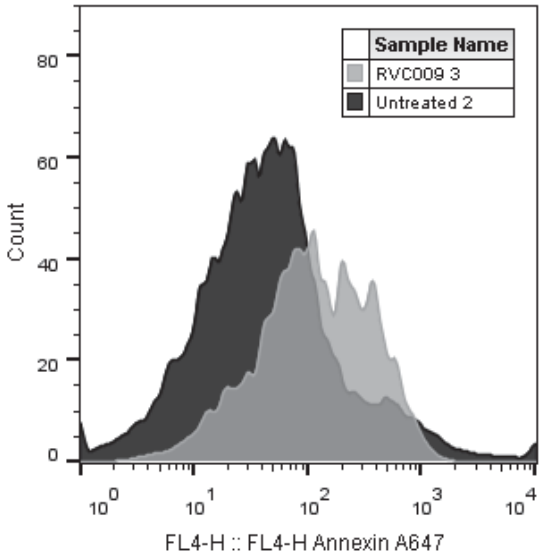
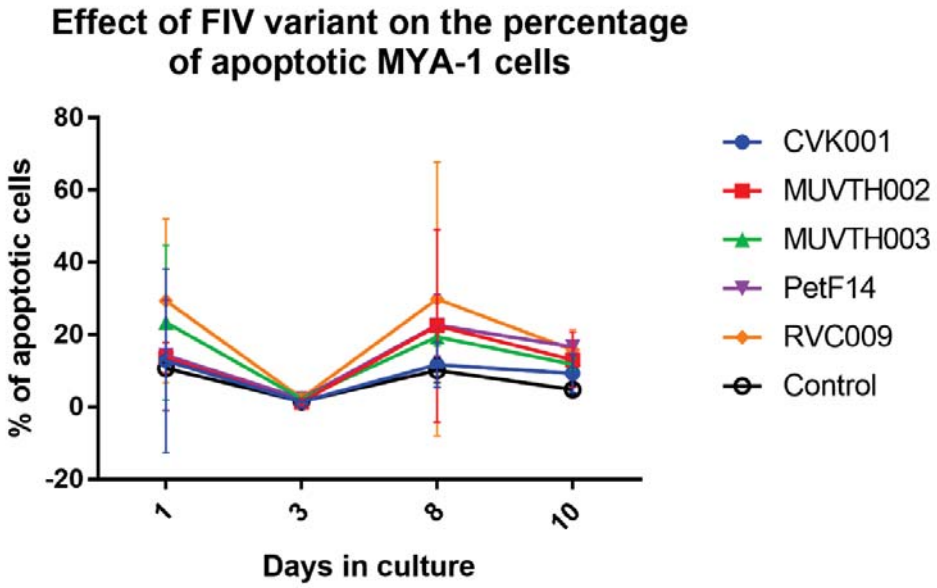
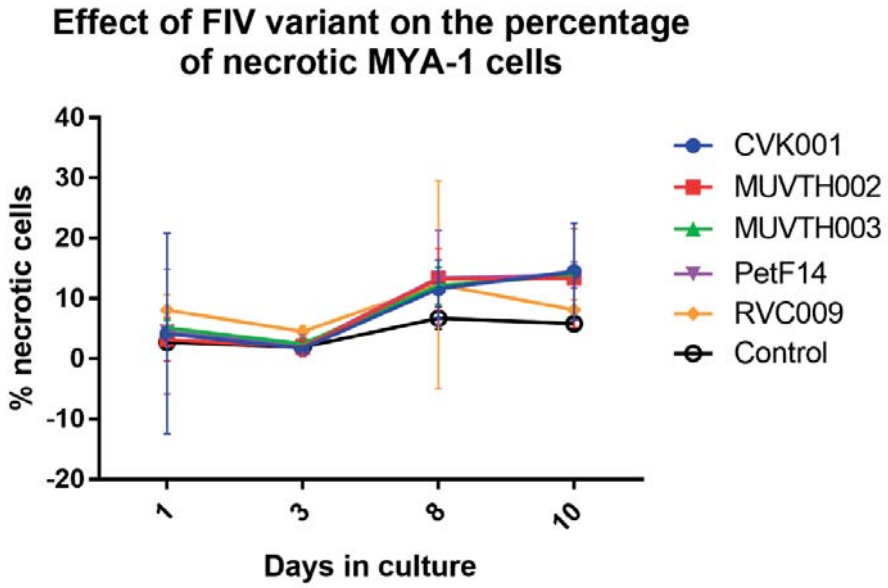


Figure 3-12 Overlay histogram demonstrating FIV-induced apoptosis at day 10.
The number of cells (y-axis) is plotted against the fluorescence intensity of Annexin-V (FL4, x-axis). Cells from the uninfected culture are shown in dark grey and cells from the RVC009 FIV infected culture are shown in light grey. The overall fluorescence of Annexin-V is increased in cells from the infected versus uninfected culture, indicating increased apoptosis in the FIV-infected culture.

There was a significant difference in the amount of apoptosis induced by each isolate of FIV (**Figure 3-13a**). Over time, MYA-1 cultures infected with RVC009 showed the highest percentage of apoptotic cells when compared to controls (mean 19.35% versus 6.78%, p=0.00), and this was also significantly increased compared to the CVK001 and MUVTH002 isolates (**Figure 3-14**). Similarly, the MFI of Annexin-V was increased in RVC009 infected cultures compared to the controls and the CVK001 and MUVTH002 isolates (413.09 versus 172.21, p=0.00). The CVK001 isolate induced the least apoptosis in MYA-1 cells, showing a slight increase in the percentage of apoptotic cells and no increase in the MFI of Annexin-V when compared to uninfected controls (mean apoptosis 8.87% versus 6.78%, p=0.024; MFI Annexin-V 205.45 versus 172.21, p=0.093). All isolates of FIV induced more necrosis in MYA-1 cells when compared to uninfected controls, as indicated by the percentage of necrotic cells (p=0.00). There were no differences between individual isolates in the percentage of necrotic cells or the MFI of 7AAD (**Figure 3-13b**). However, when results from individual time points were compared, the RVC009 isolate induced significantly more necrosis than the other isolates at days 1 (p=0.00) and 3 (p=0.00) and significantly less necrosis than the other isolates at day 10 (p=0.00, **Figure 3-15**). Regression tables are included in **Appendix 6**. These results support the hypothesis that FIV isolates differ in their ability to induce apoptosis and necrosis in MYA-1 cells *in vitro*.



a)



b)

Figure 3-13 Comparison of MYA-1 cell viability in cultures infected with different isolates of FIV.
Line plots show the percentage of cells (y-axis) undergoing **a)** apoptosis and **b)** necrosis over time (x-axis) in cultures infected with FIV. MYA-1 cells were cultured in triplicate, and infected with 5 different isolates of FIV (RVC009, PetF14, MUVTH002, MUVH003 and CVK001). Results from the 3 control conditions (as described in section 2.2.8) were pooled and shown as "control" in this graph. Dual-colour flow cytometry was performed as described in section 3.2.1.2 to assess apoptosis (Annexin-V positive / 7AAD negative) and necrosis (Annexin-V positive / 7AAD positive) in MYA-1 cultures. There was a significant increase in both apoptosis and necrosis induced by each FIV isolate over time when compared to the controls. There was also a significant difference among isolates, with RVC009 and CVK001 differing significantly (p=0.00). At day 10, the RVC009 isolate induced significantly less necrosis than the other isolates (p=0.00). Error bars represent the 95% CI for the mean.

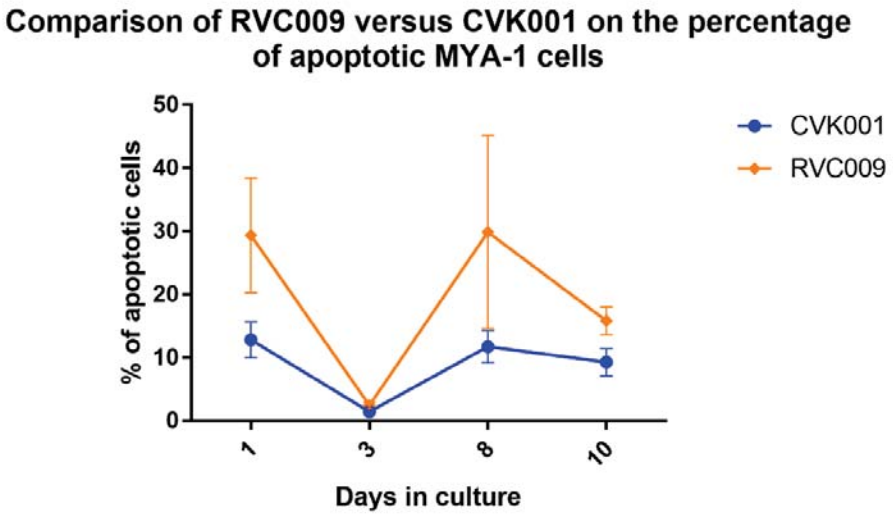


Figure 3-14 Comparison of the percentage of MYA-1 cells undergoing apoptosis over time in cultures infected with the RVC009 and CVK001 isolates of FIV.
Line plots show the percentage of cells (y-axis) undergoing apoptosis over time (x-axis) in cultures infected with the two isolates of FIV. Experimental conditions are as described in Figure 3-13. There was a significant increase in apoptosis over time in cultures infected with the RVC009 isolate compared to cultures infected with the CVK001 isolate (mean percentage of apoptotic cells over time 19.4% in RVC009 infected cultures, versus 8.9% in CVK001 infected cultures, p=0.004). Error bars represent the 95% CI for the mean.

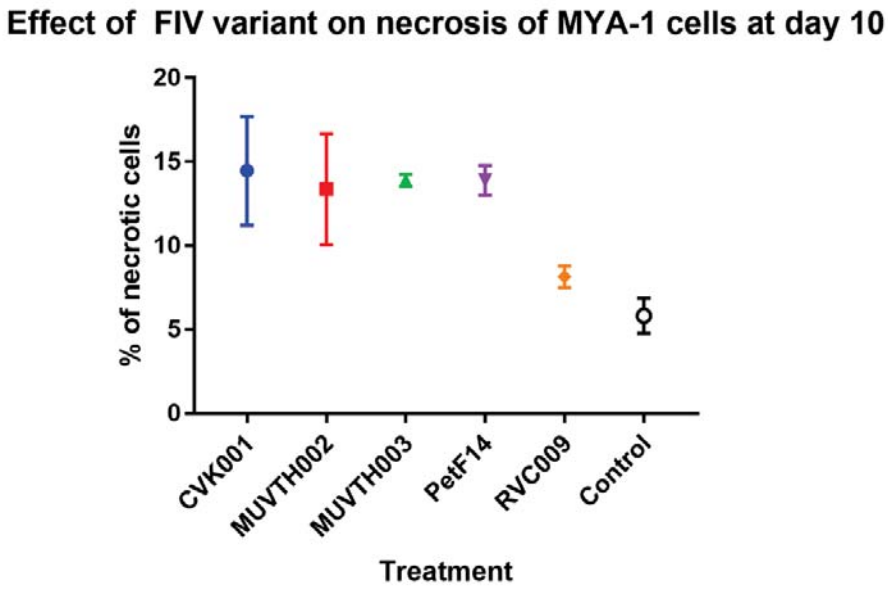
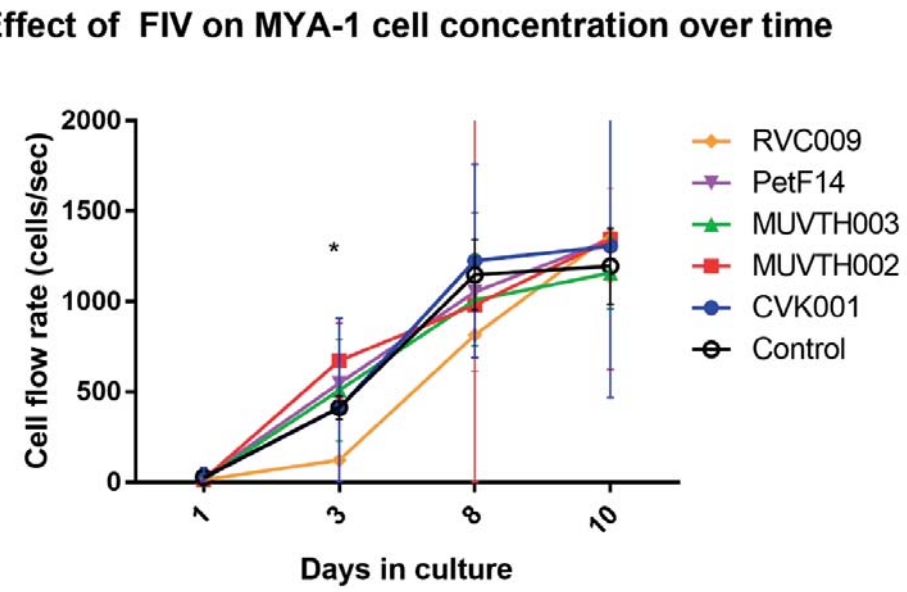


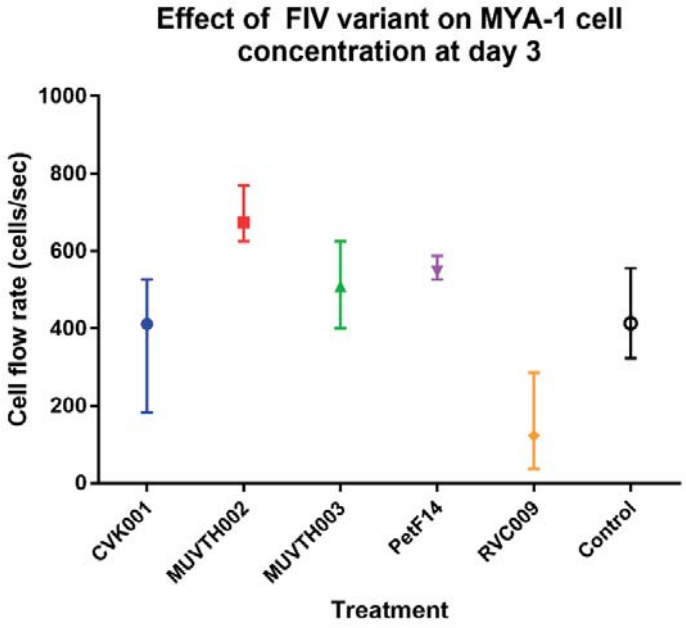
Figure 3-15 Comparison of the percentage of MYA-1 cells undergoing necrosis at day 10 in cultures infected with different isolates of FIV. the RVC009 and CVK001 isolates of FIV.
The percentage of MYA-1 cells undergoing necrosis is shown on the y-axis. Experimental conditions are as described in Figure 3-13. The RVC009 isolate induced significantly less necrosis when compared to the other isolates at this time point (p=0.00). Error bars represent the range for the mean.

3.3.3. Effect of FIV infection on MYA-1 cell concentration in culture

Subjective assessment of the cell concentration at each time point suggested a difference in the effect of each isolate of FIV on this parameter. Cell flow rate (cells/sec) was calculated using data from the flow cytometry during acquisition. Cell flow rate and therefore cell concentration increased in all cultures over time (**Figure 3-16a**). Comparison of the mean cell flow rate at each time point revealed a significant difference between viruses at day 3 (p=0.00) (**Figure 3-16b**). Cultures treated with RVC009 had the lowest cell concentration at day 3 (mean 122.74 cells/sec), and this was significantly different to cultures infected with other isolates of FIV (mean MUVTH002 673.1, MUVTH003 508.3, PetF14 547.0 cells/sec) and control cultures (mean 413.2 cells/sec). Raw data is included in **Appendix 5,** and the statistical analysis is included in **Appendix 6**. These results suggested that infection with FIV did affect MYA-1 cell concentration in culture, and this was variable depending on the individual isolate.



a)

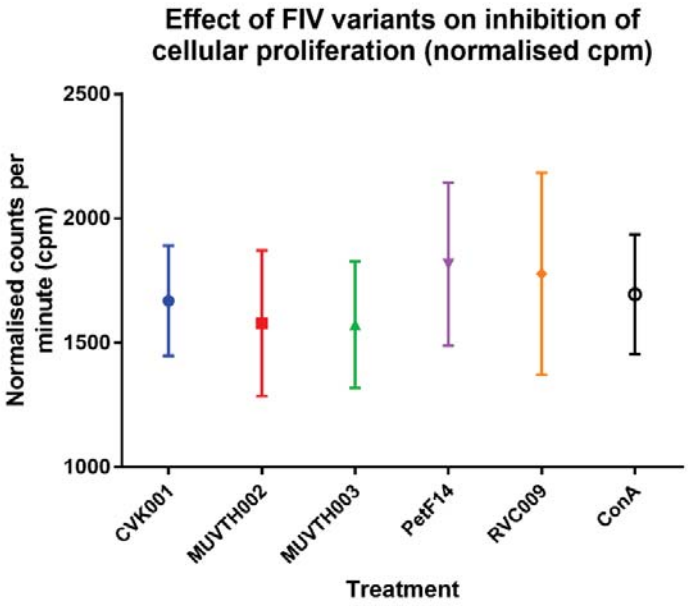


b)

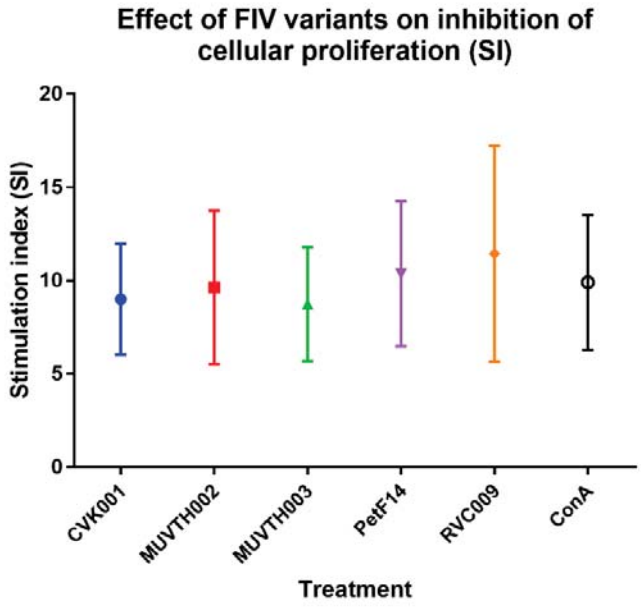
Figure 3-16 The effect of FIV infection on MYA-1 cell concentration.
Cell concentration **a)** over time and **b)** at day 3. Cell flow rate (cells/sec, y-axis) was calculated using data from the apoptosis / necrosis flow cytometry during acquisition at the different time points. As all cells in culture were counted, cell flow rate was used as a measure of cell concentration in each culture of cells infected with the 5 different isolates of FIV. There was a significant difference in cell concentration at day 3 (p=0.00), as indicated by an asterisk (*). Infection of MYA-1 cells with the RVC009 isolate of FIV resulted in a reduced cell concentration in culture at day 3. Error bars represent the range for the mean.

3.3.4. Inhibition of mitogen-induced lymphocyte proliferation by different isolates of FIV

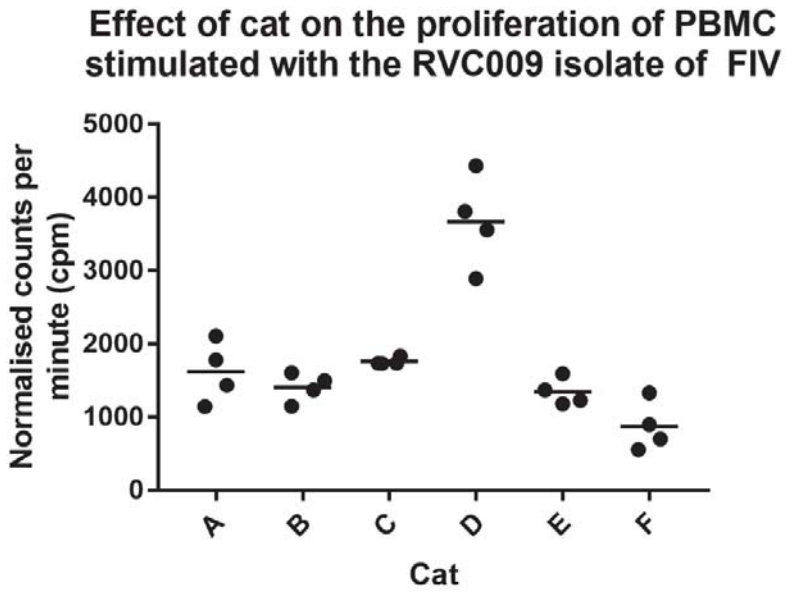
There was no effect of FIV on mitogen-induced cellular proliferation *in vitro*. Both the mean normalised cpm and the mean SI were similar for all groups, regardless of whether cells were infected with FIV in the presence of ConA, or treated with ConA alone (p=0.802 for cpm, p=0.606 for SI). Large inter-cat variation was observed among replicates (**Figure 3-17c**). Raw data and regression tables are included in **Appendix 5 and 6**.



a)



b)



c)

Figure 3-17 The effect of FIV infection on mitogen-induced lymphocyte proliferation.
Feline PBMC were stimulated with ConA, infected with 1 of 5 different isolates of FIV and cultured in quadruplicate for 2 days post-infection. The effect of FIV infection on inhibiting ConA-induced lymphocyte proliferation was examined by culturing cells for a further 18 hours with [H^3] and measuring **a)** counts per minute and **b)** stimulation index, using unstimulated PBMC as control cells. Graph **c)** is an example of the individual results for PBMC from each cat (A-F) following stimulation with FIV (RVC009 shown), highlighting the inter-cat variation observed.

3.4. Discussion

The purpose of this study was to investigate whether the pathogenicity of FIV isolates differ *in vitro.* Cells were infected with 5 different isolates of FIV (4 NZ field isolates and the Petaluma vaccine strain) and the effects of infection on cellular apoptosis, necrosis and mitogen-induced proliferation were studied. Results demonstrated that there were differences among these isolates in their ability to induce apoptosis and necrosis of MYA-1 cells *in vitro*. In addition, there was a variable effect of infection on MYA-1 cell concentration in culture, suggesting that some isolates may have been able to suppress MYA-1 cell proliferation.

MYA-1 cell cultures infected with the subtype A RVC009 isolate of FIV showed the highest percentage of apoptotic cells, and this was significantly greater than the controls and the subtype C isolates, CVK001 and MUVTH002. The percentage of necrotic cells in cultures infected with the RVC009 isolate at individual time points also differed when compared to control cultures and cultures infected with the other isolates of FIV. Early after infection (days 1 and 3),

the RVC009 isolate induced more necrosis in MYA-1 cells when compared to the other isolates. However, by day 10, less necrosis was detected in cultures infected with this isolate compared to those infected with the other isolates. Finally, the concentration of MYA-1 cells at day 3 in cultures infected with the RVC009 isolate was reduced compared to cultures infected with other isolates. The differences observed for the RVC009 isolate for these 3 effects (apoptosis, necrosis and MYA-1 cell concentration) supports the hypothesis that the pathogenicity of FIV isolates differ *in vitro*.

Phylogenetic analysis of the 5 isolates used in this study was performed in chapter 2. Comparison of the sequenced region of the *env* gene showed that the RVC009 isolate differed by 21% at this region when compared to the 3 other NZ field isolates of FIV. The RVC009 isolate was more similar to the subtype A, Petaluma isolate, showing 90.7% nucleotide similarity at the sequenced region of the *env* gene. Accordingly, the RVC009 isolate was classified as subtype A, whereas the other NZ isolates were classified as subtype C. Differences in apoptosis induced by the different isolates did not clearly reflect subtype classification. Although cells infected with the 2 subtype A isolates (RVC009 and PetF14) showed the highest mean percentages of apoptotic cells, there were no apparent differences between the PetF14 infected cultures and the subtype C infected cultures. These findings highlight that subtype classification does not necessarily reflect viral phenotype. Only a small region of the *env* gene was sequenced, so it is possible that viruses belonging to the same subtype in this study may have differed substantially at other regions of the genome.

The structure of the envelope glycoprotein is relevant when considering the apoptotic effect of FIV. As discussed in section 1.4.1.2, the mechanism of FIV-induced apoptosis is likely to be multifactorial, but one suggested mechanism involves fusion of uninfected lymphocytes with the transmembrane portion of gp35 on the surface of virions. This results in destabilisation of the cell membrane and activation of the caspase-3 pathway (Garg and Blumenthal, 2006). The role of the envelope glycoprotein in this pathway is supported by *in vitro* studies demonstrating apoptosis of activated PBMC following exposure to CRFK cells expressing the envelope glycoprotein (Garg

et al., 2004a). Some authors have suggested that exposure to soluble envelope glycoprotein and other viral pro-apoptotic factors may also induce apoptosis in lymphocytes, probably via upregulation of various apoptosis signalling molecules (Ahr *et al.*, 2004, Tompkins *et al.*, 2002, Mizuno *et al.*, 2003, Folkl *et al.*, 2008). Given the proposed role of the envelope glycoprotein in inducing apoptosis, it is feasible that sequence variation of the *env* gene among these isolates could be responsible for the differences observed in their apoptotic potential.

The 5 viruses used in this study were harvested from the supernatant of infected MYA-1 cell cultures following virus isolation from naturally infected cats (RVC009, MUVTH002, MUVH003, CVK001) or production of virus from an infectious molecular clone (PetF14). Virus stock was then concentrated by ultrafiltration and quantified as described in section 2.2.8 and 2.2.9. Therefore, as the virus stock contained media from MYA-1 cell cultures, the possibility of non-viral factors (such as excessive lactic acid present in exhausted media) inducing apoptosis or necrosis in this study had to be considered. For this reason, 3 control conditions were tested – fresh media, conditioned media from uninfected MYA-1 cell cultures, and filtrate obtained following ultrafiltration of supernatant from virally infected MYA-1 cell cultures. The latter media may have contained viral proteins ≤100kDa in size, which could have included any soluble envelope glycoprotein present in infected MYA-1 cell cultures. No differences were observed between these 3 control conditions in any parameter measured. This suggests that if viral factors are responsible for apoptosis, then they are likely to be larger than 100 kDa, which may include whole virions, cell associated envelope glycoprotein, or soluble envelope glycoprotein existing as multimers. Further studies using inactivated FIV and purified envelope glycoprotein would be useful to elucidate the factors involved in FIV-induced apoptosis.

The virus used to induce apoptosis in MYA-1 cells was standardised according to infectivity ($TCID_{50}$/cell) rather than protein concentration. Therefore, the possibility that differences in apoptosis induced by the viruses reflected variation in the amount of envelope glycoprotein present in each preparation, rather than inherent differences among each isolate, cannot be excluded. Virus preparation containing some non-infectious virus (perhaps inactivated

during the storage or filtration process) would contain a disproportionately high amount of viral protein and nucleic acid compared to virus stock of the same titre, consisting of only live, infectious virus. Comparison of apoptosis induced by the different isolates of FIV showed that the RVC009 virus induced more apoptosis than the CVK001 virus at a given $TCID_{50}$/cell. This implies that either the RVC009 virus is inherently more apoptotic than the CVK001 virus, or that the RVC009 stock contained more pro-apoptotic factors than the CVK001 stock, perhaps in the form of soluble viral proteins. The gag p24 protein concentration had been previously measured for each virus stock (as described in section 2.2.9.3). Following standardisation according to virus titre, the RVC009 and CVK001 stocks contained a similar concentration of this protein (6 – 7 ng/mL). In contrast, the PetF14 isolate contained more than twice the amount of p24 protein (16 ng/mL), suggesting the presence of non-infectious virus in this stock. If it is assumed that the concentration of envelope glycoprotein in the virus preparation is proportional to the concentration of p24, then the differences in apoptosis cannot be explained by a difference in protein concentration between the RVC009 and CVK001 viruses. However, if the stock contained soluble envelope glycoprotein existing as multimers [and too large (>100 kDa) to be filtered out in the ultrafiltration process used to concentrate the virus stocks], then there may be differences in the concentration of envelope glycoprotein between the virus stocks. Further studies using viruses that have been standardised according to envelope glycoprotein concentration would be useful to confirm that the differences observed in this study were the result of inherent differences in the apoptotic potential of the various isolates, rather than differences in viral protein concentration.

Infection with all viruses resulted in increased numbers of necrotic cells when compared to controls. In addition, the amount of necrosis in each culture differed, depending on which isolate it was infected with. The amount of necrosis in culture did not correlate with the amount of apoptosis in all cultures. For example, the highest number of apoptotic cells was observed in cultures infected with the RVC009 isolate, but these cultures also showed the lowest number of necrotic cells by day 10. As apoptotic cells eventually undergo secondary necrosis *in vitro*, one

would expect increased necrosis in cultures that contain a high proportion of cells undergoing apoptosis. Results from this study suggest that there may be mechanisms other than apoptosis that are responsible for necrosis in virus infected cultures. Recent studies examining the effect of HIV on lymphocytes have indicated that CD4+ T cell loss occurs as a result of pyroptosis as well as apoptosis (Doitsh *et al.*, 2014). Pyroptosis is a form of programmed cell death caused by activation of the caspase-1 pathway. In contrast to apoptosis, pyroptosis leads to the formation of pores in the cell membrane, resulting in cell swelling, rupture and release of inflammatory cytosolic contents into the extracellular space. Cells undergoing pyroptosis would therefore be positive for Annexin-V and 7AAD, and thus indistinguishable from cells undergoing necrosis secondary to apoptosis (Miao *et al.*, 2011). If infection with FIV also results in pyroptosis or primary necrosis of lymphocytes, then this would explain the poor correlation between apoptosis and necrosis of MYA-1 cells seen in this study.

The effect of FIV on MYA-1 cell apoptosis and necrosis was evident only at days 1, 8 and 10. At day 3, no effect of virus infection was apparent, and the cell viability was increased compared to other time points. MYA-1 cells proliferate rapidly in culture, and in support of this, the cell flow rate (and thus cell concentration in culture) increased over time. A consequence of cell proliferation over time was that the concentration of virus per cell would not have remained constant at each time point. There is a lag time of approximately 4 days before RT is detected in cultures of MYA-1 cells infected with FIV (Sutton *et al.*, 2005). Rapid proliferation of MYA-1 cells in cultures prior to virus replication would have resulted in a relatively lower concentration of virus per cell in cultures on day 3 compared to day 1. This may explain the increased cell viability at day 3 compared to other time points, followed by the subsequent decrease in viability after this time point (coinciding with an increased virus concentration of virus per cell, assuming virus replication occurred after day 3). Measurement of virus load in culture would have been useful to study how the virus kinetics in culture related to its apoptotic effects over time.

The effect of FIV on mitogen-induced lymphocyte proliferation was also investigated in this study. This was prompted by an observation that MYA-1 cultures infected with different

viruses had variable cell concentrations, with cultures infected with the RVC009 isolate showing the lowest cell concentration (as estimated by the calculation of cell flow rate during the apoptosis experiment). Although these differences may have been attributed to increased cell death in these cultures, there was also the possibility that there was a direct inhibitory effect of the virus on MYA-1 cell proliferation. Such an effect has been reported previously in mitogen treated, uninfected PBMC exposed to HIV *in vitro* (Amadori *et al.*, 1988). No similar studies have been reported for FIV *in vitro*, however PBMC from FIV infected cats do show reduced proliferative responses when cultured in the presence of mitogens (Hara *et al.*, 1990, Lin *et al.*, 1990, Taniguchi *et al.*, 1990, Torten *et al.*, 1991, Bishop *et al.*, 1992, Lawrence *et al.*, 1992, Barlough *et al.*, 1991). Although this is probably explained by T cell exhaustion of lymphocytes from infected cats, the *in vitro* effects of HIV suggest that there may also be a direct suppression of lymphocyte proliferation in FIV infected cultures. The purpose of this experiment was therefore to assess whether exposure to FIV *in vitro* inhibited feline lymphocyte proliferation, and if so, whether there was a difference between isolates of FIV.

Despite the suspected effect of the virus on MYA-1 cell proliferation during the apoptosis experiment, there was no effect of FIV on mitogen-induced lymphocyte proliferation at the conditions studied. It is possible that FIV does not have the same direct inhibitory effect on cell proliferation that has been shown for HIV. Alternatively, the failure to confirm an inhibitory effect of FIV on mitogen-induced proliferation of PBMC in this study may have been due to insufficient concentration of the virus in cultures, or insufficient time for the virus to affect this parameter in culture. MYA-1 cells may be more sensitive than PBMC to such an effect, accounting for the reduced cell concentrations observed in the apoptosis experiment but not the study using PBMC, despite infection of cells with the same concentration of virus. One important difference in MYA-1 cells compared to PBMC is their reliance on exogenous IL-2 (Miyazawa *et al.*, 1989b). The transmembrane portion of the FIV envelope glycoprotein shares a similar three-dimensional structure to the IL-2 molecule (Serres, 2000). If this resulted in competitive inhibition of IL-2 binding, there may be suppression of proliferation of IL-2 dependent cell lines. In contrast, PBMC

utilise endogenous IL-2 once stimulated, perhaps producing enough to overcome competitive inhibition by the virus when present at low concentrations. In support of a dose-dependent effect, the previous study investigating the effect of HIV on PBMC showed that minimal inhibition of cell proliferation occurred when the p24 concentration was <3.1 μg/mL (Amadori *et al.*, 1988). The concentration of virus used in this study was much lower than this, ranging from between 0.6-1.54 ng/mL of p24. This study aimed to assess *in vitro* markers of pathogenicity, so a low concentration of virus was chosen to reflect plasma virus load within a host (Raboud *et al.*, 1996). Given these findings, it is likely that mechanisms other than a direct effect of the virus are more important in impairing lymphocyte proliferation in FIV infected hosts. The differences in cell concentration of MYA-1 cells observed in the apoptosis experiment may therefore have been due to increased cell death in culture rather than suppression of proliferation, a processing artefact, or may reflect an increased susceptibility of MYA-1 cells to a suppressive effect of the virus, as discussed above.

The apoptosis assay was initially developed on feline PBMC. These cells were chosen in order to mimic the *in vivo* effects of the virus as closely as possible. During the optimisation process, 2 methods were investigated to confirm the quality of PBMC separation. CPT™ vacutainers were chosen as one method, due to the reduced labour required and the increased stability of samples during transit. When compared to Ficoll density gradient medium separation however, the PBMC isolated using CPT™ vacutainers were largely contaminated by granulocytes. This was evident on the FSC/SSC profiles of cells analysed by flow cytometry immediately after centrifugation, with almost 63% of separated cells being small in size with high granularity, and therefore consistent with granulocytes. As far as the author is aware, there have been no previous studies assessing the use of CPT™ vacutainers to separate feline PBMC, although studies using human blood report similar results when comparing these tubes to Ficoll density gradient medium (Schlenke *et al.*, 1998). Possible causes for granulocyte contamination include prolonged storage prior to centrifugation and storage at temperatures less than 18 °C. As samples separated using both methods in this study were stored at room temperature and

centrifuged immediately, these factors are unlikely to be responsible for the differences observed. Centrifugation at suboptimal speeds can also increase granulocyte contamination. As samples for each method were centrifuged using different machines, it is possible that the samples collected into the CPT™ vacutainers were not centrifuged at the correct speed due to a malfunction of the centrifuge, although this also seems unlikely. Finally, activation of granulocytes can affect their buoyancy and result in granulocyte contamination of the PBMC layer (Mallone *et al.*, 2011). It is possible that use of the CPT™ vacutainers resulted in granulocyte activation, perhaps as a result of contact with the polyester gel used to separate the anticoagulant from the Ficoll solution. Further studies using CPT™ vacutainers for separation of feline PBMC are indicated to determine their suitability for use in this species.

Low cell viability of feline PBMC was a problem in this study. The cytotoxic effect of ConA on these cells was therefore investigated as one possible cause of cell death. PBMC require activation with ConA to render them permissible to FIV infection, as resting T cells do not express the necessary co-receptor, CXCR4 (Willett *et al.*, 2003). ConA is an α-mannose / α-glucose binding lectin that cross-links the T- cell receptor, resulting in cellular activation (Lindahlk K, 1972). ConA mediated cytotoxicity has been reported previously in lymphocytes from rats, where doses of 5 µg/mL resulted in a reduction of cell viability to 60% after 24 hrs (Takigawa and Waksman, 1981). Cell death in ConA treated cultures is probably due to increased calcium influx, causing increased apoptosis and necrosis (Mattson and Chan, 2003, Ozato *et al.*, 1977). In this study, the effect of increasing concentrations of ConA on feline PBMC viability over 24 hours was examined. Cellular activation was also assessed via CD25 expression, in order to determine the optimal concentration of ConA that activated cells without compromising cell viability. Results confirmed dose-dependent cytotoxicity of feline PBMC treated with ConA, with cell viability declining from 81.6% in untreated cultures to <50% at concentrations >3 µg/mL. CD25 expression increased from 53.3% in cells treated with 1 µg/mL of ConA to 74.2% in cells treated with 5 µg/mL of conA. A concentration of 1 µg/mL was considered optimal, and was used in all further experiments using feline PBMC.

Attempts to optimise the apoptosis assay using PBMC remained unsuccessful due to cell death in culture. Therefore, the feline lymphoblast cell line, MYA-1, was subsequently used to investigate FIV-induced apoptosis. These cells are highly permissible to FIV infection without the requirement for pre-treatment with ConA, thereby reducing the amount of apoptosis in uninfected controls (Miyazawa *et al.*, 1989b). IL-2 dependent feline T cell lines, such as MYA-1 cells, have been used previously to demonstrate FIV-induced apoptosis (Ohno *et al.*, 1994, Johnson *et al.*, 1996, Sutton *et al.*, 2005, Mizuno *et al.*, 2003). They were therefore considered a suitable alternative to PBMC for assessing the apoptotic effect of FIV in culture. The use of an immortal cell line for the apoptosis experiment may not accurately represent the effect of apoptosis on lymphocytes *in vivo*. However, these cells are reminiscent of activated lymphocytes, as they express IL-2Rα and remain reliant on IL-2 for survival (Miyazawa *et al.*, 1989b). As PBMC from FIV infected cats show increased activation *in vivo* (as shown by increased expression of IL-2Rα and MHC class II), the MYA-1 cells used in this study may actually be very similar to the populations of cells affected by FIV *in vivo* (Ohno *et al.*, 1992b, Rideout *et al.*, 1992).

The obvious limitation of this study is the use of *in vitro* characteristics as markers for pathogenicity *in vivo*. To highlight differences between isolates of FIV, *in vitro* models were necessary to avoid the confounding effects of host and environmental factors on results. However, one consequence of this approach, is the difficulty in determining the *in vivo* significance of differences observed between viruses *in vitro*. The effect of FIV on cellular apoptosis and suppression of mitogen-induced lymphocyte proliferation were chosen specifically for their relationship to clinical disease in the host, as these mechanisms at least partially explain the $CD4^+$ T cell depletion and impaired immunity that has been reported as a result of FIV infection. If FIV isolates differed in their ability to induce apoptosis in lymphocytes, then this may account for variability in the rate of $CD4^+$ T cell decline observed in FIV infected cats. As such a difference was observed in this study, it is very likely that FIV variants do differ in pathogenicity. It is interesting to note that the RVC009 induced the more apoptosis over time when compared to most

other isolates, and that this virus was isolated from an asymptomatic cat. No conclusions can be drawn from this however, as the current study was not designed to compare *in vivo* pathogenicity between isolates. From the clinical data collected, it is impossible to know how long each cat was infected with FIV, and therefore the time frame between infection and developing clinical signs is unknown. The length of the asymptomatic period in FIV infected cats is one measure of pathogenicity. The asymptomatic cats recruited in this study may still have been infected with a highly virulent isolate of FIV, if they were sampled shortly after infection (i.e. not long enough after infection for disease to develop). Likewise, the sick cats in this study may have experienced a prolonged asymptomatic period prior to clinical signs occurring, which would actually suggest infection with a less pathogenic isolate of FIV. As with all studies describing disease in FIV infected cats, it cannot be assumed that the clinical signs recorded in their submission form history are actually related to their FIV infection.

