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Virus evolution in the progression of natural feline immunodeficiency virus infection

Submitted in the fulfilment of the requirements of the degree of
Doctor of Philosophy

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Abstract

Feline immunodeficiency virus (FIV) is an important pathogen of domestic cats which in some cases can lead to feline AIDS. It shares many similarities with its human counterpart and is studied to understand correlates of immune-protection and mechanisms of disease progression in cats, both to improve the welfare of infected cats and as an animal model for the pathogenesis of HIV infection in humans.

FIV is believed to evolve during the course of infection as a result of the error prone nature of reverse transcriptase and recombination between viral variants, but relatively little is known about this process in naturally occurring infection. Ultimately, it remains unknown why some infected cats remain healthy while others progress to AIDS rapidly.

The studies reported in this thesis addressed this lack of knowledge by examining sequential blood samples obtained during the course of natural FIV infection in a population of 44 privately owned domestic cats.

Employing Bayesian coalescent framework, it was demonstrated that the FIV *env* gene is relatively stable genetically. Although not necessary a prerequisite, this is likely to explain why many naturally infected cats can remain healthy and do not progress to AIDS. By determining the cell tropism of isolated viral variants, it was shown that sick cats were more likely to harbour viruses of the "late" phenotype than healthy animals, similar to the co-receptor switch observed during the progression of HIV infection. Intra-host diversity analyses highlighted a likely role for the leader region of the *env* gene in viral pathogenesis. Furthermore, recombination was demonstrated to be abundant in natural infection, indicating a requirement for the current phylogenetic classification of FIV to be revised.

By assessing the strength and breadth of neutralising antibodies (NAbs), it was shown that NAbs did not appear to influence the course of natural FIV infection, arguing against a role in controlling infection and disease progression.

Following an examination of samples collected from a group of privately owned Australian vaccinates, it was shown that the Fel-O-Vax FIV vaccine did not induce cross-reactive neutralising antibodies. Furthermore, in the country where commercial FIV vaccine is licenced, we identified and characterised the virus strain which was likely able to establish infection in vaccinated cat and raised concerns of vaccine's efficacy.

Overall this study broadens our understanding of natural FIV infection, and highlights that much can be learned, not from the similarities but rather by studying the differences between the feline and human lentiviruses. Such comparative studies are likely to contribute to design of highly desirable, safe and fully efficacious lentiviral vaccines.

Acknowledgement

Finally, I would like to express my sincere acknowledgement to mum, dad and my brother for their love, care, support and encouragement throughout the years and it is to them that I dedicate this thesis – to my beloved parents and brother.

To my beloved parents and brother

Author's Declaration

I declare that this thesis is the result of my own work, unless specifically referenced and has not been submitted for any other degree at the University of Glasgow or any other institution.

Paweł Bęczkowski
December 2012

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2. S1V_Sydney_Alignment.FASTA – alignment of full length *env* sequences of the Australian strain of FIV which likely overcame vaccine induced protection.

3. Clinical_Data.XLS – Excel file comprising worksheets containing: detailed signalement, clinical history, physical examination and body weight data collected from cats recruited in this study.

List of abbreviations

Abbreviation	Meaning
ABCD	The Advisory Board on Cat Diseases
Ab	Antibody
AC	Asymptomatic carrier
ADCVI	Antibody-dependent cell mediated viral inhibition
ADE	Antibody-dependent enhancement
AIDS	Acquired immunodeficiency syndrome
Ala	Alanine
ARC	AIDS related complex
B2542	B2542 strain of FIV
BEAST	Bayesian evolutionary analysis sampling trees
BIV	Bovine immunodeficiency virus
bp	Base pair
BSA	Bovine serum albumin
CA	Capsid
CAEV	Caprine arthritis and encephalitis virus
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CID50	50% cat infectious dose
CPG-41	CPG-41 strain of FIV
CRD	Cysteine rich domain
CRF	Circulating recombinant form
Cr-NAbs	Cross-reactive neutralising antibodies
CTL	Cytotoxic T lymphocyte
DENV	Dengue virus
DLH	Domestic long hair
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DSH	Domestic short hair
dUTPase	Deoxyuracil triposphatase

EBV	Epstein-Barr virus
EC	Elite controllers
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine infectious anaemia virus
ELISA	Enzyme-linked immunosorbent assay
ESS	Effective sample size
FC-1	FC-1 (Florida) strain of FIV
FCV	Feline calicivirus
FeLV	Feline leukaemia virus
FeSFV	Feline syncytia forming virus
FIV-Fca	FIV strain of domestic cat (*Felis catus*)
GARD	Genetic algorithm recombination detection
GL-8	Glasgow 8 strain of FIV
Glu	Glutamic acid
HCM	Hypertrophic cardiomyopathy
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHV-6	Human herpes virus 6
HIV	Human immunodeficiency virus
HPD	Highest posterior density
HRP	Horseradish peroxidase
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IL-2	Interleukin 2
IN	Integrase
ISCOM	Immune stimulating complex
K	Thousand
K2P	Kimura-2-Parameter
KKS	KKS strain of FIV
LB	Luria Bertani
LTNP	Long-time non-progressor
LTR	Long terminal repeat
luc	Firefly luciferase
Lys	Lysine
M2 PET	M2PET strain of FIV
MA	Matrix

MCMC	Markov Chain Monte Carlo
MDV	Marek's disease virus
MHC	Major histocompatibility complex
ML	Maximum likelihood
MMTV	Mouse mammary tumour virus
MuLV	Murine leukaemia virus
MVV	Maedi-visna virus
NAb	Neutralising antibody
NCSU	NCSU (North Carolina State University) strain of FIV
NJ	Neighbour joining
NK	Natural killer cells
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Primer binding site
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNGS	Potential N-linked glycosylation site
PPR	PPR strain of FIV
PR	Protease
PVL	Plasma viral load
R5	Strain of HIV utilizing CCR5 co-receptor
R5X4	Strain of HIV utilizing both CCR5 and CXCR4 co-receptors
RDP	Recombination detection program
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RRE	Rev response element
RT	Reverse transcriptase
SBP	Single breakpoint
SGA	Single genome amplification
SIV	Simian immunodeficiency virus
SLAC	Single likelihood ancestor counting
SOC	Super optimal broth
SPF	Specific pathogen free

SU	Surface domain of Env
Thr	Threonine
TM	Trans membrane domain of Env
TMA	Thrombotic micro-angiopathy
TRIM	Tripartite motif protein
Tris	Hydroxymethyl aminomethane
Tween 20	Poloxyethylene-sobitanmonolaurate
URI	Upper respiratory infection
URT	Upper respiratory tract
USDA	United States Department of Agriculture
UV	Ultra-violet
V	Volt
V loop	Variable loop
VNA	Virus neutralisation assay
X4	Strain of HIV utilizing CXCR4 co-receptor

Chapter 1. Introduction

1.1 Overview

Feline immunodeficiency virus (FIV) is a lentivirus of domestic cats (*Felis catus)*, which in its natural host induces acquired immunodeficiency syndrome (AIDS), mirroring human immunodeficiency virus (HIV) infection (Elder et al., 2010). Both FIV and HIV induce a progressive decline in circulating CD4+ T lymphocytes, lead to inversion of the CD4:CD8 ratio and subsequently cause immunosuppression that paves the way to opportunistic infections, neoplasia and death. Therefore, as well as being an important pathogen of domestic cats, FIV has significant comparative value to better understand correlates of immune-protection and mechanisms of disease progression in HIV infected humans.

1.2 Discovery and origins

FIV, initially described as feline T-lymphotropic virus, was first isolated in Petaluma, California, USA in 1986 from a domestic cat suffering from multiple symptoms consistent with immunodeficiency like syndrome in humans (Pedersen et al., 1987). Since its discovery, FIV infection of the domestic cat has been shown to be distinct amongst the non-primate lentiviruses, namely caprine arthritis-encephalitis virus (CAEV), Maedi Visna virus (MVV), equine infectious anaemia virus (EIAV) and bovine immunodeficiency virus (BIV), since FIV alone may cause an AIDS like disease in its natural host.

FIV most likely originated between 3.1 and 0.7 million years ago among wild felids in Africa and disseminated through species within the Panthera lineage, subsequently infecting ancestors of the African lion, jaguar and eventually spreading among New World cats as species-specific strains (Pecon-Slattery et al., 2008). The evolution of FIV-Fca (FIV of *Felis catus)* is characterized by a long monophyletic branch (Pecon-Slattery et al., 2008). Taken together with the relatively recent origin of the cat (which was domesticated around 10,000 years ago in the Middle East (Driscoll et al., 2007)), it has been suggested that acquisition of the virus was much more recent for the domestic cat compared to the lion or puma. Subsequent world-wide dissemination of the virus was likely triggered by the domestication process (Pecon-Slattery et al., 2008).

Furthermore, an analysis of viral sequence divergence and disease pathogenesis amongst the Felidae, suggested different periods of virus-host co-evolution (Troyer et al., 2008). From this perspective, in the majority of cases, the least pathogenic viruses co-evolve with their hosts for longer periods of time, whereas more virulent strains have more recent origins. This pattern seems to be reflected in the disease inducing potential of different species specific FIVs, with FIV-Pco (FIV of the puma) being the least pathogenic while FIV-Fca is the most pathogenic. Although FIV infection in pumas and lions is believed to be asymptomatic, recent evidence suggests that infected lions may exhibit mild immunodeficiency symptoms, subsequently affecting the lifespans of infected individuals (Roelke et al., 2009). Similar observations have been made recently in SIV-infected chimpanzees, which had previously been regarded as asymptomatic carriers of the virus (Terio et al., 2011). Nevertheless, the evolutionary evidence, together with the pathogenic potential of FIV-Fca, is suggestive of a rather short period of virus-host coadaptation and relatively recent acquisition of the virus by domestic cats (Pecon-Slattery et al., 2008).