Another limitation of this study is the small number of replicates used to investigate the apoptotic effect of FIV on MYA-1 cells. Only 3 samples were measured at each time point, limiting the power of the statistical analysis used in the study. However, significant differences were still observed among viruses, despite the limited power of the study. This supports the conclusions of these findings, reinforcing that FIV isolates do differ in their ability to induce apoptosis and necrosis in MYA-1 cells. As only a small region of the *env* gene was sequenced in this study, it cannot be concluded that differences among isolates are due to variation at the *env* gene. Further studies comparing whole genome sequences of these isolates would be useful to confirm which genes are important in the mechanism of FIV-induced apoptosis and necrosis.

3.5. Conclusion

This study showed that there was a difference in the amount of apoptosis and necrosis observed in a feline lymphoblastic cell line following treatment with FIV, with one isolate in particular (RVC009), inducing more apoptosis than the others. In addition, the apoptosis assay

developed in this study provided an *in vitro* method to evaluate viral pathogenicity, potentially enabling larger scale evaluation of viral pathogenicity in future studies.

Virally-induced apoptosis is at least partially responsible for the loss of $CD4^+$ T cells in infected cats, contributing to the immune dysfunction associated with FIV infection (Garg *et al.*, 2004b, Muro-Cacho *et al.*, 1995, Finkel *et al.*, 1995, Sarli *et al.*, 1998, Tompkins *et al.*, 2002). It is therefore feasible that the differences in apoptosis observed *in vitro* in this study may reflect differences in pathogenicity among field variants of FIV. Based on these findings, viral factors should be considered in addition to host and environmental factors as an explanation for the vast diversity of FIV-associated disease.

The FIV genome mutates frequently during replication within the host, and as a result, FIV is continually evolving and responding to a range of selection pressures. If FIV variants differ in pathogenicity *in vivo*, then the spectrum of FIV-associated disease may also change over time as the virus evolves. Widespread use of a vaccine against FIV, such as the Fel-O-Vax® FIV vaccine, may introduce additional selection pressures that offer an advantage to certain variants of FIV. The likelihood of this depends on the cross-reactivity of the vaccine-induced immune response, with poor cross-reactivity increasing the chances that vaccine-resistant variants may emerge. As has been shown with this study, FIV isolates differ in pathogenicity *in vitro*, so it is also possible that the structure of key epitopes may differ among these isolates, affecting the efficacy of the vaccine. Further studies are therefore indicated to investigate the cross-reactivity of the immune response following vaccination with the Fel-O-Vax® FIV vaccine, against the field isolates of FIV used in this study.

CHAPTER FOUR

In vitro cross-reactivity of the Fel-O-Vax® FIV vaccine-induced immune response against NZ isolates of FIV

4.1. Introduction

In 2004, a vaccine marketed to protect against FIV infection was introduced to NZ veterinarians. The Fel-O-Vax® FIV vaccine contains paraformaldehyde inactivated whole virus and killed cells infected with 2 different strains of FIV (the subtype A virus, FIV-Petaluma, and the subtype D virus, FIV-Shizuoka) (Uhl *et al.*, 2002). The inclusion of 2 diverse strains of FIV in the vaccine was thought to broaden the specificity of the vaccine-induced immune response beyond that of a monovalent vaccine (Yamamoto *et al.*, 2007). Based on interpretation of previous efficacy studies, the preventable fraction (PF) for the Fel-O-Vax® FIV vaccine is often reported as 68.1% (Hohdatsu *et al.*, 1997b, Pu *et al.*, 2001, Pu *et al.*, 2005, Kusuhara *et al.*, 2005, Dunham *et al.*, 2006b, Huang *et al.*, 2004, Huang *et al.*, 2010, Omori *et al.*, 2004). The PF of the vaccine is defined as the percentage of vaccinated versus unvaccinated cats that are protected from FIV infection following challenge with the virus.[7] Some of the Fel-O-Vax® FIV vaccine efficacy studies have demonstrated excellent protection (100%) of vaccinated cats against challenge with vaccine strains of the virus, as well as some other field isolates of subtypes A and B (Hohdatsu *et al.*, 1997a, Pu *et al.*, 2001, Yamamoto *et al.*, 2010, Omori *et al.*, 2004, Coleman *et al.*, 2014, Pu *et al.*, 2005, Kusuhara *et al.*, 2005). However, others have demonstrated poor efficacy of the vaccine. For example, Dunham et al (2006) reported a complete lack of efficacy when vaccinated cats were challenged with a subtype A field isolate of FIV from the U.K. (FIV-GL8) (Dunham *et al.*, 2006b). In 2 studies, vaccinated cats were challenged with a NZ field isolate of FIV (NZ-1[8]) of a recombinant C/F' subtype (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014). In these studies, a PF of only 40 to 44.4% was reported. Although all of the reported studies have differed in regards to challenge dose, route of infection and potentially other (e.g. host-related) factors, another source of variation was the use of different challenge viruses for each study. The latter raises the

[7] Preventable fraction (PF) is calculated using the following equation: PF(%) = (% controls infected - % vaccinates infected) / % controls infected x 100.
[8] FIV-NZ1 is a recombinant virus, with the *env* gene clustering with subtype C, and the *pol* and *gag* genes belonging to an unknown subtype (designated F')

possibility that different isolates of FIV are not equally controlled by the vaccine-induced immune response.

FIV is a virus that mutates frequently due to the error-prone nature of RT. The majority of variation of the FIV genome occurs at the *env* gene, that encodes the envelope glycoprotein expressed on the surface of virions and FIV-infected cells (Pancino *et al.*, 1993b). Based on variation at this location, FIV variants are classified phylogenetically into at least six different subtypes (A-F) (Kakinuma *et al.*, 1995, Pecoraro *et al.*, 1996, Weaver, 2010). However, even within a subtype, up to 15% variation of nucleotide sequence is expected at the *env* gene, affording the possibility that the biological properties of viruses within the same subtype may also differ (Sodora *et al.*, 1994). The extensive genetic variation of FIV is one of the major obstacles in vaccine development. To be effective against a range of viruses in the field, the vaccine-induced immune response must either target conserved epitopes on the virus, or have broad specificity against different antigens.

The Fel-O-Vax® FIV vaccine elicits both humoral and cell-mediated responses, although the relative importance of each mechanism in protecting the host against infection remains unknown. Some of the antibodies generated neutralise FIV *in vitro*, however, their specificity is narrow, apparently effective only against the vaccine strains of FIV (Pu *et al.*, 2001, Hohdatsu *et al.*, 1997a). Despite this, protection against infection with non-vaccine isolates of FIV has been demonstrated, suggesting the importance of other components of the vaccine-induced immune response (Hohdatsu *et al.*, 1997a, Pu *et al.*, 2001). Few studies have investigated the cell-mediated vaccine-induced immune response against FIV. In one study, an anti-FIV cytotoxic response against the Petaluma vaccine strain of FIV was described in PBMC from cats vaccinated with the Fel-O-Vax® FIV vaccine (Omori *et al.*, 2004). In another study, adoptive transfer of purified PBMC from Fel-O-Vax® FIV vaccinated cats resulted in some protection of unvaccinated cats from challenge with the Petaluma vaccine strain of FIV (Pu *et al.*, 2006). No study has investigated the cross-reactivity of the cell-mediated vaccine-induced immune response against field isolates of FIV.

Designing and executing appropriate vaccine efficacy studies to test a range of field viruses presents numerous challenges in terms of ethics, cost and resources. Demonstration of efficacy against a single challenge virus does not imply efficacy of the vaccine against a range of field viruses, due to the vast genetic diversity of the virus. An *in vitro* model investigating the specificity of the vaccine-induced immune response is an attractive alternative to *in vivo* challenge studies, also offering the opportunity to further characterise the response. One example of such an assay is flow cytometric detection of antigen-specific memory cells. This approach has been used previously in pigs to determine the specificity of the vaccine-induced immune response against swine influenza. In that study, flow cytometry was used to measure CD25 expression in porcine PBMC stimulated with swine influenza antigens (Kappes *et al.*, 2012). Similarly, CD25 expression has been used to detect allergen-specific T cells in PBMC from atopic dogs (Simpson *et al.*, 2009). The CD25 molecule is one subunit (the alpha chain) of the IL-2 receptor. Although it is expressed constitutively on T regulatory cells, it is only expressed on other T cells following activation (Kindt T.J., 2007, Smith, 1988). In addition (at least in humans), CD25 is also expressed on activated B cells, monocytes and NK cells (Kindt T.J., 2007). Results from assays measuring CD25 expression on human PBMC stimulated with various antigens (phytohaemaglutinin, Staphylococcal enterotoxin B, tetanus toxoid and influenza A antigen) showed that the assay was equally effective as traditional lymphocyte proliferation assays in discriminating between the presence and absence of an antigen-specific immune response (Caruso *et al.*, 1997).

The DTH response has also been used previously to predict immunity to a pathogen following vaccination. Intradermal injection of an antigen in sensitised individuals leads to the development of a firm cutaneous reaction at the injection site (Crowle, 1975). A positive response requires the presence of memory T cells, as well as functional antigen processing, presentation, cytokine production and effector responses (Tizard, 2009, Pier, 2004). The DTH response test has been used for many years to diagnose and assess vaccine-induced immunity against tuberculosis in cattle and humans, and it has also been used in mice following vaccination against

Cryptococcus neoformans (Anders, 1900, Delepine, 1900, Nichols *et al.*, 2002, Lee *et al.*, 2011). In cats, a DTH response was elicited in vaccinated cats following inoculation of inactivated feline herpesvirus and antigens from a polyvalent vaccine (Fargeaud *et al.*, 1984, Otto *et al.*, 1993, Cave *et al.*, 2007). In addition to providing a way to assess the *in vivo* response to specific antigens, another benefit of measuring the DTH response is the ability to test immunity against multiple antigens in the same animal. It is therefore a promising test to predict a vaccine-induced immune response against FIV, where cross-reactivity of the response must be assessed across a range of field viruses.

The purpose of this study was to determine whether T cells generated following vaccination with the Fel-O-Vax® FIV vaccine recognise antigens from field isolates of FIV. The null hypothesis was that there would be no difference in the magnitude of the DTH response generated, and in the expression of CD25 in response to stimulation with the NZ field isolates of FIV prepared in chapter 2.

4.2. Materials and methods

4.2.1. Animals

A total of 15 domestic shorthair cats were vaccinated against FIV using the dual-subtype, Fel-O-Vax® FIV vaccine according to the manufacturer's directions (3 doses of vaccine subcutaneously, each 2 weeks apart). Of the 15 cats, 4 had been vaccinated 1 year previously and had also received a booster vaccine 2 weeks prior to sampling for the CD25 assay (designated "revaccinated cats"). The remaining 11 cats received their third dose of vaccine 2 weeks prior to sampling ("newly vaccinated cats"). An additional 4 cats remained unvaccinated for use as controls in the CD25 assay and for the purposes of assay optimisation. The allocation of cats to various experimental groups is outlined in **Figure 4-1**. All cats were classified as healthy based on physical examination and stable bodyweight for the preceding 3 months, were less than 4 years of age, and were desexed. They were housed at the MUFNU (Palmerston North, NZ). The

experimental procedure was reviewed and approved by the MUAEC (protocols #13/10 and 11/15).

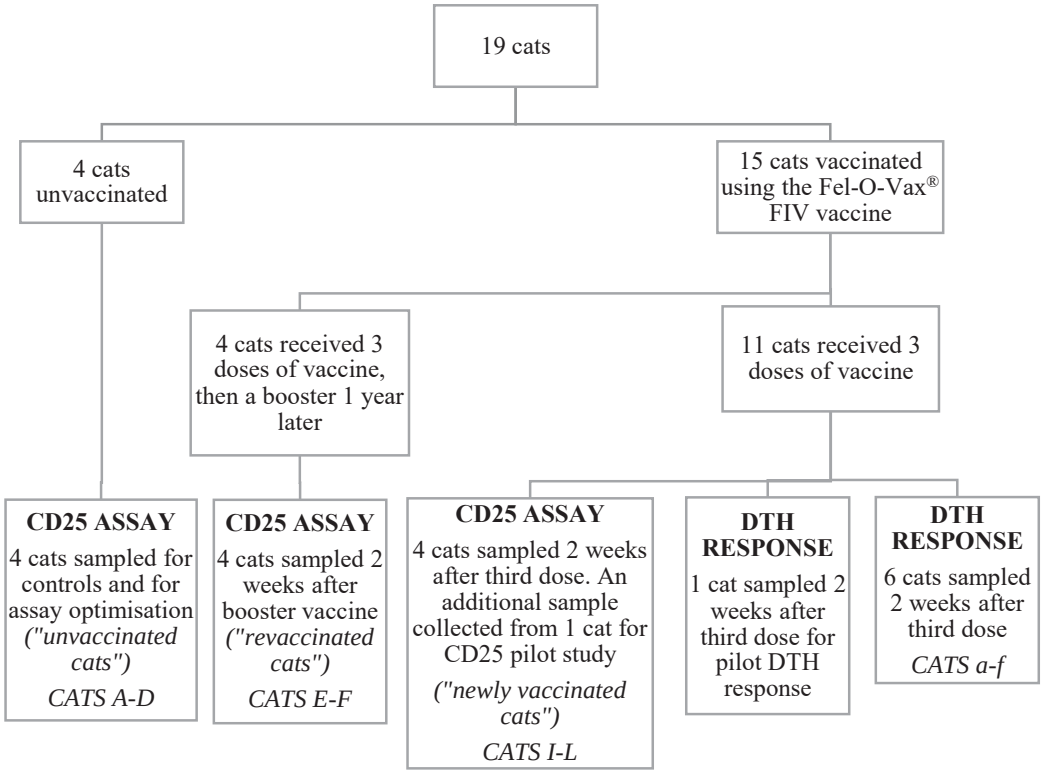


Figure 4-1 Flow chart depicting the allocation of cats to experimental groups.
A total of 19 cats were used in this study. 15 of these cats were vaccinated with the dual-subtype Fel-O-Vax® FIV vaccine, and 4 remained unvaccinated. 12 cats in total were sampled for the CD25 assay (4 cats were "unvaccinated", 4 were "revaccinated" following a booster 1 year after the initial course of vaccinations, and 4 cats were "newly vaccinated" 6,4 and 2 weeks prior to sampling). 7 cats in total were used for the DTH study (including 1 cat sampled for the pilot study).

4.2.2. Preparation of viral antigen

Viral antigen for challenge comprised inactivated FIV of 4 different isolates. Two of the viruses were NZ field isolates belonging to subtype C (MUVTH002 and CVK001), 1 was a NZ field isolate belonging to subtype A (RVC009) and 1 was a laboratory adapted virus obtained from an infectious molecular clone of the subtype A, Petaluma strain (PetF14). These viruses were isolated and prepared as described in chapter 2, with subtypes confirmed by sequencing the PCR fragment amplified from the *env* gene. Viruses were diluted in RPMI media for standardisation according to p24 concentration, to produce a final concentration of 42 ng/mL p24

for the CD25 assay and 14 ng/mL p24 for the DTH response. Diluted virus preparations were inactivated thermally (56 °C for 30 mins) and then irradiated under UV light for 15 mins [as per the protocol described by Omori and others (2004)].

4.2.3. CD25 assay

4.2.3.1. FIV-specific cellular activation pilot study

A pilot study was designed to test the hypothesis that CD25 expression increased in PBMC from Fel-O-Vax® FIV vaccinated cats compared to PBMC from unvaccinated cats, following stimulation with FIV antigens. Heparinised whole blood (2 mL) was collected from 2 cats (1 vaccinated and 1 unvaccinated). PBMC were separated using density gradient medium (Ficoll-Paque, GE Healthcare Life Sciences) as described in section 2.2.6.1. Cells were cultured in 24-well plates, at a density of $1.5x10^6$ cells/mL, in a total volume of 500 µL RPMI media (GIBCO®) supplemented with 10% FBS (GIBCO®), 1% penicillin / streptomycin (GIBCO®), and 1% glutamax (GIBCO®) and maintained in a humidified environment at 37 °C and 5% CO_2 for a period of 1 day. Cells were incubated in duplicate in the presence of inactivated FIV (100 µL supernatant collected from MUVTH002 infected MYA-1 cell cultures as described in section 2.2.6.3, and inactivated as described in section 4.2.2). Controls consisted of PBMC stimulated with ConA at a final concentration of 1 µg/mL, and cells treated with 100 µL of fresh supplemented RPMI as described above. Following incubation, cells were transferred to microcentrifuge tubes and centrifuged at 400 x g for 5 minutes. Supernatant was discarded and cells were resuspended in 1 mL of PBS, centrifuged at 400 x g for 5 minutes and resuspended in 100 µL of PBS. Cells were then incubated for 30 minutes in the dark with 0.5 µL of non-purified cell culture supernatant containing a FITC conjugated anti-feline CD25 antibody[9] (Fogle *et al.*, 2010). PBS (1 mL) was then added to each sample, and cells were centrifuged at 400 x g for 5 minutes. Finally, the pellet was resuspended in 200 µL of PBS and analysed immediately by flow cytometry using FACScalibur (BD Biosciences). Cells were gated based on FSC/SSC graphs to

[9] Generously gifted by Dr. Mary Tompkins, Department of Microbiology, Pathology and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC.

include only single lymphocytes, and the percentage of CD25 positive lymphocytes was calculated using FlowJo® software (TreeStar, Inc., Ashland, OR).

4.2.3.2. Optimisation of the CD25 assay

4.2.3.2.i. Cell culture

For optimisation of the CD25 assay, PBMC were prepared as follows: Heparinised whole blood (2 mL) was collected from 1 unvaccinated cat and PBMC were separated using a density gradient medium (Ficoll-Paque, GE Healthcare Life Sciences) as described in section 2.2.6.1. Cells were cultured for 1 day in 96-well plates at a density of $1.5x10^6$ cells/mL in a total volume of 200 µL of supplemented RPMI as described in 4.2.3.1, with the addition of ConA to a final concentration of 1 µg/mL. Following culture, cells were centrifuged at 800 x g for 2 minutes. The supernatant was discarded, and cells were resuspended in 1 mL of PBS and centrifuged again at 800 x g for 2 minutes. The supernatant was discarded, and the pellet resuspended in 50 µL of PBS.

4.2.3.2.ii. Antibody titrations

An unconjugated, monoclonal mouse anti-feline CD25 antibody (9F23[10]) (Ohno *et al.*, 1992a) was used to detect CD25 expression in all experiments subsequent to the pilot study. A rabbit anti-mouse IgG antibody conjugated to Alexa-fluor 647 (Invitrogen NZ Ltd, cat no. A-21239) was used as a secondary antibody for CD25 detection. Commercially available mouse antibodies directed against the feline CD4 antigen (conjugated to FITC, AbD Serotec®, MCA1346F) and the CD8 antigen (conjugated to R-PE, Abcam®, ab25417) were used. Two-fold serial dilutions of each antibody were prepared in PBS, and 20 µL of the diluted antibody was added to PBMC (prepared as described in section 4.2.3.2.i) to give a final antibody dilution in PBS of 1/5, 1/10, 1/20 and 1/40 for anti-CD4 and anti-CD8 antibodies, and 2/5, 1/5, 1/10, 1/20 for the anti-CD25 antibody in a total volume of 50 µL. Cells were incubated with each antibody separately in the dark and at room temperature for 30 minutes. Cells were then centrifuged at 800

[10] Generously gifted from Dr. Yuko Goto-Koshino (Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan).

x g for 2 minutes, washed in 1 mL of PBS, pelleted by centrifugation (800 x g for 2 minutes), the supernatant discarded and the pellet resuspended in 50 µL of PBS. The CD25 samples were then incubated with 10 µL of the anti-IgG antibody (diluted 1:10 in PBS) for 20 minutes and washed again in PBS as described above. All cells were then resuspended in 50 µL PBS and incubated with 10 µL of the fixable cell viability dye (eFluor 780, eBioscience, diluted 1:100 in PBS) for 20 minutes to enable exclusion of dead cells from the analysis. Cells were washed once with PBS and resuspended in 100 µL of 4% paraformaldehyde (Cytofix Fixation Buffer, BD Biosciences) to fix each sample for 15 minutes, prior to a final wash in PBS followed by resuspension in 150 µL of PBS. Cells were stored in the dark at 4 °C until analysis by flow cytometry (FACSverse, BD Biosciences) on the same day.

Cells were gated to include only single, eFluor 780 negative lymphocytes and analysis was performed using FlowJo® software (TreeStar, Inc., Ashland, OR). The MFI and the percentage of antibody positive cells were calculated for each sample. The optimal dilution of each antibody was selected as the lowest concentration required to discretely separate the antibody positive cells from the antibody negative cells.

4.2.3.2.iii. Lymphocyte subset analysis of CD25$^+$ cells

A 4-colour flow-cytometric assay was developed to measure CD25, CD4, CD8 and eFluor 780 fluorescence on feline PBMC using the optimal dilution of each antibody as determined in section 4.2.3.2.ii. PBMC were prepared as described in section 4.2.3.2.i, and incubated with 20 µL of the non-purified supernatant containing the anti-CD25 antibody supernatant for 30 minutes (at a final dilution of 2/5 in PBS containing PBMC), followed by the secondary antibody (1/100) for 20 minutes, and lastly with a combination of anti-CD4 antibody (1/40), anti-CD8 antibody (1/40) and efluor 780 (1/500) for 20 minutes. Cells were washed in 1 mL of PBS between incubations with each antibody, pelleted by centrifugation (800 x g for 2 minutes), fixed in 100 µL of 4% paraformaldehyde, washed in 1 mL of PBS and finally resuspended in 150 µL of PBS as described in section 4.2.3.2.ii. Unstained, single-stained and fluorescence minus one (FMO) controls were prepared for each antibody.

4.2.3.3. Cross-reactivity of Fel-O-Vax® FIV vaccine-induced antigen-specific cellular activation

PBMC from the 4 unvaccinated cats and 8 vaccinated cats (including 4 "newly vaccinated cats" and 4 "revaccinated cats") were isolated from 5 mL of heparinised blood using a density gradient medium (Ficoll-Paque, GE Healthcare Life Sciences) as described in section 2.2.6.1. Cells were resuspended in supplemented RPMI as described in section 4.2.3.1 and transferred to 96-well plates in a total volume of 200 µL and at a density of $1.5x10^6$ cells/mL. Cells were cultured in triplicate at 37 ˚C in 5% CO_2 in the presence of an equal volume of 1 of the 4 inactivated FIV isolates (CVK001, RVC009, MUVTH002, PetF14; p24 concentration 3.2 ng/mL), ConA (final concentration 1 µg/mL) or fresh media.

Cells were harvested after 20 hours and prepared for 4-colour flow cytometry as described in section 4.2.3.2.iii. Cells were gated to include only single, eFluor 780 negative lymphocytes, and analysis was performed using FlowJo® software (TreeStar, Inc., Ashland, OR). The MFI of CD25 expression was then calculated for each lymphocyte subset: $CD4^+/CD8^-$, $CD4^-/CD8^+$ cells and $CD4^-/CD8^-$ cells.

4.2.4. DTH response

4.2.4.1. Pilot study

To assess the non-specific effect of vaccine adjuvant on wheal diameter, the DTH response was initially performed on 1 cat pre- and post-vaccination with the Fel-O-Vax® FIV vaccine. The cat was sedated diazepam (0.25 mg/kg) and ketamine (5 mg/kg) intravenously to effect and the right chest wall was clipped and cleaned in preparation for the procedure. Two sites were marked with permanent ink to enable subsequent identification of test sites for measurement. Prior to vaccination, saline (0.9% NaCl) was injected intradermally (100 µL) at 1 test site, using a 100 IU insulin syringe with attached needle, and the same volume of undiluted Fel-O-Vax® FIV vaccine was injected intradermally at the other site, approximately 5 cm apart. The diameter of the wheal (r) was measured using calipers. The wheal thickness was also determined by pinching the affected skin and using calipers to measure the thickness of the wheal and the underlying

unaffected pinched skin (double skin thickness). These parameters were measured at each test site on days 1, 2 and 3 post-inoculation (PI). The wheal volume was calculated using the following formula:

Wheal volume = $\pi r^2 h$

(h= double skin thickness at challenge site minus skin thickness at control site)

Following the initial DTH response, the cat was then vaccinated with the Fel-O-Vax® FIV vaccine as per the manufacturer's directions (3 vaccinations, each 3 weeks apart). Two weeks after the final vaccination, a DTH response to the Fel-O-Vax® FIV vaccine was repeated as described above. The DTH response to one isolate of FIV was also measured post-vaccination (this was not tested pre-vaccination so as to avoid sensitisation of the cat with field virus). The FIV test antigen consisted of 100 µL of undiluted supernatant from MYA-1 cells infected with the isolate MUVTH002 (unquantified virus stock).

4.2.4.2. Cross-reactivity of the Fel-O-Vax® FIV vaccine-induced DTH response

The DTH response was determined for 6 additional "newly vaccinated" cats, 2 weeks after their third Fel-O-Vax® FIV vaccine. Cats were sedated and the chest wall on both sides was prepared as for the pilot study. Each cat was challenged intradermally with 100 µL of 6 different antigens (3 on each side), including 4 inactivated viruses containing 14 ng/mL of p24 protein (CVK001, MUVTH002, RVC009 and PetF14, prepared as described in chapter 2 and inactivated as described in section 4.2.2), a positive control (undiluted, unquantified Fel-O-Vax® FIV vaccine) and a negative control (RPMI cell culture media collected from uninfected MYA-1 cell maintained for 3 days in culture). Wheal volume was calculated as described in section 4.2.4.1, using wheal diameter and double skin thickness measurements taken at days 1, 2 and 3 PI.

4.2.5. Statistical analysis

Data was assessed for normality using the Shapiro-Wilk test. To assess the effect of FIV exposure on PBMC from unvaccinated cats, a paired t-test was used. Results from each

unvaccinated cat were first pooled to create an overall treatment group (3 replicates for each virus) and a control group for each cat (*n*=4) (**Table 4-1**). The effect of treatment versus control on PBMC from unvaccinated cats was compared for each parameter (MFI for CD25 and the percentage of $CD25^+$ cells in each subset) using a paired t-test.

Table 4-1 An example to demonstrate the grouping of cells from unvaccinated cats for statistical analysis.
Results from each replicate of PBMC exposed to FIV were averaged to form an overall pooled treatment group for each unvaccinated cat. Similarly, results from each replicate of PBMC exposed to media only were averaged to form an overall pooled control group for each cat. A paired t-test was used to compare the MFI for CD25 and the percentage of $CD25^+$ cells in each lymphocyte subset for treated versus control groups. The table shows grouping for the unvaccinated cat A. Results from cats B – D were grouped in a similar fashion, in order to obtain a pooled treatment group and a control group for each unvaccinated cat.

Vaccination status	Cat	Antigen	Replicate	Pooled groups
Unvaccinated	A	PetF14	1	Cat A treatment group
			2	
			3	
		RVC009	1	
			2	
			3	
		CVK001	1	
			2	
			3	
		MUVTH002	1	
			2	
			3	
		Control	1	Cat A control group
			2	
			3	

The effect of vaccination status on CD25 expression was then assessed using a 2-sample t-test. As the experiment was performed over 3 separate days, data were normalised to unstimulated cells to eliminate batch variation in fluorescence, by subtracting the mean MFI CD25 or the percentage of $CD25^+$ unstimulated cells from the values obtained from each virus stimulated replicate (**Table 4-2**). The effect of vaccination versus non-vaccination on each parameter was then compared within the FIV stimulated group using a 2-sample t-test.

Table 4-2 The method used to pool results for statistical analysis of CD25 expression.
Firstly, the results for each parameter (MFI for CD25 or percentage of CD25+) in unstimulated cells were averaged to form a mean control group for each cat. These values were then subtracted from the corresponding value obtained from measurement of each parameter for each replicate of virus stimulated cells. The normalised values for each replicate were then pooled to form an overall normalised treatment group. The table shows the grouping for the unvaccinated cat A and the vaccinated cat E. Results from unvaccinated cats B – D and vaccinated cats F - L were grouped in a similar fashion, in order to obtain a normalised treatment group for each cat.

Vaccination status	Cat	Antigen	Rep.	Normalised treatment group	Pooled normalised treatment groups
Unvaccinated (*n*=4)	A	PetF14	1	1 – mean cat A control	Cat A normalised treatment group
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		RVC009	1	1 – mean cat A control	
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		CVK001	1	1 – mean cat A control	
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		MUVTH002	1	1 – mean cat A control	
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		Control	1	Mean cat A control group	
			2		
			3		
Vaccinated (*n*=8)	E	PetF14	1	1 – mean cat A control	Cat E normalised treatment group
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		RVC009	1	1 – mean cat A control	
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		CVK001	1	1 – mean cat A control	
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		MUVTH002	1	1 – mean cat A control	
			2	2 – mean cat A control	
			3	3 – mean cat A control	
		Control	1	Mean cat A control group	
			2		
			3		

Within the vaccinated cat group, the percentage of antigen-specific PBMC was then estimated by calculating the increase in the percentage of $CD25^+$ cells from each subset affected by FIV stimulation. Finally, within the vaccinated cat group, the effect of vaccination protocol ("newly vaccinated" versus "revaccinated") and treatment (each virus) were compared for each parameter, using a linear regression model. "Newly vaccinated" cats and the Petaluma (PetF14) virus were selected as reference groups for comparison.

Results from the DTH response were checked for outliers using Grubb's test. The DTH response was compared for each virus at each time point (days 1, 2 and 3 PI) using a general linear model of ANOVA, with wheal volume as the response. Factors assessed included cat (as a

random factor) and virus (fixed factor), and the response to the Petaluma virus (PetF14) was selected as a reference group. A Bonferroni comparison was used to determine which means were different.

All statistical analyses were performed using R statistical software (R: A Language and Environment for Statistical Computing, R foundation for Statistical Computing, Vienna) and Minitab® Statistical Software (Version 17, Minitab Inc., Pennsylvania, U.S.A). A *p*-value of less than 0.05 was considered significant.

4.3. Results

4.3.1. CD25 assay

4.3.1.1. FIV-specific cellular activation pilot study

Stimulation of cells with inactivated FIV resulted in upregulation of the CD25 molecule on PBMC from the vaccinated, but not unvaccinated cat, as shown by binding of the FITC-conjugated anti-feline CD25 antibody. A 2.2-fold increase in MFI for CD25 was seen in FIV stimulated PBMC versus unstimulated PBMC from the vaccinated cat, compared to a 1.1-fold increase in FIV stimulated PBMC from the unvaccinated cat (**Figure 4-2** and **Figure 4-3**). As expected, stimulation with ConA resulted in an even greater increase in CD25 expression, with a 26-32-fold increase in MFI of CD25 in ConA-stimulated PBMC versus unstimulated cells from both cats (data not shown).

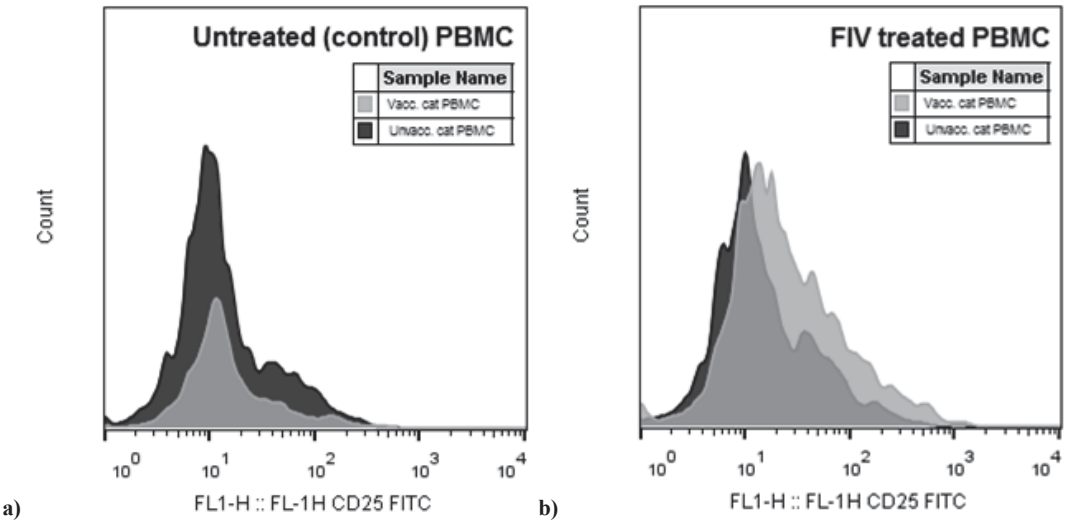


Figure 4-2 Overlay histograms showing the effect of vaccination on CD25 expression.
Feline PBMC from a vaccinated and unvaccinated cat were cultured in the presence of cell culture supernatant containing inactivated FIV of the subtype C isolate, MUVTH002. Controls consisted of cells from the same cats, treated with fresh media only. The MFI for CD25 is shown along the x-axis, and represents lymphocyte activation. The y-axis represents the number of cells at each fluorescence intensity. Graph **a)** shows that the MFI of CD25 in PBMC from the vaccinated cat (light grey) and unvaccinated cat (dark grey) are similar (15.8 versus 13.8 respectively) when cells are treated with fresh media only. In contrast, graph **b)** shows that the MFI of CD25 is increased in PBMC from the vaccinated cat (25.0) versus the unvaccinated cat (15.2).

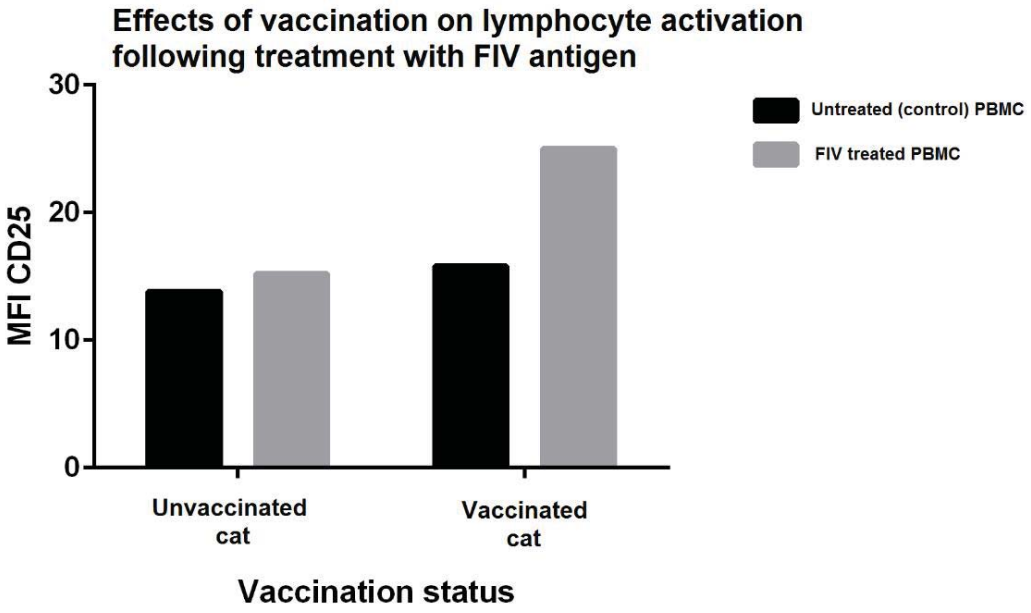


Figure 4-3 The effect of vaccination on activation of FIV-specific lymphocytes.
PBMC from the vaccinated and unvaccinated cat were exposed to fresh media (control) or the subtype C, MUVTH002 isolate of FIV and CD25 expression was measured (shown as the MFI of CD25 on the x-axis). Cells from the vaccinated cat show increased activation following exposure to FIV when compared to PBMC from the unvaccinated cat.

4.3.1.2. Optimisation of the CD25 assay

4.3.1.2.i. Antibody titrations

The optimal dilution of the unconjugated CD25 antibody was 2/5. Non-specific binding of the secondary (anti-mouse IgG) antibody was evident on some cells. This was shown by 3 peaks of CD25 fluorescence, consistent with 3 populations of cells: cells binding both the CD25 antibody and the secondary antibody (true $CD25^+$ cells), cells binding only the secondary antibody, and cells negative for both (**Figure 4-4**). Increasing concentrations of the CD25 antibody resulted in an increase in the MFI for CD25, allowing differentiation of true CD25 positive cells from those binding the secondary antibody non-specifically.

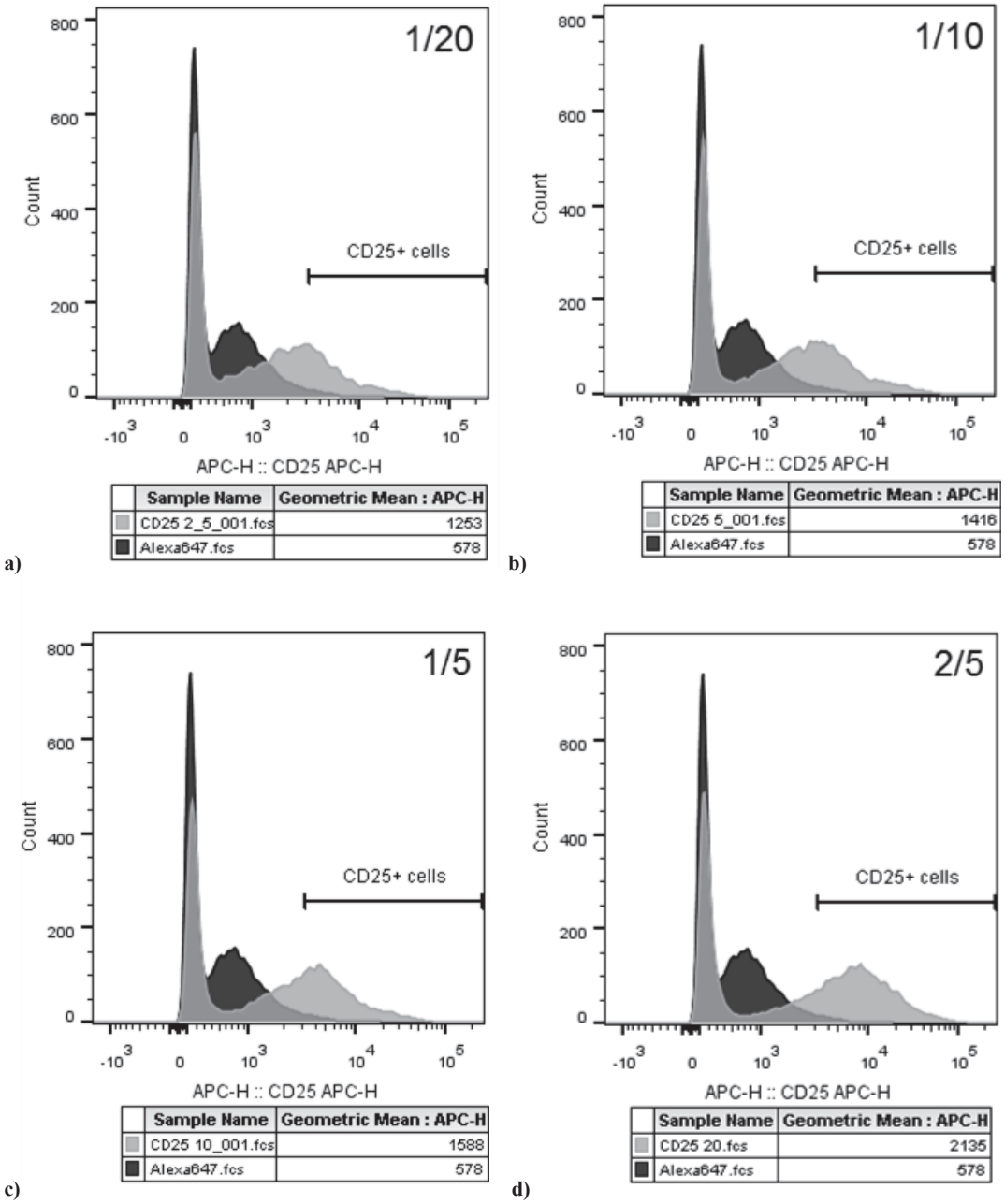


Figure 4-4 Results from anti-CD25 antibody titration.
Overlay histograms showing the MFI for CD25 of ConA activated PBMC incubated with the secondary antibody alone (dark grey) versus the anti-CD25 and secondary antibodies (light grey). The left and right light grey peaks indicate the $CD25^-$ and $CD25^+$ cell populations, respectively. The left and right dark grey peaks respectively indicate the cell population negative for the anti-IgG antibody and the cell population binding the anti-IgG antibody non-specifically. Each graph depicts a decreasing dilution of CD25 antibody: **a)** 1/20, **b)** 1/10, **c)** 1/5 and **d)** 2/5. The MFI of CD25 increased with increasing concentrations of the CD25 antibody. A dilution of 2/5 was optimal for separating the true CD25 positive cells from those cells non-specifically binding the secondary antibody.

$CD4^+$ cells were easily differentiated from $CD4^-$ cells even at the highest dilution of antibody tested (1/40) (**Figure 4-5**). A final dilution of 1/40 was therefore used in all subsequent experiments. The optimal dilution of the anti-CD8 antibody was also determined to be 1/40 (**Figure 4-6**).

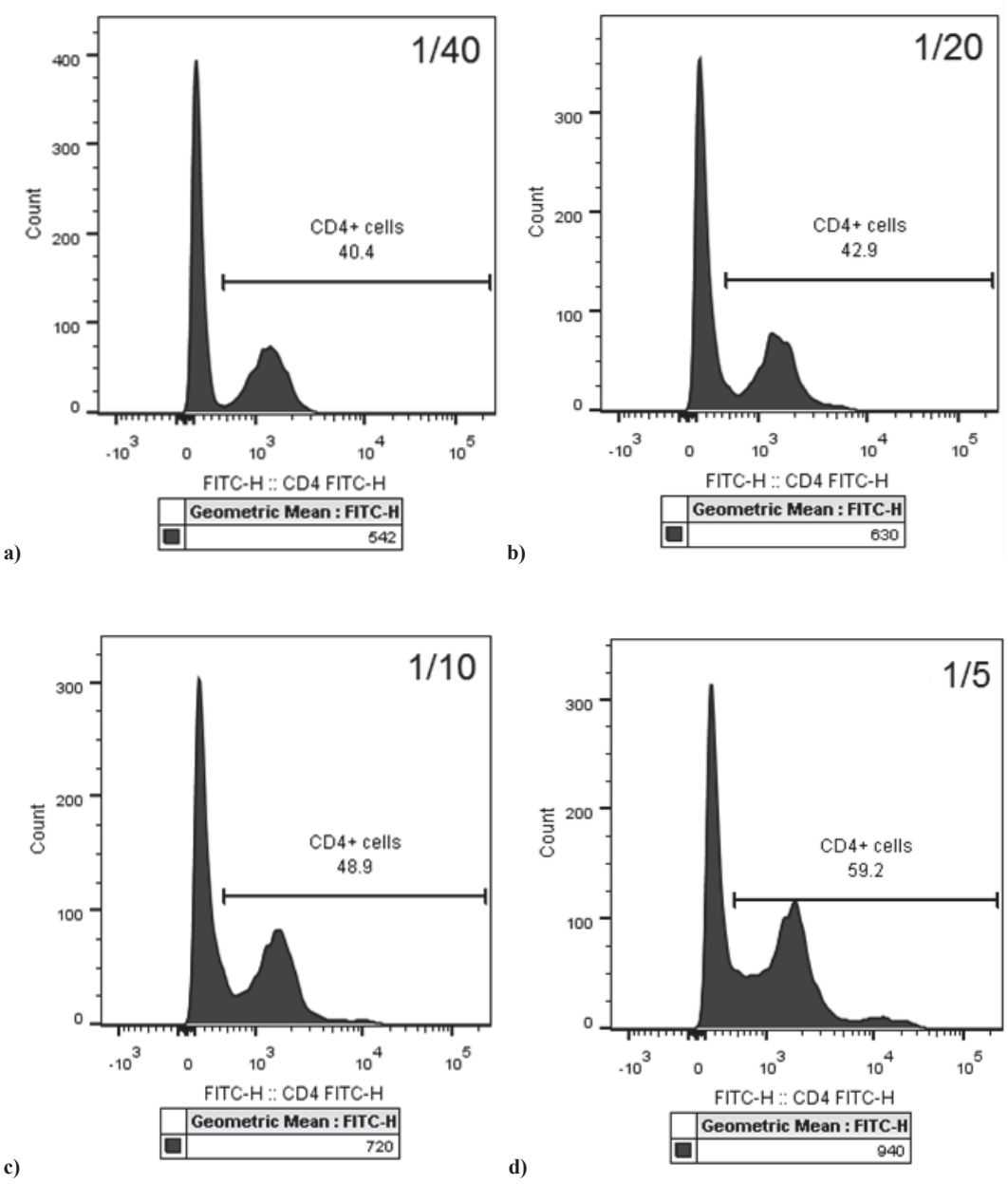


Figure 4-5 Results from anti-CD4 antibody titration.
Histograms showing CD4 fluorescence of ConA activated PBMC incubated with the CD4 antibody. Left and right peaks represent the $CD4^-$ and $CD4^+$ cell populations, respectively. Each graph depicts a decreasing dilution of CD4 antibody: **a)** 1/40, **b)** 1/20, **c)** 1/10 and **d)** 1/5. The MFI of CD4 increased only slightly with increasing concentrations of CD4 antibody. A dilution of 1/40 was therefore adequate to identify $CD4^+$ cells.

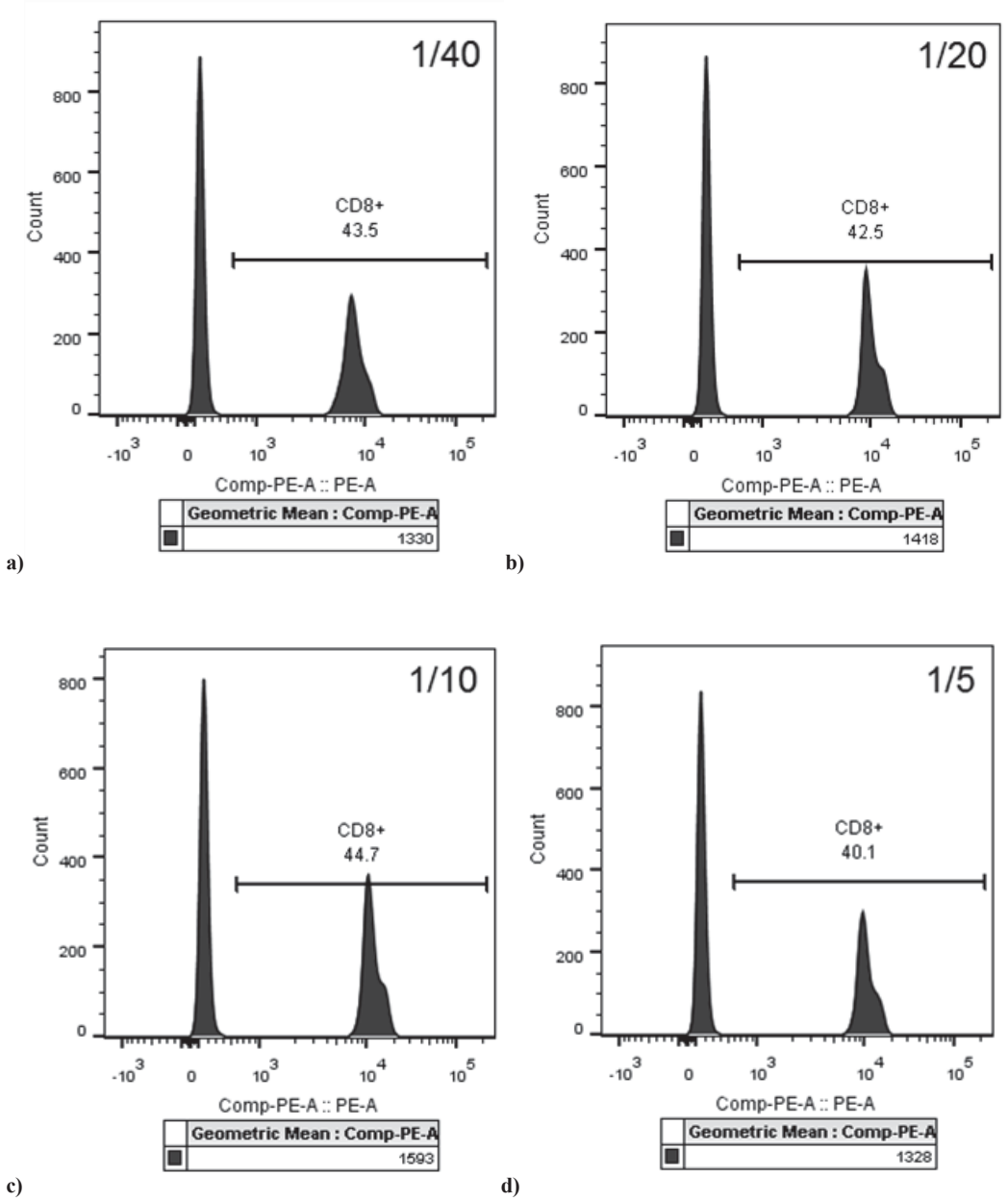


Figure 4-6 Results from anti-CD8 antibody titration.
Histograms showing CD8 fluorescence of ConA activated PBMC incubated with the CD8 antibody. Left and right peaks represent the $CD8^-$ and $CD8^+$ cell populations, respectively. Each graph depicts a decreasing dilution of CD8 antibody: **a)** 1/40, **b)** 1/20, **c)** 1/10 and **d)** 1/5. A dilution of 1/40 was adequate to identify $CD8^+$ cells.

4.3.1.2.ii. Lymphocyte subset analysis of $CD25^+$ cells

Four-colour flow cytometry was used for lymphocyte subset analysis on all eFluor 780 negative, single lymphocytes and on the $CD25^+$ portion of these cells. In the unstimulated samples of PBMC (fresh media only), a mean 26.6% of lymphocytes were $CD8^+$ and a mean 29.8% were $CD4^+$ cells. As expected, the overall MFI of CD25 increased in response to ConA stimulation

(from 1457 to 2153). ConA activated lymphocytes consisted of both $CD8^+$ cells (mean 10.2%) and $CD4^+$ cells (mean 18.2%). The remaining $CD25^+$ cells were mostly $CD4^-/CD8^-$ cells (**Figure 4-7**).

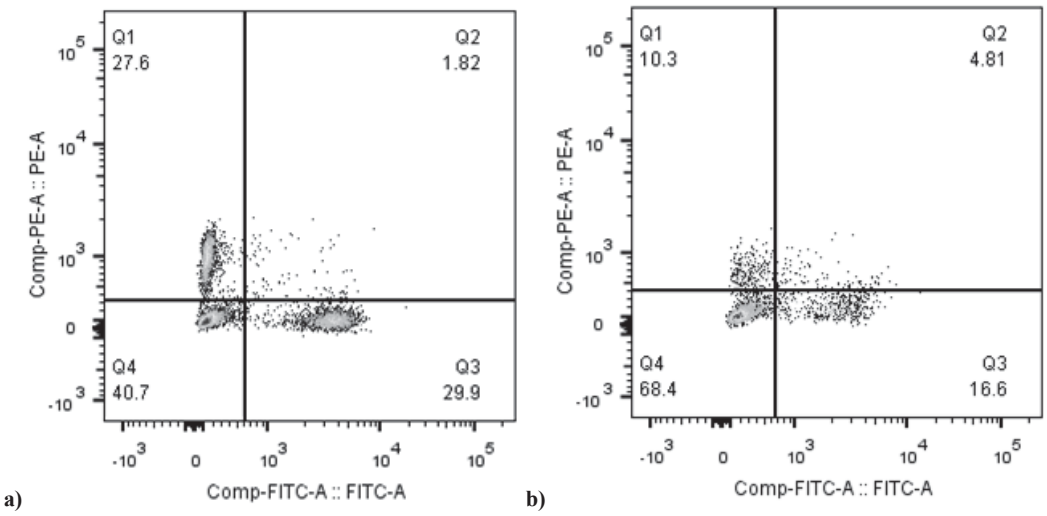


Figure 4-7 Four-colour flow cytometry on ConA activated PBMC.
a) FCS graph showing CD4 fluorescence (FITC, x-axis) versus CD8 fluorescence (PE, y-axis) on live (eFluor 780 negative) lymphocytes from a sample of unstimulated PBMC maintained for 1 day in culture. Quadrant 1 represents the $CD8^+$ cells and quadrant 3 represents the $CD4^+$ cells. Cells in quadrant 4 are negative for both CD4 and CD8, including B cells and $CD4^-/CD8^-$ T cells.
b) FCS graph showing CD4 fluorescence (FITC, x-axis) versus CD8 fluorescence (PE, y-axis) on $CD25^+$ lymphocytes from the same sample of ConA stimulated PBMC as shown in figure a. Quadrant 1 represents the $CD25^+CD4^-CD8^+$ cells and quadrant 3 represents the $CD25^+CD4^+CD8^-$ cells. $CD25^+CD4^-CD8^-$ cells are shown in quadrant 4, including activated B cells and activated $CD4^-/CD8^-$ T cells.

4.3.1.3. Cross-reactivity of Fel-O-Vax® FIV vaccine-induced antigen-specific cellular activation

PBMC from unvaccinated cats were not activated in response to *in vitro* stimulation with FIV. This was evident when comparing CD25 expression (MFI for CD25) on stimulated versus unstimulated PBMC (p=0.801), $CD4^+CD8^-$ cells (p=0.600), $CD4^-CD8^+$ cells (p=0.362) and $CD4^-CD8^-$ cells (p=0.931). Similarly, the percentage of activated ($CD25^+$) cells within each lymphocyte subset did not increase significantly in response to stimulation with FIV ($CD25^+CD4^+CD8^-$ p=0.673, $CD25^+CD4^-CD8^+$ p=0.812, $CD25^+CD4^-CD8^-$ p=0.747) (**Figure 4-8**).

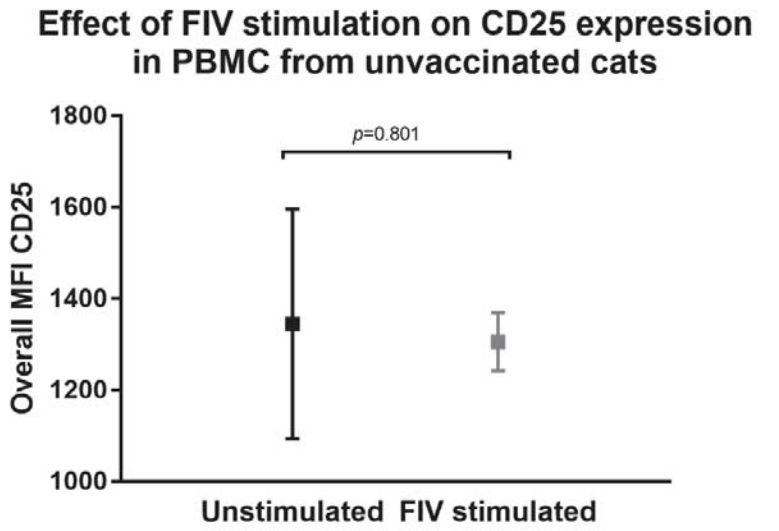


Figure 4-8 The effect of FIV on CD25 expression in PBMC from unvaccinated cats.
The overall MFI for CD25 in cells exposed to virus (all 4 viruses, 3 replicates per virus, pooled to create the "FIV stimulated" group) was averaged for each cat within the unvaccinated group (n=4). A paired t-test was then used to compare the mean for virus-stimulated cells versus unstimulated (control) cells. There was no significant difference in the means (p=0.801), indicating that overall, stimulation with FIV did not cause cellular activation in PBMC from unvaccinated cats.

Vaccination resulted in increased activation of $CD4^+$ cells following *in vitro* stimulation of PBMC with FIV (**Figure 4-9**). Compared to $CD4^+$ cells from unvaccinated cats, the normalised MFI for CD25 of $CD4^+$ cells from vaccinated cats was increased (p=0.031, square root transformed data). Similarly, there was a trend for an increased percentage of $CD25^+CD4^+$ cells in FIV stimulated PBMC from vaccinated versus unvaccinated cats (mean 25.02% in unvaccinated PBMC versus mean 35.09% in vaccinated PBMC, p=0.078) (**Figure 4-10**). There was no difference in the normalised MFI for CD25 in FIV stimulated $CD4^-CD8^+$ or $CD4^-CD8^-$ cells from vaccinated versus unvaccinated cats (p=0.280 and p=0.119). Similarly, there was no difference in the percentage of $CD25^+CD4^-CD8^+$ cells or $CD25^+CD4^-CD8^-$ cells from vaccinated versus unvaccinated cats following *in vitro* stimulation with FIV ($CD25^+CD4^-CD8^+$: mean 22.18% versus 29.99%, p=0.143; $CD25^+CD4^-CD8^-$: mean 50.54% versus 48.42%, p=0.625). The percentage of FIV-specific $CD4^+$ cells was estimated to be 8.2% (mean of 26.9% $CD25^+CD4^+CD8^-$ cells in the unstimulated, vaccinated group versus 35.1% in the FIV stimulated, vaccinated group).

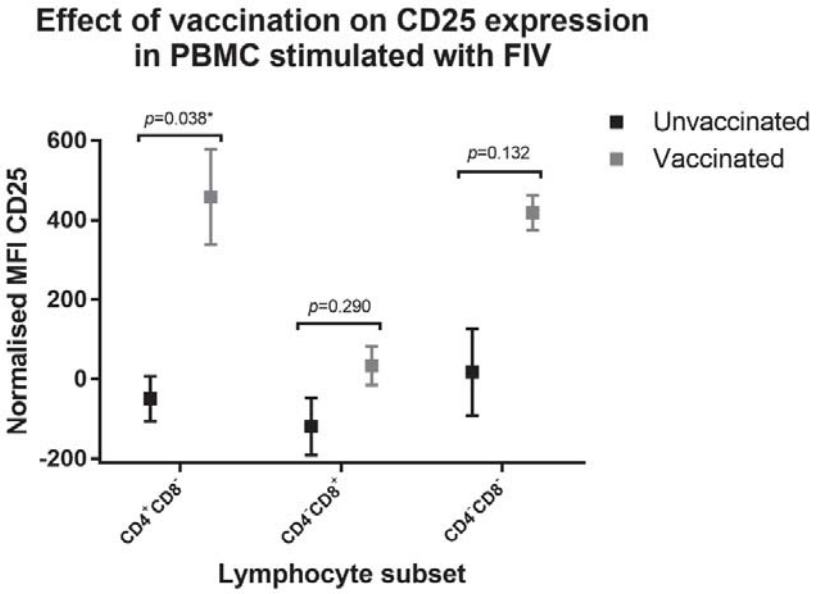


Figure 4-9 The effect of vaccination on CD25 expression in PBMC stimulated with FIV.
The normalised MFI for CD25 in FIV stimulated cells (3 replicates for each of the 4 viruses) was averaged from each cat. The mean from the vaccinated cats (n=8) was then compared to the mean from the unvaccinated cats (n=4) within each subset of lymphocytes. $CD4^+$ cells from vaccinated cats expressed significantly more CD25 (p=0.031) than $CD4^+$ cells from unvaccinated cats. There was no difference in CD25 expression in $CD8^+$ or $CD4^-/CD8^-$ cells from vaccinated versus unvaccinated cats (p=0.280 and p=0.132). Error bars represent 95% CI for the mean.

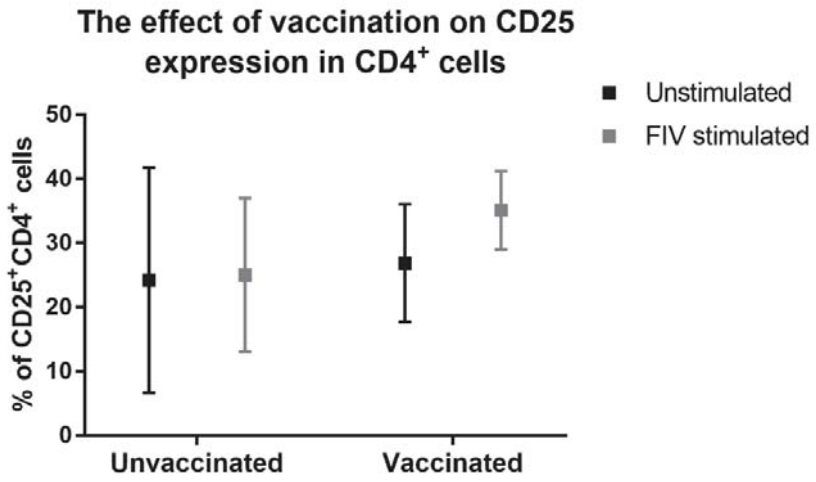
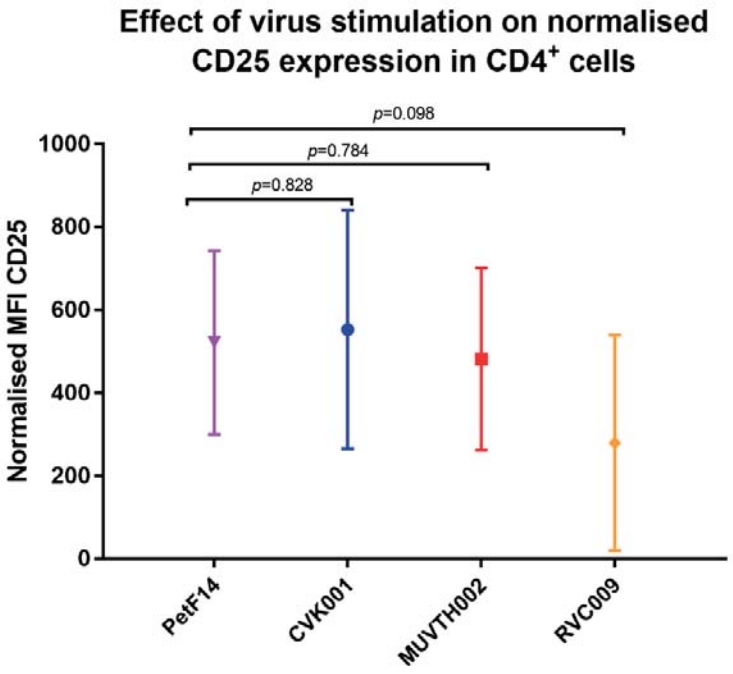
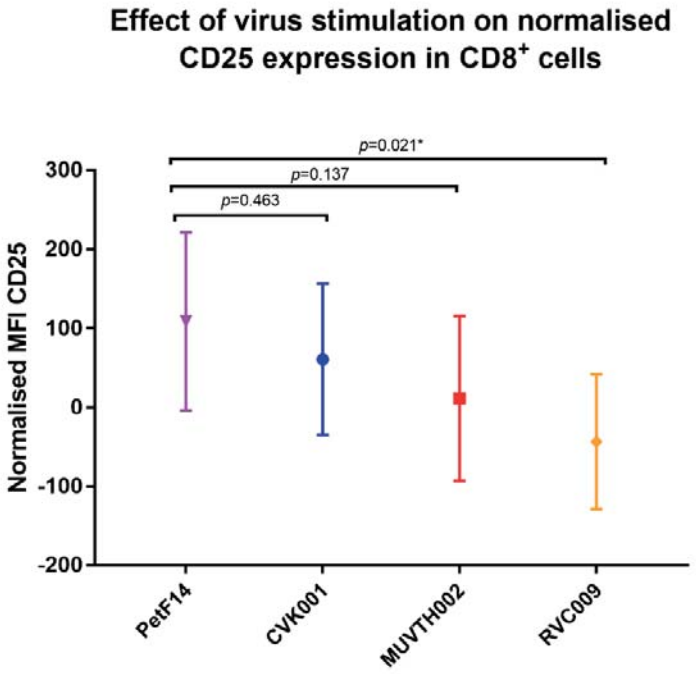


Figure 4-10 The effect of vaccination on CD25 expression in T cells.
The percentage of $CD25^+CD4^+$cells in all virus stimulated cultures (3 replicates for each of the 4 viruses) was averaged for each cat to obtain a pooled treatment group. The mean from the vaccinated cats (n=8) was then compared to the mean from the unvaccinated cats (n=4). There was a trend for an increased percentage of $CD25^+CD4^+$ cells in FIV stimulated PBMC from vaccinated versus unvaccinated cats (mean 25.02% in unvaccinated PBMC versus mean 35.09% in vaccinated PBMC, p=0.078). Error bars represent 95% CI for the mean.

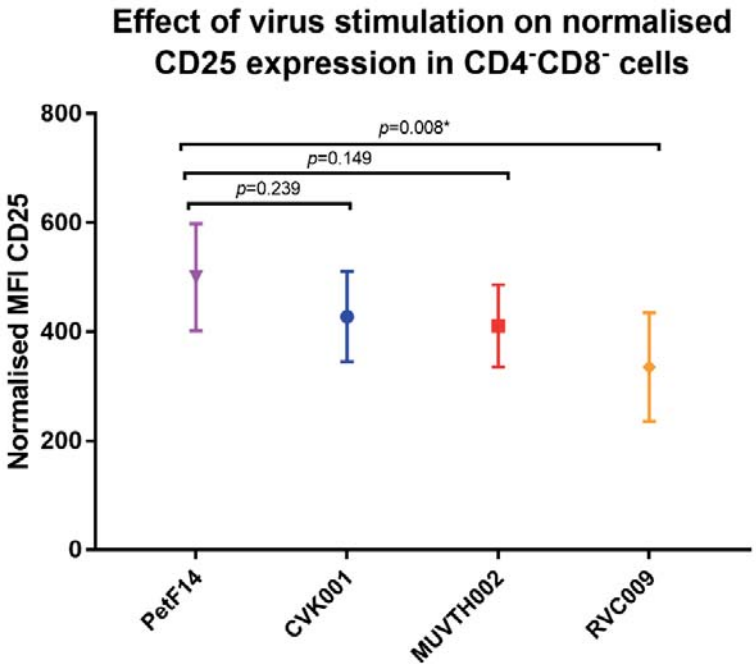
Within the vaccinated cat group, there were significant differences in CD25 expression (normalised MFI) on PBMC in response to *in vitro* stimulation with the different viruses. Both $CD4^{-}CD8^{+}$ and $CD4^{-}CD8^{-}$ cells stimulated with the subtype A, RVC009 virus expressed less CD25 compared to those cells stimulated with the PetF14 virus (p=0.021 and 0.008). There was also a trend for reduced CD25 expression on $CD4^{+}$ cells stimulated with the RVC009 virus (p=0.098). There was no difference in CD25 expression on PBMC stimulated with the 2 other NZ viruses compared to the PetF14 virus (**Figure 4-11**).



a)



b)



c)

Figure 4-11 The effect of virus on CD25 expression in lymphocyte subsets.
Box plots show the normalised MFI for CD25 in **a)** $CD4^+CD8^-$cells, **b)** $CD4^-CD8^+$ cells and **c)** $CD4^-CD8^-$ cells stimulated with each NZ isolate of FIV and PetF14. The normalised MFI for CD25 was calculated by subtracting the MFI of unstimulated cells from the MFI of virus stimulated cells. A linear regression model was used to compare results for cells stimulated with each NZ isolate to cells stimulated with the Petaluma (PetF14) vaccine strain of FIV. The *p*-value is shown for results from stimulation with each virus compared to PetF14, and an asterisk is included when the difference is significant ($p<0.05$). Within the $CD4^-CD8^+$ and $CD4^-CD8^-$ subsets, there was a difference in the response elicited by the different isolates of FIV. The RVC009 isolate resulted in less CD25 expression when compared to the PetF14 isolate. There was no difference in the responses elicited by the CVK001 and MUVTH002 isolates when compared to the PetF14 isolate within these subsets. There was no difference among viruses within the $CD4^+CD8^-$ subset of cells. Error bars represent 95% CI for the mean.

There was a significant difference in the response of PBMC from "newly vaccinated" cats versus "revaccinated" cats. For all FIV stimulated cells, $CD4^+$ cells and $CD8^+$ cells from the "newly vaccinated" cats showed increased CD25 expression (normalised MFI for CD25) compared to cells from "revaccinated" cats (p=0.01 and p=0.001, respectively). The timing of vaccination did not affect CD25 expression in the $CD4^-CD8^-$ cells (p=0.230) (**Figure 4-12**). The estimated percentage of activated $CD4^+$ cells from "newly vaccinated" cats was 13.6%, versus 2.9% from "revaccinated" cats. (See **Appendix 7 and 8** for raw data and regression tables.)

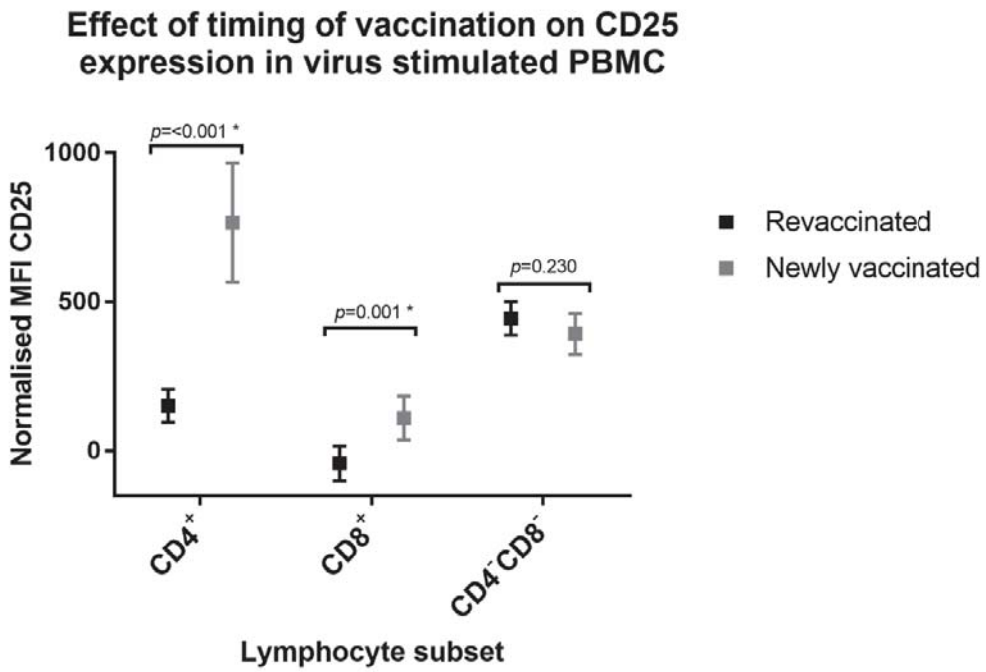


Figure 4-12 The effect of timing of vaccination on CD25 expression in lymphocytes.
A linear regression model was used to compare the MFI for CD25 from FIV-stimulated cells of each subset from cats that were vaccinated 2 weeks previously ("newly vaccinated") versus cats that had been vaccinated one year previously, with a booster vaccine administered 2 weeks prior to sampling ("revaccinated"). $CD4^+$ and $CD8^+$ cells from "newly vaccinated" cats expressed significantly more CD25 than $CD4^+$ and $CD8^+$ cells from "revaccinated" cats (p<0.001 and p=0.001, respectively). There was no difference in CD25 expression in $CD4^-CD8^-$ negative cells from each group. Error bars represent 95% CI for the mean.

4.3.2. DTH response

4.3.2.1. Pilot study

Intradermal inoculation of the Fel-O-Vax® FIV vaccine into an unvaccinated cat resulted in almost no wheal formation (3.1 mm^3 at days 1 and 2 PI, with complete resolution by day 3 PI).

No wheal was detected at the site of the saline injection. Following vaccination of the cat, a considerably larger wheal formed at the vaccine injection site (wheal volume 100.5 mm^3 at the peak response at day 2 PI). Inoculation with the MUVTH002 virus following vaccination also resulted in wheal formation (volume 28.3 mm^3 at the peak at day 2 PI) (**Figure 4-13**).

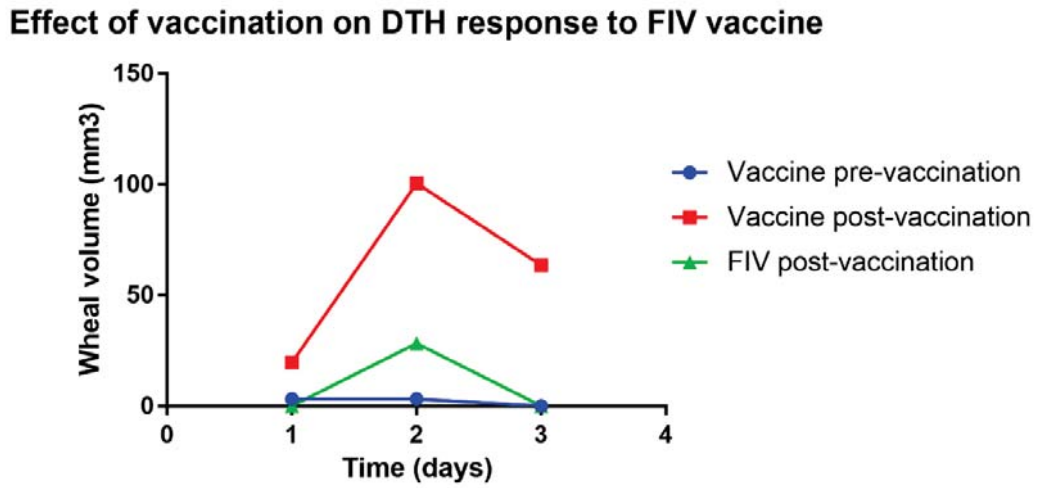


Figure 4-13 The effect of vaccination on the vaccine-induced DTH response in a single cat.
The DTH response to Fel-O-Vax® FIV vaccine antigen (undiluted vaccine) was measured in an unvaccinated cat. The cat was then vaccinated subcutaneously with the Fel-O-Vax® FIV vaccine according to manufacturer's directions (3 vaccines, each 2 weeks apart). The DTH response was repeated 2 weeks after the final vaccine. For the post-vaccination response, test antigen included the Fel-O-Vax® FIV vaccine antigen (undiluted vaccine) and supernatant from MYA-1 cells infected with the MUVH002 isolate of FIV (unquantified virus stock). No response was seen prior to vaccination, confirming that the response was FIV-specific. A positive response was seen following intradermal injection of vaccine and field FIV antigen following vaccination.

4.3.2.2. Cross-reactivity of the Fel-O-Vax® FIV vaccine-induced DTH response

The DTH response to each virus was assessed in 6 cats over 3 time points (days 1, 2 and 3 PI). A positive response was seen for all viruses (but not control), with the peak response occurring at day 2 PI. All field isolates of FIV elicited a DTH response that was at least equal to the response elicited by the vaccine virus, PetF14. In 4/6 cats, the DTH response was greater for the MUVTH002 and RVC009 isolates at day 3 PI when compared to the PetF14 isolate (p=0.011 and p=0.009), and there was also a trend for this at day 2 PI (p=0.069 and p=0.100) (**Figure 4-14** and **Figure 4-15**). Comparison of wheal volumes for each virus at each time point revealed large inter-cat variation, which was a significant factor in the model at the day 1 PI time point (p=0.008), limiting the power of the study. Retrospective power analysis showed that at a power

of 80%, and assuming an overall standard deviation of 25 mm with a maximum expected difference of 20 mm in wheal volume, 36 cats would have been required to detect a significant difference between viruses. Nevertheless, results showed that vaccination with the Fel-O-Vax® FIV vaccine did induce a DTH response against the Petaluma vaccine strain of FIV, and also suggested that there was considerable cross-reactivity of this response against the 3 FIV field isolates used in this study. (See **Appendix 7 and 8** for raw data and ANOVA tables.)

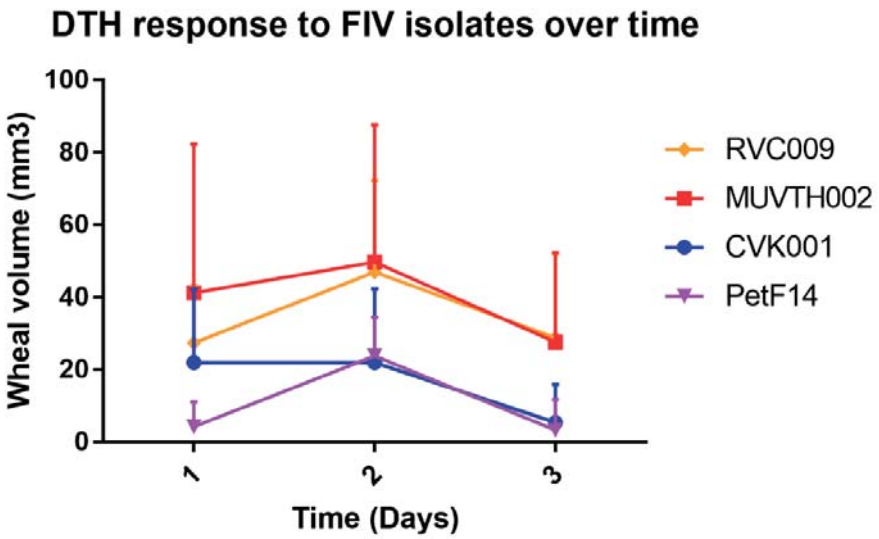


Figure 4-14 Comparison of the DTH elicited by each FIV isolate.
Data points represent the mean wheal volume (y-axis) over time (x-axis) of 6 Fel-O-Vax® FIV vaccinated cats following intradermal inoculation with the vaccine strain of FIV (PetF14) and 3 NZ isolates of FIV (RVC009, MUVTH002 and CVK001). The peak wheal volume in response to all viruses was seen at day 2 PI. The isolates, MUVTH002 and RVC009, elicited a greater response at day 3 PI, when compared to the PetF14 isolate (p=0.011 and p=0.009) and there was also a trend for an increased response to these two viruses at day 2 PI (p=0.069 and p=0.100). Error bars represent the 95% CI for the mean.

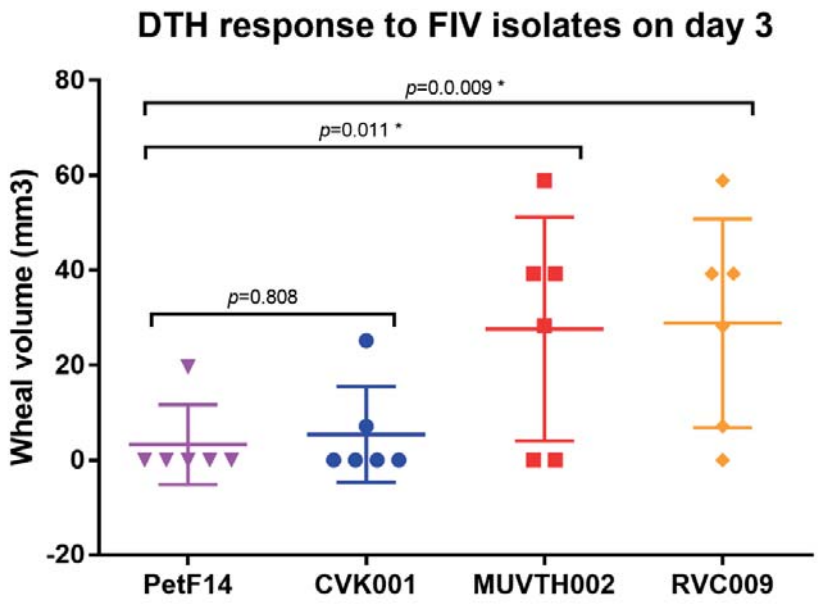


Figure 4-15 The DTH response for each cat at day 3.
Each data point represents wheal volume for each cat at day 3 following inoculation with the 4 different viruses, 2 weeks after final vaccination with the Fel-O-Vax® FIV vaccine. The mean wheal volume and 95% confidence intervals are represented by bars. The MUVTH002 and RVC009 isolates elicited a greater response than the PetF14 isolate (p=0.011 and p=0.009). There was no difference in the response elicited by the CVK001 isolate and PetF14. Note the large inter-cat variation seen in the responses.

4.4. Discussion

The purpose of this study was to determine whether T cells generated following vaccination with the Fel-O-Vax® FIV vaccine recognise antigens from field isolates of FIV. The results of this study demonstrated that there is at least some cross-reactivity of the response, as lymphocytes from Fel-O-Vax® FIV vaccinated cats became activated following stimulation with the 3 different field isolates of FIV. This was shown by increased expression of the cell surface activation marker CD25 on FIV stimulated PBMC from vaccinated, but not unvaccinated cats. In addition, a DTH response developed in vaccinated cats, following inoculation with each of the 3 field isolates of FIV. However, both the CD25 expression assay and the DTH response test showed that there was a difference in the magnitude of responses elicited by each field isolate, suggesting a degree of antigenic variation between the FIV isolates tested.