1.3 Virus biology

1.3.1 Genomic organisation

FIV shares a similar genomic organisation with other retroviruses (Miyazawa et al., 1994) which consists of three major open reading frames (ORFs): *gag, pol* and *env,* accompanied by three accessory genes: *vif, ref* and *orf-a,* all bounded by long terminal repeats (LTRs) within the provirus (Talbott et al., 1989), (Miyazawa et al., 1994), (Figure 1-1). The genome contains approximately 9400 nucleotides and sequence analysis revealed that, despite the fixed pattern of genomic organisation, FIV is more closely related to equine infectious anaemia virus (EIAV), bovine immunodeficiency virus (BIV) or caprine arthritis-encephalitis virus (CAEV) than to the primate immunodeficiency viruses (SIV and HIV) (Olmsted et al., 1989). Similarly to other retroviruses, the *gag, pol* and *env* genes encode structural, enzymatic and envelope polyproteins; respectively these are cleaved and further proteolytically processed into proteins composing mature virions (Miyazawa et al., 1994). The FIV genome contains additional accessory and regulatory genes: *vif, orf-A* and *rev* (Miyazawa et al., 1994). The *vif* gene encodes a highly conserved hydrophilic protein which counteracts

cytidine deamination by the APOBEC3G host restriction factor (Zielonka et al., 2010). It also has a role for FIV replication; viruses with a deleted *vif* gene are unable to replicate *in vivo* (Shen et al., 2007). The protein encoded by the regulatory *rev* gene is involved in trafficking of viral RNA from the nucleus to the cytoplasm by binding to the Rev response element (RRE) on incompletely spliced viral mRNA (Malim et al., 1989), (Na et al., 2010). O*rf-A* has multiple functions including influencing the cell cycle, increasing translation and mediating virus release from the cell (de Parseval and Elder, 1999), (Gemeniano et al., 2003), (Gemeniano et al., 2004). The accessory genes: *vpr, vpu* and *nef* (which are present in HIV-1) are absent in FIV particles (Miyazawa et al., 1994).

Figure 1-1 Genomic organisation of FIV proviral DNA. The genome of FIV consists of 3 major open reading frames (ORFs): *gag* – encoding: matrix (MA), capsid (CA) and nucleocapsid (NC), *pol* – encoding: protease (PR), reverse transcriptase (RT), dUTPaze (DU) and integrase (IN) and *env* – encoding: surface unit domain (SU) and transmembrane domain (TM) of the envelope glycoprotein. Additionally, in close proximity to the *env* gene, there are 2 accessory genes: *vif, orf-a* as well as one regulatory gene: *rev*. Within the provirus, the ORFs are flanked by long terminal repeats (LTRs).

The Long Terminal Repeats (LTRs) of the retroviral genome consist of U3 (unique 3' end), R (repeat) and U5 (unique 5' end) non-coding regions and flank the provirus at both ends. The U3 region of the LTR contains promoter and enhancer elements which are involved in regulating viral gene expression in infected cells (Sparger et al., 1992), (Miyazawa et al., 1994).

1.3.2 Virion structure

The FIV virion (Figure 1-2) is a spherical particle of approximately 120 nm in diameter containing two copies of positive, single stranded RNA (Talbott et al., 1989). Structural (Gag), enzymatic (Pol) and envelope (Env) precursor products of the three major retroviral genes are proteolytically processed and assemble

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into the mature virions. The Gag polyprotein consist of: the nucleocapsid NC (p7), matrix MA (p17) and capsid CA (p24) proteins. The viral core consists of the p24 CA protein which surrounds the viral RNA, bound to zinc finger motifs of NC (Elder et al., 1992). MA builds the outer shell facing the lipid bilayer. Protease PR (p13), reverse transcriptase RT (p51), integrase IN (p31) and, unique to FIV, deoxyuridine pyrophosphatase DU (p14) are contained within the viral capsid and are enzymatic products of *pol* (Talbott et al., 1989). The RT enzyme facilitates DNA synthesis from viral RNA (Goff, 1990). IN is responsible for the integration of proviral DNA into the DNA of the host cell, while PR cleaves the precursor proteins.
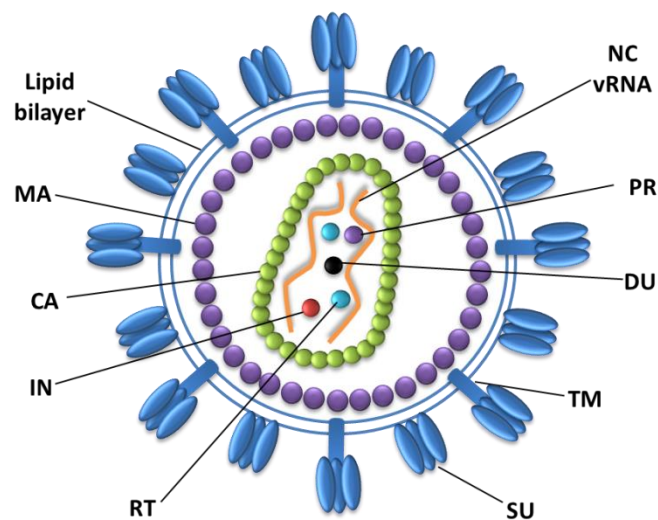
Figure 1-2 Schematic structure of FIV virion. vRNA, viral RNA; MA, matrix; CA capsid; NC nucleocapsid; RT, reverse transcriptase; IN, integrase; PR, protease; DU, deoxyuridine pyrophosphatase; SU and TM; surface and transmembrane units of the envelope glycoprotein.

DU is involved in preventing the misincorporation of dUTP into DNA and facilitates infection of non-dividing cells with low levels of endogenous DU, such as macrophages (Lerner et al., 1995). Hence FIV DU deletion mutants achieve lower burdens in macrophages compared to dividing T cells (Lerner et al., 1995).

1.3.2.1 Envelope glycoprotein

The envelope glycoprotein (Env) is exposed on the viral surface and influences almost every aspect of virus biology. Polyprotein Env precursors are synthesized on the rough endoplasmic reticulum (RER) and further processed and

glycosylated in the lumen of the RER (Leonard et al., 1990). Subsequently, the Env precursor is cleaved in the Golgi apparatus at the furin site to form the surface (SU) and the transmembrane proteins (TM) (McCune et al., 1988). TM and SU are non-covalently associated and form the viral envelope. SU is responsible for virus-host interactions, mediates viral entry through its interplay with CD134 and CXCR4 and (since it is heavily glycosylated) also facilitates immune evasion (Willett et al., 2008), (Samman et al., 2010). Structurally, the SU glycoprotein has five variable regions (V1 to V5) distributed amongst five relatively conserved regions (Pancino et al., 1993b). The TM domain holds SU within the viral membrane and plays an essential role, via its fusion peptide (FP), in the fusion of viral and cellular membranes (Bosch et al., 1989), (Freed et al., 1992). The TM comprises approximately 340 amino acids and consists of an ecto-domain, a highly conserved transmembrane domain (TMD) and a cytoplasmic tail (CT) (Checkley et al., 2011). Conserved, yet prone to some variability (Jobes et al., 2006), a number of cystein residues forming disulphide bonds is distributed throughout SU and TM and these residues are integral in the formation of the three dimensional structure of the glycoprotein (Leonard et al., 1990). The FIV Env is heavily glycosylated with host derived glycans that encompass exposed epitopes, thus contributing to protecting the virus from host immune response (Stephens et al., 1991).

1.3.3 The retroviral life cycle

The FIV replication cycle resembles that of HIV and other retroviruses (Freed, 2012) (Figure 1-3). However, there are several unique features of the FIV life cycle attributable to its genomic organisation (Talbott et al., 1989), characteristics of its RT (Huisman et al., 2008b) and, most importantly, its initial interaction with target cells (Shimojima et al., 2004).

1.3.3.1 Virus-cell interaction

Retroviral infection commences with the interaction of the viral Env with specific receptors on the surface of susceptible cells. The HIV Env preferentially binds, via the CD4 molecule, T helper lymphocytes and cells of the myeloid linage such as monocytes and macrophages (Maddon et al., 1986), (Sattentau and Weiss, 1988). The interaction of the viral envelope glycoprotein with CD4

alone is not sufficient for fusion of viral and cellular membranes; subsequent viral entry is facilitated by a secondary interaction with a chemokine co-receptor such as CXCR4 (Feng et al., 1996), (Bleul et al., 1997) or CCR5 (Alkhatib et al., 1996), (Trkola et al., 1996a). The utilisation of these two major co-receptors partially explains the differential cell tropism of the virus. Generally, X4 strains are T-cell tropic (Correa and Munoz-Fernandez, 2001), while R5 dependent strains preferentially target memory CD45RA+ T cells and macrophages (Collman et al., 1989). Nevertheless, macrophage tropic X4 strains also have been identified (Simmons et al., 1998), highlighting that other determinants within Env play a role in cell tropism.
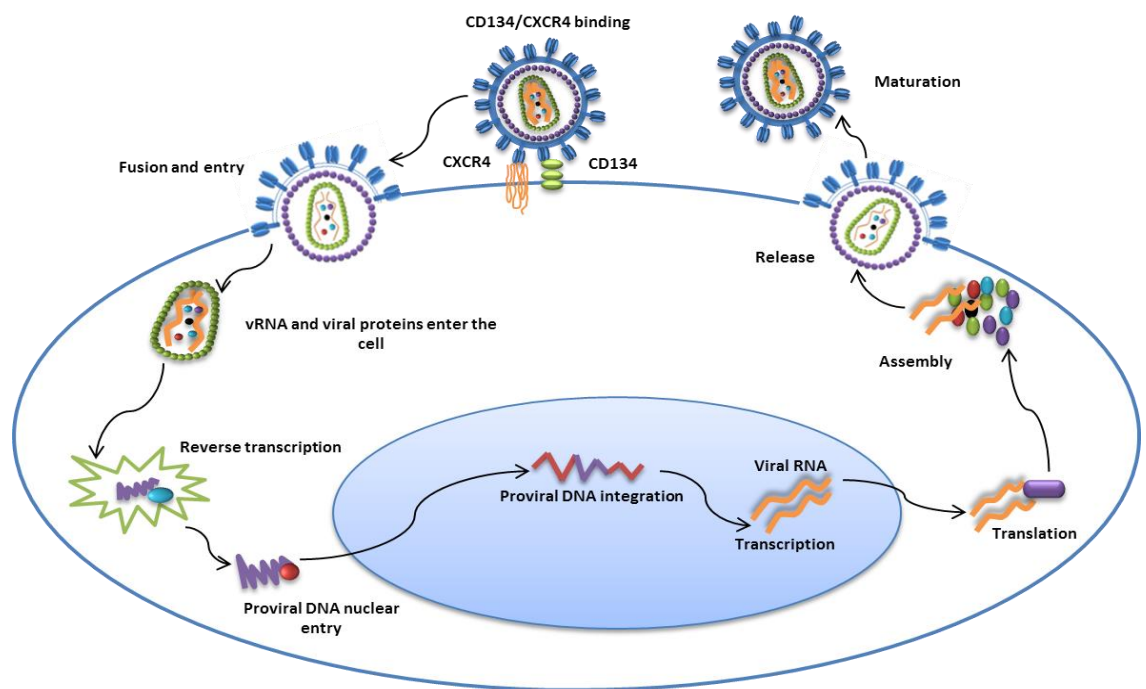
Figure 1-3 The FIV replication cycle: binding of the primary CD134 and the CXCR4 co-receptor; membrane fusion and viral entry; reverse transcription within the pre-integration complex; proviral DNA-integrase nuclear entry and integration; transcription; translation; assembly at the plasma membrane, virion release and maturation.