Three different field isolates of FIV were used in this study to evaluate the extent of cross-reactivity of the Fel-O-Vax® FIV vaccine-induced immune response. Previous phylogenetic

analysis (described in section 2.3.3) classified these viruses as belonging to either subtype A (RVC009) or C (MUVTH002, CVK001). Each of these viruses was isolated from naturally infected cats in NZ, representing the type of viruses that vaccinated cats may encounter in the field. Significant differences were encountered when the magnitude of CD25 upregulation in response to stimulation with these different isolates was compared. In particular, stimulation of PBMC with the subtype A, RVC009 isolate resulted in less lymphocyte activation when compared to PBMC stimulated with the Petaluma virus. Similarly, a difference was observed in the magnitude of the DTH response elicited by each isolate of FIV. The MUVTH002 and RVC009 isolates elicited a greater response (as measured by wheal volume) when compared to the Petaluma virus. These results suggest that the differences in antigenicity between isolates of FIV leads to a difference in the magnitude of the immune response elicited by the virus, which may be functionally significant in regards to protection against field isolates of FIV. Based on previous sequencing of a small section of the *env* gene (V3-V6 region) in chapter 2, the NZ field isolates used in this study were genetically different to the Petaluma vaccine strain (9.3%, 21.2% and 22% nucleotide pairwise distance between PetF14 and RVC009, CVK001 and MUVTH002, respectively, at the 859 bp sequenced region of the genome). The *env* gene encodes the envelope glycoprotein, which is expressed on the exterior of the virion. This glycoprotein contains epitopes that are important in vaccine-induced humoral immunity (Hohdatsu *et al.*, 1997b, Yamamoto *et al.*, 1993). Genetic variation at this location may therefore influence antigenic recognition by vaccine-induced memory B and $CD4^+$ Th2 cells. Full genome sequencing of the viruses was not attempted in this study, but it is possible that these field isolates were also genetically different to the Petaluma virus at other immunodominant locations. This may have contributed to the variation in the magnitude of the responses observed in this study.

Two assays were used in this study to assess different aspects of the vaccine-induced immune response. The CD25 assay was a measure of lymphocyte activation in response to FIV antigen, and also provided information on the lymphocyte subsets involved in this process. CD25 is expressed predominantly on activated lymphocytes, so expression following antigen

stimulation suggested the presence of FIV-specific lymphocytes in this study. The possibility of non-specific lymphocyte activation by FIV antigen was also considered, as viruses may contain superantigens that activate T cells by binding directly to the MHC class II molecule and a conserved region of the T cell receptor (Llewelyn and Cohen, 2002). To investigate this, PBMC from unvaccinated, uninfected cats were stimulated with the different isolates of FIV. As predicted, there was no increase in CD25 expression within any of the lymphocyte subsets examined. In contrast, lymphocytes from Fel-O-Vax® FIV vaccinated cats did become activated following stimulation with FIV. These results suggest an effect of vaccination in inducing CD25 expression following stimulation with FIV antigen. This is most likely due to the presence of FIV-specific T cells generated in response to vaccination. However, vaccination has also been shown to increase innate immunity to pathogens not included in the vaccine, and vaccine-induced memory T cells may also become activated non-specifically by heterologous antigen [reviewed by Welsh & Selin (2002)]. The possibility that this occurred in the current study cannot be excluded, however the effect of vaccination-induced non-specific cellular activation would be expected to be similar for all isolates tested. Therefore, differences in the magnitude of the response to the different isolates should still reflect differences in the number of FIV-specific T cells.

The DTH response test was also used to demonstrate an FIV-specific immune response following vaccination. In contrast to the CD25 expression assay, which only evaluated antigen-specific cellular activation, the DTH response test also assessed antigen processing, cellular recruitment and proliferation of memory cells into effector cells (Pier, 2004). Following intradermal inoculation, Langerhan's cells process and present the antigen to local memory T cells. Neutrophils initially accumulate within the site, but within 12 hrs, a mixture of $CD4^+$ T cells, $CD8^+$ T cells and monocytes predominate. Th1 and possibly Th2 $CD4^+$ T cells are capable of initiating this reaction and causing the characteristic wheal that lasts for 2 – 3 days (Muller *et al.*, 1993, Cher and Mosmann, 1987). In this study, the DTH response to the CVK001 virus occurred early, reaching a peak at 24 hours. Tissue trauma may occur with intradermal injection,

causing early wheal formation, however it would be unlikely for this to occur in enough of the cats to significantly affect the mean. There have been limited studies assessing the DTH response in cats, so the expected response (i.e. maximal at 48 hours) has been extrapolated from results in different species. A previous study did demonstrate a maximal response to one antigen at 24 hours (Cave *et al.*, 2007), so it is possible that the response occurs more rapidly in cats. Alternatively, the CVK001 virus stock may have contained a substance that induced a stronger innate response compared to the other virus stocks.

As the CD25 assay and the DTH response test assessed different aspects of the vaccine-induced cell-mediated immune responses, results across the two assays cannot be quantitatively compared. It was interesting that the isolate producing the lowest CD25 response was also the isolate producing the highest DTH response (RVC009). As the DTH response is an indicator of a cell mediated response (compared to the CD25 assay which is a measure of both humoral and cell mediated immune responses), perhaps the response to the RVC009 isolate is predominantly cell mediated rather than humoral. However, although the CD25 assay and DTH response test were both a measure of antigen recognition, the DTH response also evaluated antigen presentation and processing *in vivo*, and may have been influenced by other local inflammatory factors contained within the inoculum. In addition, as both assays are influenced by host factors, biological variation is expected, potentially contributing to the accuracy of quantitative comparisons. In particular, significant inter-cat variation was observed for the DTH response, limiting the usefulness of this assay in predicting immunity to the individual virus and also limiting the power of the test.

The DTH response elicited by the field isolates RVC009 and MUVTH002 was greater in magnitude when compared to that elicited by the vaccine strain, PetF14. If the DTH response was entirely FIV-specific, and assuming the concentration of virus in each inocula is the same, then one would expect the vaccine strain to generate at least an equal response in magnitude compared to the field isolates. As this was not the case, there may have been other factors within the inocula (such as cellular debris and chemokines) that contributed to wheal volume via an innate response. In an attempt to control for these factors, cell culture supernatant collected from uninfected MYA-

1 cell cultures after 3 days was also inoculated into each cat and this did not result in a positive DTH response. However, it is possible that supernatant from virally infected MYA-1 cell cultures versus uninfected cultures may have contained more of these factors due to virally induced cell necrosis. Furthermore, the quantity of these factors may have differed between isolates, depending on the degree of cell necrosis induced by each virus. Results described in section 3.3.2 demonstrated a difference in the percentage of cell necrosis in MYA-1 cultures infected with the different isolates of FIV. However, at the day 10 time point (which is closest to the time point at which virus was harvested for preparation of virus stock in section 2.2.6.3), the RVC009 isolate induced the least amount of necrosis when compared to the PetF14, so it is difficult to explain the increased DTH response by an increased amount of cellular debris in the RVC009 virus inoculum compared to the PetF14 virus inoculum. Another possible explanation is that the amount of protein contained in each inoculum was not equal. Although virus stock was standardised according to p24 concentration, the concentration of other proteins (such as the envelope glycoprotein, which may have contained immunodominant epitopes) was not measured. Quantitative comparison of the DTH response between viruses should therefore be interpreted with caution. The more important finding in this study was that inoculation of all of the field viruses into vaccinated cats resulted in the generation of a DTH response, demonstrating at least some cross-reactivity of the vaccine-induced cellular immune response.

The use of flow cytometry to measure CD25 expression enabled concurrent identification of the lymphocyte subsets involved in the response. The exact nature of the Fel-O-Vax® FIV vaccine-induced immune response remains elusive, based on results from previous studies. There is clearly a humoral response, as shown by the generation of antibodies against *env* and *gag* proteins (Hosie *et al.*, 1995, Mazzetti *et al.*, 1999, Hohdatsu *et al.*, 1997b, Giannecchini *et al.*, 2001, Hosie and Flynn, 1996, Yamamoto *et al.*, 1993, Matteucci *et al.*, 1996). However the specificity of this response appears to be narrow, at least in regards to VNA (Pu *et al.*, 2001, Hohdatsu *et al.*, 1997a). Previous authors have suggested that an anti-FIV cytotoxic T cell response may also occur in cats vaccinated with the Fel-O-Vax® FIV vaccine. Omori and others

(2004) demonstrated increased mRNA expression of IL-2, IFN-γ and perforin when T cells from vaccinated cats were stimulated *in vitro* with vaccine strains of FIV, implying the presence of FIV-specific Th1 $CD4^+$ and $CD8^+$ T cells. Results from this study demonstrated FIV-specific activation of $CD4^+$ T cells in PBMC from Fel-O-Vax® FIV vaccinated cats, as shown by an increase in the normalised MFI for CD25 of $CD4^+$ T cells and a trend (p=0.078) for an increased percentage of $CD25^+CD4^+$ cells in FIV stimulated PBMC from vaccinated versus unvaccinated cats. It was unknown as to whether the activated $CD4^+$ T cells represent a Th1 or Th2 phenotype, as cytokine profiles were not measured in this study. In contrast, FIV-specific activation of $CD8^+$ T cells in vaccinated cats were not able to be shown in this study. Although not significant, the percentage of $CD25^+CD8^+$ cells did however increase in PBMC from vaccinated versus unvaccinated cats (29.99% versus 22.18%, p=0.143). Given the small percentage of FIV-specific $CD8^+$ T cells expected, and the large inter-cat variation observed, the current study was underpowered to confirm a cytotoxic response. Retrospective power analysis showed that at a power of 80%, at least 12 unvaccinated cats (24 vaccinated cats) would have been required to detect a difference of 7.8% in the percentage of FIV-specific $CD25^+CD8^+$ T cells in vaccinated versus unvaccinated cats. Therefore, although results did not demonstrate the presence of FIV-specific $CD8^+$ T cells following vaccination, further studies with sufficient power are required to confirm this finding.

The percentage of FIV-specific $CD4^+$ T cells in PBMC from vaccinated cats was calculated to be approximately 8.2%, which is higher than the expected proportion of circulating memory T cells [estimated to be ~1/100,000 (Welsh and Selin, 2002)]. The FIV-specific T cells identified in this study are therefore likely to represent a combination of memory and effector $CD4^+$ T cells. Vaccination results in the generation of memory cells following initial exposure to antigen. Repeated (booster) vaccination expands this population of memory cells, but also generates a population of short-lived effector cells. In addition, exposure to antigen *in vitro* results in proliferation of effector cells within hours, further expanding the number of FIV-specific T cells in culture (Welsh and Selin, 2002). A difference was observed in the percentage of FIV-specific

$CD4^+$ T cells in the "newly vaccinated" versus "revaccinated" cats in this study. The "newly vaccinated" cats that had recently received 3 doses of the Fel-O-Vax® FIV vaccine had an increased percentage of FIV-specific $CD4^+$ T cells (13.6%) when compared to the "revaccinated" cats (2.9%) that had only received 1 recent dose of the vaccine (having had 3 doses 1 year previously). This is not unexpected, given that the "newly vaccinated" cats would be expected to have an increased percentage of FIV-specific T cells in circulation, consisting of both memory and effector T cells generated by recent repeated antigen exposure. In addition, it is also possible that recently generated FIV-specific T cells in the "newly vaccinated" cats showed broader specificity and therefore more cross-reactivity towards the field isolates of FIV when compared to those from the "revaccinated cats". Affinity maturation of T cells occurs following repeated exposure to antigen, resulting in memory cells with a narrower specificity and increased affinity for the selected antigen (Busch and Pamer, 1999). As the "revaccinated" cats had increased opportunity for affinity maturation due to repeated antigen exposure over time, this may have contributed to the reduced number of FIV-specific T cells that were detected in this study, following stimulation of cells with heterologous FIV antigen.

One of the major limitations of this study is that the clinical relevance of the results (in regards efficacy of the Fel-O-Vax® FIV vaccine) is unknown. Although results do suggest quantitative differences in the responses elicited by the field isolates compared to the vaccine strain of FIV, it is unclear as to whether these differences equate to a significant effect on vaccine-induced immunity. As already discussed, the CD25 assay evaluated antigen recognition and cellular activation in response to FIV. As these processes represent only the first steps in a complex response leading to protective immunity, demonstrating FIV-specific CD25 expression does not confirm the existence of protective immunity to FIV. Similarly, a positive DTH response suggests only sensitisation of an individual to a pathogen, which does not necessarily equate to protective immunity. Concurrent *in vivo* challenge studies are necessary to determine any correlation between the magnitudes of each response and the presence of protective immunity.

This information would be required to determine if the cross-reactivity of the response observed in this study is sufficient to protect cats against infection with different field isolates of FIV.

4.5. Conclusion

Results from this study provide evidence that T cells generated following vaccination with the Fel-O-Vax® FIV do recognise antigen from field isolates of FIV. However, the magnitude of the each response was shown to vary according to the isolate tested, so the hypothesis that there would be no difference in the response elicited by each isolate of FIV tested is thereby rejected. Only the initial steps of the vaccine-induced immune response were evaluated by the assays used in this study, so the relevance of these findings in regard to protective immunity generated by vaccination is unclear. Given the differences in the magnitude of the response generated by each NZ isolate tested, further studies are indicated to determine the effect of genomic sequence variation of FIV on efficacy of the Fel-O-Vax® FIV in the NZ field.

CHAPTER FIVE

Field efficacy of the Fel-O-Vax® FIV vaccine in NZ

5.1. Introduction

The Fel-O-Vax® FIV vaccine has been available to veterinarians in NZ for the past 12 years. Although vaccine efficacy in the field remains uncertain, many veterinarians are using this vaccine routinely. In fact a recent survey showed that 35.7% of NZ veterinarians in practice recommend the Fel-O-Vax® FIV vaccine "often or always" – a substantially high percentage given the lack of evidence for efficacy in this country (Cave *et al.*, 2016). Previous vaccine efficacy studies have shown a combined average PF of 68.1% for the Fel-O-Vax® FIV vaccine (Hohdatsu *et al.*, 1997b, Pu *et al.*, 2001, Pu *et al.*, 2005, Kusuhara *et al.*, 2005, Dunham *et al.*, 2006b, Huang *et al.*, 2004, Huang *et al.*, 2010, Omori *et al.*, 2004). Two of these studies included a recombinant isolate of FIV from NZ (FIV-NZ1). Against this challenge isolate, the PF for the Fel-O-Vax® FIV vaccine was only 40 and 44% (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014).

In NZ, up to 48% of FIV variants are classified as subtype C, with the remaining viruses belonging to subtype A or unknown subtypes (designated U or F') (Kann *et al.*, 2007b, Hayward *et al.*, 2007). In the majority of efficacy studies, Fel-O-Vax® FIV vaccinated cats were challenged with subtype A or B viruses. Although subtype classification is unlikely to be biological significance in regard to susceptibility to the vaccine-induced immune response, subtype differences do infer significant genetic variation of FIV in NZ compared to that of other countries, such as Australia or the U.S.A. In addition, recombinant forms of FIV appear to be common in NZ, and results of previous studies suggested that efficacy of the Fel-O-Vax® FIV vaccine may be reduced against recombinant viruses (Yamamoto *et al.*, 2010, Coleman *et al.*, 2014). Further studies are required to confirm efficacy of Fel-O-Vax® FIV vaccine against FIV circulating in NZ.

The current prevalence of FIV in NZ pet cats is largely unknown. The most recent study to investigate this was performed in 1989, and these authors reported an overall prevalence of 14.4% in pet cats of variable health status (Swinney *et al.*, 1989). More recently, the FIV prevalence was reported to vary between 11% and 36% among feral cats in different geographical locations of

NZ (Hayward *et al.*, 2007). In North America, the prevalence of FIV in pet cats was reported to be low, with only 2.5% of cats testing positive using serology on whole blood (Levy *et al.*, 2006). Recently, a door-to-door survey was conducted in Australia, and these authors reported an FIV prevalence of 10.6% in randomly selected pet cats (Chang-Fung-Martel *et al.*, 2013). That study was unique in that PCR was used to amplify viral DNA from saliva, a less-invasive method used to maximise case recruitment (Chang-Fung-Martel *et al.*, 2013).

PCR has been recommended as a method to differentiate between FIV vaccinated and unvaccinated cats, as uninfected vaccinated cats test positive on serology (Crawford and Levy, 2007). As the Fel-O-Vax® FIV vaccine contains inactivated virus, replication and integration of the viral genome into the host cell does not occur. Despite this, some vaccinated cats were found to be FIV PCR positive on whole blood in previous studies. For example, Crawford and others (2005) demonstrated a specificity as low as 44% in vaccinated cats using a conventional PCR assay that amplified a region of the *gag* gene. In the same study, a qPCR designed to amplify a region of the FIV *gag* gene showed 95% specificity compared to virus isolation, in a population of FIV vaccinated cats. Ammersbach and others (2013) investigated the specificity of a commercial FIV qPCR assay, and they showed that 2/26 uninfected, vaccinated cats were positive for FIV DNA in whole blood samples. Most recently, Westman and others (2015) reported no repeatable false positives when using a qPCR assay to detect FIV DNA in whole blood of uninfected, vaccinated cats. Similarly, viral DNA was not detected in saliva from any of the uninfected, vaccinated cats in another study (Chang-Fung-Martel *et al.*, 2013). Use of qRT-PCR to detect FIV RNA and DNA in saliva, has been shown to be just as specific, but slightly less sensitive, than use of the same assay on whole blood in a range of vaccinated and unvaccinated cats (Westman *et al.*, 2016b). Infection of FIV vaccinated cats in the field has been documented, with one study showing that 5.6% (5/89) of vaccinated cats versus 11.8% (25/212) of unvaccinated cats were infected with FIV (as confirmed by virus isolation) (Westman *et al.*, 2016a). Interestingly, these data indicate a protective rate of only 56% for the Fel-O-Vax® FIV vaccine in the Australian field, a value much lower than that suggested by the majority of

experimental challenge studies that used virus of a subtype expected within Australia (A or B) (Kann *et al.*, 2006b).

Results from the previous chapter demonstrated that the Fel-O-Vax® FIV vaccine-induced immune response is at least partially cross-reactive against the NZ isolates of FIV tested. However, there were differences in the magnitude of the response measured for the different isolates. The clinical relevance of these quantitative differences is unknown, and concurrent challenge studies would be required to correlate these results with the presence of protective immunity in cats. However, the differences observed have provided justification for the work described in this chapter, which aimed to investigate the efficacy of the Fel-O-Vax® FIV vaccine in the NZ field. The hypothesis was that the prevalence of FIV in vaccinated pet cats would be lower than the prevalence in unvaccinated pet cats, indicating at least some protection of Fel-O-Vax® FIV vaccinated cats against FIV in the field.

5.2. Materials and methods

5.2.1. The effect of RNA stabilisation solution on detection of FIV provirus

Whole blood (2 mL in EDTA) was collected from a known FIV positive cat recruited from the MUVTH. The cat had been previously diagnosed with FIV by ELISA serology and had no history of FIV vaccination. Nucleic acid was extracted from 3 samples - 200 µL whole blood, 200 µL whole blood diluted 2:3 in RNAlater® (Sigma-Aldrich), and 200 µL whole blood diluted 2:3 in RNase-free water, using the High Pure Viral Nucleic Acid Extraction Kit (Roche) according to manufacturer's directions. Briefly, 200 µL of binding buffer containing poly(A) and proteinase K was added to the 200 µL sample, and the samples were incubated for 10 minutes at 72 °C before adding 100 µL binding buffer. The samples were transferred to a High Pure Filter Tube and collection tube, and centrifuged for 1 minute at 8,000 x g. The flow-through was discarded, and 500 µL of inhibitor removal buffer was added prior to centrifuging the sample again for 1 minute at 8,000 x g. The sample was then washed twice with 450 µL of wash buffer, and centrifuged for 1 minute at 8,000 x g each time, with the final 10 seconds at high speed (13,000 x g). The sample

was eluted in 50 µL of prewarmed elution buffer and collected after centrifugation for 1 min at 8000 xg. FIV qPCR was used to amplify proviral DNA from 2 µL of template DNA in a total volume of 10 µL, as described in section 2.2.5.3. Fel-O-Vax FIV® vaccine cDNA, previously prepared as described in section 2.2.5.1, was used as a positive control for the reaction.

5.2.2. The effect of RNA stabilisation solution at high dilutions on detection of FIV provirus

To ensure that the RNA stabilisation solution did not interfere with the nucleic acid extraction process at higher dilutions, the experiment in section 5.2.1 was repeated with the following samples – 200 µL whole blood from the known FIV positive cat described in section 5.2.1, 20 µL FIV positive whole blood diluted 1:9 in RNAlater® (Sigma-Aldrich), and 20 µL FIV positive whole blood diluted 1:9 in RNase-free water. Nucleic acid was extracted from each sample and FIV qPCR was performed to amplify proviral DNA as described in section 5.2.1 and 2.2.5.3.

5.2.3. Detection of FIV provirus in a buccal swab

A buccal swab was collected from the known FIV positive cat described in section 5.2.1. A rayon tipped sterile swab (Copan) was inserted into the cat's mouth and rubbed against the inner cheek mucosa for 30 sec. The tip of the swab was then cut off and inserted into a sterile cryovial containing 250 µL of RNAlater® (Sigma-Aldrich). The sample was stored at -80 °C prior to processing. Once thawed, the cryovial containing the swab tip was vortexed for 30 seconds and the swab was then transferred to a 0.6 mL microcentrifuge tube with a hole in the base. The swab-containing microcentrifuge tube was then inserted into a larger 1.5 mL microcentrifuge tube and the assembly was centrifuged at high speed for 1 minute. The swab tip was discarded and the flow-through combined with the remaining volume of RNA later in which the swab was initially submerged. Nucleic acid was extracted from this sample using the High Pure Viral Nucleic Acid Extraction Kit (Roche) as described in section 5.2.1. FIV qPCR was used to amplify proviral DNA as described in section 5.2.1 and 2.2.5.3. A standard curve was included in the assay, using

serial dilutions of the cDNA stock (10^8 copies/µL) produced from the Fel-O-Vax FIV® vaccine in section 2.2.5.4.

5.2.4. The prevalence of FIV in vaccinated and unvaccinated cats in the field

Veterinarians from clinics around NZ were contacted via email asking for participation in this study. Email addresses were obtained from the MUVTH referring veterinarian database. Following the initial contact, veterinarians expressing interest in the study were sent consumables and asked to collect buccal swabs from cats that had been previously vaccinated with the Fel-O-Vax FIV® vaccine. Inclusion criteria included the following:

1. Cats were to be more than 2 years of age.

2. Cats must have been vaccinated according to manufacturer's directions, with the most recent vaccination administered within the past year.

3. Cats must have been tested for FIV prior to vaccination (unless they were less than 6 months of age when they first presented for vaccination, as per the current manufacturer recommendations).

4. Unvaccinated cats were to have access to outdoors, in order to have similar exposure to FIV compared to vaccinated cats.

Detailed instructions for sample collection were provided (as described in section 5.2.3), along with a letter for each cat owner, summarising the study objectives. Submission forms accompanied all samples, and this served as a signed client permission form and provided information about the cat, including details of prior FIV testing, and vaccination history. Veterinarians were asked to label the samples clearly, and store the cryovials upright in a -20 °C freezer for a period of no longer than 1 month before shipping at room temperature using the supplied courier bags. Where a freezer was not available, veterinarians were asked to store the samples at -4 °C and ship them within 2 weeks of collection. The study protocol was approved

by the MUAEC (Protocol # 14/129). A sample of the submission form is provided in **Appendix 9.**

Nucleic acid was extracted from each sample as described in section 5.2.3. Where sample volume was insufficient, sterile PBS was added to increase the volume to 200 µL. Extracted DNA was tested for the presence of FIV DNA by qPCR (section 2.2.5.3) in duplicate. Initially, a standard template volume (2 µL) was chosen for the assay. A standard curve of Fel-O-Vax FIV® vaccine cDNA (previously produced in section 2.2.5.4) was imported into the analysis of each batch of samples tested. Samples were identified as suspect positives if the amplification plot crossed the baseline and the melting temperature of the product peaked between 79.5 °C and 83.5 °C (which was the temperature range of FIV products previously identified in this study).

All suspect positive samples were then tested for FIV DNA using conventional nested PCR. The primers described in section 2.2.2.2 were used to amplify an 859 bp region of the *env* gene from 1.0 µL of template DNA. The PCR reactions were performed in a total volume of 10 µL, consisting of 2.0 µL HOT FIREPol® Blend Master mix (Solis BioDyne), 0.1 µM each of forward and reverse primers, 1.0 µL of template DNA and 6.8 µL of H_20. For the nested reaction, 2.0 µL of the primary PCR product was used as template DNA in a 20 µL reaction. Amplification conditions for both primary and nested reactions consisted of initial denaturation at 95 ºC for 10 minutes, followed by 35 cycles of template denaturation (15 seconds at 95 ºC), primer annealing (15 seconds at 50 ºC), and elongation (1 minute at 72 ºC). Final elongation occurred at 72 ºC for 5 minutes. PCR products were identified by gel electrophoresis (100 V for 40 minutes), using a 1% agarose gel stained with ethidium bromide. A molecular weight marker (DNA Molecular Weight Marker XIV, Roche) was included for estimation of the product's size. The bands of the expected size (859 bp) were excised from the gel and sequenced as described in section 2.2.3. Sequence reads were assessed and trimmed using Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ) and aligned with an FIV subtype C reference sequences downloaded from GenBank® (AF474246.1).

Suspect FIV positive samples that tested negative on conventional PCR were tested again with the FIV qPCR reaction described above, using 4 µL of template DNA. Samples were confirmed to be positive for FIV if they produced an amplification curve with a melting peak between 79.5 °C and 83.5 °C. Quantitative results were presented as copies of FIV DNA per µL of template.

All samples negative for FIV DNA were tested to confirm that they contained amplifiable DNA with feline ribosomal RNA (rRNA) housekeeping gene primers (**Table 5-1**) (Helps *et al.*, 2003). The reaction was performed in a total volume of 10 µL, consisting of 2.0 µL HOT FIREPol® Blend Master mix (Solis BioDyne), 0.5 µM of forward primer, 0.4 µM of reverse primer, 2.0 µL of template nucleic acid and 5.1 µL of H_2O. Amplification conditions consisted of initial denaturation at 95 ºC for 5 minutes, then 45 cycles of template denaturation (95 ºC for 5 seconds), primer annealing (60 ºC for 20 seconds), and elongation (72 ºC for 15 seconds). The protocol for sample testing is outlined in **Figure 5-1**.

Table 5-1 Housekeeping gene PCR primers.
Primer sequences used for amplification of a 97 bp region of feline 28S rRNA gene by qPCR. Primer sequences were originally designed by Helps and others (Helps *et al.*, 2003).

Primer	Product size	Sequence (5' to 3')
Forward Reverse	97 bp	CGCTAATAGGGAATGTGAGCTAGG TGTCTGAACCTCCAGTTTCTCTGG

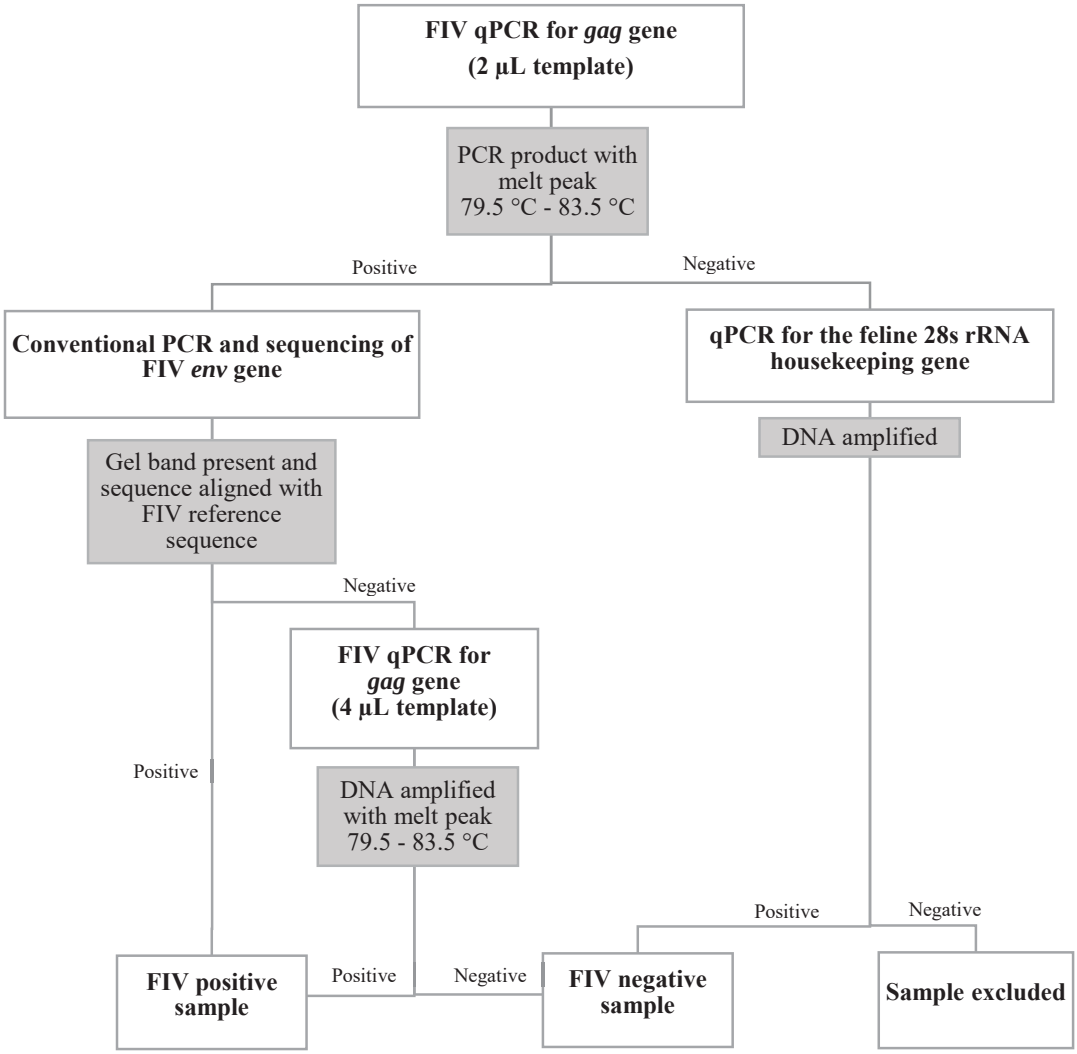


Figure 5-1 Flow chart describing the protocol for sample testing.
All samples were initially screened using the FIV qPCR (section 2.2.5.3) to amplify a region of the FIV *gag* gene from 2 µL of template DNA. Negative samples were then tested for the presence of DNA using a qPCR reaction to amplify the feline 28S rRNA housekeeping gene. Samples negative on this PCR were excluded from the study on the basis that they did not contain amplifiable DNA. Samples positive on the screening FIV qPCR were suspected to be positive for FIV and subjected to further analysis. Conventional FIV PCR was performed and positive products were sequenced to confirm the presence of FIV DNA. Negative samples were further tested using the FIV qPCR described above, using 4 µL of template DNA. Samples testing positive on either conventional FIV PCR or the confirmatory FIV qPCR were considered FIV positive.

5.2.5. Statistical analysis

Submission forms were reviewed and any samples with an incomplete history were excluded. A logistic regression model was created, with FIV status as the outcome variable. Interaction terms included vaccination status, as well as sex and age to assess for bias in the sample populations. The analysis was then repeated following exclusion of all cats that had not been tested for FIV prior to vaccination (if they were vaccinated as kittens). All statistical analyses were performed using R statistical software (R: A Language and Environment for Statistical Computing, R foundation for Statistical Computing, Vienna). A *p*-value of less than 0.05 was considered significant.

5.3. Results

5.3.1. The effect of RNA stabilisation solution on detection of FIV provirus

As expected, FIV proviral DNA was amplified from the nucleic acid extracted from undiluted whole blood from the FIV positive cat (**Figure 5-2**). The melting temperature of the product was 81.7 °C, consistent with the 164 bp FIV product previously amplified using this qPCR (section 2.2.5.3). A similar product with a higher Cq value (compared to the whole blood) was amplified from the nucleic acid extracted from each of the samples of diluted whole blood (mean Cq 22.5 for whole blood sample, versus Cq 25.9 for RNA later® diluted sample and Cq 25.8 for water diluted sample). There was no difference in the quantity or melting temperature of the products obtained from whole blood diluted in RNAlater® versus water. These findings confirmed that RNAlater® did not interfere with detection of FIV provirus in samples.

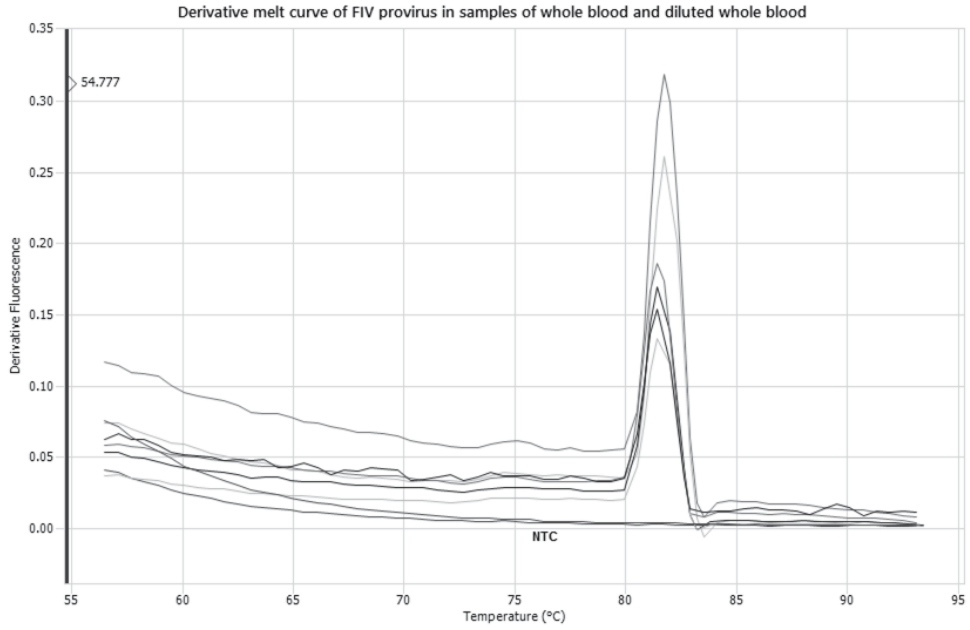


Figure 5-2 Effect of sample dilution on FIV qPCR results.
Derivative melt curves of the *gag* FIV product amplified from blood of an FIV positive cat using the qPCR described in section 2.2.5.3. All products (including duplicates of whole blood, whole blood diluted 2:3 in RNAlater® and whole blood diluted 2:3 in water) had melting peaks at approximately 81.6 °C, consistent with the previously amplified FIV products using this qPCR reaction. The negative (no template) control is represented as NTC.

5.3.2. The effect of RNA stabilisation solution at high dilutions on detection of FIV provirus

Similar to the previous experiment in section 5.3.1, FIV provirus was detected in all samples tested when the nucleic acid was diluted 10-fold in either RNAlater® or water (**Figure 5-3**). There was no clear difference in the melting temperature or Cq value of the products detected from the RNAlater® diluted sample versus the water diluted sample. These results indicated that RNAlater® did not interfere with DNA extraction and subsequent qPCR amplification of FIV DNA.

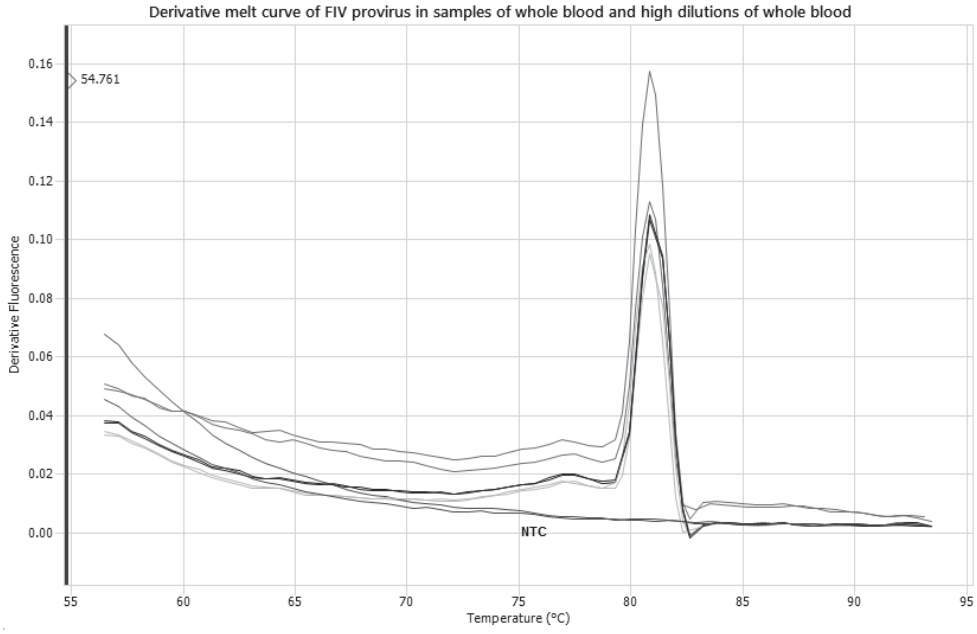
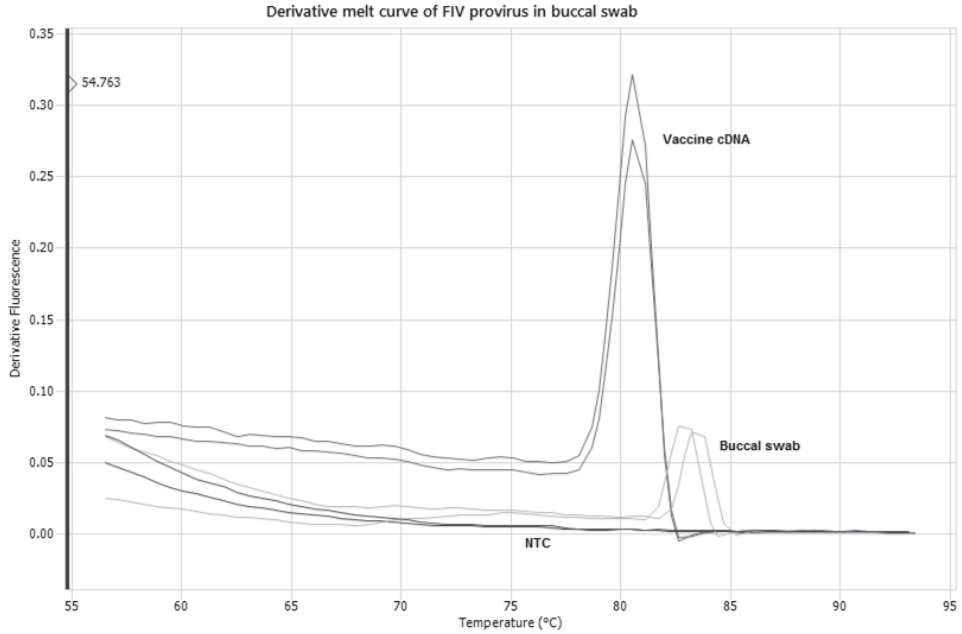


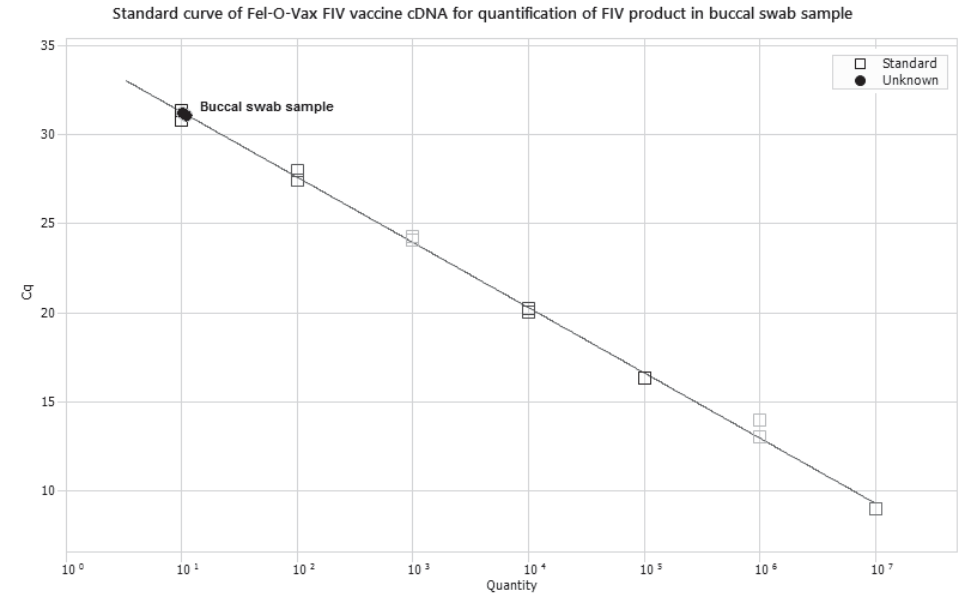
Figure 5-3 The effect of high sample dilution on FIV qPCR results.
Derivative melt curves of the *gag* FIV product amplified from blood of an FIV positive cat using the FIV qPCR described in section 2.2.5.3. All products (including duplicates of whole blood, whole blood diluted 1:9 in RNAlater® and whole blood diluted 1:9 in water) had melting peaks at approximately 81.6 °C, consistent with the previously amplified FIV products using this qPCR reaction. The negative (no template) control is represented as NTC.