Indeed dual tropic R5X4 strains, which utilize both co-receptors, also have been described (Yi et al., 1999).

FIV has a similar cell tropism to HIV, but unlike its human counterpart it utilizes feline CD134 as its primary entry receptor (Shimojima et al., 2004) and CXCR4 as a coreceptor (Willett et al., 1997b), (Richardson et al., 1999). CD134 belongs to the tumour necrosis factor/nerve growth factor receptor superfamily

(TNFR/NGFR) (Locksley et al., 2001) and is expressed on feline CD4+T cells *in vitro* (Shimojima et al., 2004) and *in vivo* (Joshi et al., 2005), concordant with viral tropism and the progressive depletion of the CD4+ lymphocyte subset that occurs during the course of infection. The structure of CD134 resembles that of classical TNFR molecules, with an extracellular binding domain comprising three cysteine rich domains (CRD1, CRD2, CRD3) and an additional C-terminal domain (Bodmer et al., 2002).

Various strains of FIV differ in the affinity of their interaction with the primary receptor (Willett et al., 2006a). The PPR strain of FIV achieves productive infection following an interaction with the cysteine rich domain 1 (CRD1) of CD134 (de Parseval et al., 2005), while primary strains such as GL-8 require a more stringent interaction involving determinants on the cysteine rich domain 2 (CRD2) of CD134 (Willett et al., 2006a), (Willett et al., 2006b). It has been proposed that viral variants such as GL-8, isolated in early stage disease require a complex interaction with the receptor, while chronic infections are accompanied by the emergence of strains (such as PPR or B2542) requiring less stringent interactions with CD134, resulting in a broader cell tropism (Willett et al., 2010).

Fusion of viral and cellular membranes permits the viral core to enter the host cytoplasm where, in common with all retroviruses, reverse transcription takes place (Gilboa et al., 1979).

1.3.3.2 Reverse transcription

Reverse transcription of the viral RNA (vRNA) into a double stranded DNA copy (known as the provirus) occurs within the pre-integration complex (PIC) while vRNA is still virion associated. Reverse transcription commences shortly after budding (Oude Essink et al., 1996), (Huang et al., 1997) and is catalysed by the virion associated, error-prone RT enzyme (Temin and Mizutani, 1970). Reverse transcription is primed by the formation of RT–cellular tRNA complex (Harada et al., 1975), (Mak and Kleiman, 1997), which subsequently binds to the primer binding site (PBS) located close to the 5' end of the viral genome (Figure 1-4 A). The 3' end of the tRNA primes synthesis of single strand cDNA until it reaches the end of the 5' R region of the viral genome. This mid-product is termed

antisense strong stop DNA (minus ssDNA). At the same time, the RNase H domain of RT mediates degradation of the RNA template annealed to the minus ssDNA, thus allowing its release (Figure 1-4 A).
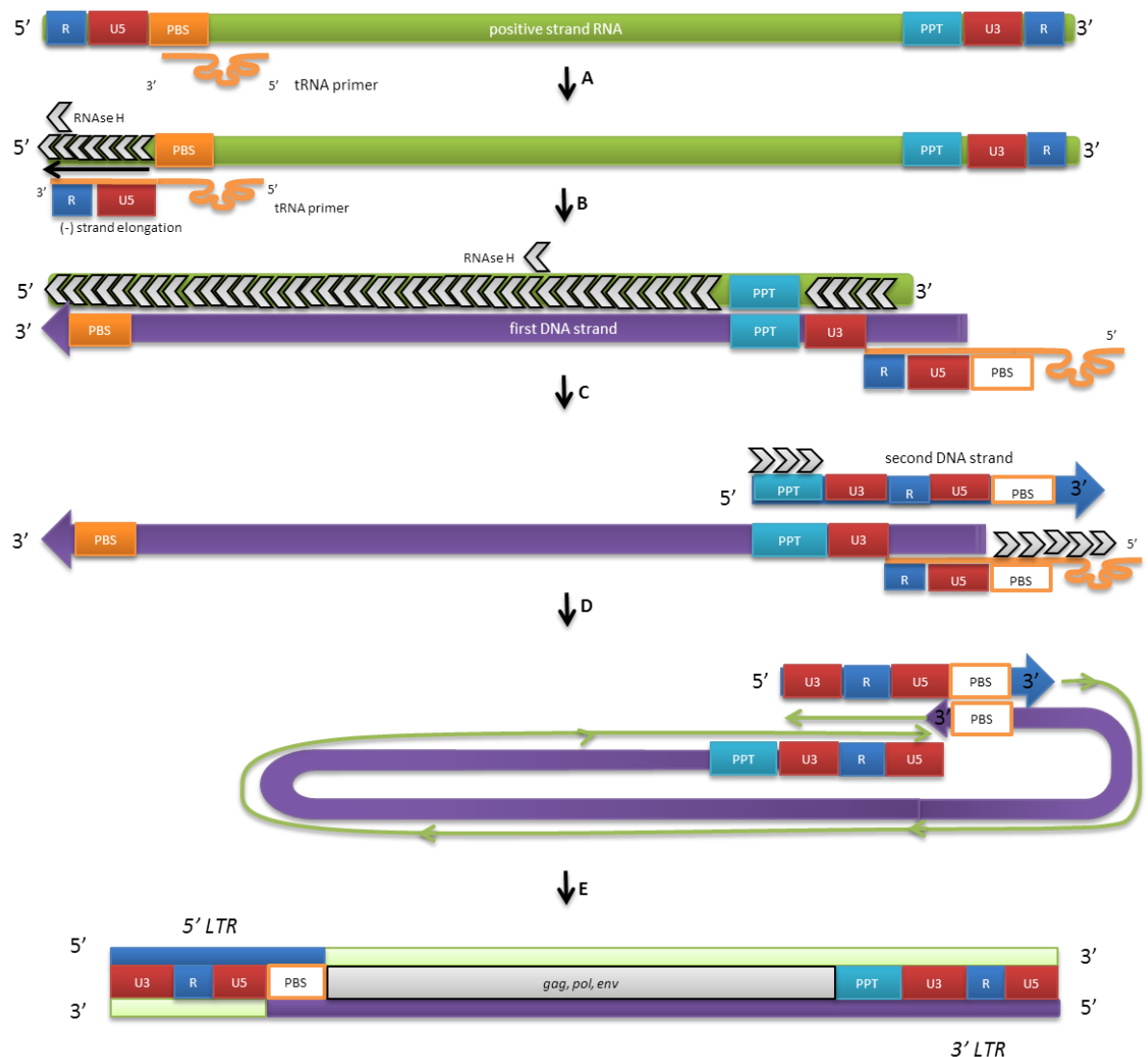
Figure 1-4 Schematic diagram of reverse transcription. For a description of each step (A-E) please refer to the text. RNase H, Ribonuclease H; tRNA, transfer RNA; first DNA strand = (-) strand DNA in purple; second DNA strand = (+) strand DNA in blue and light green. Modified from (Sarafianos et al., 2001).

Released minus ssDNA in the process of first strand transfer binds to the 3' R region of the viral genome and subsequently acts as a primer for RT, allowing the (-) strand DNA synthesis. Meanwhile, the RNase H domain of RT digests the RNA template, with the exception of the resistant polypurine regions (PPT) (Figure 1-4 B). The PPT primes subsequent (+) strand DNA synthesis, which is carried by another RT-tRNA complex (Figure 1-4 C). Thus synthesised, the

positive sense strong stop DNA (plus ssDNA) in the process of second strand transfer binds to the 3' end of (-) strand DNA (Fig 1-4 D). Reverse transcription then continues until the complementary strand is synthesised, leading via the strand displacement process to synthesis of double stranded DNA, flanked at each end by long terminal repeats (LTRs) (Figure 1-4 D, E).

Reverse transcription occurs within the virion and the resulting proviral DNA is later trafficked into the nucleus and incorporated into the host cell genome by the virally encoded integrase (IN).

1.3.3.3 Integration

Similarly to reverse transcription, integration is well conserved among retroviruses. In the course of 3' end processing, the integrase (IN) removes two nucleotides from both 3' ends of the proviral DNA and subsequently cleaves the host cellular DNA. In the process of strand transfer, the 3' ends of previously prepared proviral DNA are connected to the ends of cleaved cellular DNA. This process is completed by host repair enzymes which fill the gaps in the insertion site.

Following integration, the proviral DNA serves as a template for the synthesis of viral RNA which is subsequently used by the cell machinery for translation.

1.3.3.4 Assembly and release

The synthesis of viral proteins is followed by viral assembly at the plasma membrane of the host cell, a process mediated by the Gag precursor polyprotein (Luttge and Freed, 2010). Gag domains: matrix (MA), capsid (CA), nucleocapsid (NC) and C-terminal peptide (p2) (Lin et al., 2006) each play an important role in this process. The MA domain directs Gag to the plasma membrane and allows Env incorporation into virions (Freed, 1998). CA advances formation of the conical core surrounding the viral RNA and *pol*-encoded enzymes. The NC domain recognizes one or more packaging signals (Ψ) within the viral RNA and mediates its trafficking into the assembling virion (Browning et al., 2003). FIV Gag, despite differences in the sequences between viruses, is able to package SIV and HIV-1 RNA containing Ψ sites (Browning et al., 2001), suggesting that the structure of the Ψ sites plays a role. Indeed it has been demonstrated that FIV

genomic RNA can adopt two conformations (Kenyon et al., 2011). One of the conformations has an exposed dimerization initiation site and hence may facilitate RNA packaging (Kenyon et al., 2011).

Budding, the final step of assembly and release, is also mediated by Gag (Luttge and Freed, 2010). Unique to FIV, the p2 peptide and its highly conserved PSAP motif interact directly with the ESCRT (endosomal sorting complex required for transport) machinery and is essential for budding of the virion (Luttge et al., 2008).

Virion maturation, mediated by the viral protease (PR), proceeds shortly after virion release (Kohl et al., 1988). It involves cleavage of the Gag-Pol polyprotein precursors and formation of the respective mature proteins. Maturation is associated with a change of morphology such that virions become fully infectious.

1.4 Epidemiology

1.4.1 Prevalence

FIV-Fca is a host specific virus that is distributed world-wide among domestic cats. The prevalence of FIV infection varies significantly between geographic areas (Hosie and Beatty, 2007) and various cat populations, with estimates ranging from 1% in healthy, low risk cats in Germany to 44% in sick, high-risk populations in Japan (Yamamoto et al., 1989), (Ishida et al., 1989), (Hartmann, 1998). Adult, male cats with outdoor access have a significantly higher risk of infection (Yamamoto et al., 1989), (Gleich and Hartmann, 2009). It has been estimated that approximately 11% of cats world-wide may be infected with FIV (Courchamp and Pontier, 1994).

1.4.2 Transmission

FIV spreads mainly via the inoculation of virus in saliva during biting, associated with mating and territorial fights (Pedersen et al., 1989). Virus can also be transmitted vertically from infected queens to kittens (O'Neil et al., 1996). Sexual, trans-mucosal transmission has been reported only experimentally

(Bishop et al., 1996), and although FIV can be isolated from semen (Jordan et al., 1998), the role of this route in natural infection is unknown.

1.4.3 Diversity and classification

Based on sequence diversity, FIV is currently classified into five distinct subtypes or clades (A to E) with up to 26% variability in the V3-V5 region of the *env* gene (Sodora et al., 1994), (Kakinuma et al., 1995), (Pecoraro et al., 1996). Despite its world-wide distribution, geographical clustering of FIV is apparent (Figure 1-5). Subtype A is the only subtype identified to date in the UK (Samman et al., 2011) and is the most prevalent subtype in Australia (Kann et al., 2006), the west coast of USA, South Africa (Sodora et al., 1994), (Bachmann et al., 1997) and northern Japan (Kakinuma et al., 1995).
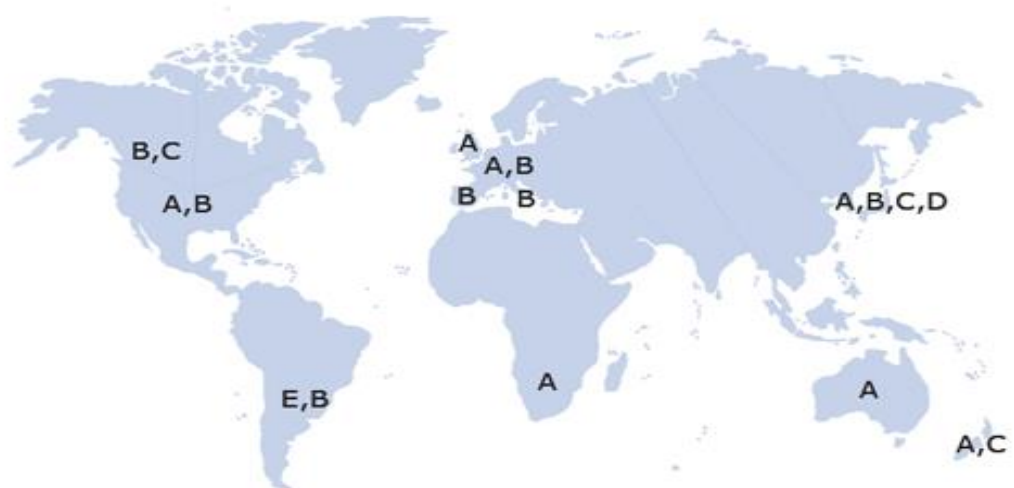
Figure 1-5 Worldwide distribution of FIV subtypes. Adopted from (Hosie et al., 2009).

Subtype B has been isolated from cats in eastern Japan (Nishimura et al., 1998), the east coast of USA (Bachmann et al., 1997), Brazil (Teixeira et al., 2012) and in the south of Europe (Pistello et al., 1997), (Steinrigl and Klein, 2003), (Duarte and Tavares, 2006). Subtype C has been described in Canada (Reggeti and Bienzle, 2004), New Zealand (Hayward and Rodrigo, 2008) and Taiwan (Kurosawa et al., 1999) while subtypes D and E have been identified only in Japan (Nishimura et al., 1998) and Argentina (Pecoraro et al., 1996) respectively.

Several studies identified cats infected with intra-clade recombinants in Canada (A/B), USA (A/C, A/B/C), Japan (A/B, B/D), New Zealand (A/C) and even the UK (A/C) (Reggeti and Bienzle, 2004), (Bachmann et al., 1997), (Carpenter et al., 1998) (Hayward and Rodrigo, 2008) and (Samman et al., 2011). These findings highlighted the substantial limitations of the current subtype A-E classification, which is based on a short V3-V5 fragment of the *env* gene (Hayward and Rodrigo, 2008). Moreover, the existence of recombinant viruses has implications for molecular diagnosis of FIV infection. Furthermore, in the USA, New Zealand and Japan (where the current FIV vaccine is licensed), the existence of recombinant viruses has implications for vaccine efficacy.

1.4.4 Detection and diagnosis

FIV infection is usually suspected in outdoor, adult, male, sick cats with bite wounds and various degrees of gingivo-stomatitis (Hartmann, 1998). Such cats, as well as high risk animals living in shelters, newly acquired cats of unknown origin and cats to be vaccinated, should be tested routinely for FIV (Little et al., 2011). Once cats have acquired FIV infection, they remain infected for life; cats subsequently produce antibodies against the virus and therefore ELISA based screening tests for antibodies against p24 or p15 viral proteins are sufficient for diagnosis in unvaccinated cats (Hartmann, 1998). Antibodies against the virus can be detected in some cats 2 to 4 weeks post experimental infection (Yamamoto et al., 1988) but the majority of cats will seroconvert within 60 days following exposure (Barr, 1996).

The IDEXX SNAP® FIV/FeLV Combo PetCheck FIV antibody test is most commonly used in the clinical setting and has been shown to have very high sensitivity and specificity (Levy et al., 2004b); however it is recommended that in-house results should be confirmed, especially for low risk cats, where the likelihood of false positive results is higher (Jacobson, 1991). There are several confirmatory tests available, with virus isolation considered the gold standard; however, it is not used routinely due to high labour costs (Levy et al., 2004b). Additional soluble antibody testing, immuno-fluorescence assay (IFA) or Western blot analysis were shown to have variable performances (Levy et al., 2004b) and are used as confirmatory measures in different countries based on their availability.

Positive serology results in kittens from infected and vaccinated queens should be interpreted with caution, since maternally derived antibodies (MDA) may persist for up to 20 weeks (MacDonald et al., 2004). In such cases, kittens should be re-tested after 6 months of age (Richards, 2005).

The FIV vaccine (Fel-O-Vax, Fort Dodge) currently available in the USA, Australia, Japan and New Zealand confounds the diagnosis of infection (Little et al., 2011). Cats sero-convert within a few weeks of vaccination; vaccine induced antibodies can persist for up to four years (Levy et al., 2008a) and cannot be distinguished from those produced against naturally acquired viruses. It is a significant challenge for practitioners to differentiate infected cats from those which have been vaccinated, as well as cats which were vaccinated and subsequently become infected. An experimental discriminatory ELISA test has been developed (Kusuhara et al., 2007). It is based on differences in reactivity of sera from vaccinated and FIV infected cats to a synthetic TM peptide and formalin-treated whole FIV antigen (Kusuhara et al., 2007). This test was shown to accurately distinguish infected from vaccinated cats, regardless of vaccination status, with 97.1% specificity (Levy et al., 2008b). Nevertheless, this diagnostic approach has been tested only in an experimental setting and is still not available commercially. There are numerous PCR assays of variable performance to aid FIV diagnosis, with sensitivities and specificities ranging from 41% to 98% (Bienzle et al., 2004), (Crawford et al., 2005). Research is being carried out to improve their sensitivity; nevertheless the diagnosis of FIV infection in countries where vaccination is widespread poses a significant challenge.

1.5 Pathogenesis

1.5.1 Course of disease

Despite the differences between the feline and human lentiviruses, the disease course following FIV infection is very similar to that of HIV (Willett et al., 1997a) (Figure 1-6). Following infection, the primary acute phase (lasting from 2 to 8 weeks) is characterised by a peak of viral replication and various non-specific clinical signs such as anorexia, lethargy and fever (del Fierro et al., 1995). Complete blood counts may disclose neutropenia (Pedersen et al., 1989).