5.3.3. Detection of FIV provirus in a buccal swab

Proviral DNA was detected in nucleic acid extracted from the buccal swab of a known FIV positive cat (**Figure 5-4a**). Quantification of FIV DNA load in this sample indicated an approximate concentration of 10 copies/µL of template (Cq=31.18) (**Figure 5-4b**). The melting temperature of the product amplified from the buccal swab sample was higher than that of the product amplified from the Fel-O-Vax FIV® vaccine (Tm 83.2 °C versus 80.8 °C). This was suggestive of sequence variation between the field virus and the vaccine strains of FIV. These results showed that FIV provirus was successfully amplified from a buccal swab of an FIV positive cat. Results also indicated that the quantity of DNA present in buccal swabs was low, at least in the single sample tested.



a)



b)

Figure 5-4 Quantification of FIV provirus in buccal swab samples.
a) Derivative melt curves of the *gag* product amplified from a buccal swab of an FIV positive cat using the FIV qPCR (section 2.2.5.3). The buccal swab sample had a mean melting peak of 83.2 °C, which was higher than that of the product amplified from Fel-O-Vax FIV® cDNA (80.8 °C). The no template (negative) control is shown as NTC.
b) Standard curve generated using the FIV qPCR reaction for quantification of template in the buccal swab sample. The standards comprised an 874 bp product amplified from Fel-O-Vax FIV® vaccine cDNA that included the target 164 bp sequence (section 2.2.5.4). The mean Cq value of the buccal swab sample (shown as dots) was 31.18, giving a concentration of 10 copies/µL of template.

5.3.4. The prevalence of FIV in vaccinated versus unvaccinated cats in the field

A total of 493 veterinary clinics were contacted via email and asked to participate in this study. Of these clinics, 42 responded and were sent supplies to collect samples. A total of 191 samples were received from 15 different veterinary clinics from the North Island (60% of samples) and the South Island (40% of samples) of NZ (**Figure 5-5**). Two samples were excluded due to insufficient information provided on the submission form. Of the remaining 189 samples, 107 were from cats vaccinated against FIV, and 82 were from cats not vaccinated against FIV. Of the 107 vaccinated cats, 53 were male (49.5%) and 54 were female (50.5%). Of the 82 unvaccinated cats, 51 were male (62.2%) and 31 were female (37.8%). The age of the cats ranged from 2 – 18 years, with a median age of 5 years for vaccinated cats and 5 years for unvaccinated cats. Review of the clinical data provided on the submission form showed that the majority of cats sampled were healthy (165/189). Within the unvaccinated cat group, 17/82 (20.7%) cats were 'sick', whereas only 7/107 (6.5%) of vaccinated cats were 'sick'.

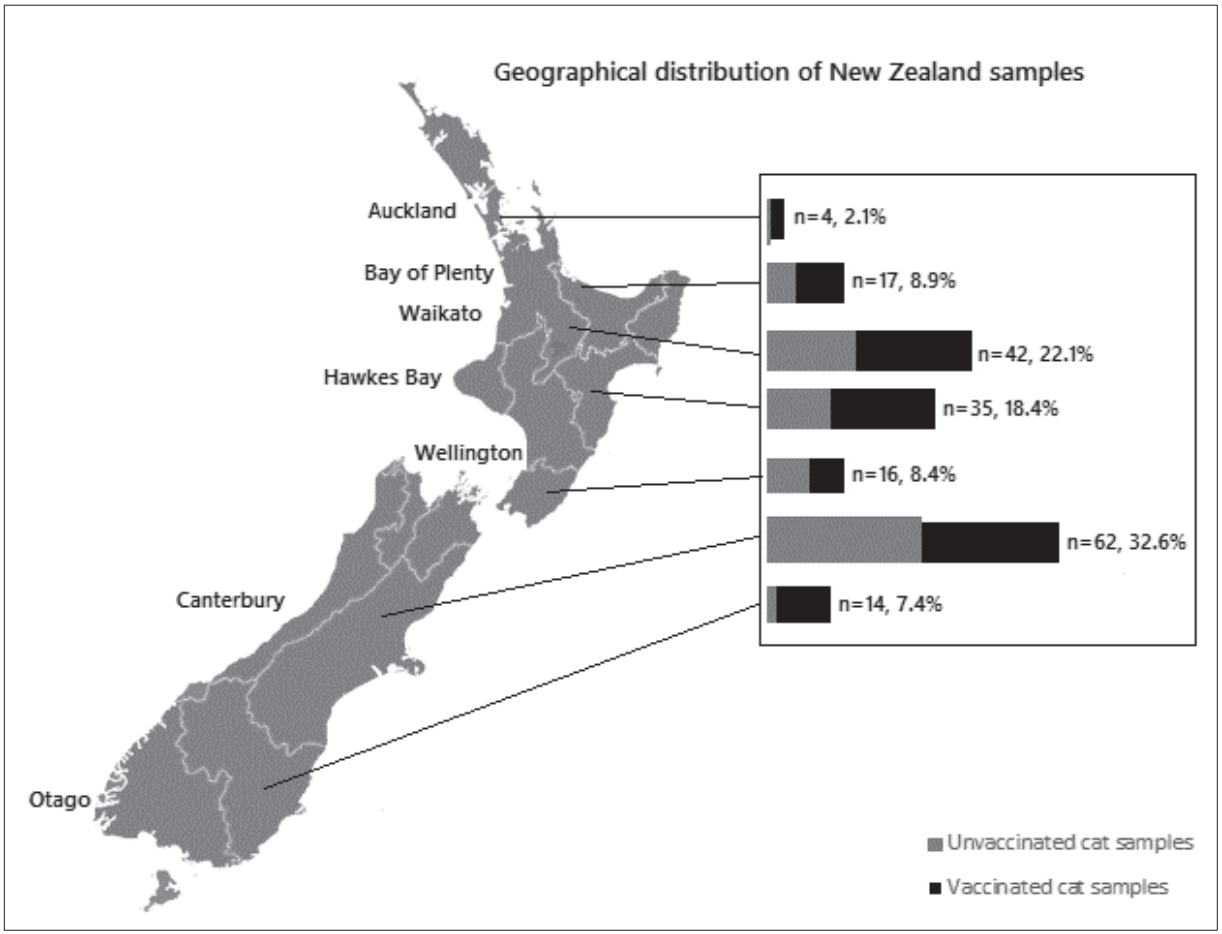


Figure 5-5 Geographical distribution of samples collected from different regions of NZ.
Each region is represented by a bar that represents the number of samples collected from vaccinated cats (black) and unvaccinated cats (grey). The total number of samples and the percentage of samples from each region are also displayed alongside each bar.

In the FIV qPCR with 2 µL of template, 78/189 (41.0%) samples were identified as suspect positives. The calculated concentration of FIV DNA present in the samples ranged from 3 to 116,331 copies/µL, however a large proportion of the suspect positive samples (33/78) contained less than 100 copies of FIV DNA per µL of template. Melt analysis revealed multiple peaks in many of the samples, suggestive of non-specific amplification of DNA. Only those samples with at least one of the melt peaks between 79.5 – 83.5 °C were considered suspect positive for FIV DNA, with further testing required to confirm their FIV status. **Figure 5-6** shows melt curves representative of the suspect positive samples showing the typical double melt peak present in many of the samples.

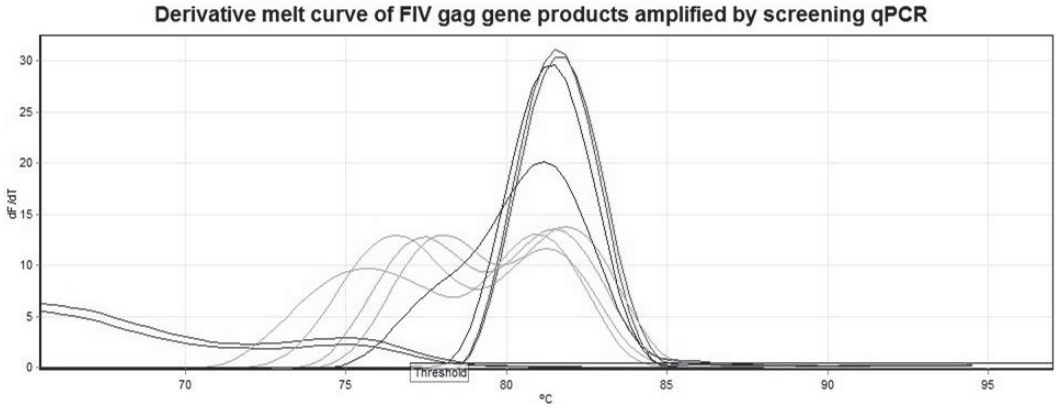


Figure 5-6 Representative melt curve analysis from the screening FIV qPCR assay.
Derivative melt curves from amplification of the target region of the FIV *gag* gene using the FIV qPCR as described in section 2.2.5.3 and 2 µL template DNA. Samples shown are representative of the 189 samples tested. Two of the samples displayed (one of which is comprised of Fel-O-Vax FIV® vaccine DNA) are clearly positive for FIV DNA, with a single melt peak within the expected range for the product (79.5 – 83.5 °C). One sample displayed is negative for FIV DNA. The remaining samples are suspect positive samples, with double melt peaks suggesting amplification of non-target DNA. The purple sample is represents a negative sample.

Conventional PCR was performed on the 78 suspect FIV positive samples. Of these, 5 samples (2 from unvaccinated cats, and 3 from vaccinated cats) produced a PCR product consistent in size with the 859 bp FIV env product targeted. The identity of all 5 bands was further confirmed by sequencing. A further 6 samples were suspected to be positive (based on the presence of a band 700-800 bp in size), however sequencing of these products showed that they were not consistent with the target sequence. Of the 5 confirmed positive samples, 4 showed a high concentration of FIV DNA (>800 copies / µL) using the initial screening FIV qPCR. **Figure**

5-7 shows representative results from gel electrophoresis, and **Figure 5-8** shows the alignment of the sequences with an FIV subtype C reference sequence.

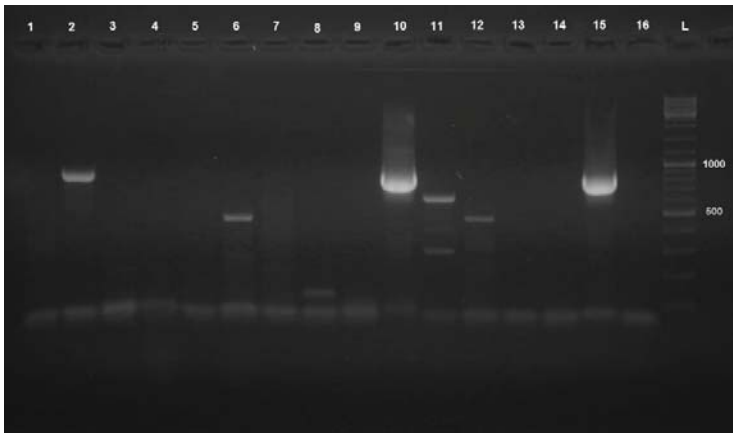


Figure 5-7 Gel electrophoresis of PCR products amplified from suspect FIV positive samples.
Results showed a discrete product, approximately 860 bp in 2 of the 14 samples (lanes 2 and 10) and the positive control (lane 15) in this representative batch. The sample in lanes 1, 3 - 5, 7 - 9, and 11 - 14 were negative for FIV DNA in this reaction. Lane 16 contained no template control. The products in lanes 2 and 10 (cats VAH012U and U01U) were subsequently sequenced and found to align with the FIV reference sequence.

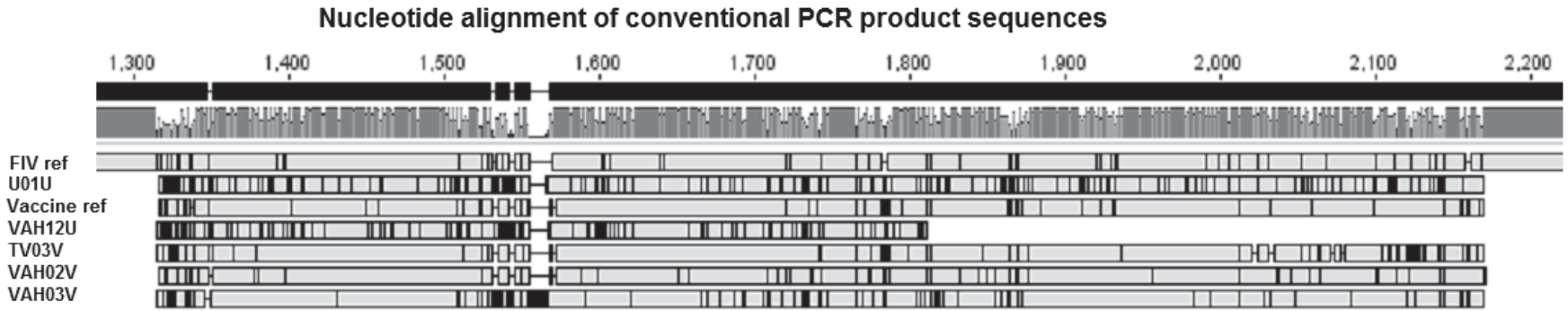


Figure 5-8 Nucleotide alignment of the PCR products.
The target region of the *env* gene from a subtype C FIV reference sequence was aligned with the 5 sequenced conventional PCR products (from samples U01U, VAH12U, TV03V, VAH02V and VAH03V) that were amplified using the conventional PCR described in section 5.2.4. A product amplified from Fel-O-Vax® FIV vaccine (Vaccine ref) was also included in the alignment. Similarity to the reference sequence is indicated by the bar along the top, with the height of the bar at each location indicative of similarity between the sequences. All sample sequences aligned with the FIV subtype C reference sequence, confirming amplification of the target DNA. Alignment was generated using the Geneious Pro 4.8.5 software (Biomatters Ltd, 2009, Auckland, NZ). The FIV reference sequence was downloaded from GenBank® (AF474246.1). A subtype C reference sequence was chosen for its expected similarity to NZ field isolates of FIV.

Using an FIV qPCR with 4 µL template DNA, a specific product was amplified from 31 of the remaining 73 suspect FIV positive samples. Use of the higher template concentration resulted in less non-specific amplification, enabling much clearer differentiation of positive versus negative samples, despite the concentration of DNA remaining very low. **Figure 5-9** shows a representative melt curve from positive and negative samples.

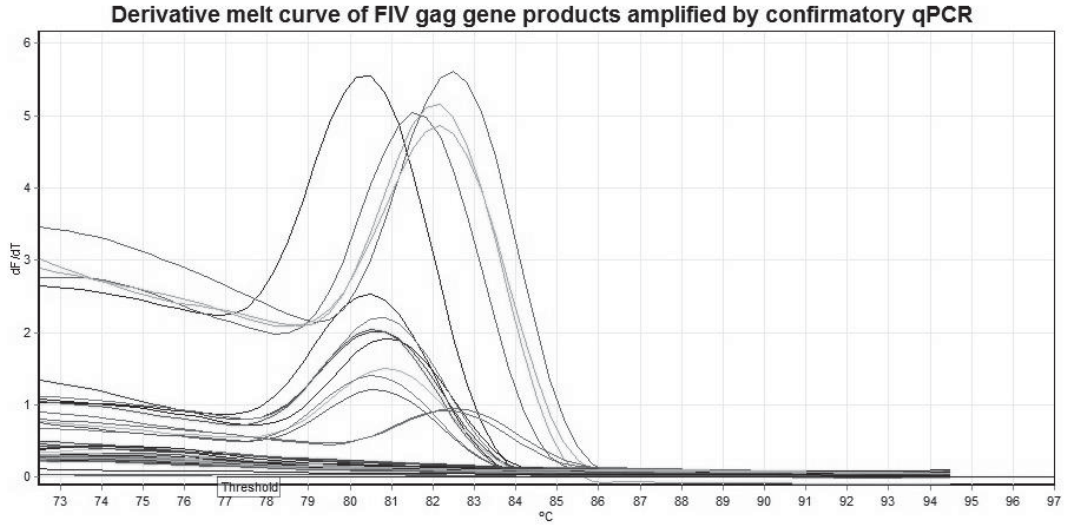


Figure 5-9 Representative melt curve analysis from confirmatory FIV qPCR.
Derivative melt curves following amplification of the target region of the FIV *gag* gene from 4 µL template DNA, using the qPCR described in section 2.2.5.3. Samples shown are representative of the 73 samples tested. Each positive sample has a single melt peak within the expected range for this product (79.5 – 83.5 °C). Negative samples are those without a melt peak present, and results show minimal non-specific amplification. The melting temperature is represented along the x-axis, and the relative fluorescence is shown on the y-axis.

The qPCR for the feline 28s rRNA housekeeping gene was positive for all 111 of the samples tested. Melt curves from a representative batch of samples are shown in **Figure 5-10**. As they were all positive, no samples were excluded from the analysis on the basis of insufficient amplifiable DNA in the swabs.

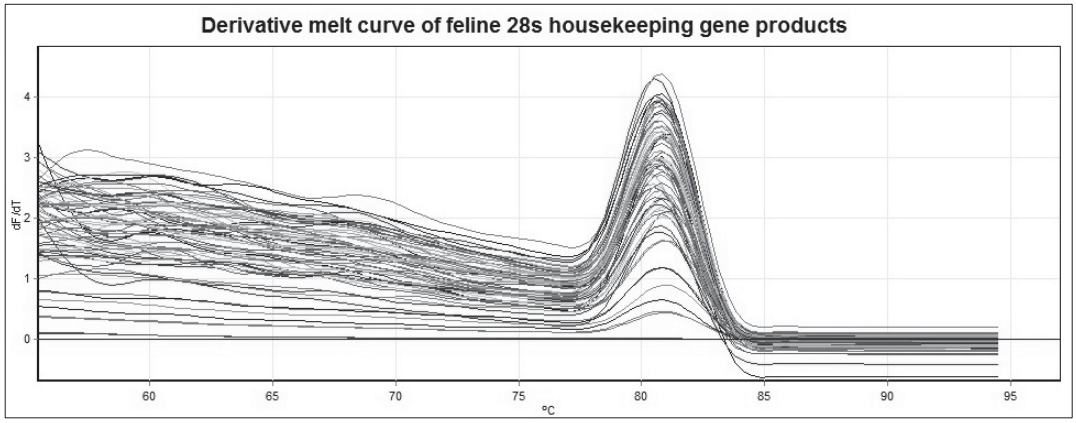


Figure 5-10 Representative melt curve analysis for the housekeeping gene qPCR assay.
Derivative melt curves following amplification of the feline 28s rRNA housekeeping gene using the qPCR reaction described in section 5.2.4. Samples shown are a representative batch of the 111 samples tested. All samples (apart from the no template controls) were positive for target DNA, as indicated by the presence of amplified DNA with a specific melt peak. The melting temperature is represented along the x-axis, and the relative fluorescence is shown on the y-axis.

Overall, 36/189 (19.0%) of the buccal swab samples were considered positive for FIV (**Figure 5-11**). Within the unvaccinated group of cats, 11/82 samples were FIV positive, giving a prevalence of 13.4% within the population sampled. For the vaccinated cat samples, 25/107 were considered positive, giving an FIV prevalence of 23.4% within this group. Results are summarised in **Table 5-2**. Logistic regression analysis showed that vaccination status did not significantly affect the outcome of FIV status, however there was a trend for vaccination to increase the risk for a positive FIV PCR result (p=0.08). Age and sex were included in the model to assess for sampling bias between the 2 populations of vaccinated and unvaccinated cats, and there was no effect of these factors on FIV status (p=0.49 and 0.75 respectively). For a complete summary of individual results, please see **Appendix 10**. Regression tables are provided in **Appendix 11**.

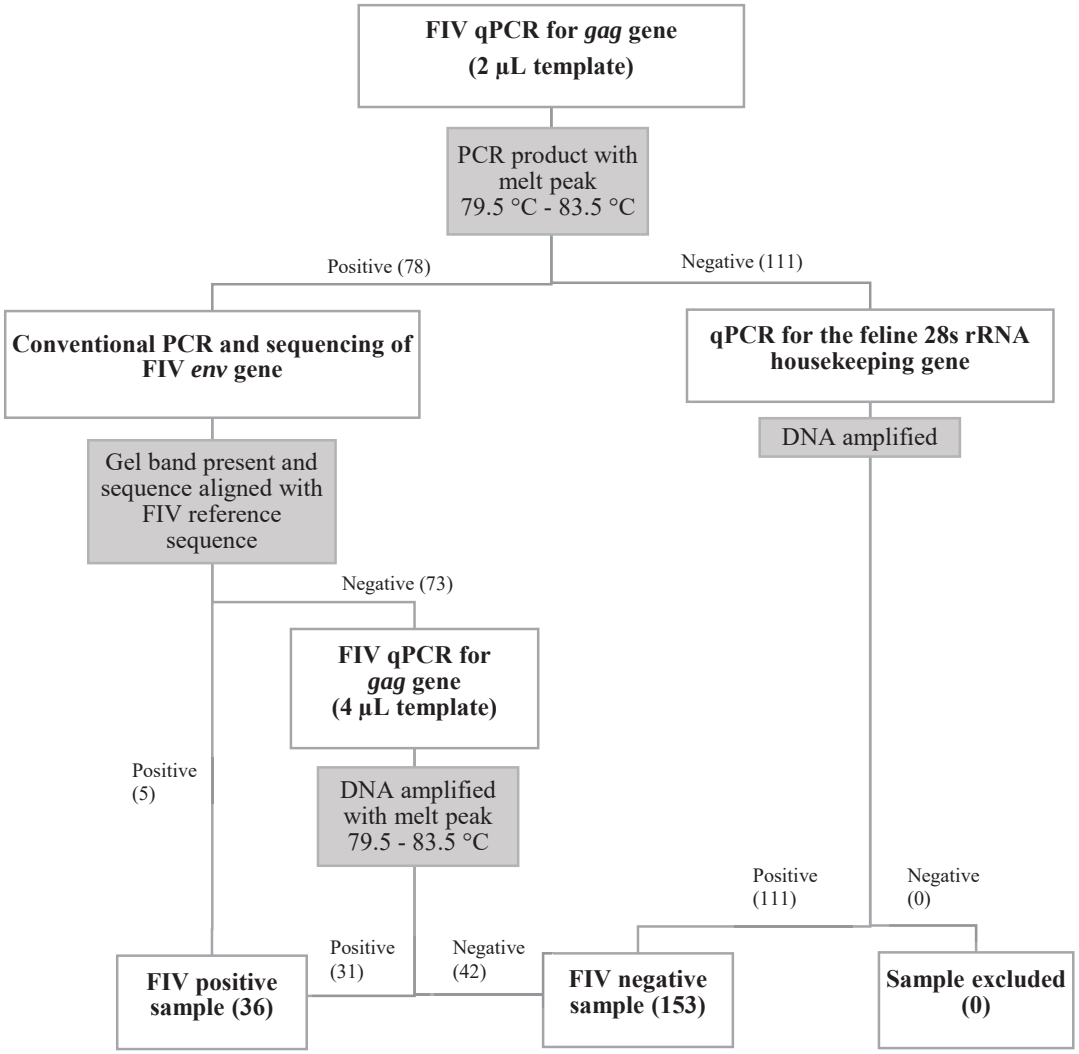


Figure 5-11 Flow chart showing the results following testing of all buccal swab samples.
A total of 189 buccal swab samples were tested for FIV in this study. 78/189 were suspect FIV positive, based on results from the initial screening FIV qPCR. 5/78 of these samples were positive for a specific FIV product using conventional PCR. Of the remaining 73 suspect FIV positive samples, 31 were confirmed to be FIV positive based on results from the confirmatory FIV qPCR. Of the 111 samples that were FIV negative on the screening FIV qPCR, all of these were positive for the rRNA housekeeping gene product on qPCR. Overall, 36/189 (19.0%) of buccal swab samples were FIV positive.

Table 5-2 Summary of results from all vaccinated and unvaccinated cats.
Cats were considered positive for FIV if the buccal sample was positive for FIV DNA on the screening FIV qPCR and confirmed with a positive result on conventional PCR and sequencing, or on repeat FIV qPCR with increased template concentration. There was no significant effect of vaccination on FIV status, however there was a trend for vaccination to increase the risk of a positive FIV PCR result (p=0.08).

	FIV+	FIV-	Total	Percentage
Vaccinated cats	25	82	107	23.4
Unvaccinated cats	11	71	82	13.4
Total	36	153	189	

A complete vaccination history was provided for all 25 FIV positive vaccinated cats, confirming that they had all been vaccinated according to the manufacturer's protocol and were current for FIV vaccination. Review of the submission form data revealed that 13 of the 25 FIV positive cats had been vaccinated as kittens and therefore not tested prior to vaccination (as per the manufacturer's recommendations). The analysis was repeated following exclusion of these 13 cats, as we could not confirm their FIV status prior to vaccination. Results showed that there was no effect of vaccination on FIV status, with an FIV prevalence of 12.8% (12/94) in the vaccinated cat population versus 13.4% (11/82) in the unvaccinated cat population (p=0.91). Results are summarised in **Table 5-3**. See **Appendix 11** for regression tables.

Table 5-3 Summary of results from FIV-tested vaccinated cats and unvaccinated cats.
FIV positive vaccinated cats that had not tested for FIV prior to vaccination were excluded from this analysis (n=13). Cats were considered positive for FIV if the buccal sample was positive for FIV DNA on the screening FIV qPCR and confirmed with a positive result on conventional PCR and sequencing, or on repeat FIV qPCR with increased template concentration. There was no significant effect of vaccination on FIV status (p=0.91).

	FIV+	FIV-	Total	Percentage
Vaccinated cats	12	82	94	12.8
Unvaccinated cats	11	71	82	13.4
Total	23	153	176	

5.4. Discussion

Results from this study showed that there was no difference in the prevalence of FIV in vaccinated, versus age- and sex-matched unvaccinated cats. The hypothesis that the prevalence of FIV would be lower in vaccinated cats is thereby rejected. These results suggest that vaccination of cats with the Fel-O-Vax® FIV vaccine does not reduce the risk of FIV infection in the NZ field.

Few studies have investigated efficacy of the Fel-O-Vax® FIV vaccine in the field. The majority of previous studies have compared infection rates in vaccinated versus unvaccinated cats following experimental challenge with a single isolate of FIV. In these studies, the reported PF for the Fel-O-Vax® FIV vaccine in the experimental cat population ranged from 0 to 100%, with sequence variation of the challenge virus likely to account for much of this variation (Hohdatsu *et al.*, 1997b, Pu *et al.*, 2001, Pu *et al.*, 2005, Kusuhara *et al.*, 2005, Dunham *et al.*, 2006b, Huang *et al.*, 2004, Huang *et al.*, 2010, Omori *et al.*, 2004). The only studies investigating efficacy against a NZ isolate of FIV showed a relatively low efficacy, with the PF reported as 40 to 44% (Coleman *et al.*, 2014, Yamamoto *et al.*, 2010). The current study differed from previous challenge studies, as it aimed to investigate vaccine efficacy in the field by testing a vaccinated and unvaccinated population of cats naturally exposed to FIV. Results showed that vaccine efficacy under NZ conditions appears to be even lower than that reported in experimental studies, with no evidence of prevention. A similar method was used previously to investigate efficacy of the Fel-O-Vax® FIV vaccine among Australian cats. FIV infection was confirmed in 5.6% of vaccinated cats versus 11.8% of unvaccinated cats, giving a protective rate of 56% for the vaccine (Westman *et al.*, 2016a) – much lower than that expected based on comparable data from experimental studies (Huang *et al.*, 2010, Huang *et al.*, 2004, Kusuhara *et al.*, 2005, Pu *et al.*, 2005).

In the majority of experimental studies, cats were challenged with laboratory adapted isolates of FIV or field viruses isolated from symptomatic cats. It has been suggested that these viruses are not representative of circulating field viruses, as they differ in cell tropism and are more susceptible to virus neutralisation *in vitro* (Hosie *et al.*, 2011, Beczkowski *et al.*, 2014). This appears to be related to their requirement for a CD134 interaction, with viruses isolated from cats in acute or asymptomatic stages of infection (such as FIV-GL8 and FIV-CPGammar) often requiring a more complex interaction with the CD134 molecule compared to laboratory isolates of FIV (Willett *et al.*, 2006b). In one study, vaccinated cats were not protected against challenge with a virus more typical of field FIV (FIV-GL8). Instead, it appeared that vaccination enhanced

the infection (Dunham *et al.*, 2006b). These studies highlight the importance of testing vaccine efficacy against field viruses. Results from this current study and the comparative Australian study (Westman *et al.*, 2016a) are likely to be more representative of the true efficacy of the Fel-O-Vax® FIV vaccine when compared to experimental studies.

In addition to the differences between field and laboratory adapted viruses, there are likely to be geographical differences in vaccine efficacy relating to sequence variation among FIV circulating in different regions. The dual-subtype vaccine was developed in an attempt to broaden the specificity of the vaccine induced-immune response and improve efficacy against the spectrum of field viruses, as prototype single subtype vaccines were shown to be ineffective against non-vaccine viruses [reviewed by Leocollinet & Richardson (2008)]. The Fel-O-Vax® FIV vaccine therefore contains whole virus of subtype A (Petaluma strain) and subtype D (Shizuoka). As NZ FIV belongs predominantly to subtype C or other unclassified subtypes, there is large variation (approximately 20-23%) between the *env* sequences of typical NZ field viruses and those contained within the Fel-O-Vax® FIV vaccine (Hayward and Rodrigo, 2008). Therefore, vaccine efficacy against field virus in NZ may be even further reduced compared to the efficacy in countries such as Australia where the predominant circulating viruses (subtype A) are more similar to the vaccine strains of FIV (Kann *et al.*, 2006b, Iwata and Holloway, 2008).

An alternative explanation for the apparent poor efficacy of the Fel-O-Vax® FIV vaccine in this study is vaccine failure due to poor compliance, infection during the primary course of vaccination, or infection of cats prior to vaccination. Inclusion criteria for this study specified that cats must have received a complete course of vaccination according to the manufacturer's directions (3 vaccines 2-4 weeks apart and annual boosters), with the most recent vaccine administered within the past 12 months. Review of submission data confirmed this to be true in each of the vaccinated cats sampled, eliminating poor compliance as a likely explanation for poor vaccine efficacy. Current manufacturer recommendations are that adult cats (>6 months of age) are tested for FIV prior to vaccination, and that the vaccine is not administered to cats testing

positive.[11] The manufacturer does not recommend testing cats less than 6 months of age prior to vaccination. This is because kittens may acquire antibodies from the colostrum of an infected or vaccinated queen, interfering with serological tests (MacDonald *et al.*, 2004). Kittens are also thought to be at low risk of FIV infection, as fighting is the major route of transmission and this is unlikely to occur in kittens. However, there are experimental reports of up to 70% transmission of the virus to kittens when the queen is acutely infected whilst pregnant (O'Neil *et al.*, 1995). Although the risk seems to be low in the natural environment, the possibility that some of the FIV positive cats in this study were infected prior to vaccination cannot be excluded. A total of 13/25 infected vaccinated cats were identified in this study as having received their first FIV vaccination at <6 months of age without prior FIV testing. Analysis was therefore repeated to exclude these cats on the basis that it could not be certain that these cats represented true vaccine failures. Results showed that there was still no effect of vaccination on FIV status, with no difference in the prevalence of FIV infection in vaccinated versus unvaccinated cats.

Buccal swabs were used in this study as a less invasive method of sampling (compared to venepuncture), in order to increase the number of recruited cats. The FIV qPCR used was designed to detect proviral DNA, requiring the presence of infected lymphocytes within the saliva and buccal mucosa sampled. As lymphocytes are more abundant in blood samples, the quantity of FIV DNA present in the buccal samples was expected to be low compared to blood. The sensitivity of the assay in detecting proviral DNA from swabs of known infected cats was not assessed in this study, so there is a possibility that the number of FIV positive cats was underestimated in this study. However, as the aim of the study was to compare the prevalence of FIV among vaccinated and unvaccinated cats, not to determine the overall prevalence of FIV, the actual sensitivity of the assay was considered less important, provided that vaccination did not affect the amount of virus present in the saliva of FIV infected cats. One study has shown an increased viral load in blood from vaccinated, FIV infected cats compared to unvaccinated, FIV infected cats (Dunham *et al.*, 2006b). If this were true, and vaccination also resulted in an

[11] http://www.zoetis.co.nz/species-products/cats/fel-o-vax-fiv-vaccine.aspx

increased salivary viral load, then the sensitivity of the qPCR assay used in this study may have been higher in the vaccinated cat population. This may account for the apparent trend for vaccination to increase the risk for a positive FIV test result.

Initially, the qPCR was performed on all samples using a standard template volume of 2 µL. Melt peak analysis of results showed significant amounts of non-specific amplification, as indicated by multiple melt peaks, including those outside the expected range for the FIV target product. Non-specific amplification is more likely when the concentration of target DNA is very low, as more primers are available for non-specific binding. Given the expected low concentration of FIV DNA in these samples, the FIV qPCR was repeated on suspect positive samples using twice as much template. Samples were clearly identified as positive or negative on repeat analysis, with single melt peaks present in positive samples and very little non-specific amplification. The assay was not repeated on the negative samples due to limited reagents available for this study. For these reasons, the initial qPCR assay was labelled a 'screening' test, with the subsequent assay using 4 µL labelled 'confirmatory'.

All suspect positive samples were also tested using conventional PCR and sequencing to confirm FIV status. Of the 78 samples tested, only 5 were confirmed to contain FIV DNA. The low number of positive samples is likely to be a reflection of reduced sensitivity of conventional PCR assay compared to the FIV qPCR. This is supported by 4/5 of the conventional PCR positive samples having much higher concentrations of DNA detected on the initial FIV qPCR. Alternatively, the inability to confirm FIV status on conventional PCR may indicate a high number of false positive results using the FIV qPCR. As the specificity of the assay was not assessed in the current study, this possibility cannot be excluded. False positives on qPCR are most likely due to contamination, but previous authors have also suggested that the assay may detect FIV DNA from the vaccine or that non-FIV components in the vaccine may increase the likelihood of non-specific amplification occurring (Ammersbach *et al.*, 2013). Review of the literature reveals that the incidence of false positives using qPCR for the detection of FIV appears to be low, with 2 independent studies reporting a specificity of 95 and 99% in FIV vaccinated cats

for commercially available qPCR assays (Westman *et al.*, 2015, Crawford *et al.*, 2005). In contrast, false positives are more common using conventional PCR assays, and the specificity is lowest in populations of vaccinated cats (44-66% specificity) (Crawford *et al.*, 2005). In the current study, the FIV qPCR was repeated to reduce the risk of false positives due to contamination, and melt curve analysis was used to try to exclude any false positives due to non-specific amplification. The use of buccal swabs rather than blood samples is also likely to be more specific, as FIV DNA would not be expected in the mouth of a vaccinated cat unless the cat was also infected with the virus. Nevertheless, as conventional PCR and sequencing was not successful on the majority of samples in this study, the specificity of the assay remains uncertain, and infection of these cats cannot be confirmed. Given the expected high impact of these results on use of the vaccine in NZ, follow-up testing of samples using a probe based assay is planned prior to disseminated of these results.

The main limitation of this study is the possibility of bias as a result of the recruitment method. In order to attribute the difference in prevalence of FIV in vaccinated versus unvaccinated cats to the effectiveness of the vaccine in the field, the risk of exposure to FIV in both groups must be comparable. Veterinarians were provided with strict inclusion criteria stipulating that unvaccinated cats were only to be included if they had access to outdoors, but the validity of the data from this study does depend on the participating veterinarians following these guidelines. Prior to administering a non-core vaccine, current vaccine guidelines encourage the clinician to consider the risk of infection for a particular individual (Day *et al.*, 2016). Therefore, veterinarians using the Fel-O-Vax® FIV vaccine in a rational manner, would therefore be expected to have access to fewer unvaccinated, versus vaccinated cats, who had exposure to an outdoor environment. This however does assume good client compliance, and there will always be clients who choose not to follow practice vaccine recommendations, especially when given a choice. Nevertheless, veterinarians probably experienced some difficulty in recruiting unvaccinated, versus vaccinated cats for this study, and this was reflected in the samples received (107 samples from vaccinated cats, versus 82 from unvaccinated cats). In the recent Australian study

investigating the protective rate of the Fel-O-Vax® FIV vaccine in the field (Westman *et al.*, 2016a), veterinarians and owners were asked to complete a questionnaire detailing the amount of time each cat spent outdoors, as well as the number of suspected cat fights the cat had been involved in previously. Inclusion of this information on the submission form for this study would have been useful, so as to eliminate this as a potential source of bias. Examination of the clinical data revealed that a number of unvaccinated cats were presented to the veterinarian with cat bite wounds (7/82, data not shown), and although very small numbers, it does support the assumption that the unvaccinated cats sampled in this study were in fact exposed to FIV in the field. Examination of the clinical data in this study also revealed that there were more 'sick' cats in the unvaccinated versus vaccinated group (20.7% versus 6.5%). Again, this is likely to reflect the difficulty in recruiting unvaccinated cats for this study, as there would be less of these cats presenting for routine health checks. This is another potential source of bias, as 'sick' FIV infected cats may have higher viral loads, thereby increasing the sensitivity of the assay in this group of cats. However, if this were true, differences would bias the results towards showing that the vaccine is more, rather than less, effective in the field.

5.5. Conclusion

Results from this study are highly suggestive that the Fel-O-Vax® FIV vaccine is not effective against FIV under NZ conditions. Further work should aim to confirm these findings, by using an FIV probe to confirm specificity of the results, or by resampling each of the cats testing positive in this study and using conventional PCR and sequencing of the product from blood. Phylogenetic analysis would also be useful to compare the subtype distribution of FIV in vaccinated versus unvaccinated infected cats, in order to determine the relevance of sequence variation on vaccine efficacy.

CHAPTER SIX

Concluding remarks

The overall aim of the research in this thesis was to determine the relevance of the variability of FIV, in regard to pathogenicity and vaccination in NZ. FIV in NZ is genetically distinct compared to FIV in other countries, so overseas studies investigating these questions are not necessarily relevant to the situation in NZ. Little is known about viral factors that influence the pathogenicity of FIV and the reasons why some cats develop severe disease whilst others never progress to AIDS. There are also serious questions regarding the efficacy of the Fel-O-Vax® FIV vaccine, with few studies investigating its value in preventing disease in the field. The research in this thesis makes a significant contribution to the current literature on FIV, by investigating the influence of viral factors on *in vitro* pathogenicity, and investigating the efficacy of the Fel-O-Vax® FIV vaccine against FIV isolates from NZ.