Generalized lymphadenopathy develops during this period as a result of stimulation of the germinal centres in lymph nodes and can persist for up to 9 months post exposure (Pedersen et al., 1989), (del Fierro et al., 1995). The virus replicates to high levels in dendritic cells, macrophages and CD4+ T lymphocytes, reaching peak levels in plasma 8 to 12 weeks post infection.
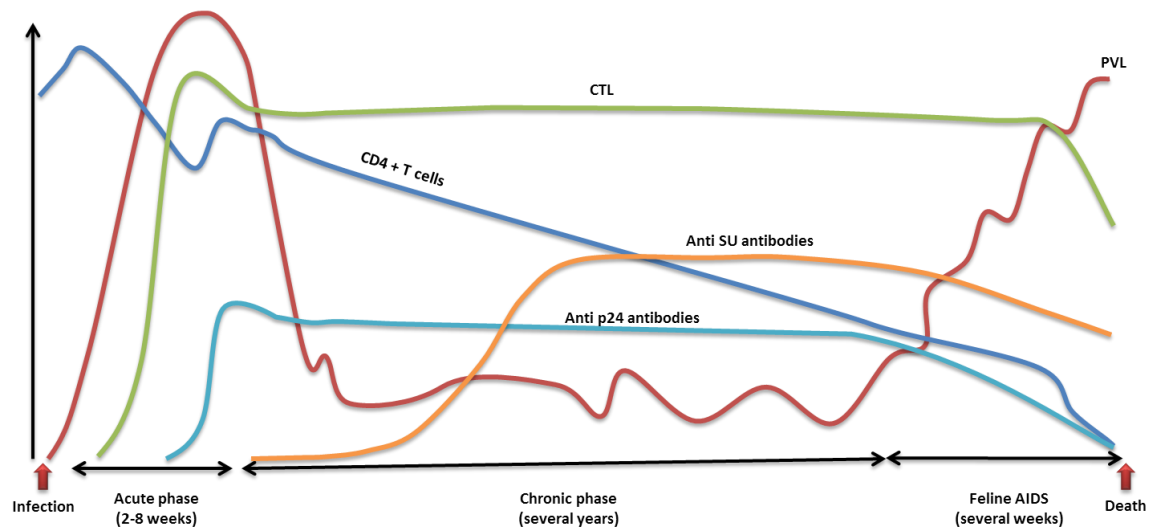
Figure 1-6 Course of disease. Following FIV infection there is an acute peak viremia which is restricted by the adaptive immune response within approximately 8 weeks. The chronic, "asymptomatic" phase follows, lasts for several years and is characterized by a robust CTL response, relatively low viral loads, a progressive decline in CD4+T lymphocytes and the emergence of anti-Env neutralising antibodies. Eventually, disease leads to the onset of an immunodeficiency like syndrome which lasts for several weeks and ultimately leads to death. PVL, plasma viral load; CTL, cytotoxic T lymphocytes; SU, surface unit of the Env glycoprotein; p24, viral capsid protein. Adapted from (Weiss, 1993).

During this phase, the numbers of CD4+ and CD8+ lymphocytes decline, but inversion of the CD4:CD8 ratio (which is likely to persist during the lifetime of the cat) is associated with the subsequent excess production of a subset of CD8+ T cells in response to infection, aimed at clearing the pathogen (Egberink and Horzinek, 1992), (Pantaleo and Koup, 2004), (Yamamoto et al., 2007). The acute phase of infection is followed by the silent phase with no clinical signs, which may last several years or be lifelong; this chronic phase is typically marked by a lack of clinical signs and supressed plasma viral loads. It is not known why some cats remain "asymptomatic" for their life time, while others progress and develop disease rapidly (Kahler, 1996), (Addie et al., 2000). Nevertheless, FIV infection may lead to immunodepletion, affecting cell-mediated immunity in particular and associated with an increased frequency of opportunistic

infections, the onset of chronic inflammatory conditions, neoplasia and subsequently death (Shelton et al., 1989b), (Hutson et al., 1991), (Gabor et al., 2001b).

1.6 Correlates of immune protection

The immunopathogenesis of FIV in many aspects resembles that of HIV infection, with both viruses targeting CD4+ T cells, CD4+CD25+ T regulatory cells, monocytes, macrophages, glial cells, CD8+ T cells and B cells (Dow et al., 1990), (English et al., 1993), (Hein et al., 2000), (Vahlenkamp et al., 2004), (Joshi et al., 2005). FIV infection elicits an early vigorous innate immune response, followed by adaptive, cell mediated and humoral responses which fail to clear the virus (Beatty et al., 1996). Loss of CD4+ T cells results in dysfunction of B and T cells and subsequently (months to years post infection) leads to the well documented failure of the immune system (Ackley et al., 1990), (Torten et al., 1991), (Taniguchi et al., 1991), (Hoffmann-Fezer et al., 1992). A paradox of lentiviral infection is the combination of immunosuppression together with prolonged, global immune hyper-activation (Tompkins and Tompkins, 2008).

1.6.1 Cell mediated immunity

Cytotoxic CD8+ T-lymphocytes (CTLs) develop as a specific response to the virus as early as 2 weeks post exposure, prior to the development of the antibody response (Beatty et al., 1996). The vigorous response is correlated with down regulation of viral replication and the decline in plasma viral load during the stage of infection with no clinical signs (Bucci et al., 1998), (Hohdatsu et al., 2003). The antiviral activity of CD8+ T cells is mediated through cytotoxic and non-cytotoxic mechanisms. Classical, FIV specific, MHC class I restricted cytotoxic T lymphocytes primed against the Gag, Pol and Env proteins were detected in the peripheral blood during the acute stage of infection (Flynn et al., 1995a), (Beatty et al., 1996), while precursors of those cells were identified in lymph nodes and spleen during the "asymptomatic" phase (Song et al., 1992), (Song et al., 1995). Populations of non-specific, CD8+ T cells targeting the virus via non cytotoxic, either contact-dependent (Bucci et al., 1998), (Gebhard et al., 1999) or contact-independent (Hohdatsu et al., 1998), (Choi et al., 2000) mechanisms have also been described. Such non-antigen specific CD8+ T cells

were shown to appear before MHC restricted cytotoxic lymphocytes, as early as one week post infection (Flynn et al., 2002) and were associated with decreased levels of cell associated virus by 12 weeks post infection (Bucci et al., 1998). Increased numbers of CD8+ T-cells are maintained throughout infection, declining only at the terminal stages of disease (Ackley et al., 1990), (English et al., 1994).

1.6.2 Humoral immunity

Infection with FIV elicits an antibody response directed against Gag and Env proteins within 2 to 4 weeks post infection (Yamamoto et al., 1988), (Egberink et al., 1992), (English et al., 1994). Virus neutralizing antibodies (VNAb) develop soon after the antigen specific CTL response and provide additional immunity against the virus (Inoshima et al., 1996). Antigen specific B cell hyper stimulation is manifested by increased plasma immunoglobulin G (IgG) levels (Ackley et al., 1990). Although VNAb titres increase progressively during the first 30 weeks post infection (Inoshima et al., 1996), it has been suggested that autologous and heterologous VNAb in HIV infected individuals may take up to 8 months to fully develop (Moog et al., 1997), (Bower et al., 2004). VNAbs are believed to have an important role in controlling FIV infection. This is attributed to the interaction of immunoglobulins with the SU trimers on the virion surface and subsequent blockage of the interaction between the virus and its receptor, thus preventing infection (Spenlehauer et al., 2001), (Labrijn et al., 2003). An additional mechanism of protection has been associated with the presence of autoantibodies recognizing the primary receptor for FIV, CD134 (Grant et al., 2009). The presence of autoantibodies against CD134 correlated with improved health status and lower viral load amongst FIV infected cats (Grant et al., 2009). Passively transferred maternal antibodies have been shown to protect kittens against challenge with laboratory adapted strains of FIV (Pu et al., 1995). However, antibody dependent enhancement, rather than restriction of infection, has also been reported (Siebelink et al., 1995). Thus, the role of VNAbs *in* vivo may be either harmful or beneficial. Nevertheless, while VNAbs may neutralise FIV *in vitro*, in the majority of cases there does not appear to be any correlation with clinical signs of disease *in vivo* (Yamamoto et al., 1991), (Tozzini et al., 1993).

1.6.3 Intrinsic immunity

Recently discovered retroviral restriction factors have a role in innate immunity that control almost every stage of the retroviral life cycle (Bieniasz and Cullen, 2000), (Huthoff and Towers, 2008), (Malim and Bieniasz, 2012). Whereas adaptive immunity requires time to fully develop, intrinsic resistance factors act immediately following infection, directly blocking viral replication and assembly (Goff, 2004), (Huthoff and Towers, 2008). Intrinsic immunity to HIV is dependent on the activity of the host's interferon stimulated genes (ISGs) that encode proteins such as: APOBEC3G (apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3 G) (Sheehy et al., 2002), Trim5α (tripartite-motif-containing 5α) (Stremlau et al., 2004) and Tetherin/BST-2 (bone marrow stromal cell antigen 2) (Neil et al., 2008). Of these, the cat genome only encodes functional feline APOBEC3G (McEwan et al., 2009) and Tetherin/BST-2 proteins (Dietrich et al., 2011a).

APOBEC3G is a cytidine deaminase, which acts during reverse transcription, editing cytidine to uracil in the negative strand viral cDNA, resulting in an accumulation of guanosine to adenosine substitutions in the positive strand viral DNA (Bishop et al., 2008) which is often seen as an accumulation of premature stop codons (Vartanian et al., 1991).

Feline Tetherin/BST-2 prevents the release of the FIV virion, retaining it on the cell surface (Dietrich et al., 2011b). However, despite tethering the virion on the cell surface, tetherin is unable to prevent cell-cell spread of FIV infection (Dietrich et al., 2011b).

1.6.4 Immune dysfunction

Despite the activity of the innate, intrinsic and adaptive immune responses, FIV infection results in exhaustion of the immune system and subsequently leads to immunodeficiency. T cell dysfunction (including impaired proliferative responses) in FIV infection is more pronounced than B cell dysfunction, which only appears to fail to respond to T dependent antigens (Siebelink et al., 1990). Weakened cell mediated immunity is attributed to the diminished number of CD4+ T cells and consequently decreased release of cytokines such as IFN-γ and

IL-2 (Davidson et al., 1993b). Thus, FIV infected cats are not only more prone to neoplasia (Terry et al., 1995) but exhibit more pronounced symptoms associated with intra-cellular pathogens such as *Listeria monocytogenes* (Dean et al., 1998) or *Toxoplasma gondii* (Davidson et al., 1993a).

The pathogenesis of FIV infection and subsequent immunodeficiency cannot be attributed solely to the depletion of CD4+ T cells and impaired cellular immunity. Immune activation and the impaired regenerative capacity of the progenitor immune cells appear to be as important in the development of immunodeficiency as direct targeting of CD4+ T cells by the virus (Appay and Sauce, 2008), (Khaitan and Unutmaz, 2011).

1.7 Prognostic indicators for FIV infection

Predicting the likely outcome of an infection is of great significance in designing an appropriate therapeutic approach. Surrogate markers for HIV infection have been well described and are valuable in monitoring both responses to therapy as well as the likelihood of disease progression (Churchill, 1997), (Smith and Stein, 2002), (Shaunak and Teo, 2003), (Katzenstein, 2003), (Okonji et al., 2012).

In contrast, relatively few studies have focused on the course of naturally acquired FIV infection and prognostic indicators of disease progression are not well established. The staging of natural infection is complex, because of the variable pathogenicity of infecting strains (Pedersen et al., 2001) and there remains uncertainty as to the role of FIV as a causative agent of morbidity and mortality in cats (Hofmann-Lehmann et al., 1997), (Kohmoto et al., 1998b), (Addie et al., 2000).

Nevertheless, in the clinical setting there is a substantial need for surrogate markers, which would aid the identification of animals which are likely to remain free of clinical signs or progress rapidly to immunodeficiency.

Haematological abnormalities such as anaemia, thrombocytopenia, neutropenia and lymphopenia are very common in FIV infected cats and were proposed as potential indicators of advanced disease (Shelton et al., 1990), (Fleming et al., 1991), (Callanan et al., 1992), (Sparkes et al., 1993), (Hart and Nolte, 1994).

However, the value of these parameters is confounded by the non-specificity of cytopenias, which may be associated with other non-viral conditions. Biochemical abnormalities include hypergammaglobulinaemia (Hopper et al., 1989), (Miro et al., 2007), (Takano et al., 2012), although the potential prognostic value may be problematic due to the chronic nature of polyclonal B cell activation.

Analysis of CD4 and CD8 lymphocyte subsets revealed that the CD4:CD8 ratio remains inverted throughout FIV infection but, in contrast to HIV infection (Uppal et al., 2003), several studies found no correlation between alterations of lymphocyte subsets and disease progression (Hoffmann-Fezer et al., 1992), (Walker et al., 1994), (Walker et al., 1996).

Other markers, such as the degree of p24 antigenaemia (Lombardi et al., 1994b) or alterations in neopterin levels (Beatty et al., 1997), have been evaluated, but showed no correlation with disease progression.

Extrapolating from HIV infection, the plasma viral load (PVL) has been postulated as a potential prognostic indicator for FIV infection. A study focusing upon disease progression in SPF cats experimentally infected with two strains of FIV of different virulence observed a correlation between disease progression and increased plasma viral loads (Diehl and Hoover, 1995). Similarly, a relationship between PVL, disease progression and survival time was found in a study of naturally infected cats in Japan (Goto et al., 2002).

As outlined above, an accurate assessment of the likely outcome of infection in individual cats is problematic due to the lack of reliable prognostic indicators. Differences between FIV and its human counterpart often make it difficult to use similar surrogate markers of disease progression; specific factors may perform better. A recent study demonstrated that the presence of autologous antibodies targeting a cryptic epitope on CD134, which is only exposed following binding of the FIV SU, correlated with detachment of the virion from the cell surface and inhibition of infection. Anti-receptor antibodies were found in 63% (143/226) of the examined cats and their presence correlated with decreased PVL, improved health and survival of FIV infected cats (Grant et al., 2009). Although these

results need to be further evaluated in different cohorts, they have potential for the development of ELISA based approaches to FIV prognosis.

1.8 Treatment

It is recommended that FIV infected cats should be examined every 6 months together with an assessment of complete blood counts (CBC) and body mass (Hosie et al., 2009). Depending on the severity of the abnormalities detected, animals should receive supportive treatment, which may be accompanied by the inclusion of antiretrovirals and immune modulators (Hosie et al., 2009).

Supportive treatment will depend on the presentation of clinical problems, taking into account that treatment of FIV infected cats may require higher therapeutic doses along with extended courses of therapy. The use of corticosteroids to treat oral lesions in FIV infected cats is controversial and against the accepted wisdom that such drugs themselves cause immunosuppression (Wallis, 2007). Nevertheless, as suggested for the long term management of HIV patients, low doses of prednisolone may be clinically beneficial, leading to increased numbers of circulating CD4+ lymphocytes (Andrieu et al., 1995), (Andrieu and Lu, 2004).

Anaemias that often present in FIV infected cats with chronic renal failure can be successfully treated with human EPO (erythropoietin) (Arai et al., 2000). Another therapy yet to be validated in natural infection, but which could potentially be beneficial in younger cats, is the use of insulin-like growth factor - 1 (IGF-1) to stimulate thymic regeneration of peripheral T-cells with (Woo et al., 1999).

Therapy with feline interferon-ω may contribute to slight increases in CBC values, but further evaluation of its efficacy in the field is needed (de Mari et al., 2004). Better documented is the antiviral effect of human interferon-α to treat FIV infected cats (Tompkins, 1999), (Pedretti et al., 2006), although efficacy is lost following the development of VNAbs three to seven weeks after its administration (Zeidner et al., 1990).

Classical antiviral drugs, such as Zidovudine and Plerixafor used in HIV infected patients, can also be used for the treatment of FIV infected cats. Zidovudine, a thymidine analogue that inhibits the reverse transcriptase, is well tolerated by cats and was shown to be effective in the therapy of sick cats (Hartmann et al., 1992). The bicyclam derivative, Plerixafor blocks the CXCR4 chemokine co-receptor and was shown to significantly decrease proviral load in FIV infected cats, with no side effects reported (Hartmann et al., 2012).

1.9 FIV vaccination

Studying FIV pathogenesis in its natural host provides a remarkable opportunity to learn about correlates of immune protection and gives hope for the development of a fully protective vaccine against the virus and subsequently its human counterpart, HIV. To date, various FIV vaccine studies resulted in a number of significant discoveries, embraced by the release of the first commercial FIV vaccine in 2002 (Uhl et al., 2002). Nevertheless, full protection against the virus remains elusive (Dunham et al., 2006). Vaccine development is confounded by several significant challenges attributed to the peculiar features of the virus biology as well as host dependent factors.

1.9.1 Challenges to FIV vaccine development

A major obstacle to FIV vaccine development is the remarkable genetic diversity of the viral Env glycoprotein, which on a nucleotide level varies up to 15% within a specific subtype and up to 26% between subtypes (Burkhard and Dean, 2003). Owing to the error-prone nature of the retroviral reverse transcriptase, the within-host level FIV quasispecies variability is up to 3.7% over the V3-V5 region of the *env* gene (Sodora et al., 1994). Intra-host diversity can be further elevated following superinfection and recombination between co-infecting viruses, potentially resulting in the production of highly diverse quasispecies (Kyaw-Tanner et al., 1994), (Hayward and Rodrigo, 2008). This enormous plasticity of the viral Env glycoprotein is reflected in the diverse, evolving number of antigenic determinants on the viral SU protein and the emergence of viral escape mutants during the course of infection. In addition, antigenic determinants on the viral surface are heavily glycosylated by host derived glycans which render them inaccessible to antibodies (Dacheux et al., 2004).

The ability of lentiviruses to infect memory and non-dividing cells leads to the establishment of latent infection; latently integrated provirus is not accessible to either the humoral or the cellular immune systems (Curran et al., 2000). When a cell harbouring proviral DNA becomes activated, it sheds infectious virions capable of infecting other targets, subsequently broadening the pool of latently infected cells which remain unseen by the immune system. Latently infected cells in immune privileged sites such as the central nervous system (CNS) are practically inaccessible for either immune surveillance or the majority of therapeutic drugs.

Immunization induced enhancement of FIV infection is poorly understood and is a major concern for the development of safe, effective vaccines. Several FIV vaccine candidates failed to protect cats; rather vaccination rendered cats more susceptible to infection (Hosie et al., 1992), (Richardson et al., 1997), (Karlas et al., 1998), (Karlas et al., 1999), (Giannecchini et al., 2002), (Dunham et al., 2006). It has been demonstrated that passive transfer of plasma from vaccinated cats displaying enhanced infection led to enhanced viral infection in unvaccinated controls, suggesting that antibodies lie at the basis of this mechanism, presumably enhancing virus entry into target cells (Siebelink et al., 1995). One possible explanation is that VNAbs target epitopes on the Env glycoprotein, altering its conformation and facilitating virus binding directly to the co-receptor without requiring an interaction with the primary receptor molecule (Guillon et al., 2002). Another possible mechanism is associated with vaccine-induced activation of cell mediated immunity and quantitative enhancement of susceptible memory T cells, expanding the target population for the challenge virus (Wahl and Orenstein, 1997). Vaccine induced enhancement accounted for the recent clinical failure of Merck's prototype adenovirus based HIV-1 vaccine (Hanke, 2008), highlighting the importance of this phenomenon in the development and testing of prospective vaccine candidates.

FIV infection, despite inducing strong immune responses, is never completely eradicated and cats remain infected for life (Ishida et al., 1989). Spontaneous recovery from infection has not been documented and therefore it is tempting to speculate that FIV infection cannot be cleared by vaccine approaches mimicking natural adaptive immune responses (Garber and Feinberg, 2003). Nevertheless,

based on our current understanding of the correlates of immune protection, it is believed that an efficacious vaccine should achieve full protection by stimulating both humoral and cell mediated responses (Patterson et al., 2004). The more recently discovered antiviral intrinsic immunity (Bieniasz and Cullen, 2000), extends our understanding of immunity against viruses and offers an interesting approach that could be exploited to aid the development of effective retroviral control strategies (Poeschla, 2011).