Review of the literature in chapter 1 highlighted the diversity of the FIV genome. Very few studies examined the biological effect of this genomic variation, so it was unclear from the literature as to the relevance of this to FIV-associated disease and vaccination. The information presented in chapter 1 also highlighted the unpredictable clinical course of FIV. The results of many experimental studies showed that disease develops in FIV infected cats, often resulting in increased mortality of infected cats (Beczkowski *et al.*, 2015c, Diehl *et al.*, 1995b, Ishida *et al.*, 1992). However, results from studies of naturally infected cats proved little to no association between FIV and disease (Addie *et al.*, 2000, Kohmoto *et al.*, 1998, Liem *et al.*, 2013, Ravi *et al.*, 2010). Given the genetic variation of FIV, it is likely that viral factors at least contribute to this variation in clinical disease. Chapter 1 included a summary of the efficacy data currently available for the Fel-O-Vax® FIV vaccine. The reported preventable fraction for the vaccine was shown to range from 0% to 100% (Hohdatsu *et al.*, 1997b, Pu *et al.*, 2001, 2010, Omori *et al.*, 2004, Huang *et al.*, 2004, Dunham *et al.*, 2006b, Pu *et al.*, 2005, Huang *et al.*, 2010, Kusuhara *et al.*, 2005, Coleman *et al.*, 2014). Furthermore, results from one study suggested that the efficacy of the vaccine in the field may be lower than what could be expected in experimental situation (Westman *et al.*, 2016a). The reasons for such unpredictable efficacy were unclear from the literature, raising

the question as to the relevance of genomic variation of the virus on susceptibility to the vaccine-induced immune response.

Chapter 2 described the preparation of NZ FIV stock of a known titre. Phylogenetic analysis of the small number of viruses identified in this study was consistent with the previously reported subtype distribution in this country, with viruses of subtypes A and C detected, as well as an A/C recombinant virus. Virus stocks of 3 subtype C isolates and 1 subtype A isolate were produced for use in subsequent studies described in this thesis. In addition, stock of the Petaluma strain of FIV was produced from an infectious molecular clone, for its use in subsequent studies investigating cross-reactivity of Fel-O-Vax® FIV vaccine-induced immune response.

A number of laboratory techniques were used in this chapter to identify, sequence, isolate, and quantify virus from NZ cats. In addition, infectious molecular clones of FIV were obtained from colleagues overseas and transfection methods were employed to generate infectious virus of these strains. Lentiviruses are difficult to isolate and propagate, and extensive troubleshooting was required in order to successfully prepare virus stock. The RT-qPCR assay also required considerable optimisation before it could be used to quantify virus in this chapter. Despite recruiting over 20 cats for the purposes of virus isolation, only 4 NZ isolates of virus were available for use in subsequent chapters of this thesis. This reflected the difficulty in the work carried out, as well as the problems associated with resampling pet cats when virus isolation was unsuccessful. The work in this chapter contributes to the literature by generating awareness of the challenges when working with lentiviruses, and the labour and resources required for such a task.

The aim of the work presented in chapter 3 was to investigate whether FIV variants differ in pathogenicity. Two laboratory based assays (measuring FIV-induced apoptosis and FIV-induced inhibition of mitogen proliferation) were developed to compare *in vitro* characteristics among the isolates of FIV prepared in chapter 2. Results from chapter 3 supported the hypothesis that selected characteristics of FIV differ *in vitro*, and this suggests that FIV variants may also differ in pathogenicity. *In vivo* studies investigating FIV pathogenicity in naturally infected cats

are inherently difficult to interpret, as it is impossible to separate the contribution of viral factors, from the influence of host and environmental factors on disease expression. For this reason, *in vitro* effects of FIV were chosen as markers of pathogenicity. Of course, this led to the challenge of deciding which viral characteristics would be most appropriate to study. Following extensive review of the literature, especially regarding the effect of FIV on the immune system, virally-induced apoptosis and inhibition of lymphocyte proliferation stood out as important factors affecting the progression of disease in FIV infected cats. The lymphocytes from cats with AIDS have been shown to undergo significant apoptosis *in vivo*, and be poorly responsive to mitogens *ex vivo* (Barlough *et al.*, 1991, Bishop *et al.*, 1992, Finkel *et al.*, 1995, Garg *et al.*, 2004b, Hara *et al.*, 1990, Lawrence *et al.*, 1992, Lin *et al.*, 1990, Muro-Cacho *et al.*, 1995, Sarli *et al.*, 1998, Taniguchi *et al.*, 1990, Tompkins *et al.*, 2002, Torten *et al.*, 1991). For the apoptosis assay, feline PBMC were initially chosen to demonstrate this effect, as these are the cells affected *in vivo* in FIV infected cats. Unfortunately, despite extensive trouble-shooting, the assay was not sensitive enough to show an effect of the virus on PBMC, as even under control conditions, these cells underwent extensive apoptosis *ex vivo*. For this reason, the feline lymphoblast cell line was used instead, and given that these cells are reminiscent of activated lymphocytes, this seemed to be an appropriate choice. For both the apoptosis and lymphocyte proliferation assays, the amount of virus used was kept to a concentration similar to that expected in the plasma of infected cats. Probably because of this, FIV was not shown to inhibit lymphocyte proliferation *in vitro*. However, the use of non-physiological concentrations of virus would have limited the application of the assay as a marker of pathogenicity. Use of the term '*in vitro* pathogenicity' was unconventional, but carefully contemplated. It seemed to accurately reflect the effect of the virus on cell health, the *in vitro* characteristic most relevant to pathogenicity.

The differences in apoptosis elicited by each isolate are perhaps not unexpected, given the wide genomic variation of FIV at the *env* gene. Results from chapter 3 support findings from previous studies that showed variable apoptosis in cell cultures infected with FIV from two feline species (Sutton *et al.*, 2005), and those studies showing variation in certain other *in vitro*

characteristics (de Rozieres *et al.*, 2008, Hohdatsu *et al.*, 1996, Miller *et al.*, 2011). Results from chapter 3 also supported findings from previous studies that showed a difference in disease progression in cats infected with genetically distinct isolates of FIV (Kohmoto *et al.*, 1998, Pedersen *et al.*, 2001). To the author's knowledge, this is the first study to demonstrate differences between FIV variants as a result of viral properties, using a marker that is biologically relevant to disease progression *in vivo*. This contributes to the current understanding of FIV-associated disease, as there is now evidence to suggest that viral characteristics, as well as host and environmental factors, are likely to be involved in determining the length of the asymptomatic period in FIV infected cats. Further work could focus on developing markers of pathogenicity for FIV, identifying the genetic sequences that influence the apoptotic potential of the virus. For a virus that has an unpredictable clinical course, and does not always cause disease in infected cats, this information could impact significantly on how FIV positive cats are managed in clinical practice.

The aim of the work presented in chapter 4 was to investigate whether vaccine-induced memory and effector T cells recognise antigen from field isolates of FIV. For this purpose, an *in vitro* assay was designed to measured antigen-specific activation of T cells from Fel-O-Vax® FIV vaccinated cats. In addition, a DTH response was measured in vaccinated cats following inoculation of the NZ isolates of FIV prepared in chapter 2. Results indicated that the Fel-O-Vax® FIV vaccine-induced immune response was at least partially cross-reactive against the NZ isolates of FIV tested. However, there were differences in the magnitude of the responses elicited, suggesting variability of the vaccine-induced immune response against each isolate *in vitro* and *in vivo*.

For some isolates, the vaccine-induced response was greater in magnitude when compared to that directed against the vaccine strain of FIV. In addition, the isolates generating the highest CD25 expression did not always elicit the strongest DTH response. These findings highlighted the fact that each assay demonstrated a different aspect of the immune response, and represented only the initial steps to an effective immune response. Although results suggested effective

recognition of antigen from field viruses, this is not necessarily predictive of protective immunity. For this reason, further investigation of vaccine efficacy under NZ conditions was indicated.

The hypothesis from chapter 5 was that the prevalence of FIV infection in vaccinated pet cats would be lower than the prevalence in unvaccinated pet cats, indicating at least some protection of Fel-O-Vax® FIV vaccinated cats against FIV in the field. Results indicated that this was not the case, suggesting that there was no protective effect of vaccination on the prevalence of FIV infection in the field. Given the difficulty in sequencing virus from the cats suspected to be infected with FIV, the results remain unconfirmed at this point, and further work is necessary to demonstrate the specificity of the qPCR assay used in this study. However, if these results are true, this information will undoubtedly impact use of the Fel-O-Vax® FIV vaccine in NZ.

Results presented in chapter 5 support the recent research by Westman *et al.* (2016a), which indicated poor effectiveness of the Fel-O-Vax® FIV vaccine in the field. However, results also suggested that the effectiveness of the vaccine in the NZ may be even lower when compared to the Australian field. If susceptibility of the vaccine-induced immune response is affected by genomic variation of the virus, this could explain the difference between the research presented in this thesis and the study in Australia. Phylogenetic analysis of FIV shows that Australian and NZ virus are genetically distinct, at least at the *env* gene (Greene *et al.*, 1993, Hayward *et al.*, 2007). The epitopes important for generating protective immunity are yet to be elucidated, however if the envelope glycoprotein is involved, as suspected, then genetic variation at the *env* gene could explain the apparent difference in vaccine efficacy between these two countries. Another point worth mentioning is the difference in recruitment method between the study by Westman *et al.* (2016a), and the work presented in chapter 5 of this thesis. Although the inclusion criteria stipulated that cats must have access to an outdoor environment to qualify for the study in chapter 5, this information was not specifically recorded on the submission form as it was for the Australian study. Therefore, differences in exposure to FIV between the vaccinated and unvaccinated cats cannot be excluded as a potential bias in chapter 5, so comparison between these two studies should be made with caution.

Reduced efficacy of the Fel-O-Vax® FIV vaccine against field virus compared to experimental isolates of FIV may be due to differences in viral characteristics. Laboratory passage of FIV favours the generation of CD134-independent virus. Similarly, viruses isolated from sick cats late in infection (which is often the origin of experimental isolates) are also often CD134-independent, and these viruses have been shown to be more susceptible to virus neutralising antibodies (Hosie *et al.*, 2011). In the field, cats are generally exposed to virus from asymptomatic FIV infected cats, as cats in the advanced stages of AIDS are less likely to be fighting with other cats. For this reason, as well as the variation in antigenicity among FIV variants, it is perhaps not surprising that retrospective field studies indicate reduced effectiveness of the vaccine compared to experimental challenge studies.

The research presented in this thesis suggested that the Fel-O-Vax® FIV vaccine is ineffective in preventing infection with FIV in NZ, however, the worth of a vaccine does not depend solely on its ability to generate sterilising immunity. One example of a widely recommended vaccine that produces non-sterilising immunity is the feline herpesvirus vaccine, which significantly reduces the severity of disease in infected cats (Gaskell *et al.*, 2007). Given this, perhaps the veterinary community should revise their expectations of the Fel-O-Vax® FIV vaccine, and consider its use in preventing progression of disease in cats infected with FIV, and in reducing transmission of the virus. In naturally infected cats, $CD8^+$ T cells are involved in maintaining a low viral load in the asymptomatic stage of infection, and this is at least one factor that limits progression of disease (Hohdatsu *et al.*, 2005, Hohdatsu *et al.*, 2003b). The results in Chapter 4 of this thesis failed to demonstrate the presence of FIV-specific $CD8^+$ T cells. As discussed, this may in part be due to low power of the study in detecting such a small number of cells. However, it is equally possible that the vaccine does not elicit such a response. Although cross-presentation of antigen to MHC I can occur with inactivated vaccines, the cytotoxic response is likely to be weaker when compared to the humoral response. Further study is needed to determine the efficacy of the Fel-O-Vax® FIV vaccine in reducing disease progression and

transmission of the virus, and in determining whether humoral factors are also involved in prolonging the asymptomatic phase of infection.

When considering efficacy of the Fel-O-Vax® FIV vaccine for preventing disease rather than infection, the possibility of vaccine enhancement of infection also needs to be evaluated. Infection-enhancing antibodies have been described in patients infected with HIV (Subbramanian *et al.*, 2002), and previous recombinant DNA vaccines for FIV have also been unsuccessful due to antibody dependent enhancement of infection (Siebelink *et al.*, 1995d, Richardson *et al.*, 1997). Results from the study by Dunham *et al.* (2006b) suggested vaccine-enhancement of infection, as increased viral loads were demonstrated in the vaccinated FIV-infected cats compared to the unvaccinated infected cats. In chapter 5, although not statistically significant, the prevalence of FIV was actually higher in the vaccinated, compared to the unvaccinated cat population. Potential mechanisms for vaccine enhancement of lentivirus infection include antibody-dependent enhancement, activation of $CD4^+$ T cells and activation of dendritic cells (Huisman *et al.*, 2009). Further studies are required to investigate whether enhancement of infection occurs in FIV infected, vaccinated cats, prior to considering use of the Fel-O-Vax® FIV vaccine for generating non-sterilising immunity.

Apart from the current lack of evidence for efficacy of the Fel-O-Vax® FIV vaccine in reducing disease progression in FIV infected cats, there are other issues associated with use of this vaccine that warrant careful consideration. A vaccine with partial efficacy in preventing infection could favour the expansion of vaccine-resistant variants of FIV in the cat population. Results from chapter 3 suggested that FIV variants differ in pathogenicity. Use of the Fel-O-Vax® FIV vaccine could therefore potentially alter the spectrum of FIV-associated disease, as a result of vaccine-induced selection pressure. Another factor to consider when deciding whether to administer the Fel-O-Vax® FIV vaccine, is the risk of injection site sarcomas. This vaccine contains inactivated virus, so an adjuvant is included to augment the immune response generated by the antigen. The use of adjuvanted vaccines have been linked to the development of feline injection site sarcomas, due to the local inflammatory response that is generated by such vaccines

(Day *et al.*, 2007). Current guidelines recommend the use of non-adjuvanted vaccines in cats where possible. If the Fel-O-Vax® FIV vaccine is to be recommended in cats at risk of FIV infection, then there should be substantial evidence that the benefit of this vaccine outweighs the risks of developing an injection site sarcoma.

The experiments in this thesis were not designed to investigate differences between subtypes of FIV, however results do highlight that subtype classification of FIV is not a clinical useful characterisation, as it does not predict pathogenicity or susceptibility to the vaccine-induced immunity. At least one commercial laboratory (Idexx Laboratories Pty Ltd., Australia) is now providing subtype classification of viruses detected using qPCR. This could be useful from an academic point of view, as it gives an idea of the degree of genetic variation between two viruses (i.e. >15% variation if belonging to different subtypes), but it really provides no useful information to the clinician requesting the test.

In summary, the research presented in this thesis suggests that FIV variants differ in pathogenicity, at least *in vitro*. Further study is indicated to investigate which viral factors influence pathogenicity, and the mechanisms by which they do so. In addition, the research presented in this thesis research failed to provide evidence that the Fel-O-Vax® FIV vaccine prevents infection with FIV in the NZ field. Further studies are required to determine the efficacy of the vaccine in minimising disease progression, and exclude the possibility of vaccine enhancement of infection. Until such work is performed, use of the Fel-O-Vax® FIV vaccine cannot currently be recommended in NZ. There is still much to learn about the relevance of genomic variation on clinical aspects of FIV infection, however there is now at least some evidence to suggest that viral factors influence the pathogenicity and susceptibility of FIV to the vaccine-induced immune response. Hopefully this work will provide the foundation to further study in this area, enhancing our overall understanding of FIV infection.

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Appendix 1

Submission form for recruitment of FIV positive cats

MASSEY UNIVERSITY - FIV STUDY

Participation Consent Form

I, _____ consent to participating in the FIV study currently being undertaken at Massey University, Palmerston Nth.

I understand that my cat, _____ will be required to visit my local vet clinic on two separate occasions. On each occasion, a blood sample will be collected for submission to Massey University.

I agree to my veterinarian supplying you with my cat's clinical records, and to being contacted directly by Massey University if additional details are required for the purposes of the study.

Signed _____ Date _____

Contact details:

Name	
Address	
Phone number (home) (mobile)	
Email	

Appendix 2

Summary of results from FIV seropositive cats recruited from various veterinary practices

Isolate	PCR result	Subtype	Clinical signs
TVF001	Positive	Not sequenced	Lymphoma
MUVTH001	Positive	C	Euthanased for unknown illness
MUVTH002	Positive	C	Chronic kidney disease
MUVTH003	Positive	C	Nasopharyngeal polyp
MUVTH004	Positive	Not sequenced	Unknown
CVK001	Positive	C	Asymptomatic
CVK002	Positive	Not sequenced	Asymptomatic
CVK003	Positive	C	Chronic rhinitis
RVC001	Positive	A/C recombinant	Asymptomatic
RVC002	Positive	C	Asymptomatic
RVC003	Negative	Not sequenced	Asymptomatic
RVC004	Positive	C	Asymptomatic
RVC005	Positive	A	Euthanased for unknown illness
RVC006	Positive	Not sequenced	Asymptomatic
RVC007	Positive	C	Asymptomatic
RVC008	Positive	C	Asymptomatic
RVC009	Positive	A	Asymptomatic
CSV001	Positive	Not sequenced	Cellulitis
SRV001	Positive	C	Asymptomatic
SRV002	Negative	Not sequenced	Asymptomatic
SRV003	Positive	Not sequenced	Asymptomatic
SRV004	Positive	Not sequenced	Asymptomatic

Appendix 3

Summary of troubleshooting process for real-time *gag* PCR reaction

Reverse transcrip. Method	**Enzyme (5.0µL)**	**Temp. vol.(µ/ 10µL)**	**Primers**	**Primr vol. (µ of 20µM)**	**Cycle conditions**						**Results**
					RT step	**Initial denat.**	**Denat.**	**Anneal.**	**Elong.**	**No.**	
Two-step	SYTO 9 [12]	2.0	FIVf FIVr	0.15 0.15	N/A	95ºC 5min	95ºC 5s	64ºC 10s	70ºC 8s	50	Correct amplification of product, but non-specific amplification of NTC
One-step [13] (0.5µL)	SYTO 9	2.0	FIVf FIVr	0.15 0.15	50ºC 10min	95ºC 5min	95ºC 5s	64-58ºC 10s	70ºC 10s	50	No amplification
Two-step (RT cycle with **no enzyme**)	SYTO 9	2.0	FIVf FIVr	0.15 0.15	50ºC 10min	95ºC 5min	95ºC 5s	64-58ºC 10s	70ºC 10s	50	Amplification
One-step[14] (0.5µL RT)	SYBR green[15]	2.0	FIVf FIVr	0.15 0.15	50ºC 10min	95ºC 5min	95ºC 5s	64-58ºC 20s	70ºC 10s	50	No amplification
One-step (**0.2µL** RT)	SYBR green	2.0	FIVf FIVr	0.15 0.15	50ºC 40min	95ºC 10min	95ºC 10s	64-58ºC 20s	70ºC 30s	35	No amplification
One-step (0.2µL)	SYBR green	4.0	FIVf FIVr	0.15 0.15	50ºC 10min	95ºC 5min	95ºC 10s	64-58ºC 20s	70ºC 30s	35	No amplification
One-step (0.2µL)	SYBR green	2.0	FIVf FIVr	0.30 0.30	50ºC 10min	95ºC 5min	95ºC 10s	64-58ºC 20s	70ºC 30s	35	Non-specific amplification confirmed by gel electrophoresis
Two-step	SYBR green	2.0	FIVf FIVr	0.15 0.15	N/A	95ºC 5min	95ºC 10s	64-58ºC 20s	70ºC 30s	35	Amplification with minor non-specific amplification of NTC

[12] Quanta Accumelt HRM supermix
[13] Transcriptor High Fidelity Reverse Transcriptase
[14] Quanta qScript one-step SYBR green RT-PCR kit
[15] Quanta qScript one-step SYBR green RT-PCR kit

One-step (0.2µL)	SYBR green	2.0	FIVf FIVr	1.0 0.1	50ºC 10min	95ºC 5min	95ºC 10s	64-58ºC 20s	70ºC 30s	50	Amplification of product but also significant non-specific amplification (confirmed by gel electrophoresis)
One-step (0.2µL)	SYBR green	2.0	FIVf FIVr	**Primer matrix** (FIVf:FIVr) 4:1[16] 4:1[17] 2:1[18] 1:2[19] 1:2[20] 1:4[21] 1:4[22]	50ºC 10min	95ºC 5min	95ºC 10s	64-58ºC 20s	70ºC 30s	50	Some (delayed) amplification of product with 4:1 ratio of FIVf:FIVr but non-specific amplification with all conditions, confirmed by gel electrophoresis
One-step (0.2µL)	SYBR green +/- random hexa-mers	2.0	FIVf FIVr	0.2 0.05	50ºC 10min	95ºC 5min	95ºC 10s	64-58ºC 10s	72ºC 10s	45	Minor specific amplification but mostly non-specific as confirmed by gel electrophoresis
Two-step	SYBR green	2.0	FIVf FIVr	0.15 0.15	N/A	95ºC 5min	95ºC 10s	64-58ºC 20s	72ºC 30s	45	Amplification of product with minor non-specific amplification as confirmed on gel electrophoresis
Two-step	SYBR green OR **SYTO 9** (for each primer conc)	2.0	FIVf FIVr	FIVf :FIVr (µL) 0.1:0.1 0.2:0.2 0.3:0.3 0.4:0.4	N/A	95ºC 5min 95ºC 5min	95ºC 10s 95ºC 5s	64-58ºC 20s 64-58ºC 10s	72ºC 30s 72ºC 10s	45	SYBR green reactions show non-specific amplification compared to SYTO 9 reactions. Asymmetric primer vol. of FIVf:FIVr 0.1:0.5µL resulted in specific amplification of product

[16] FIVf : FIVr = 800µM : 200µM
[17] FIVf : FIVr = 400µM : 100µM
[18] FIVf : FIVr = 200µM : 100µM
[19] FIVf : FIVr = 100µM : 200µM
[20] FIVf : FIVr = 200µM : 400µM
[21] FIVf : FIVr = 100µM : 400µM
[22] FIVf : FIVr = 200µM : 800µM

				0.5:0.5 0.1:0.5 0.5:0.1 0.3:0.5 0.5:0.3							
Two-step (standard curve)	SYTO 9	2.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	64-58ºC 10s	72ºC 10s	45	Efficiency **107%** R^2 value **0.991**
Two-step (standard curve repeated on **new thermo-cycler**)	SYTO 9	2.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	58ºC 10s	72ºC 10s	45	Efficiency **59.5%** R^2 value **0.911** Non-specific amplification of NTC
Two-step (using **new reagents** to rule-out contamin.)	SYTO 9	2.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	58ºC 10s	72ºC 10s	45	Non-specific amplification of NTC
Two-step (standard curve)	SYTO 9	2.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	58ºC 10s	72ºC 10s	35	Amplification of product only. Efficiency **78.2%** R^2 value **0.987**
Two-step (standard curve)	SYTO 9	2.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	58ºC 13s	72ºC 15s	40	Non-specific amplification. Efficiency **98.6%** R^2 value **0.963**
Two-step (standard curve)	SYTO 9	4.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	58ºC 13s	72ºC 15s	35	Non-specific amplification. Efficiency **159%** R^2 value **0.750**
Two-step (standard curve)	SYTO 9	2.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	58ºC 13s	72ºC 15s	35	Amplification of products. Efficiency **86.4%** R^2 value **0.999**
Two-step (standard curve)	SYTO 9	2.0	FIVf FIVr	0.1 0.5	N/A	95ºC 5min	95ºC 5s	58ºC 16s	72ºC 15s	35	Amplification of products. Efficiency **92.2%** R^2 value **0.999**

Appendix 4

Chapter 2 raw data

Endpoint dilution assay results

PetF14		10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Presence of FIV DNA	Live virus	+	+	+	+	+	-	-	-	-	-	-	-	-
		+	+	+	+	-	-	-	-	-	-	-	-	-
	Inact. virus	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
		(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
% infected		100	100	100	100	50	0	0	0	0	0	0	0	0

Proportionate distance	0
50% endpoint dilution	10^{-4}
$TCID_{50}$ / ml	$10^{5.3}$

MUVTH002		10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Presence of FIV DNA	Live virus	+	+	+	+	+	-	-	-	-	-	-	-	-
		+	+	+	+	+	-	-	-	-	-	-	-	-
	Inact. virus	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
		(+)	(+)	-	-	-	-	-	-	-	-	-	-	-
% infected		100	100	100	100	100	0	0	0	0	0	0	0	0

Proportionate distance	0.5
50% endpoint dilution	$10^{-4.5}$
$TCID_{50}$ / ml	$10^{5.8}$

MUVTH003		10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Presence of FIV DNA	Live virus	+	+	+	+	-	-	-	-	-	-	-	-	-
		+	+	+	+	-	-	-	-	-	-	-	-	-
	Inact. virus	(+)	(+)	-	-	-	-	-	-	-	-	-	-	-
		(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
% infected		100	100	100	100	0	0	0	0	0	0	0	0	0

Proportionate distance	0.5
50% endpoint dilution	$10^{-3.5}$
$TCID_{50}$ / ml	$10^{4.8}$

CVK001		**10**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**	**10^{-11}**	**10^{-12}**
Presence of FIV DNA	Live virus	+	+	+	+	+	-	-	-	-	-	-	-	-
		+	+	+	+	+	-	-	-	-	-	-	-	-
	Inact. virus	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
		(+)	(+)	-	-	-	-	-	-	-	-	-	-	-
% infected		100	100	100	100	100	0	0	0	0	0	0	0	0

Proportionate distance	0.5
50% endpoint dilution	$10^{-4.5}$
$TCID_{50}$ / ml	$10^{5.8}$

RVC009		**10**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**	**10^{-11}**	**10^{-12}**
Presence of FIV DNA	Live virus	+	+	+	+	+	-	-	-	-	-	-	-	-
		+	+	+	+	+	-	-	-	-	-	-	-	-
	Inact. virus	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-
		(+)	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-
% infected		100	100	100	100	100	0	0	0	0	0	0	0	0

Proportionate distance	0.5
50% endpoint dilution	$10^{-4.5}$
$TCID_{50}$ / ml	$10^{5.8}$

Appendix 5

Chapter 3 raw data

Comparison of apoptosis and necrosis induced by different isolates of FIV

Day	Treatment	% necrotic cells	% apoptotic cells	% viable cells	MFI 7AAD	MFI Annexin-V
1	CVK001 1	2.88	10.8	85.7	7.12	579
1	CVK001 2	5.5	14.8	78.9	7.25	720
1	MUVTH002 1	4.5	15.6	79.2	7.62	721
1	MUVTH002 2	1.65	12.7	84.9	5.75	612
1	MUVTH002 3	3.45	12.6	82.8	6.12	645
1	MUVTH003 1	4.74	18.1	75.8	7.14	832
1	MUVTH003 2	5.71	33.3	57.9	7.4	1454
1	MUVTH003 3	4.96	18.7	75.9	6.21	880
1	PetF14 1	*33.3*	*33.3*	*0*	*438*	*3254*
1	PetF14 2	5.31	15.5	78.4	8.29	735
1	PetF14 3	3.68	13.1	82.9	7.73	681
1	RVC009 1	8.64	38.8	52	6.85	1818
1	RVC009 2	8.66	28.6	62.1	9.92	1381
1	RVC009 3	6.91	20.6	71.4	9.11	897
1	Untreated 1	1.86	7.86	89.7	7.33	484
1	Untreated 2	2.43	9.78	87.2	7.76	499
1	Untreated 3	4.48	12.3	82.7	8.92	589
1	Used media 1	2.55	12	84.9	6.67	578
1	Used media 2	1.99	11.1	86.5	6.21	570
1	Used media 3	3.59	11.6	84.2	7.4	623
1	Virus filtrate 1	1.5	7.4	90.7	6.01	482
1	Virus filtrate 2	3.76	13.7	82.1	9.22	658
1	Virus filtrate 3	2.32	10.3	86.6	6.81	582
3	CVK001 1	1.68	1.36	96.4	26	35.2
3	CVK001 2	1.59	1.81	96.2	25.7	31.8
3	CVK001 3	1.87	1.17	96.4	25.1	26.5
3	MUVTH002 1	1.38	1.87	96.4	23.2	37.3
3	MUVTH002 2	1.99	1.35	96.1	24.3	33.1
3	MUVTH002 3	2.46	1.02	95.8	25.6	27.8
3	MUVTH003 1	2.61	2.37	94.5	23	57.5
3	MUVTH003 2	2.02	2.16	95.2	25	47.5
3	MUVTH003 3	2.74	2.36	94.5	25.1	48.4
3	Pet F14 1	2.99	2.44	94	25	45.8
3	Pet F14 2	1.63	2.26	95.6	22.6	38.5
3	Pet F14 3	1.78	2.15	95.4	23.3	40.4
3	RVC009 1	4.76	2.56	92.3	28.9	57.7
3	RVC009 2	4.1	1.87	93.7	27	41.9
3	RVC009 3	4.77	2.88	91.9	26.6	35.2
3	Untreated 1	1.53	1.89	96.2	23	48.7
3	Untreated 2	1.54	1.89	96.2	24.6	42.3
3	Untreated 3	1.43	1.54	96.7	24.2	46.5
3	Media 1	2.35	1.74	95.6	26	47.8
3	Media 2	2.39	1.56	95.6	25.2	34.5
3	Media 3	1.91	1.64	95.9	22.2	43.6

Day	Treatment	% necrotic cells	% apoptotic cells	% viable cells	MFI 7AAD	MFI Annexin-V
3	Virus filtrate 1	1.96	1.31	96.2	25.1	41.6
3	Virus filtrate 2	1.38	1.02	97.4	24.2	36.8
3	Virus filtrate 3	2.68	1.59	95.2	26.3	42.3
8	CVK001 1	9.56	9.17	76.7	36.5	34
8	CVK001 2	11.9	11.8	73.6	40.4	42.8
8	CVK001 3	13.4	14.2	69.7	41.2	52.5
8	MUVTH002 1	15.5	34.7	49	41.3	154
8	MUVTH002 2	11.5	17.7	66.9	44.5	60.1
8	MUVTH002 3	12.6	14.9	66.6	50	61.6
8	MUVTH003 1	13.4	18.3	65.7	48	73.4
8	MUVTH003 2	12.1	19.5	65.9	46.8	76
8	MUVTH003 3	10.9	20.3	66.2	42.2	70.2
8	PetF14 1	15.5	24.2	57.9	46	94.2
8	PetF14 2	15	25	57.9	46	89.8
8	PetF14 3	9.78	18.7	67.2	43.1	59.9
8	RVC009 1	19.9	43.2	36.3	53	253
8	RVC009 2	6.31	13.2	78.2	29.1	48.8
8	RVC009 3	10.7	33.2	55.2	36.1	117
8	Untreated 1	8.83	15.9	74.9	36.1	58.8
8	Untreated 2	4.54	6.2	87.6	25.5	23.8
8	Untreated 3	6.3	7.18	85.2	30.7	31.8
8	Media 1	4.58	8.87	85.6	29	30.8
8	Media 2	5.35	8.15	85.6	30.2	31.7
8	Media 3	7.31	7.95	83.7	31.5	32.9
8	Virus filtrate 1	5.32	11.4	82.4	29.8	38.1
8	Virus filtrate 2	12.1	18.8	68.7	39.7	77.6
8	Virus filtrate 3	6.16	6.38	86.7	29.5	31
10	CVK001 1	12.7	8.8	73	38.9	58.8
10	CVK001 2	12.5	7.41	73	37.9	61.9
10	CVK001 3	18.2	11.6	65.3	44	84.7
10	MUVTH002 1	10.1	9.56	66	48.3	83.1
10	MUVTH002 2	16.7	15.2	62.1	46.2	115
10	MUVTH002 3	13.3	14.4	63.4	43.1	104
10	MUVTH003 1	14	12	67.7	44.6	94.8
10	MUVTH003 2	14.2	12.3	67.2	45.3	101
10	MUVTH003 3	13.5	11.1	67.2	46.7	94.8
10	PetF14 1	12.9	17.2	65	35.3	93.3
10	PetF14 2	14.5	16.7	61.1	44.8	115
10	PetF14 3	14.3	15.9	61.5	48	111
10	RVC009 1	7.54	17.9	70.8	27.5	106
10	RVC009 2	8.08	13.5	73.6	29.1	93.5
10	RVC009 3	8.83	16	71.4	28.1	108
10	Untreated 1	4.88	4.58	88.9	23.5	41.9
10	Untreated 2	7.34	3.86	87	26.3	45.4
10	Untreated 3	7.24	8.27	83.9	28	63.8
10	Media 1	4.97	5.67	88.1	23.8	46.5
10	Media 2	4.27	4.59	90.2	22.4	40.9
10	Media 3	5.72	3.87	89	23.9	39.1
10	Virus filtrate 1	5.88	4.44	88.3	21.7	38.4
10	Virus filtrate 2	5.67	4.33	88.8	24	37.9
10	Virus filtrate 3	6.49	3.4	88.6	22.2	40.2

Effect of FIV infection on MYA-1 cell concentration in culture

Day	Treatment	Cell flow rate (cells/sec)
1	Untreated	47.39
1	Untreated	35.09
1	Untreated	36.10
1	Media	22.01
1	Media	21.61
1	Media	22.24
1	Virus filtrate	25.01
1	Virus filtrate	20.30
1	Virus filtrate	21.99
1	CVK001	29.15
1	CVK001	20.46
1	MUVTH002	17.37
1	MUVTH002	17.29
1	MUVTH002	14.76
1	MUVTH003	14.45
1	MUVTH003	21.29
1	MUVTH003	7.60
1	PetF14	20.03
1	PetF14	28.74
1	RVC009	7.32
1	RVC009	15.47
1	RVC009	17.22
3	Untreated	333.33
3	Untreated	322.58
3	Untreated	454.55
3	Media	384.62
3	Media	416.67
3	Media	357.14
3	Virus filtrate	370.37
3	Virus filtrate	555.56
3	Virus filtrate	526.32
3	CVK001	526.32
3	CVK001	181.82
3	CVK001	526.32
3	MUVTH002	625.00
3	MUVTH002	625.00
3	MUVTH002	769.23
3	MUVTH003	500.00
3	MUVTH003	625.00
3	MUVTH003	400.00
3	PetF14	526.32
3	PetF14	526.32

Day	Treatment	Cell flow rate (cells/sec)
3	PetF14	588.24
3	RVC009	45.87
3	RVC009	285.71
3	RVC009	36.50
8	Untreated	833.33
8	Untreated	1250.00
8	Media	1250.00
8	Media	1111.11
8	Media	1428.57
8	Virus filtrate	909.09
8	Virus filtrate	1250.00
8	CVK001	1428.57
8	CVK001	1250.00
8	CVK001	1000.00
8	MUVTH002	263.16
8	MUVTH002	1428.57
8	MUVTH002	1250.00
8	MUVTH003	1000.00
8	MUVTH003	1111.11
8	MUVTH003	909.09
8	PetF14	909.09
8	PetF14	1000.00
8	PetF14	1250.00
8	RVC009	108.70
8	RVC009	1666.67
8	RVC009	666.67
10	Untreated	909.09
10	Untreated	1428.57
10	Untreated	666.67
10	Media	1111.11
10	Media	1428.57
10	Media	1428.57
10	Virus filtrate	1428.57
10	Virus filtrate	1250.00
10	Virus filtrate	1111.11
10	CVK001	1250.00
10	CVK001	1666.67
10	CVK001	1000.00
10	MUVTH002	1666.67
10	MUVTH002	1111.11
10	MUVTH002	1250.00
10	MUVTH003	1111.11
10	MUVTH003	1250.00
10	MUVTH003	1111.11

Day	Treatment	Cell flow rate (cells/sec)
10	PetF14	1111.11
10	PetF14	1666.67
10	PetF14	1250.00
10	RVC009	1428.57
10	RVC009	1250.00
10	RVC009	1428.57

Inhibition of mitogen-induced lymphocyte proliferation by different isolates of FIV

Treatment	Cat	Raw cpm	Mean cpm unstimulated	Normalised cpm	SI (raw cpm/mean cpm unstimated)
PetF14	A	3380	237.5	3142.5	14.23158
PetF14	A	1595	237.5	1357.5	6.715789
PetF14	A	1887	237.5	1649.5	7.945263
PetF14	A	1613	237.5	1375.5	6.791579
PetF14	B	4351	650.25	3700.75	6.691273
PetF14	B	3043	650.25	2392.75	4.679739
PetF14	B	2946	650.25	2295.75	4.530565
PetF14	B	2641	650.25	1990.75	4.061515
PetF14	C	1263	462.5	800.5	2.730811
PetF14	C	1377	462.5	914.5	2.977297
PetF14	C	2065	462.5	1602.5	4.464865
PetF14	C	2269	462.5	1806.5	4.905946
PetF14	D	3234	92.75	3141.25	34.86792
PetF14	D	2209	92.75	2116.25	23.81671
PetF14	D	2700	92.75	2607.25	29.11051
PetF14	D	2615	92.75	2522.25	28.19407
PetF14	E	1983	383.25	1599.75	5.174168
PetF14	E	1311	383.25	927.75	3.420744
PetF14	E	1447	383.25	1063.75	3.775603
PetF14	E	1662	383.25	1278.75	4.336595
PetF14	F	1395	128	1267	10.89844
PetF14	F	1526	128	1398	11.92188
PetF14	F	1315	128	1187	10.27344
PetF14	F	1561	128	1433	12.19531
MUVTH002	A	2011	237.5	1773.5	8.467368
MUVTH002	A	1841	237.5	1603.5	7.751579
MUVTH002	A	1630	237.5	1392.5	6.863158
MUVTH002	A	1520	237.5	1282.5	6.4
MUVTH002	B	2416	650.25	1765.75	3.715494
MUVTH002	B	1401	650.25	750.75	2.154556
MUVTH002	B	3019	650.25	2368.75	4.64283
MUVTH002	B	1843	650.25	1192.75	2.834295
MUVTH002	C	2034	462.5	1571.5	4.397838
MUVTH002	C	1805	462.5	1342.5	3.902703
MUVTH002	C	1920	462.5	1457.5	4.151351
MUVTH002	C	2677	462.5	2214.5	5.788108
MUVTH002	D	2140	92.75	2047.25	23.07278
MUVTH002	D	3886	92.75	3793.25	41.89757
MUVTH002	D	2320	92.75	2227.25	25.01348
MUVTH002	D	2397	92.75	2304.25	25.84367
MUVTH002	E	1711	383.25	1327.75	4.464449
MUVTH002	E	1384	383.25	1000.75	3.61122
MUVTH002	E	1845	383.25	1461.75	4.81409
MUVTH002	E	881	383.25	497.75	2.298761
MUVTH002	F	1179	128	1051	9.210938
MUVTH002	F	1153	128	1025	9.007813
MUVTH002	F	871	128	743	6.804688
MUVTH002	F	1808	128	1680	14.125
MUVTH003	A	2097	237.5	1859.5	8.829474

Treatment	Cat	Raw cpm	Mean cpm unstimulated	Normalised cpm	SI (raw cpm/mean cpm unstimated)
MUVTH003	A	1768	237.5	1530.5	7.444211
MUVTH003	A	1359	237.5	1121.5	5.722105
MUVTH003	A	1447	237.5	1209.5	6.092632
MUVTH003	B	2070	650.25	1419.75	3.183391
MUVTH003	B	3027	650.25	2376.75	4.655133
MUVTH003	B	1078	650.25	427.75	1.657824
MUVTH003	B	1695	650.25	1044.75	2.60669
MUVTH003	C	2355	462.5	1892.5	5.091892
MUVTH003	C	2824	462.5	2361.5	6.105946
MUVTH003	C	2696	462.5	2233.5	5.829189
MUVTH003	C	2585	462.5	2122.5	5.589189
MUVTH003	D	1638	92.75	1545.25	17.66038
MUVTH003	D	2927	92.75	2834.25	31.55795
MUVTH003	D	2123	92.75	2030.25	22.88949
MUVTH003	D	1900	92.75	1807.25	20.48518
MUVTH003	E	2582	383.25	2198.75	6.737117
MUVTH003	E	1417	383.25	1033.75	3.697326
MUVTH003	E	1563	383.25	1179.75	4.078278
MUVTH003	E	1961	383.25	1577.75	5.116765
MUVTH003	F	1161	128	1033	9.070313
MUVTH003	F	1086	128	958	8.484375
MUVTH003	F	883	128	755	6.898438
MUVTH003	F	1313	128	1185	10.25781
CVK001	A	1896	237.5	1658.5	7.983158
CVK001	A	1772	237.5	1534.5	7.461053
CVK001	A	1748	237.5	1510.5	7.36
CVK001	A	1985	237.5	1747.5	8.357895
CVK001	B	2654	650.25	2003.75	4.081507
CVK001	B	1417	650.25	766.75	2.179162
CVK001	B	2124	650.25	1473.75	3.266436
CVK001	B	2728	650.25	2077.75	4.195309
CVK001	C	2353	462.5	1890.5	5.087568
CVK001	C	2454	462.5	1991.5	5.305946
CVK001	C	3025	462.5	2562.5	6.540541
CVK001	C	2755	462.5	2292.5	5.956757
CVK001	D	1849	92.75	1756.25	19.93531
CVK001	D	2008	92.75	1915.25	21.6496
CVK001	D	2617	92.75	2524.25	28.21563
CVK001	D	2241	92.75	2148.25	24.16173
CVK001	E	1853	383.25	1469.75	4.834964
CVK001	E	1825	383.25	1441.75	4.761905
CVK001	E	1933	383.25	1549.75	5.043705
CVK001	E	2348	383.25	1964.75	6.126549
CVK001	F	889	128	761	6.945313
CVK001	F	1668	128	1540	13.03125
CVK001	F	775	128	647	6.054688
CVK001	F	952	128	824	7.4375
RVC009	A	1672	237.5	1434.5	7.04
RVC009	A	1382	237.5	1144.5	5.818947
RVC009	A	2344	237.5	2106.5	9.869474