Finally, the experimental design of vaccine trials is crucial to deliver informative and comparative data for prospective vaccines. Candidate vaccines should be evaluated in conditions most closely resembling natural exposure to the virus, including the route of infection, the dose and nature of the challenge inoculum. Unfortunately, in the majority of studies, vaccinated cats have been challenged with virus by intravenous or intraperitoneal inoculation, routes that do not reflect the natural route of transmission. Similarly, the dose of challenge inoculum used in those studies usually exceeded that of natural infection (Pu et al., 1997). The biggest difficulty, however, lies in the choice of challenge inoculum. Very often viruses used in vaccine challenge studies did not reflect those circulating in the field but resembled less pathogenic, laboratory adapted strains. It is important to emphasize that only field trials, in which cats are either free roaming or housed together, would determine the true efficacy of candidate vaccines (Matteucci et al., 2000), (Kusuhara et al., 2005b).

1.9.2 Proposed FIV vaccines

Several types of vaccines, ranging from subunit through recombinant to live attenuated virus vaccines, have been tested in the feline lentiviral model system, yielding variable outcomes that ranged from full protection to enhancement of infection. Interpretation of these data is difficult, due to the variation in experimental design between laboratories. Variables include the choice of challenge inoculum, as well as the methodology used for the evaluation of candidate vaccines.

1.9.2.1 Subunit vaccines

FIV subunit vaccines, composed of either the entire or fragments of viral structural proteins such as p24 CA protein, SU and TM glycoproteins, have been tested, sometimes in combination with immunostimulating complexes (ISCOMs). Despite numerous FIV vaccine studies employing the subunit approach (Hosie et al., 1992), (Lombardi et al., 1994a), (Flynn et al., 1995b), (Verschoor et al., 1996), (Hosie et al., 1996), (Richardson et al., 1998), (Finerty et al., 2000), (Pistello et al., 2006), the majority did not confer protective immunity, with some vaccine candidates rather inducing immune enhancement of infection (Hosie et al., 1992), (Siebelink et al., 1995), (Osterhaus et al., 1996), (Huisman et al., 1998).

1.9.2.2 Recombinant vaccines

Immunogenic viral proteins or their fragments can be synthesised endogenously following transgene delivery by gene transfer vectors (plasmid DNA, viruses and bacteria). Major FIV transgenes used in recombinant vaccines included the entire *env* (Richardson et al., 1997), (Richardson et al., 2002) or fragments encoding SU glycoproteins (Cuisinier et al., 1997), (Cuisinier et al., 1999), (Boretti et al., 2000), (Leutenegger et al., 2000). Several studies employed the gene transfer technique to deliver whole proviral DNA, bearing deletions in various viral genes (Flynn et al., 2000), (Lockridge et al., 2000), (Gupta et al., 2007). The outcomes of those trials, depending on the challenge strain, were diverse, ranging from partial protection and suppression of viral replication (Hosie et al., 1998) to enhancement of infection (Cuisinier et al., 1997). Bacterial (Tijhaar et al., 1997), (Stevens et al., 2004) and viral vectors (Gonin et al., 1995), (Tellier et al., 1998) have been tested in FIV vaccines studies; however, neither approach offered satisfactory protection.

1.9.2.3 Live attenuated vaccines

Live attenuated vaccines display reduced virulence but are still capable of replication. Attenuated viruses can revert to full virulence through mutation and recombination with live viruses and therefore this type of vaccine (due to safety concerns) has received less attention in FIV research. Indeed, one study documented reversion to virulence of the attenuated Petaluma strain of FIV

following challenge with the clade B, FIV M2 virus (Pistello et al., 1999). Although a few studies documented some degree of protection, depending on the route of challenge (Kohmoto et al., 1998a), (Pistello et al., 2003), (Pistello et al., 2005), the use of live attenuated vaccines in confining FIV infection is limited, due to potential safety concerns.

1.9.2.4 Inactivated vaccines

Inactivated vaccines have been the most promising FIV vaccine candidates to date. The inactivation process results in the production of killed, non-infectious viruses, capable of inducing immune responses. Inactivated vaccines include whole inactivated: i) cell free, and ii) cell associated, virus vaccines. Cell associated whole inactivated virus compositions can be purified more cost effectively. Several studies have been conducted using inactivated vaccines (Yamamoto et al., 1991), (Yamamoto et al., 1993), (Hosie et al., 1995), (Hosie and Flynn, 1996), (Matteucci et al., 1996), (Matteucci et al., 1997) with results ranging from protection to enhancement as described for other types of vaccines. Nevertheless, those studies informed the development of the commercial dual subtype inactivated vaccine (Fel-O-Vax), which consists of the clade A Petaluma and clade D Shizuoka strains of FIV, derived from FL4 and FeT-1 cells respectively (Pu et al., 2001). The Fel-O-Vax vaccine has been shown to protect cats against homologous clade A and heterologous clade B challenges (Pu et al., 2001), (Pu et al., 2005), (Kusuhara et al., 2005b) but not against the more virulent GL-8 isolate of FIV (Dunham et al., 2006). Lack of protection against this European isolate, which shares properties with the viruses most likely to be transmitted in the field, poses questions about the efficacy of Fel-O-Vax against field strains of FIV. The vaccine remains to be rigorously evaluated in countries where the vaccine is licenced.

1.10 Viral quasispecies theory

The quasispecies theory was first proposed to model the early evolution of mutation prone, self-replicating macromolecules which are believed to give the origin of life on Earth (Eigen, 1971), (Eigen and Schuster, 1977). Later, the principles of this theoretical framework were adopted to model the evolution of rapidly replicating RNA viruses (Holland et al., 1982).

Accordingly, at the intra-host level, RNA viruses are present not in the form of a single genotype, but rather as an assembly of closely related viral variants, called a quasispecies (Holland et al., 1982), (Holland et al., 1992), (Eigen, 1993), (Domingo and Holland, 1997). Quasispecies theory assumes that those viral variants resemble a cloud of closely connected genotypes which jointly contribute to their characteristics (Biebricher and Eigen, 2005) (Figure 1-7). The generation of polymorphic populations and the potentially high intra-host diversity of RNA viruses are features attributable to their biology as well as the unique characteristics of retroviral reverse transcription (Domingo et al., 1978), (Domingo, 2000).
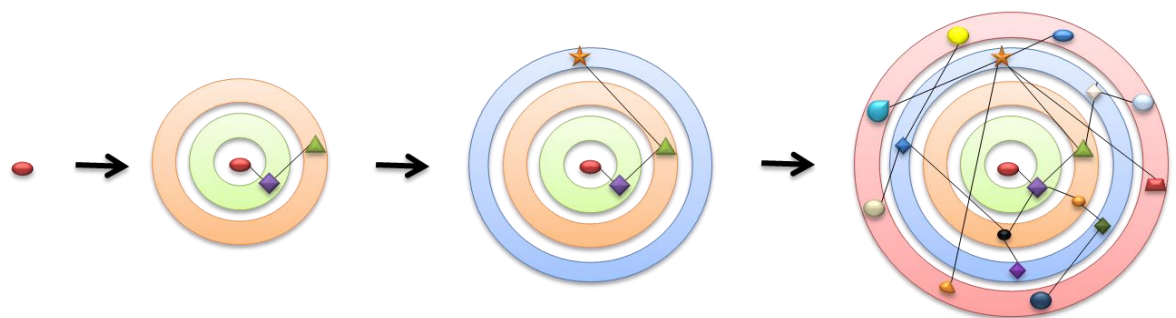
Figure 1-7 Simplified schematic illustration of viral quasispecies. Quasispecies are represented as a cloud of closely related genotypes linked through mutation to parental master sequence. Each circle corresponds to one viral replication cycle. (Modified from (Lauring and Andino, 2010)).

Owing to the error prone RT and viral recombination, many potentially beneficial mutations can arise, lending retroviruses the tremendous ability to adapt to the challenging landscape within the host. Nevertheless, viral variants are subjected to scrutinizing selection processes and only very few of the generated mutations become fixed within the quasispecies (Coffin, 1995), (Anderson et al., 2004), (Biebricher and Eigen, 2005). The theory assumes that mutations occur constantly at a high rate (Domingo, 1998), (Domingo et al., 2006). Therefore, the Darwinian fitness of an individual variant becomes insignificant and is replaced by the connectedness of the quasispecies cloud (Bull et al., 2005). A well-connected cloud is represented by viral variants that arise during replication and are viable and capable of generating successors and further contributing to diversity within the cloud. In contrast, a poorly connected cloud is characterised by the generation of viral particles unable to

produce further progeny. Within the hypothetical framework of the quasispecies theory, it is the cloud of quasispecies rather than the individual genome that comprises the unit of selection (Domingo, 1998).

The quasispecies framework makes several theoretical assumptions, thought to be valid to describe fast self-replicating molecules, and has been described as the only well placed theory to model the evolution of RNA viruses (Domingo, 2000). At the same time, this concept received wide criticism, being regarded as causing confusion in describing viral evolution (Moya et al., 2000), (Comas et al., 2005). Nevertheless, the evidence shows that the quasispecies theory principle fits well into the mutation and selection concept of population genetics (Wilke, 2005). It seems to be more in agreement than in conflict with Darwinian evolution, and provides an additional theoretical framework for understanding the evolution of asexual, haploid, self-replicating RNA viruses (Wilke, 2005), (Lauring and Andino, 2010).

1.10.1.1 Quasispecies diversity and pathogenicity

Intra-host diversity and the plasticity and dynamics of quasispecies are important for disease pathogenesis (Lauring and Andino, 2010). In HIV infection, the enormous potential to adopt to changing environments is reflected by the existence of R5, X4 and R5X4 viral variants with different cell tropisms (Karlsson et al., 2004), (Clevestig et al., 2005), (Gorry et al., 2005). Similarly, HIV immune escape mutants emerge during the course infection, providing further evidence for the plasticity of quasispecies in response to landscape alterations within the host (McKeating et al., 1989), (Arendrup et al., 1992), (Guillon et al., 2006).

In FIV, VNAb escape mutants were shown to emerge during the course of infection (Samman et al., 2010), (Willett et al., 2010) as well as viral variants displaying different cell tropisms (Willett et al., 2006b), (Willett et al., 2008), (Willett et al., 2010), suggesting that FIV quasispecies also possess significant plasticity in adapting to a challenging host environment.