Treatment	Cat	Raw cpm	Mean cpm unstimulated	Normalised cpm	SI (raw cpm/mean cpm unstimated)
RVC009	A	2020	237.5	1782.5	8.505263
RVC009	B	2148	650.25	1497.75	3.303345
RVC009	B	1799	650.25	1148.75	2.766628
RVC009	B	2018	650.25	1367.75	3.103422
RVC009	B	2255	650.25	1604.75	3.467897
RVC009	C	2198	462.5	1735.5	4.752432
RVC009	C	2195	462.5	1732.5	4.745946
RVC009	C	2200	462.5	1737.5	4.756757
RVC009	C	2292	462.5	1829.5	4.955676
RVC009	D	3648	92.75	3555.25	39.33154
RVC009	D	3898	92.75	3805.25	42.02695
RVC009	D	2983	92.75	2890.25	32.16173
RVC009	D	4522	92.75	4429.25	48.75472
RVC009	E	1565	383.25	1181.75	4.083496
RVC009	E	1979	383.25	1595.75	5.163731
RVC009	E	1610	383.25	1226.75	4.200913
RVC009	E	1750	383.25	1366.75	4.56621
RVC009	F	1025	128	897	8.007813
RVC009	F	829	128	701	6.476563
RVC009	F	1460	128	1332	11.40625
RVC009	F	685	128	557	5.351563
ConA	A	2646	237.5	2408.5	11.14105
ConA	A	2219	237.5	1981.5	9.343158
ConA	A	2053	237.5	1815.5	8.644211
ConA	A	1807	237.5	1569.5	7.608421
ConA	B	2275	650.25	1624.75	3.498654
ConA	B	2659	650.25	2008.75	4.089196
ConA	B	1791	650.25	1140.75	2.754325
ConA	B	1387	650.25	736.75	2.133026
ConA	C	1434	462.5	971.5	3.100541
ConA	C	2164	462.5	1701.5	4.678919
ConA	C	2158	462.5	1695.5	4.665946
ConA	C	2858	462.5	2395.5	6.179459
ConA	D	2880	92.75	2787.25	31.05121
ConA	D	2185	92.75	2092.25	23.55795
ConA	D	2015	92.75	1922.25	21.72507
ConA	D	2985	92.75	2892.25	32.18329
ConA	E	1978	383.25	1594.75	5.161122
ConA	E	2406	383.25	2022.75	6.277886
ConA	E	1832	383.25	1448.75	4.78017
ConA	E	1477	383.25	1093.75	3.853881
ConA	F	1050	128	922	8.203125
ConA	F	1253	128	1125	9.789063
ConA	F	1415	128	1287	11.05469
ConA	F	1559	128	1431	12.17969

Appendix 6

Chapter 3 statistics

Comparison of apoptosis and necrosis induced by different variants of FIV

General linear model of ANOVA tables examining the effect of control conditions on apoptosis and necrosis

Statistic – Percentage of apoptotic (single positive) cells

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)	3	514.820	171.607	24.62	0.000
Treatment (control condition)	2	1.185	0.593	0.09	0.919
Error	30	209.077	6.969		
Lack-of-Fit	6	29.547	4.925	0.66	0.683
Pure Error	24	179.529	7.480		
Total	35	725.082			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
2.63993	71.17%	66.36%	58.48%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		6.779	0.440	15.41	0.000	
Time (day)	1	3.892	0.762	5.11	0.000	1.50
	3	-5.204	0.762	-6.83	0.000	1.50
	8	3.313	0.762	4.35	0.000	1.50
Treatment	Untreated	-0.009	0.622	-0.01	0.989	1.33
(control cond.)	Used media	-0.218	0.622	-0.35	0.729	1.33

Statistic – Percentage of necrotic (double positive) cells

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)	3	147.765	49.255	23.42	0.000
Treatment (control condition)	2	2.923	1.461	0.70	0.507
Error	30	63.082	2.103		
Lack-of-Fit	6	8.477	1.413	0.62	0.712
Pure Error	24	54.605	2.275	24	54.605
Total	35	213.770			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
1.45008	70.49%	65.57%	57.51%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		4.294	0.242	17.77	0.000	
Time (day)	1	-1.574	0.419	-3.76	0.001	1.50
	3	-2.387	0.419	-5.70	0.000	1.50
	8	2.427	0.419	5.80	0.000	1.50
Treatment	Untreated	0.072	0.342	0.21	0.834	1.33
(control cond.)	Used media	-0.379	0.342	-1.11	0.276	1.33

Statistic – MFI for Annexin-V

Analysis of variance						
Source		**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)		3	1830566	610189	557.01	0.000
Treatment (control condition)		2	1055	527	0.48	0.623
Error		30	32864	1095		
Lack-of-Fit		6	6821	1137	1.05	0.420
Pure Error		24	26043	1085		
Total		35	1864485			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
33.0980	98.24%	97.94%	97.46%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		172.21	5.52	31.22	0.000	
Time (day)	1	390.56	9.55	40.88	0.000	1.50
	3	-129.54	9.55	-13.56	0.000	1.50
	8	-132.60	9.55	-13.88	0.000	1.50
Treatment	Untreated	-7.63	7.80	-0.98	0.336	1.33
(control cond.)	Used media	4.35	7.80	0.56	0.581	1.33

Statistic – MFI for 7AAD

Analysis of variance						
Source		**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)		3	2801.91	933.969	147.59	0.000
Treatment (control condition)		2	6.49	3.246	0.51	0.604
Error		30	189.84	6.328		
Lack-of-Fit		6	29.20	4.866	0.73	0.632
Pure Error		24	160.64	6.693		
Total		35	2998.24			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
2.51556	93.67%	92.61%	90.88%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		21.804	0.419	52.00	0.000	
Time (day)	1	-14.434	0.726	-19.88	0.000	1.50
	3	2.730	0.726	3.76	0.001	1.50
	8	9.530	0.726	13.12	0.000	1.50
Treatment	Untreated	0.356	0.593	0.60	0.553	1.33
(control cond.)	Used media	-0.597	0.593	-1.01	0.322	1.33

General linear model of ANOVA tables examining the effect of FIV isolates on apoptosis and necrosis

Statistic – Percentage of apoptotic (single positive) cells

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)	3	3440.1	1146.69	43.58	0.000
Treatment (virus)	5	1804.1	360.83	13.71	0.000
Error	85	2236.7	26.31		
Lack-of-Fit	15	920.3	61.36	3.26	0.000
Pure Error	70	1316.4	18.81		
Total	93	7476.5			

Model Summary		
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
5.12974	70.08%	67.27%	62.56%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		12.700	0.580	21.89	0.000	
Time (day)	1	4.690	0.939	4.99	0.000	1.54
	3	-9.395	0.910	-10.32	0.000	1.51
	8	5.817	0.910	6.39	0.000	1.51
Treatment	CVK001	-3.83	1.39	-2.75	0.007	2.97
(virus)	MUVTH002	-0.07	1.34	-0.05	0.960	2.86
	MUVTH003	1.51	1.34	1.12	0.264	2.86
	PetF14	1.65	1.39	1.19	0.239	2.97
	RVC009	6.66	1.34	4.97	0.000	2.86

Statistic – Percentage of necrotic (double positive) cells

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)	3	1195.4	398.473	62.22	0.000
Treatment (virus)	5	353.1	70.624	11.03	0.000
Error	85	544.3	6.404		
Lack-of-Fit	15	283.8	18.918	5.08	0.000
Pure Error	70	260.5	3.722		
Total	93	2110.3			

Model Summary		
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
2.53056	74.21%	71.78%	68.01%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		7.606	0.286	26.57	0.000	
Time (day)	1	-2.494	0.463	-5.38	0.000	1.54
	3	-4.464	0.449	-9.94	0.000	1.51
	8	3.578	0.449	7.97	0.000	1.51
Treatment	CVK001	0.511	0.686	0.74	0.459	2.97
(virus)	MUVTH002	0.321	0.662	0.49	0.628	2.86
	MUVTH003	0.800	0.662	1.21	0.230	2.86
	PetF14	1.019	0.686	1.49	0.141	2.97
	RVC009	0.660	0.662	1.00	0.321	2.86

Statistic – MFI for Annexin-V

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)	3	8477769	2825923	123.78	0.000
Treatment (virus)	5	617438	123488	5.41	0.000
Error	85	1940615	22831		
Lack-of-Fit	15	1194938	79663	7.48	0.000
Pure Error	70	745677	10653		
Total	93	11112001			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
151.099	82.54%	80.89%	77.99%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		261.8	17.1	15.32	0.000	
Time (day)	1	532.7	27.7	19.25	0.000	1.54
	3	-198.2	26.8	-7.39	0.000	1.51
	8	-170.9	26.8	-6.37	0.000	1.51
Treatment	CVK001	-56.3	41.0	-1.38	0.173	2.97
(virus)	MUVTH002	-40.6	39.5	-1.03	0.307	2.86
	MUVTH003	57.3	39.5	1.45	0.150	2.86
	PetF14	-22.1	41.0	-0.54	0.591	2.97
	RVC009	151.3	39.5	3.83	0.000	2.86

Statistic – MFI for 7AAD

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Time (days)	3	12790.7	4263.58	148.37	0.000
Treatment (virus)	5	1369.4	273.88	9.53	0.000
Error	85	2442.6	28.74		
Lack-of-Fit	15	1722.5	114.84	11.16	0.000
Pure Error	70	720.1	10.29		
Total	93	16870.7			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
5.36069	85.52%	84.16%	82.23%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		27.883	0.606	45.98	0.000	
Time (day)	1	-18.702	0.982	-19.05	0.000	1.54
	3	-1.480	0.951	-1.56	0.124	1.51
	8	12.228	0.951	12.85	0.000	1.51
Treatment	CVK001	0.42	1.45	0.29	0.772	2.97
(virus)	MUVTH002	2.62	1.40	1.87	0.065	2.86
	MUVTH003	2.74	1.40	1.95	0.054	2.86
	PetF14	2.25	1.45	1.55	0.126	2.97
	RVC009	-1.94	1.40	-1.39	0.169	2.86

Effect of FIV infection on MYA-1 cell concentration in culture

One-way ANOVA tables examining the effect of FIV variant on cell flow rate at each time point

Statistic – Cell flow rate day 1

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Treatment	5	835.8	167.17	2.86	0.050
Error	16	935.8	58.49		
Total	21	1771.7			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
7.64790	47.18%	30.67%	12.91%

Means				
Treatment	**N**	**Mean**	**StDev**	**95% CI**
CVK001	2	24.81	6.15	(13.34, 36.27)
MUVTH002	3	16.476	1.485	(7.116, 25.837)
MUVTH003	3	14.45	6.85	(5.08, 23.81)
PetF14	2	24.38	6.16	(12.92, 35.85)
RVC009	3	13.34	5.28	(3.98, 22.70)
Control	9	27.97	9.40	(22.57, 33.38)

Statistic – Cell flow rate day 3

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Treatment	5	522021	104404	8.70	0.000
Error	18	216033	12002		
Total	23	738054			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
109.553	70.73%	62.60%	41.50%

Means				
Treatment	**N**	**Mean**	**StDev**	**95% CI**
CVK001	3	411	199	(279, 544)
MUVTH002	3	673.1	83.3	(540.2, 806.0)
MUVTH003	3	508.3	112.7	(375.4, 641.2)
PetF14	3	547.0	35.7	(414.1, 679.8)
RVC009	3	122.7	141.3	(-10.2, 255.6)
Control	9	413.5	83.0	(336.7, 490.2)

Statistic – Cell flow rate day 8

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Treatment	5	345401	69080	0.45	0.810
Error	16	2477052	154816		
Total	21	2822453			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
393.466	12.24%	0.00%	0.00%

Means				
Treatment	**N**	**Mean**	**StDev**	**95% CI**
CVK001	3	1226	215	(745, 1708)
MUVTH002	3	981	628	(499, 1462)
MUVTH003	3	1006.7	101.2	(525.2, 1488.3)
PetF14	3	1053	177	(571, 1535)
RVC009	3	814	789	(332, 1296)
Control	7	1147.4	211.1	(832.2, 1462.7)

Statistic – Cell flow rate day 10

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Treatment	5	151264	30253	0.46	0.803
Error	18	1191529	66196		
Total	23	1342793			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
257.286	11.26%	0.00%	0.00%

Means				
Treatment	**N**	**Mean**	**StDev**	**95% CI**
CVK001	3	1306	337	(993, 1618)
MUVTH002	3	1343	289	(1031, 1655)
MUVTH003	3	1157.4	80.2	(845.3, 1469.5)
PetF14	3	1343	289	(1031, 1655)
RVC009	3	1369.0	103.1	(1057.0, 1681.1)
Control	9	1195.8	273.0	(1015.6, 1376.0)

FIV-induced inhibition of mitogen-induced lymphocyte proliferation

General linear model of ANOVA tables examining the effect of FIV on inhibition of cellular proliferation

Statistic – Normalised counts per minute

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Treatment	5	1200568	240114	0.84	0.525
Cat	5	30386712	6077342	21.22	0.000
Error	133	38089044	286384		
Lack-of-Fit	25	14931689	597268	2.79	0.000
Pure Error	108	23157355	214420		
Total	143	69676324			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
535.148	45.33%	41.22%	35.92%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		1684.5	44.6	37.77	0.000	
Treatment	ConA	10.1	99.7	0.10	0.920	1.67
	CVK001	-15.6	99.7	-0.16	0.876	1.67
	MUVTH002	-106.4	99.7	-1.07	0.288	1.6
	MUVTH003	-112.1	99.7	-1.12	0.263	1.67
	PetF14	131.0	99.7	1.31	0.191	1.67
Cat	A	-18.1	99.7	-0.18	0.856	*
	B	-52.0	99.7	-0.52	0.603	*
	C	101.2	99.7	1.01	0.312	*
	D	886.1	99.7	8.89	0.000	*
	E	-305.1	99.7 -	3.06	0.003	*

Statistic – Stimulation index

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Treatment	5	115.3	23.07	1.88	0.102
Cat	5	10851.2	2170.23	176.66	0.000
Error	133	1633.9	12.28		
Lack-of-Fit	25	767.8	30.71	3.83	0.000
Pure Error	108	866.0	8.02		
Total	143	12600.4			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
3.50495	87.03%	86.06%	84.80%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		9.847	0.292	33.71	0.000	
Treatment	ConA	0.056	0.653	0.08	0.932	1.67
	CVK001	-0.848	0.653	-1.30	0.196	1.67
	MUVTH002	-0.212	0.653	-0.32	0.746	1.67
	MUVTH003	-1.108	0.653	-1.70	0.092	1.67
	PetF14	0.516	0.653	0.79	0.431	1.67
Cat	A	-1.831	0.653	-2.80	0.006	*
	B	-6.336	0.653	-9.70	0.000	*
	C	-4.986	0.653	-7.63	0.000	*
	D	18.868	0.653	28.89	0.000	*
	E	-5.248	0.653	-8.03	0.000	*

Appendix 7

Chapter 4 raw data

Cross-reactivity of Fel-O-Vax® FIV vaccine-induced antigen-specific cellular activation

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
A	Unvacc	CVK001	27.5	16.2	50.3	24.5	28	53.8	1366	-233	660	-314	1040	-239	1898	-214
A	Unvacc	CVK001	27.9	16.3	49	27	29.8	45.8	1207	-392	685	-289	1021	-258	1482	-630
A	Unvacc	CVK001	28.9	14.5	49.3	36.7	33.1	57.9	1734	135	1113	139	1155	-124	2203	91
A	Unvacc	MUVTH002	28.6	15.6	50.5	30	31	60.9	1685	86	897	-77	1181	-98	2377	265
A	Unvacc	MUVTH002	27.8	15.5	51.6	24.1	23.7	59.5	1454	-145	704	-270	982	-297	2166	54
A	Unvacc	MUVTH002	27.7	14.6	51.6	27.5	20.7	59.3	1517	-82	831	-143	946	-333	2166	54
A	Unvacc	PetF14	28.1	16.5	48.9	30.2	29.1	61.6	1674	75	851	-123	981	-298	2496	384
A	Unvacc	PetF14	29.6	15.4	47.9	29.6	24.9	61.8	1594	-5	813	-161	976	-303	2397	285
A	Unvacc	PetF14	30.2	15.2	46	29.6	24.4	59.9	1508	-91	764	-210	886	-393	2235	123
A	Unvacc	RVC009	28.3	15.3	51.6	28.9	33.9	59.2	1614	15	871	-103	1230	-49	2251	139
A	Unvacc	RVC009	28.4	15.9	50.3	25	24.9	57.2	1358	-241	671	-303	926	-353	2026	-86
A	Unvacc	RVC009	28.5	15.1	50.6	35.1	41.4	57.4	1925	326	1331	357	1874	595	2181	69
A	Unvacc	ConA	28.9	16	48.9	30	35.2	61.2	1669		931		1308		2314	
A	Unvacc	ConA	25.5	16.6	51.1	32.8	38.6	60.3	1720		911		1337		2278	
A	Unvacc	ConA	27.4	16.1	50.2	28.9	33.2	54	1462		808		1181		1884	
A	Unvacc	Unstim	28.9	16.7	47.7	22.1	20.8	61.2	1482		799		1101		2222	
A	Unvacc	Unstim	28.6	15.8	48.2	31.1	38.5	54.2	1567		977		1510		1882	

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
A	Unvacc	Unstim	29.5	15.9	47.2	36	31.9	60.1	1749		1146		1225		2233	
B	Unvacc	CVK001	27.8	16.1	51	39.8	37.6	52.1	1568	-217	1308	-14	1252	-135	1762	-343
B	Unvacc	CVK001	27.1	15	52.5	31.7	28.8	47.8	1280	-505	960	-362	943	-444	1528	-577
B	Unvacc	CVK001	28	14.9	51.5	35.5	29.4	50.7	1417	-368	1164	-158	1003	-384	1664	-441
B	Unvacc	MUVTH002	28.3	15.2	52.8	28.8	19.3	49.3	1229	-556	922	-400	757	-630	1601	-504
B	Unvacc	MUVTH002	27.2	14.8	53.6	30.7	25.3	42.3	1161	-624	942	-380	850	-537	1335	-770
B	Unvacc	MUVTH002	27.1	16	52.2	33.6	27.8	51.6	1428	-357	1131	-191	950	-437	1711	-394
B	Unvacc	PetF14	28.9	17	49.6	30.9	25.9	47	1255	-530	974	-348	955	-432	1509	-596
B	Unvacc	PetF14	29.5	17.9	48.8	33.8	26.9	50.5	1339	-446	1085	-237	956	-431	1659	-446
B	Unvacc	PetF14	28.8	17.3	49.4	30.3	23.8	42.5	1159	-626	963	-359	859	-528	1348	-757
B	Unvacc	RVC009	27.4	16.3	51.4	36.6	35.5	48	1449	-336	1234	-88	1236	-151	1560	-545
B	Unvacc	RVC009	26.2	16	53.5	35	31.6	49	1395	-390	1102	-220	1071	-316	1614	-491
B	Unvacc	RVC009	26.3	15.1	53.5	36.6	35.9	50.8	1533	-252	1198	-124	1219	-168	1718	-387
B	Unvacc	ConA	26.1	16.2	53.9	37.1	35.6	57.8	1732		1301		1282		2093	
B	Unvacc	ConA	25.8	16.5	54	38.6	33.1	58.4	1697		1330		1079		2104	
B	Unvacc	ConA	24.4	17.1	55.7	28.3	19.8	53	1299		924		779		1734	
B	Unvacc	Unstim	26.5	14.5	53.6	34.3	37.2	50.8	1542		1167		1267		1727	
B	Unvacc	Unstim	27.5	14.6	53	37.9	43.9	61.3	1956		1382		1579		2338	
B	Unvacc	Unstim	28	15.3	52	38.9	37.8	60.5	1858		1416		1314		2249	
C	Unvacc	CVK001	37.8	14.4	44.3	18.9	18.6	45.3	1094	209	766	123	975	156	1427	354
C	Unvacc	CVK001	35.8	14.2	46.1	19	20	39.2	1014	129	723	80	982	163	1258	185
C	Unvacc	CVK001	36.1	14.5	45.4	15.9	10.6	36.3	891	6	638	-5	805	-14	1124	51
C	Unvacc	MUVTH002	38.1	13.6	44.4	20.1	18.8	45.7	1131	246	791	148	994	175	1461	388

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
C	Unvacc	MUVTH002	37.2	14.1	44.7	18.7	14.5	44.1	1062	177	745	102	884	65	1401	328
C	Unvacc	MUVTH002	35.7	15.1	39.8	21.8	22.4	36.4	1034	149	781	138	995	176	1141	68
C	Unvacc	PetF14	36.5	13.8	45.7	18.9	17.1	41.8	1048	163	749	106	945	126	1305	232
C	Unvacc	PetF14	35.5	13.9	46.5	18.5	17	40.1	1021	136	727	84	933	114	1262	189
C	Unvacc	PetF14	36.3	14	43.5	24.5	12.4	49.4	1296	411	970	327	977	158	1636	563
C	Unvacc	RVC009	36.3	14.4	44.5	20.2	14.9	46.8	1251	366	958	315	1111	292	1526	453
C	Unvacc	RVC009	35.1	13.9	46.4	16.5	12.3	42	1096	211	825	182	1019	200	1315	242
C	Unvacc	RVC009	34.9	14.6	45.8	17.6	11.5	40.6	1060	175	779	136	966	147	1296	223
C	Unvacc	ConA	33.8	17.1	44.5	27.1	10.5	42.4	1094		963		730		1353	
C	Unvacc	ConA	32.1	15	47.7	27.6	9.62	35.7	1054		1093		896		1120	
C	Unvacc	ConA	30	13	51.2	40.7	20.2	37.1	1297		1625		1113		1180	
C	Unvacc	Unstim	36.8	16.9	39.1	15.2	9.33	36.8	927		680		804		1157	
C	Unvacc	Unstim	37.4	15.8	39.9	13.5	9.63	32.1	861		638		859		1029	
C	Unvacc	Unstim	34.8	15.4	40.6	14.6	9.6	32.1	867		610		794		1032	
D	Unvacc	CVK001	28.1	13.8	55.4	17.6	11.7	55.3	1270	162	702	33	839	-106	1777	333
D	Unvacc	CVK001	28.5	12.9	55.9	15.1	8.89	55	1212	104	630	-39	796	-149	1738	294
D	Unvacc	CVK001	27.9	13.4	55.8	16.4	12.8	50.9	1171	63	652	-17	831	-114	1599	155
D	Unvacc	MUVTH002	31.7	13.6	52.3	22.3	21	59.8	1410	302	814	145	989	44	2074	630
D	Unvacc	MUVTH002	29.2	13.2	54.7	20.2	20	53.5	1257	149	706	37	940	-5	1730	286
D	Unvacc	MUVTH002	29.6	13.1	54.7	19.7	18.5	50.1	1154	46	668	-1	885	-60	1562	118
D	Unvacc	PetF14	28.3	13.1	55.8	18.1	19.1	51.1	1176	68	645	-24	907	-38	1592	148
D	Unvacc	PetF14	28.5	13.2	55.6	16.1	13.3	49.4	1098	-10	591	-78	807	-138	1506	62
D	Unvacc	PetF14	28.7	12.9	55.1	21	21.3	47.7	1174	66	711	42	957	12	1494	50

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
D	Unvacc	RVC009	30	13.6	53.5	18.6	10.9	57.6	1324	216	774	105	890	-55	1910	466
D	Unvacc	RVC009	28.4	13.4	55.3	16.8	11.5	57.2	1306	198	702	33	888	-57	1871	427
D	Unvacc	RVC009	28.2	13.2	55.1	17.2	13	54.9	1271	163	710	41	895	-50	1757	313
D	Unvacc	ConA	23.1	11.7	60.5	32.6	14.6	54	1596		1280		1026		1790	
D	Unvacc	ConA	22.1	11.3	61.8	32.8	14.1	48.9	1431		1177		1024		1536	
D	Unvacc	ConA	22.1	8.63	63.2	53.9	20.9	57.9	2112		2544		1098		2055	
D	Unvacc	Unstim	28.9	15.8	51.9	15.2	11.4	47.1	1118		673		953		1459	
D	Unvacc	Unstim	29.7	15.7	50.9	15	11	46.1	1062		646		897		1409	
D	Unvacc	Unstim	29.3	14.8	51.8	16.3	16.4	47.1	1145		689		986		1464	
E	Revacc	CVK001	50.1	15.8	26.8	21.9	25.8	44.7	1602	179	1152	-130	1435	-152	1469	537
E	Revacc	CVK001	48.6	15.5	29.4	22.8	24.8	44.1	1625	202	1263	-19	1469	-118	1458	526
E	Revacc	CVK001	48.5	13.3	30.8	29.1	32	49.8	2029	606	1570	288	1772	185	1725	793
E	Revacc	MUVTH002	47.5	13.3	30.8	27.4	25.1	43.1	1822	399	1465	183	1496	-91	1390	458
E	Revacc	MUVTH002	46.4	13.9	31.2	26.4	24	43.2	1647	224	1307	25	1301	-286	1363	431
E	Revacc	MUVTH002	46.9	14.7	32.1	23.6	25.2	37.5	1535	112	1337	55	1492	-95	1227	295
E	Revacc	PetF14	43.5	9.51	31.6	36.4	39	54.9	3276	1853	2156	874	2067	480	2057	1125
E	Revacc	PetF14	46.3	12.6	32.3	28.1	29.3	48.4	2035	612	1562	280	1750	163	1647	715
E	Revacc	PetF14	46.9	13.3	32	25	25.4	46.9	1832	409	1461	179	1589	2	1536	604
E	Revacc	RVC009	47	13.3	29.5	22.8	24.9	40.6	1843	420	1239	-43	1386	-201	1368	436
E	Revacc	RVC009	47.5	15.1	30.1	24.8	26.2	43.1	1602	179	1299	17	1390	-197	1405	473
E	Revacc	RVC009	47.1	15.4	30.9	23.2	21.5	42.4	1548	125	1263	-19	1366	-221	1380	448
E	Revacc	ConA	47.5	15.1	29.3	29.5	42.9	39.6	2109		1873		2071		1344	
E	Revacc	ConA	48.4	15.3	29.4	27.1	25.5	36.4	1660		1490		1631		1150	

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
E	Revacc	ConA	47.8	15.5	30	30	30.4	34.2	1812		1801		1824		1050	
E	Revacc	Unstim	47.2	15.2	31.5	22.6	27.2	33	1411		1261		1552		965	
E	Revacc	Unstim	47.9	12.6	32.3	26	32.1	31.2	1504		1333		1679		962	
E	Revacc	Unstim	46.7	13.9	33.2	23.7	24.7	30	1353		1252		1529		868	
F	Revacc	CVK001	31	15.8	44.5	31.1	29.3	43.1	1588	318	1601	195	1268	43	1338	338
F	Revacc	CVK001	32	15.4	45.3	27.8	24.6	42	1489	219	1466	60	1228	3	1317	317
F	Revacc	CVK001	31.2	15.2	46.4	26.9	28.3	42.4	1496	226	1447	41	1303	78	1326	326
F	Revacc	MUVTH002	30.3	16	46.3	27.8	28.2	42.4	1488	218	1462	56	1257	32	1318	318
F	Revacc	MUVTH002	30.5	16.6	45.5	30.5	31.3	46.6	1671	401	1674	268	1444	219	1475	475
F	Revacc	MUVTH002	30	16.4	45.5	30.3	25.3	41.6	1443	173	1643	237	1092	-133	1265	265
F	Revacc	PetF14	31.5	16.6	44.6	27.8	28.3	44.7	1649	379	1517	111	1386	161	1453	453
F	Revacc	PetF14	32.4	16.7	44.2	25	29.1	43.4	1512	242	1326	-80	1376	151	1374	374
F	Revacc	PetF14	33	14.6	46.2	23.8	23	40	1361	91	1304	-102	1224	-1	1213	213
F	Revacc	RVC009	30.8	15.8	44.1	29.8	31.2	45.9	1744	474	1600	194	1427	202	1475	475
F	Revacc	RVC009	30.2	16.1	45.8	28.2	22.4	39.8	1406	136	1429	23	1112	-113	1221	221
F	Revacc	RVC009	30.6	15.3	45.6	27.7	21.1	39.2	1315	45	1441	35	997	-228	1168	168
F	Revacc	ConA	27.5	15.9	49.1	58.6	35	52.2	2620		3948		1765		1839	
F	Revacc	ConA	25.2	16	52	44.8	28.5	40.5	1666		2430		1381		1224	
F	Revacc	ConA	25.1	15	53.6	46.8	26.1	40.4	1665		2614		1286		1248	
F	Revacc	Unstim	30.7	18.5	44.8	24.4	25.8	39.8	1341		1314		1273		1130	
F	Revacc	Unstim	27.9	18.8	47.1	25.1	23.9	32.4	1209		1399		1209		912	
F	Revacc	Unstim	29.2	17.3	47.3	27.9	28	34.7	1260		1505		1194		958	
G	Revacc	CVK001	47.7	9.69	37.4	34.1	35.2	57.2	2278	356	1877	236	1801	-5	2149	482

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
G	Revacc	CVK001	49.4	8.97	36.9	31.3	39.9	55.2	2046	124	1644	3	1957	151	2028	361
G	Revacc	CVK001	49	9.85	36.2	32.8	31.5	56.2	2164	242	1738	97	1700	-106	2180	513
G	Revacc	MUVTH002	45.7	6.52	41.6	37.2	29.6	56.3	2351	429	1900	259	1616	-190	2196	529
G	Revacc	MUVTH002	45.3	7.26	42.4	35.7	32.5	56.3	2325	403	1996	355	1823	17	2134	467
G	Revacc	MUVTH002	44.5	7.27	42.7	34.9	32.3	51.7	2197	275	1854	213	1832	26	1908	241
G	Revacc	PetF14	48.2	9.06	37.5	31.4	18.9	50.6	1960	38	1683	42	1366	-440	1777	110
G	Revacc	PetF14	50.2	7.84	35.3	35.3	25.3	54.9	2392	470	1914	273	1491	-315	2146	479
G	Revacc	PetF14	47.8	6.56	38	38.6	49.2	61.2	2874	952	2212	571	2236	430	2465	798
G	Revacc	RVC009	49.1	9.29	37.3	30.8	31	50.1	1877	-45	1595	-46	1677	-129	1780	113
G	Revacc	RVC009	44.5	8.65	41.5	36.8	41.2	58	2483	561	2055	414	2015	209	2230	563
G	Revacc	RVC009	47.6	8.55	39.1	31.4	29.1	52.7	2003	81	1646	5	1548	-258	1925	258
G	Revacc	ConA	45.8	5.72	36.2	56.9	57.4	60.8	4112		4063		2276		2396	
G	Revacc	ConA	48.2	8.15	33.3	46.3	41.3	52.7	2976		2749		2013		1871	
G	Revacc	ConA	47.8	7.09	36.9	53.1	45.5	62.1	3678		3553		2110		2624	
G	Revacc	Unstim	48.6	10.9	34.7	31.8	35.5	51.7	2104		1781		1839		1807	
G	Revacc	Unstim	47.1	12.1	36	28.6	28.8	46.5	1702		1464		1575		1541	
G	Revacc	Unstim	47.6	9.63	37.4	31.9	45.5	48	1960		1679		2003		1654	
H	Revacc	CVK001	37.2	9.38	45	38.9	31	52.8	2374	722	2161	451	1612	-50	1855	753
H	Revacc	CVK001	38	9.15	44.4	33.3	29.5	47.3	1951	299	1836	126	1412	-250	1511	409
H	Revacc	CVK001	39.4	12.7	40.1	27.6	25.4	43.4	1649	-3	1532	-178	1345	-317	1387	285
H	Revacc	MUVTH002	37.8	11.1	43.6	34.2	28	47.3	1855	203	1820	110	1473	-189	1469	367
H	Revacc	MUVTH002	34.1	11	47.6	34.2	26.7	46	1824	172	1874	164	1365	-297	1433	331
H	Revacc	MUVTH002	36.4	11	45.4	33.2	30.6	45.8	1819	167	1793	83	1427	-235	1462	360

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
H	Revacc	PetF14	39.2	10	43.7	35.6	28.1	49.7	1991	339	1902	192	1550	-112	1601	499
H	Revacc	PetF14	38.4	9.19	43.6	38.8	35.9	53.6	2347	695	2083	373	1818	156	1847	745
H	Revacc	PetF14	39.7	8.68	43.4	36.3	36.7	49.9	2180	528	1950	240	1891	229	1687	585
H	Revacc	RVC009	38.7	12	41.3	34.8	28	44.7	1907	255	1915	205	1416	-246	1395	293
H	Revacc	RVC009	35	10.2	47.8	35.6	33.5	46.4	1982	330	1919	209	1720	58	1487	385
H	Revacc	RVC009	38	9.44	44.8	33.2	28.6	52.4	2142	490	1863	153	1654	-8	1745	643
H	Revacc	ConA	35.4	11.7	42.7	45.7	34.2	45	2322		2737		1639		1402	
H	Revacc	ConA	37.7	13.8	40.6	36	29.8	42.4	1959		1945		1662		1370	
H	Revacc	ConA	39.1	11.5	40	34.3	37.3	41.9	1962		1874		1731		1285	
H	Revacc	Unstim	39.2	12.9	39.2	31.3	30.2	34.7	1704		1744		1657		1042	
H	Revacc	Unstim	40	12.6	41	29.5	32	37	1631		1596		1699		1149	
H	Revacc	Unstim	39.9	11.7	42.2	31.4	33.7	36.3	1620		1789		1629		1116	
I	Vacc	CVK001	38.6	12.4	40.9	50.5	32.5	60.1	3155	313	3734	430	1750	-26	2391	257
I	Vacc	CVK001	42	10.6	39.4	58.3	35.7	58.2	3463	621	4776	1472	1816	40	2243	109
I	Vacc	CVK001	39.4	11.9	40.8	51.6	35.7	57.3	2978	136	3688	384	1863	87	2155	21
I	Vacc	MUVTH002	34.2	13.7	43.6	45.5	26.1	57.7	2649	-193	3043	-261	1428	-348	2198	64
I	Vacc	MUVTH002	35.7	11.3	43.6	52.2	34.9	60.5	3183	341	3964	660	1886	110	2363	229
I	Vacc	MUVTH002	35.9	11.6	43.8	50.8	34.8	60.4	3040	198	3766	462	1856	80	2309	175
I	Vacc	PetF14	34.7	12.7	43.7	47.3	26.6	59	2793	-49	3221	-83	1390	-386	2301	167
I	Vacc	PetF14	36.1	11.2	43.6	53.3	34.3	62.6	3382	540	4216	912	1827	51	2564	430
I	Vacc	PetF14	34.3	12	44.4	49.1	35.7	59.8	2974	132	3597	293	1896	120	2279	145
I	Vacc	RVC009	37.4	14.1	41.4	46.3	28.5	57.6	2625	-217	3076	-228	1518	-258	2137	3
I	Vacc	RVC009	35.1	13.4	44.2	42.6	29.9	56.2	2491	-351	2779	-525	1644	-132	2059	-75

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
I	Vacc	RVC009	35.2	14	44	39.7	26	55.8	2298	-544	2435	-869	1532	-244	2027	-107
I	Vacc	ConA	34.5	12.1	44.9	62.5	37.1	63.7	3820		5699		1946		2582	
I	Vacc	ConA	35.7	14.1	42.5	48.9	32.8	58.1	2777		3344		1775		2124	
I	Vacc	ConA	35.4	14.8	42	48.1	29.7	59.3	2807		3459		1740		2206	
I	Vacc	Unstim	35.8	12.5	43.4	46.4	35.8	58.1	2837		3117		1868		2186	
I	Vacc	Unstim	35.2	13	43.5	45.8	32.8	53.9	2605		3036		1709		1954	
I	Vacc	Unstim	40.1	12	40.3	52.1	33.2	59	3084		3760		1750		2261	
J	Vacc	CVK001	46.2	19	20.3	38.6	39.5	46.4	2665	1182	2308	1029	1932	485	1527	527
J	Vacc	CVK001	46.6	18.4	21.2	39.5	36.4	51.4	2587	1104	2299	1020	1753	306	1728	728
J	Vacc	CVK001	46.1	19.3	21.4	35.5	26.2	41.9	2160	677	2049	770	1483	36	1330	330
J	Vacc	MUVTH002	44.8	19.8	22.1	34.4	26.2	38.4	2010	527	1874	595	1328	-119	1231	231
J	Vacc	MUVTH002	45.9	19	22	36.7	27.8	45.5	2360	877	2234	955	1620	173	1454	454
J	Vacc	MUVTH002	45	20.6	21	31.8	23.7	41	2008	525	1768	489	1387	-60	1285	285
J	Vacc	PetF14	45.5	20.1	22.3	32.2	26.2	42.8	2123	640	2012	733	1586	139	1375	375
J	Vacc	PetF14	44.4	20.6	21.4	31.7	27.1	43.1	2114	631	1934	655	1610	163	1372	372
J	Vacc	PetF14	43.7	19.2	22.9	34.2	24.6	42.6	2204	721	2123	844	1562	115	1337	337
J	Vacc	RVC009	46.1	19.8	20.7	38.1	31.7	47.7	2452	969	2243	964	1620	173	1585	585
J	Vacc	RVC009	45.8	20.4	21.4	35.5	30.1	43.6	2145	662	2011	732	1555	108	1395	395
J	Vacc	RVC009	45.3	20.8	22.2	31.7	26.1	44.9	1948	465	1783	504	1457	10	1457	457
J	Vacc	ConA	45.9	21.1	19.4	34.8	35.5	40.8	2321		2001		1850		1310	
J	Vacc	ConA	48	18.7	19.3	38.1	40.8	45.4	2597		2273		2006		1509	
J	Vacc	ConA	47.1	20.9	19.5	34.9	37.5	39.3	2189		1956		1861		1243	
J	Vacc	Unstim	47.3	20.5	18.2	31.8	34.8	40	2224		1788		1743		1278	

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
J	Vacc	Unstim	42.8	28.2	20.7	16.9	18.4	41.5	1345		1215		1341		1325	
J	Vacc	Unstim	42.2	28.7	22.3	1.83	8.34	5.96	880		834		1257		398	
K	Vacc	CVK001	49.2	18.3	24	30.8	27.6	35.9	1688	187	1647	261	1401	-108	1101	226
K	Vacc	CVK001	48.8	16	25.9	32.1	27.2	36.6	1769	268	1709	323	1372	-137	1139	264
K	Vacc	CVK001	49.8	14.5	26	33.5	32.1	40	1922	421	1791	405	1490	-19	1266	391
K	Vacc	MUVTH002	48.2	16.7	26.2	32.2	24.9	46.5	1844	343	1726	340	1353	-156	1523	648
K	Vacc	MUVTH002	48.7	15.1	26.3	35.1	31.3	46.9	2088	587	1926	540	1542	33	1539	664
K	Vacc	MUVTH002	53.6	13.2	23.7	39.8	41.8	49.1	2498	997	2403	1017	2014	505	1642	767
K	Vacc	PetF14	47.5	14.9	26.9	34.5	33.6	46.4	2182	681	2084	698	1777	268	1528	653
K	Vacc	PetF14	47.1	16	26.7	32.5	26.1	42.4	1855	354	1755	369	1428	-81	1343	468
K	Vacc	PetF14	47.4	16.7	25.8	31.5	23.6	43.9	1823	322	1712	326	1359	-150	1407	532
K	Vacc	RVC009	47.9	16	27.7	29.4	28.6	33.2	1557	56	1497	111	1375	-134	1032	157
K	Vacc	RVC009	46.1	16	30.2	27.4	27.1	27.4	1363	-138	1376	-10	1331	-178	858	-17
K	Vacc	RVC009	49.1	16.2	26.1	30.9	29.8	38	1705	204	1598	212	1399	-110	1195	320
K	Vacc	ConA	48.7	17.9	23.6	29.1	29.8	31.3	1627		1524		1538		964	
K	Vacc	ConA	49	18.1	23.2	28.7	30.1	27.2	1528		1457		1479		834	
K	Vacc	ConA	49	17.1	23.6	31.5	33.9	31.2	1729		1665		1558		980	
K	Vacc	Unstim	49.3	18.4	23.6	26.5	27.6	26.2	1403		1321		1400		816	
K	Vacc	Unstim	50	17.5	23	29	33.3	28.9	1576		1451		1547		903	
K	Vacc	Unstim	49.5	16.9	23.8	27.6	33.7	28.7	1523		1385		1580		905	
L	Vacc	CVK001	39.6	13.1	37.8	44.2	33.9	51.4	2652	1476	2998	2029	1862	452	1773	514
L	Vacc	CVK001	40.5	14.5	35.6	43.4	29.6	52.9	2647	1471	2935	1966	1713	303	1866	607
L	Vacc	CVK001	40.3	14.4	35.6	44.3	37.3	53.8	2731	1555	2972	2003	1987	577	1913	654

Cat	Vacc protocol	Treatment	%CD4+	%CD8+	%CD4-CD8-	%CD25+ (CD4+)	%CD25+ (CD8+)	%CD25+ (CD4-CD8-)	MFI CD25	nMFI CD25	MFI CD25 (CD4+)	nMFI CD25 (CD4+)	MFI CD25 (CD8+)	nMFI CD25 (CD8+)	MFI CD25 (CD4-CD8-)	nMFI CD25 (CD4-CD8-)
L	Vacc	MUVTH002	39.2	14.3	36	42.5	36	52.4	2334	1158	2745	1776	1930	520	1773	514
L	Vacc	MUVTH002	39.2	14.6	35.7	42.4	33	55	2598	1422	2668	1699	1820	410	1938	679
L	Vacc	MUVTH002	40.3	15.8	34.7	38.5	30.1	53.6	2629	1453	2240	1271	1749	339	1879	620
L	Vacc	PetF14	39.2	14.7	36.2	41.7	32.1	55.6	2610	1434	2738	1769	1828	418	1972	713
L	Vacc	PetF14	39.7	14.6	35.7	41.6	37.2	53.9	2649	1473	2819	1850	1958	548	1897	638
L	Vacc	PetF14	39.9	17.4	33.2	37.9	36.5	52.2	2234	1058	2157	1188	1910	500	1737	478
L	Vacc	RVC009	39.6	14.7	36.1	41.5	31.1	54	2629	1453	2754	1785	1782	372	1944	685
L	Vacc	RVC009	38.7	16	36.6	38.4	25.5	49.5	2185	1009	2273	1304	1481	71	1762	503
L	Vacc	RVC009	40.7	15.3	35.2	40.8	33.4	53.9	2486	1310	2546	1577	1819	409	1926	667
L	Vacc	ConA	40	18.9	32.7	37.3	31	55.6	2281		2148		1815		1982	
L	Vacc	ConA	38.7	16.4	35	40.3	36.2	53.8	2487		2498		1960		1912	
L	Vacc	ConA	40	13.7	34.9	44.5	46.3	52	2897		2883		2371		1922	
L	Vacc	Unstim	36.8	24.9	34.2	26.2	22.2	59.2	1697		1453		1485		2154	
L	Vacc	Unstim	34.6	23.1	38.8	3.23	13.5	15.8	879		718		1353		737	
L	Vacc	Unstim	33.9	23.5	39	3.28	14.6	19.2	953		735		1391		885	

Cross-reactivity of the Fel-O-Vax® FIV vaccine-induced DTH response

Cat	Virus	24 hrs	48 hrs	72 hrs
a	PetF14	12.57	12.57	0
a	CVK001	0	0	0
a	MUVTH002	12.57	19.63	0
a	RVC009	19.63	19.63	0
b	PetF14	12.57	25.13	0
b	CVK001	25.13	25.13	0
b	MUVTH002	25.13	25.13	0
b	RVC009	25.13	84.82	39.27
c	PetF14	19.63	28.27	0
c	CVK001	56.55	12.57	0
c	MUVTH002	113.1	56.55	39.27
c	RVC009	56.55	56.55	39.27
d	PetF14	25.13	39.27	0
d	CVK001	12.57	56.55	25.13
d	MUVTH002	12.57	56.55	58.9
d	RVC009	12.57	39.27	58.9
e	PetF14	12.57	25.13	19.63
e	CVK001	25.13	25.13	7.07
e	MUVTH002	58.9	25.13	39.27
e	RVC009	25.13	25.13	7.07
f	PetF14	12.57	12.57	0
f	CVK001	12.57	12.57	0
f	MUVTH002	25.13	115.45	28.27
f	RVC009	25.13	56.55	28.27

Appendix 8

Chapter 4 statistics

Cross-reactivity of Fel-O-Vax FIV vaccine-induced antigen-specific cellular activation

Paired t-test examining the effect of FIV stimulation on PBMC from unvaccinated cats.

Statistic – MFI CD25 of PBMC from unvaccinated cats

Paired t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
FIV	4	1306	198	99
Control	4	1344	419	209
Difference	4	-39	283	141

95% CI for mean difference: (-489, 411)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = -0.27 P-Value = 0.801

Statistic - MFI CD25 of $CD4^+$ T cells from unvaccinated cats

Paired t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
FIV	4	853	166	83
Control	4	902	318	159
Difference	4	-49.2	168.2	84.1

95% CI for mean difference: (-316.9, 218.5)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = -0.58 P-Value = 0.600

Statistic - MFI CD25 of $CD8^+$ T cells from unvaccinated cats

Paired t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
FIV	4	989	89	45
Control	4	1107	269	134
Difference	4	-119	221	111

95% CI for mean difference: (-471, 233)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = -1.07 P-Value = 0.362

Statistic - MFI CD25 of $CD4^-CD8^-$ T cells from unvaccinated cats

Paired t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
FIV	4	1701	340	170
Control	4	1683	514	257
Difference	4	18	375	187

95% CI for mean difference: (-579, 614)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 0.09 P-Value = 0.931

Statistic – Percentage of $CD25^+CD4^+$ cells from unvaccinated cats

Paired t-test

Treatment	N	Mean	StDev	SE Mean
FIV	4	24.18	11.05	5.53
Control	4	25.02	7.51	3.75
Difference	4	-0.85	3.64	1.82

95% CI for mean difference: (-6.65, 4.95)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = -0.47 P-Value = 0.673

Statistic - Percentage of $CD25^{+}CD8^{+}$ cells from unvaccinated cats

Paired t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
FIV	4	23.12	14.31	7.16
Control	4	22.18	7.72	3.86
Difference	4	0.94	7.25	3.62

95% CI for mean difference: (-10.59, 12.47)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 0.26 P-Value = 0.812

Statistic - Percentage of $CD25^{+}CD4^{-}CD8^{-}$ cells from unvaccinated cats

Paired t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
FIV	4	49.12	11.59	5.80
Control	4	50.54	6.70	3.35
Difference	4	-1.43	8.06	4.03

95% CI for mean difference: (-14.26, 11.40)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = -0.35 P-Value = 0.747

2-sample t-test examining the effect of vaccination on FIV stimulated PBMC from each subset

Statistic - Normalised MFI CD25 of FIV stimulated CD4+ cells

2-sample t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
Unvaccinated	4	-49	168	84
Vaccinated	8	459	541	191

Difference				
Estimate	**95% CI**	**T-value**	**P-value**	**DF**
-508	(-981, -35)	-2.43	0.031	9

Statistic - Normalised MFI CD25 of FIV stimulated CD8+ cells

2-sample t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
Unvaccinated	4	-119	222	111
Vaccinated	8	34	168	59

Difference				
Estimate	**95% CI**	**T-value**	**P-value**	**DF**
-153	(-502, 196)	-1.22	0.280	4

Statistic - Normalised MFI CD25 of FIV stimulated $CD4^{-}CD8^{-}$ cells

2-sample t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**

Unvaccinated	4	18	375	187
Vaccinated	8	419	151	53

Difference				
Estimate	**95% CI**	**T-value**	**P-value**	**DF**
-401	(-1021, 219)	-2.06	0.119	3

Statistic – Percentage of $CD25^{+}CD4^{+}$ FIV stimulated cells

2-sample t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
Unvaccinated	4	25.02	7.51	3.8
Vaccinated	8	35.09	7.30	2.6

Difference				
Estimate	**95% CI**	**T-value**	**P-value**	**DF**
-10.06	(-21.77, 1.65)	-2.21	0.078	5

Statistic – Percentage of $CD25^{+}CD8^{+}$ FIV stimulated cells

2-sample t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
Unvaccinated	4	22.18	7.72	3.9
Vaccinated	8	29.99	2.44	0.86

Difference				
Estimate	**95% CI**	**T-value**	**P-value**	**DF**
-7.80	(-20.39, 4.78)	-1.97	0.143	3

Statistic – Percentage of $CD25^{+}CD4^{-}CD8^{-}$ FIV stimulated cells

2-sample t-test				
Treatment	**N**	**Mean**	**StDev**	**SE Mean**
Unvaccinated	4	50.54	6.70	3.3
Vaccinated	8	48.42	6.56	2.3

Difference				
Estimate	**95% CI**	**T-value**	**P-value**	**DF**
2.12	(-8.35, 12.59)	0.52	0.625	5

Linear regression tables examining the effect of vaccination protocol ('newly vaccinated' versus 'revaccinated') and virus (RVC009, CVK001, MUVTH002 versus PetF14) on stimulated PBMC from each subset

Statistic – Normalised MFI CD25 in $CD4^+$ cells

Coefficients				
	Estimate	**Std Error**	**T-value**	**Pr(>|t|)**
(Intercept)	828.08	114.29	7.245	1.36E-10
Vacc.protocol Revacc	-613.92	102.23	-6.005	3.86E-08
Treatment CVK001	31.46	144.57	0.218	0.8282
Treatment MUVTH002	-39.83	144.57	-0.276	0.7835
Treatment RVC009	-241.79	144.57	-1.672	0.0979

Model Summary	
Residual standard error	500.8 on 91 degrees of freedom
Multiple R-sq	0.3075
R-sq (adj)	0.2771
F-statistic	10.1
P-value	8.217e-07

Statistic – Normalised MFI CD25 in $CD8^+$ cells

Coefficients				
	Estimate	**Std Error**	**T-value**	**Pr(>|t|)**
(Intercept)	184.36	51.41	3.586	0.000542
Vacc.protocol Revacc	-151.31	45.98	-3.291	0.001423
Treatment CVK001	-47.96	65.03	-0.738	0.462706
Treatment MUVTH002	-97.67	65.03	-1.502	0.136573
Treatment RVC009	-152.25	65.03	-2.341	0.021398

Model Summary	
Residual standard error	225.3 on 91 degrees of freedom
Multiple R-sq	0.1566
R-sq (adj)	0.1196
F-statistic	4.225
P-value	0.003497

Statistic – Normalised MFI CD25 in $CD4^-CD8^-$ cells

Coefficients				
	Estimate	**Std Error**	**T-value**	**Pr(>|t|)**
(Intercept)	474.15	48.4	9.796	6.82E-16
Vacc.protocol Revacc	52.38	43.29	1.21	0.2295
Treatment CVK001	-72.5	61.23	-1.184	0.2394
Treatment MUVTH002	-89.21	61.23	-1.457	0.1486
Treatment RVC009	-164.96	61.23	-2.694	0.0084

Model Summary	
Residual standard error	212.1 on 91 degrees of freedom
Multiple R-sq	0.08816
R-sq (adj)	0.04808
F-statistic	2.2
P-value	0.0752

Cross-reactivity of the Fel-O-Vax FIV vaccine-induced DTH response

General linear model of ANOVA tables comparing the FIV-induced DTH response at each time point

Statistic – Wheal volume at day 1

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Cat	5	6668	1333.6	4.84	0.008
Virus	3	2110	703.4	2.55	0.095
Error	15	4135	275.6		
Total	23	12913			

Model Summary		
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
16.6026	67.98%	50.90%	18.03%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		0.4	10.2	0.04	0.967	
Cat	A	50.3	11.7	4.28	0.001	*
	B	7.7	11.7	0.65	0.524	*
	C	10.8	11.7	0.92	0.372	*
	D	4.5	11.7	0.38	0.706	*
	E	19.2	11.7	1.64	0.122	*
Virus	CVK001	6.15	9.59	0.64	0.531	1.50
	MUVTH002	25.39	9.59	2.65	0.018	1.50
	RVC009	11.52	9.59	1.20	0.248	1.50

Statistic – Wheal volume at day 2

Analysis of variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Cat	5	3957	791.4	1.51	0.245
Virus	3	3921	1307.2	2.49	0.100
Error	15	7862	524.1		
Total	23	15740			

Model Summary		
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
22.8933	50.05%	23.42%	0.00%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		1.1	14.0	0.08	0.936	
Cat	A	25.5	16.2	1.58	0.136	*
	B	36.3	16.2	2.24	0.040	*
	C	27.1	16.2	1.67	0.115	*
	D	35.0	16.2	2.16	0.047	*
	E	12.2	16.2	0.75	0.464	*
Virus	CVK001	-1.8	13.2	-0.14	0.892	1.50
	MUVTH002	25.9	13.2	1.96	0.069	1.50
	RVC009	23.2	13.2	1.75	0.100	1.50

Statistic – Wheal volume at day 3

Analysis of variance						
Source		**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Cat		5	2820	564.0	2.63	0.067
Virus		3	3441	1147.1	5.36	0.010
Error		15	3213	214.2		
Total		23	9474			

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
14.6358	66.09%	48.00%	13.18%

Coefficients						
Term		**Coef**	**SE coef**	**T-value**	**P-value**	**VIF**
Constant		-12.99	8.96	-1.45	0.168	-12.99
Cat	A	19.6	10.3	1.90	0.077	*
	B	14.1	10.3	1.37	0.192	*
	C	9.8	10.3	0.95	0.358	*
	D	35.7	10.3	3.45	0.004	*
	E	18.3	10.3	1.76	0.098	*
Virus	CVK001	2.10	8.45	0.25	0.808	1.50
	MUVTH002	24.35	8.45	2.88	0.011	1.50
	RVC009	25.53	8.45	3.02	0.009	1.50

Appendix 9

Submission form for recruitment of vaccinated and unvaccinated cats

FIV VACCINE STUDY – SAMPLE SUBMISSION & CONSENT FORM
Client participation consent:

I, _____ consent to participating in the FIV study currently being undertaken at Massey University, Palmerston Nth.

I understand that a saliva sample will be collected from my cat, _____, and that this sample will be tested for FIV.

I agree to my veterinarian supplying you with my cat's clinical records for the purposes of the study.

Client signature: _____ Date: _____

For veterinary use:

Cat's name:
Age / D.O.B:
(N.B. Must be more than 2yrs of age to participate)

Sex:	Male	☐	Female	☐
Neutered?	Yes	☐	No	☐
Date sample collected:				
Vaccinated against FIV?	Yes	☐	No	☐
If yes, date of most recent FIV vaccination:				
FIV testing performed prior to vaccination?	Yes	☐	No	☐
If no, was this cat first vaccinated as a kitten?	Yes	☐	No	☐

Please provide a brief summary of FIV vaccination history:

Reason for presentation to your clinic (please include a brief summary of clinical signs or diagnosis, or simply write healthy if presenting for routine vaccination):

Appendix 10

Chapter 5 raw data

Summary of PCR results in vaccinated and unvaccinated cats

				2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR			
Clinic ID	Age	Sex	Vacc status	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.	Consensus result	Clinical signs
TV11V	?	F	?	32.9	56		NEG							NEG - excluded	Asymptomatic
VPT01F	?	?	?	26.57	4,965	82.3	POS					POS	FIV	POS - excluded	Asymptomatic
CVT01U	4	M	Unvacc	32.41	49		NEG							NEG	Cat bite abscess
HVC01U	3	M	Unvacc	31.93	112	80.2	POS			88.25	NEG	NEG		NEG	Cat bite abscess
HVC02U	9	F	Unvacc				NEG							NEG	Squamous cell carcinoma
HVC03U	2	M	Unvacc	33.49	37	83.65	NEG			82.8	NEG			NEG	Cat bite abscess
NCVC01U	13	F	Unvacc	33.91	18		NEG			86.65	NEG			NEG	Asymptomatic
NCVC02U	5	M	Unvacc	32.32	55		NEG			86.65	NEG			NEG	Asymptomatic
NCVC03U	17	M	Unvacc	32.69	42		NEG			86.25	NEG			NEG	Asymptomatic
NCVC04U	7	F	Unvacc	31.62	90	77.5	NEG			86.65	NEG			NEG	Asymptomatic
NCVC05U	10	M	Unvacc	28.85	640	82.4	POS	28.92	1,328	82.1	POS	NEG		POS	Tooth root abscess
NCVC06U	9	M	Unvacc	30.84	156	80.85	POS			86.35	NEG	NEG		NEG	Asymptomatic
NCVC07U	2	M	Unvacc	31.96	71	86	NEG			86.75	NEG			NEG	Asymptomatic
NCVC08U	4	M	Unvacc	33.23	29	85.3	NEG			86.65	NEG			NEG	Cat bite abscess
NCVC09U	5	M	Unvacc	32.43	51	86.3	NEG	38.51	0	86.65	NEG			NEG	Cat bite abscess, gingivitis

				2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR			
Clinic ID	Age	Sex	Vacc status	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.	Consensus result	Clinical signs
NCVC10U	7	F	Unvacc	34.64	11	79.5	POS	33.74	13	80.5	POS	NEG		POS	Asymptomatic
NCVC11U	4	M	Unvacc	38.25	1		NEG			86	NEG			NEG	Pneumonia
NCVC12U	13	M	Unvacc	32.63	44	82.5	POS	34.76	5	82.4	POS	NEG		POS	Diabetes mellitus
NCVC13U	10	M	Unvacc	33.4	26		NEG			88.1	NEG			NEG	Asymptomatic
PD01U	4	M	Unvacc	31.02	213	83.3	POS			81.6	NEG	NEG		NEG	Asymptomatic
PD02U	5	M	Unvacc	36.83	3		NEG							NEG	Asymptomatic
TV01U	7	M	Unvacc	34.91	14	89.3	NEG			85.3				NEG	Asymptomatic
TV03U	5	F	Unvacc	31.5	152	83.3	POS			86.6	NEG	NEG		NEG	Asymptomatic
TV04U	14	F	Unvacc	30.05	423	81.8	POS			80	NEG	POS	NEG	NEG	Asymptomatic
TV05U	6	M	Unvacc	31.92	113	83.4	POS				NEG	NEG		NEG	Asymptomatic
TV06U	6	M	Unvacc	29.51	620	81.4	POS	31.82	65	81.5	POS	NEG		POS	Asymptomatic
U01U	11	M	Unvacc	28.06	1,731	80.4	POS					POS	FIV	POS	Asymptomatic
U02U	8	F	Unvacc	31.61	140	81.65	POS			86.5	NEG	POS	NEG	NEG	Lame
U03U	4	M	Unvacc	32.82	60	83.4	POS			88.1	NEG	NEG		NEG	Asymptomatic
U04U	?	F	Unvacc	37.45	2		NEG							NEG	Lame
U05U	4	F	Unvacc	36.06	6		NEG							NEG	Asymptomatic
VAH01U	5	M	Unvacc	37.04	3	80	POS			87.35	NEG	NEG		NEG	Cat bite abscess
VAH02U	10	F	Unvacc	36.19	5		NEG							NEG	Asymptomatic
VAH03U	2	F	Unvacc	33.19	46	80.25	POS			82.15	NEG	NEG		NEG	Asymptomatic
VAH04U	18	F	Unvacc	31.86	118	83.4	POS			87	NEG	NEG		NEG	Asymptomatic
VAH05U	5	M	Unvacc	36.36	5		NEG							NEG	Gingivitis
VAH06U	15	F	Unvacc	37.69	2		NEG							NEG	Asymptomatic
VAH07U	3	M	Unvacc	36.18	6		NEG							NEG	Asymptomatic

Clinic ID	Age	Sex	Vacc status	2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR		Consensus result	Clinical signs
				Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.		
VAH08U	8	F	Unvacc	32.97	53	83.25	POS			84	NEG	NEG		NEG	Asymptomatic
VAH09U	7	M	Unvacc	31.51	151	80.1	POS	31.22	116	79.8	POS	NEG		POS	Abdominal mass
VAH10U	9	F	Unvacc	31.69	133	82.15	POS	34.23	7	81.5	POS	NEG		POS	Asymptomatic
VAH11U	4	F	Unvacc	29.12	820	83.3	POS			79.45	NEG	NEG		NEG	Asymptomatic
VAH12U	15	M	Unvacc	32.74	63	81.9	POS					POS	FIV	POS	Asymptomatic
VAH13U	7	F	Unvacc	32.1	99	83.35	POS			79.2	NEG	NEG		NEG	Ocular disease
VEA01U	2	M	Unvacc	32.34	51		NEG							NEG	Asymptomatic
VEA02U	3	M	Unvacc	31.6	87		NEG							NEG	Asymptomatic
VEA03U	10	M	Unvacc	32.08	62		NEG							NEG	Asymptomatic
VEA04U	5	M	Unvacc	32.47	47		NEG							NEG	Asymptomatic
VEA05U	3	F	Unvacc	32.23	56		NEG							NEG	Asymptomatic
VEA06U	8	M	Unvacc	32.19	57	81	POS			79.45	NEG	NEG		NEG	Asymptomatic
VEA07U	13	F	Unvacc	32.97	33		NEG							NEG	Asymptomatic
VEG01U	13	M	Unvacc	31.53	148	83.2	POS			79.35	NEG	NEG		NEG	Asymptomatic
VEG02U	4	M	Unvacc	31.05	209	81.8	POS			79.5	NEG	NEG		NEG	Asymptomatic
VEG03U	10	F	Unvacc	33.13	48	83.4	POS				NEG	NEG		NEG	Asymptomatic
VEG04U	10	M	Unvacc	32.82	59	83.15	POS			81.9	NEG	NEG		NEG	Polyuria
VEG05U	8	F	Unvacc	33.7	32	79.5	POS				NEG	POS	NEG	NEG	Asymptomatic
VEVS01U	2	M	Unvacc	32.95	33	84.05	NEG			84.5	NEG			NEG	Asymptomatic
VEVS02U	3	F	Unvacc	31.64	84		NEG							NEG	Asymptomatic
VEVS03U	3	M	Unvacc	31.65	84		NEG							NEG	Asymptomatic
VFG01U	8	M	Unvacc	34.83	9		NEG							NEG	Asymptomatic
VFG02U	4	F	Unvacc	34.66	10		NEG			86.15	NEG			NEG	Asymptomatic

				2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR			
Clinic ID	Age	Sex	Vacc status	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.	Consensus result	Clinical signs
VFG03U	3	F	Unvacc	36.55	3		NEG							NEG	Asymptomatic
VFG04U	4	M	Unvacc	33.58	23	83.9	POS	35.88	1	79.5	POS	NEG		POS	Asymptomatic
VFG05U	8	F	Unvacc	32.4	52	85.15	NEG			85.15	NEG			NEG	Asymptomatic
VFG06U	4	F	Unvacc	32.74	39		NEG							NEG	Asymptomatic
VFG07U	15	M	Unvacc	32.26	55		NEG							NEG	Asymptomatic
VFG08U	3	M	Unvacc	38.26	1		NEG							NEG	Asymptomatic
VFG09U	11	M	Unvacc	30.48	192	83	POS				NEG	NEG		NEG	Asymptomatic
VPT01U	3	M	Unvacc	32.92	34		NEG							NEG	Asymptomatic
VPT02U	3	F	Unvacc	31.84	73	81.8	POS			85.35	NEG	NEG		NEG	Asymptomatic
VPT03U	5	F	Unvacc	30.62	174	81.85	POS	35.47	2	81	POS	NEG		POS	Asymptomatic
VPT04U	9	M	Unvacc	32.44	48	83.5	POS			79.2	NEG	POS	NEG	NEG	Asymptomatic
VPT05U	4	F	Unvacc	30.8	153		NEG							NEG	Asymptomatic
TV02U	5	F	Unvacc	33.02	52	83.65	NEG			79.7	NEG			NEG	Asymptomatic
WV01U	4	M	Unvacc	33.25	27		NEG							NEG	Asymptomatic
WV02U	2	M	Unvacc	33.18	28		NEG							NEG	Asymptomatic
WW01U	2	F	Unvacc	33.15	29		NEG							NEG	Cat bite abscess
WW02U	16	M	Unvacc	31.68	82		NEG							NEG	Asymptomatic
WW03U	2	M	Unvacc	32.57	44		NEG							NEG	Asymptomatic
WW04U	?	M	Unvacc	33.41	24		NEG							NEG	Asymptomatic
WW05U	5	M	Unvacc	33.1	30		NEG							NEG	Asymptomatic
WW06U	4	M	Unvacc	33.98	16		NEG							NEG	Asymptomatic
WW07U	18	M	Unvacc	33.3	26		NEG							NEG	Chronic kidney disease

Clinic ID	Age	Sex	Vacc status	2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR		Consensus result	Clinical signs
				Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.		
WW08U	4	M	Unvacc	32.07	62	82.2	POS	36.01	1	81.35	POS	POS	NEG	POS	Asymptomatic
CVT01V	16	M	Vacc	30.95	224	83.6	NEG							NEG	Asymptomatic
CVT03V	10	M	Vacc	31.35	169		NEG							NEG	Asymptomatic
HVC01V	3	M	Vacc	36.72	4		NEG							NEG	Asymptomatic
HVC02V	5	F	Vacc	32.26	89	81.5	POS	34.71	0	86	NEG	NEG		NEG	Asymptomatic
HVC03V	4	F	Vacc	34.55	18	91.7	NEG							NEG	Asymptomatic
HVC04V	6	M	Vacc	37.61	0	81.3	NEG							NEG	Asymptomatic
HVC05V	5	F	Vacc	33.91	28		NEG							NEG	Asymptomatic
HVC06V	7	M	Vacc	34.24	22	79.3	NEG							NEG	Asymptomatic
HVC07V	13	F	Vacc	36.37	5		NEG							NEG	Asymptomatic
HVC08V	4	F	Vacc	35.34	10	86.7	NEG							NEG	Asymptomatic
HVC09V	4	M	Vacc	33.46	38	92.3	NEG							NEG	Asymptomatic
HVC10V	5	M	Vacc	32.56	72	81.2	POS	30.86	16	81.25	POS	NEG		POS	Asymptomatic
HVC11V	5	M	Vacc	34.03	25	86.7	NEG							NEG	Asymptomatic
NCVC01V	2	M	Vacc	35.02	120		NEG							NEG	Asymptomatic
NCVC02V	2	M	Vacc	34.76	144		NEG							NEG	Asymptomatic
NCVC03V	2	F	Vacc	35.97	61	80.5	POS			89.45	NEG	NEG		NEG	Asymptomatic
NCVC04V	2	F	Vacc	34.52	171	79.7	POS	32.65	3	80.2	POS	NEG		POS	Asymptomatic
NCVC05V	2	F	Vacc	32.81	575	80.25	POS	31.89	6	80.15	POS	NEG		POS	Asymptomatic
NCVC06V	7	F	Vacc	34.23	209	79.5	POS			86.25	NEG	NEG		NEG	Asymptomatic
NCVC07V	7	F	Vacc	34.4	186	79.4	NEG							NEG	Asymptomatic
NCVC08V	3	F	Vacc	35.22	104	80.2	POS	34.92	0	89.7	NEG	NEG		NEG	Asymptomatic
NCVC09V	6	M	Vacc	33.93	260	80.2	POS	35.83	0	86.4	NEG	NEG		NEG	Asymptomatic

				2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR			
Clinic ID	Age	Sex	Vacc status	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.	Consensus result	Clinical signs
NCVC10V	2	M	Vacc	33.05	51		NEG							NEG	Asymptomatic
NCVC11V	2	M	Vacc	34.06	25	80.5	POS	32.81	2	86.3	NEG	NEG		NEG	Asymptomatic
PD01V	3	F	Vacc	36.35	5	81	POS	32.89	2	81.7	POS	NEG		POS	Asymptomatic
PD02V	3	F	Vacc	33.88	28	80.7	POS	31	14	80.55	POS	NEG		POS	Lymphoma
PD03V	5	F	Vacc	35.94	7		NEG							NEG	Asymptomatic
TV01V	6	M	Vacc	32.94	55		NEG							NEG	Asymptomatic
TV02V	7	F	Vacc	32.14	96		NEG							NEG	Asymptomatic
TV03V	3	F	Vacc	26.39	5,667	82	POS					POS	FIV	POS	Asymptomatic
TV04V	3	F	Vacc	32.64	68	82.5	POS	33.47	1	84.2	NEG	NEG		NEG	Asymptomatic
TV05V	4	M	Vacc	32.54	73		NEG	33.14	2	87	NEG			NEG	Asymptomatic
TV06V	4	M	Vacc	34.79	15		NEG							NEG	Asymptomatic
TV07V	3	M	Vacc	32.25	89		NEG							NEG	Asymptomatic
TV08V	2	M	Vacc	32.95	54		NEG							NEG	Asymptomatic
TV09V	2	M	Vacc				NEG							NEG	Asymptomatic
TV10V	2	M	Vacc	31.61	141		NEG							NEG	Asymptomatic
U01V	3	M	Vacc	33.82	29	84	NEG							NEG	Asymptomatic
U02V	?	M	Vacc	31.05	208	81	POS	34.26	1	86.55	NEG	NEG		NEG	Asymptomatic
U03V	6	M	Vacc	30.17	389	81.65	POS	32.25	4	89.6	NEG	NEG		NEG	Asymptomatic
U04V	6	F	Vacc	33.62	34	83.6	NEG							NEG	Asymptomatic
U05V	3	M	Vacc	32.09	100	81.3	POS	31.34	10	82	POS	NEG		POS	Asymptomatic
VAH01V	5	M	Vacc	34.71	149	79.7	POS	34.08	1	80	POS	NEG		POS	Asymptomatic
VAH02V	3	M	Vacc	32.31	818	81.25	POS					POS	FIV	POS	Asymptomatic
VAH03V	4	F	Vacc	25.3	116,331	81.8	POS					POS	FIV	POS	Asymptomatic

				2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR			
Clinic ID	Age	Sex	Vacc status	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.	Consensus result	Clinical signs
VAH04V	11	M	Vacc	34.09	231	80.5	POS			86.25	NEG	NEG		NEG	Asymptomatic
VAH05V	8	F	Vacc	34.21	212		NEG							NEG	Asymptomatic
VAH06V	10	M	Vacc	33.69	307	80.2	POS			86.5	NEG	NEG		NEG	Asymptomatic
VAH07V	8	M	Vacc	34.96	125	81	POS			86.6	NEG	NEG		NEG	Asymptomatic
VAH08V	7	M	Vacc	34.39	187	79.5	POS			86.6	NEG	NEG		NEG	Asymptomatic
VAH09V	9	F	Vacc	36.23	51		NEG							NEG	Asymptomatic
VAH10V	5	M	Vacc	35.88	65	80.5	POS			86.15	NEG	NEG		NEG	Asymptomatic
VAH11V	12	F	Vacc	36.76	35		NEG							NEG	Asymptomatic
VAH12V	5	F	Vacc	35.34	96		NEG							NEG	Asymptomatic
VAH13V	2	F	Vacc	36.27	49		NEG							NEG	Asymptomatic
VAH14V	11	F	Vacc	33.24	29		NEG							NEG	Cat bite abscess
VAH15V	4	F	Vacc	33.38	26	80	POS			87.1	NEG	NEG		NEG	Asymptomatic
VAH16V	4	F	Vacc	34.01	17		NEG			86.1	NEG			NEG	Asymptomatic
VEA01V	2	F	Vacc	30.61	284		NEG							NEG	Asymptomatic
VEA02V	3	F	Vacc	32.11	98		NEG							NEG	Asymptomatic
VEA03V	2	F	Vacc	32.06	102		NEG							NEG	Asymptomatic
VEA04V	3	F	Vacc	31.17	192	86.85	NEG							NEG	Asymptomatic
VEA05V	3	F	Vacc	31.06	208		NEG							NEG	Asymptomatic
VEA06V	3	F	Vacc	31.34	170		NEG							NEG	Asymptomatic
VEA07V	5	F	Vacc	32.18	892	80.5	POS	28.83	109	80.5	POS	NEG		POS	Asymptomatic
VEG01V	3	M	Vacc	33.57	35	87.7	NEG							NEG	Asymptomatic
VEG02V	4	M	Vacc	33.68	21	81	POS			86.65	NEG	NEG		NEG	Asymptomatic
VEG03V	4	F	Vacc	33.84	19	80.5	POS	32.61	39	80.65	POS	NEG		POS	Asymptomatic

				2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR			
Clinic ID	Age	Sex	Vacc status	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.	Consensus result	Clinical signs
VEG04V	12	F	Vacc	34.67	10	80.3	POS	33.05	26	80.7	POS	POS	NEG	POS	Hyperthyroidism
VEG05V	12	F	Vacc	34.91	9		NEG			86.2	NEG			NEG	Hyperthyroidism
VEG06V	7	M	Vacc	34.33	21	80.5	POS			84.1	NEG			NEG	Asymptomatic
VEVS01V	7	F	Vacc	33.62	34		NEG							NEG	Asymptomatic
VEVS02V	7	M	Vacc	31.67	134		NEG							NEG	Asymptomatic
VEVS03V	5	M	Vacc	34.02	26	80.3	POS	32.2	4	80.2	POS	NEG		POS	Asymptomatic
VFG01V	7	F	Vacc	32.84	560	80	POS	31.71	7	82.15	POS	NEG		POS	Asymptomatic
VFG02V	5	F	Vacc	35.03	119		NEG							NEG	Asymptomatic
VFG03V	4	M	Vacc	34.05	238	80	POS	31.74	7	82.25	POS	NEG		POS	Asymptomatic
VFG04V	8	M	Vacc	34.32	197	80.5	POS	31.64	7	80.5	POS	NEG		POS	Asymptomatic
VFG05V	2	M	Vacc	30.3	3,377	79.65	POS	29.22	75	80.65	POS	NEG		POS	Asymptomatic
VFG06V	13	F	Vacc	37.36	23		NEG							NEG	Asymptomatic
VFG07V	5	M	Vacc	35.08	115	79	NEG							NEG	Asymptomatic
VFG08V	5	F	Vacc	34.54	168	80.2	POS	32.39	4	86.4	NEG	NEG		NEG	Asymptomatic
VFG09V	5	M	Vacc	34.79	141	79.5	POS	33.31	2	86.75	NEG	NEG		NEG	Asymptomatic
VFG10V	?	F	Vacc	32.51	74		NEG							NEG	Asymptomatic
VFG11V	5	M	Vacc	33.06	50		NEG							NEG	Asymptomatic
VHN01V	11	F	Vacc	32.71	41	80.2	POS	32.45	46	80.6	POS	NEG		POS	Asymptomatic
VHN02V	7	F	Vacc	34	17		NEG			86.15	NEG			NEG	Cat bite abscess
VHN03V	5	F	Vacc	33.77	20		NEG			86.35	NEG			NEG	Asymptomatic
VHN04V	8	M	Vacc	33.08	32		NEG			87.6	NEG			NEG	Asymptomatic
VHN05V	2	M	Vacc	33.28	28		NEG			79.8	NEG			NEG	Asymptomatic
VHN06V	10	M	Vacc	32.73	41	80.3	POS	35.44	3	80.5	POS	NEG		POS	Gingivitis

Clinic ID	Age	Sex	Vacc status	2uL screening qPCR (2 µL template				Confirmatory qPCR (4 µL template)				Conventional PCR		Consensus result	Clinical signs
				Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Mean Ct	Mean calc conc (copies /uL)	Mean melt peak (°C)	Result	Result	Seq.		
VPT01V	6	F	Vacc	33.2	45		NEG							NEG	Cat bite abscess
VPT02V	3	F	Vacc				NEG							NEG	Asymptomatic
VPT03V	6	M	Vacc	35.21	11		NEG							NEG	Asymptomatic
VPT04V	6	F	Vacc	36.6	4		NEG							NEG	Asymptomatic
VPT05V	7	F	Vacc	34.7	16		NEG							NEG	Asymptomatic
VPT06V	5	M	Vacc	34.34	20	81.2	POS	33.3	2	89.1	NEG	NEG		NEG	Asymptomatic
WV01V	7	M	Vacc	31.51	150	80.1	POS	31.16	12	80.3	POS	NEG		POS	Asymptomatic
WV02V	2	M	Vacc	30.46	317	84.3	NEG							NEG	Asymptomatic
WW01V	3	M	Vacc	31.25	181	80	POS	30.31	26	80.4	POS	NEG		POS	Asymptomatic
WW02V	3	F	Vacc	32.11	99	83.6	NEG							NEG	Asymptomatic
WW03V	5	M	Vacc	32.57	71		NEG							NEG	Asymptomatic
WW04V	9	F	Vacc	31.18	190	81.35	POS	30.99	14	80.25	POS	NEG		POS	Asymptomatic
WW05V	6	M	Vacc	31.21	187	81.15	POS	31.99	5	80.5	POS	NEG		POS	Asymptomatic
WW06V	4	F	Vacc	37.98	2		NEG							NEG	Asymptomatic
WW07V	4	F	Vacc	34.55	17	81.8	POS	30.17	30	80.4	POS	NEG		POS	Asymptomatic
WW08V	3	M	Vacc				NEG							NEG	Asymptomatic

Appendix 11

Chapter 5 statistics

The effect of vaccination on FIV status in all cats

Logistic regression model examining the effect of vaccination, age and sex on the outcome variable, FIV status

Summary			
Vax_status	**FIV**	**Age**	**Sex**
No: 82 Yes: 107	Neg: 153 Pos: 36	Min: 2.000 1st Qu: 3.000 Median: 5.000 Mean: 5.984 3rd Qu: 8.000 Max: 18.000 NA's: 2	F: 85 M: 104

General linear model
Formula = fiv ~ age + sex + vax_status, family = binomial, data = fivax

Deviance residuals:				
Min	**1Q**	**Median**	**3Q**	**Max**
-0.8805	-0.7170	-0.5825	-0.5025	2.0573

Coefficients:				
	Estimate	**Std Error**	**Z value**	**P-value**
(Intercept)	-2.16747	0.56903	-3.809	0.000139
Age	0.03591	0.05240	0.685	0.493193
Sex_M	0.12057	0.38001	0.317	0.751022
Vax_status_yes	0.72490	0.41303	1.755	0.079245

The effect of vaccination on FIV status in cats tested prior to vaccination

Logistic regression model examining the effect of vaccination, age and sex on the outcome variable, FIV status

Summary			
Vax_status	**FIV**	**Age**	**Sex**
No: 82	Neg: 153	Min: 2.000	F: 80
Yes: 94	Pos: 23	1st Qu: 3.000	M: 96
		Median: 5.000	
		Mean: 6.138	
		3rd Qu: 8.000	
		Max: 18.000	
		NA's: 2	

General linear model
Formula = fiv ~ age + sex + vax_status, family = binomial, data = fivax

Deviance residuals:				
Min	**1Q**	**Median**	**3Q**	**Max**
-0.8629	-0.5469	-0.4796	-0.4386	2.2016

Coefficients:				
	Estimate	**Std Error**	**Z value**	**P-value**
(Intercept)	-2.48969	0.63678	-3.910	9.24e-05
Age	0.09409	0.05604	1.679	0.0932
Sex_M	0.08536	0.45799	-0.186	0.8521
Vax_status_yes	0.05624	0.47109	0.119	0.9050