This enormous lability is also a feature of other RNA viruses, such as influenza and hepatitis C virus (HCV) (Beigel et al., 2005), (Dawood et al., 2009), (Rong et al., 2012). The ability of these genomes to adapt to various challenges during

infection poses significant obstacles for the development of effective therapeutic and vaccine strategies (Gerrish and Garcia-Lerma, 2003). Moreover, by contrasting the ability of a single genotype of neurotropic poliovirus infecting alone with the ability of diverse quasispecies to cause disease, it has been demonstrated that the diversity of the infecting population does indeed have a profound influence on disease pathogenesis (Vignuzzi et al., 2006), (Lauring and Andino, 2010). This highlights the importance of studying naturally occurring infections, which is the focus of this thesis.

1.11 Scope and aims of this thesis

The main goal of this thesis was to assess predictors of the long term outcome and disease progression in cats naturally infected with FIV. Additionally, we evaluated humoral immune responses induced by the commercial FIV vaccine in privately owned cats.

Specific aims of the project were:

1) To define the clinical presentation of FIV infection and to assess clinically available predictors of disease progression.

2) To quantify viral evolution, diversity and quasispecies composition in cats naturally infected with FIV.

3) In functional studies, to examine naturally occurring wild type FIV variants for:

   a)   susceptibility to neutralisation by homologous plasma, and

   b)   usage of the viral receptors CXCR4 and CD134,

in order to examine the relationship between the emergence of neutralising antibody resistant variants, cell tropism and FIV disease stage.

4) To assess the humoral immune response induced by the commercial FIV vaccine against reference isolates as well as an Australian field isolate of FIV, which most likely overcame vaccine induced protection.

Chapter 2. Materials and methods

2.1 Molecular biology techniques

2.1.1 Polymerase chain reaction and primers

Amplification of full length FIV *env* genes (approx. 2500 bp) was performed using a two-step nested PCR protocol due to the high variability of wild type viruses circulating in cat population. First round PCR reactions were performed using the Phusion Blood Direct PCR Kit (Finnzymes, Hitchin, UK) followed by sequencing of PCR product to inform the primer design for the second round PCR which was performed using Roche High Fidelity Master (Roche, Mannheim, Germany).

Primers used for first round blood direct amplification were designed to bind to conserved regions of the viral genome, based on an alignment of diverse complete genome FIV sequences isolated previously in our laboratory (GL-8, TOT-1, KNG) and those available on the GenBank (accession numbers: U11820.1 (B2542), M36968.1 (PPR), NC.001482 (FIV NC), M25381.1 (Petaluma), EF455612.1 (FIV YF125), AF474246.1 (FIV C)), to ensure product amplification from all clades of FIV that occur worldwide. Primers used for first round amplification were: 2F2 and 1R4 binding from position 6203 for the forward primer and from 9148 for the reverse primer respectively of the full genome of reference FIV-PPR (accession number: M36968.1). Primers designed specifically for each cat for second round PCR had *Not1* and *Sal1* digestions sites incorporated and were designed to amplify full length FIV *env* genes, binding from position 6285 for the upstream and 8852 for the downstream primer respectively of the full length genome of reference FIV-PPR.

Primers were synthesised by Eurofins MWG (Ebersberg, Germany) and shipped in lyophilised form. Upon arrival, oligonucleotides were re-suspended in nuclease free water at a stock concentration of 100 pmol/µl and stored at -20˚C until required. Primers used for amplifications were equilibrated to a working concentration of 10 pmol/µl and were stored short term at 4˚C. Sequences of all primers used in this study are listed in Appendix 1.

2.1.1.1 Blood direct PCR

Blood direct PCR was performed using Phusion Blood II Direct PCR Kit (Finnzymes). The kit employs high fidelity Phusion Blood II DNA Polymerase to amplify directly from blood without prior sample preparation or DNA extraction. Phusion Blood II DNA is a proofreading polymerase with 25 fold greater accuracy than Taq DNA polymerase, determined with a modified lacI-based method (Frey, 1995). Blood samples collected in lithium heparin were used as templates and reactions in total volumes of 50 μl were set up using thermo-cycling conditions as per manufacturer's instructions (Appendix 2). The optimal blood template concentration was found to be 7% of total reaction volume and the optimal annealing temperature for the 2F2/1R4 primer set was 64.5°C. Following amplification, PCR products were separated using gel electrophoresis, visualised under UV light, excised with a sterile scalpel, gel purified (section 2.1.2), sequenced and subsequently used as a template for second round PCR reactions.

2.1.1.2 Roche High Fidelity PCR Master

Roche High Fidelity PCR Master (Roche, Mannheim, Germany) was selected for its inherent proofreading activity in order to amplify full length FIV *env* genes from gel purified products amplified directly from blood. The primers were specifically designed for each cat and sequences are listed in Appendix 1. *Not1* and *Sal1* digestions sites were incorporated downstream and upstream in the primer sequences respectively to facilitate cloning of amplified wild type FIV *envs*. Mastermix and thermo-cycling conditions were set as per manufacturer's instructions (listed in Appendix 2). Typically, 20 ng of gel purified blood direct PCR product was used as the template in each 50 μl reaction.

2.1.2 Gel electrophoresis and purification of PCR products

PCR and restriction digested PCR products were examined by gel electrophoresis. Generally, samples were mixed with 10x loading dye, loaded in 1% agarose gel containing ethidium bromide (10 mg/ml) and run for 1 hour at 100 V. Bands were visualised under UV light, photographed and excised using a sterile scalpel.

PCR products were purified from agarose using QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) following the manufacturer's instructions. This works on the principle of DNA being precipitated by isopropanol, then absorbed onto a silica column. Contaminants pass through the column during the ethanol washing steps and DNA is subsequently eluted in 50 μl of water.

2.1.3 Restriction enzyme digestion and ligation

Second round PCR products and vector (VR1012) (Hartikka et al., 1996) were double digested at 37°C for three hours by high fidelity *Not1HF/Sal1HF* restriction endonucleases (New England BioLabs, Hitchin, UK). Restriction endonucleases were usually used at 1 U of each enzyme per reaction but DNA concentrations were always adjusted according to manufacturer's instructions, assuming that 1 U of each restriction enzyme is able to digest 1 μg of DNA in an hour at 37°C. All reactions were set up in NEBuffer 4 buffer, as recommended by the manufacturer and brought to total volume of 20 μl. Following restriction enzyme digestion, reactions were gel electrophoresed, examined under UV light and gel purified as described above.

Double *Not I/Sal I* digested DNA (insert) was ligated into VR1012 (vector) using T4 DNA Ligase (Promega, Southampton, UK) at 1 U/μl at 14°C for 16-18 hours. For each ligation, a ratio of 1:3 (vector: insert) was used in a final volume of 20 μl. Ligated constructs were directly used for transformation. Negative controls, containing empty vector were set up alongside to assess ligation efficiency in subsequent transformations.

2.1.4 Transformation

Escherichia Coli MAX Efficiency® DH5α™ Competent Cells (genotype F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-,mk+) phoA supE44 λ-thi-1 gyrA96 relA1) (Invitrogen) were transformed using the heat shock protocol as per manufacturer's instructions. Briefly, 30 μl DH5α™ Competent Cells were thawed on ice, 0.5-1 μg of ligated construct added, incubated for 30 minutes on ice followed by heat shock at 42°C for 40 seconds and further 2 minute incubation on ice. Transformed competent cells were diluted in 400 μl of SOC medium and incubated for 1 hour at 37°C on orbital shaker at 225 rpm.

Cultures were spread on freshly prepared LB agar plates containing kanamycin (Sigma Aldrich, Gillingham, UK) at 50 µg/ml. Plates were incubated for 16-48 hours at 30°C due to the inherent instability of primary FIV *envs*. Colonies were picked and screened for the desired vector/insert constructs by double *Not I/Sal I* restriction digestion of the isolated DNA. Alternatively screening was performed on a large scale by colony PCR.

2.1.5 Colony screening

2.1.5.1 Colony PCR

Colony screening PCR was performed using GoTaq Hot Start DNA Polymerase (Promega). Colonies were picked from agar plates, re-suspended in 20 µl of nuclease free water and immediately moved to 4°C. Mastermix and thermo-cycling conditions (Appendix 2) were set up as per manufacturer's instructions with minor modifications. In brief, PCR reactions contained 2 µl of re-suspended colonies, 10 µl 5x GoTaq Flexi Green Buffer, 1 µl 2mM dNTPs, 3 µl of $MgCl_2$, 10 mM of each primer, 0.25 µl (1U) GoTaq hot start polymerase and nuclease free water to final volume of 50 µl. Primers VR1012_fwd and VR1012_rev were used to amplify ~ 2500 bp *env* insert desired in positive transformants. Primer sequences are listed in Appendix 1. Positive colonies were identified following gel electrophoresis and the visualisation of PCR products under UV light. Colonies were classified as positive when PCR products approximately 2500 bp in size were observed. This method proved to be robust and time efficient and was mainly used in the 96 well plate format to achieve high throughput screening of transformed colonies.

2.1.5.2 Small scale DNA preparation

The QIAquick Minikit (Qiagen) was used according to the manufacturer's instructions to isolate plasmid DNA for small scale colony screening. The QIAquick Minikit works on the principle of plasmid DNA selectively binding under appropriate salt and pH conditions to the anion-exchange tip of the spin column. RNA, proteins, metabolites, and other impurities are removed in washing steps. Finally plasmid DNA is eluted in 50 µl high-salt buffer. Isolated plasmid DNA was subjected to double *Not1/Sal1* restriction digest for 1 hour at 37°C, the products were gel electrophoresed for 40 minutes at 100 V and analysed under UV light.

Positive transformants were identified by comparing the digestion pattern to that of the positive control run in parallel.

2.1.6 Midi plasmid DNA preparation

The PureLink HiPure Plasmid Midiprep Kit (Invitrogen) was used for the isolation of larger amounts of plasmid DNA, necessary for downstream applications. The HiPure Midiprep columns, similar to Miniprep columns, are anion-exchange chromatography columns.

Following initial screening, as described above, positive transformants were inoculated in 4 ml of LB Broth containing kanamycin (Sigma Aldrich) at 50 mg/ml, and incubated overnight at 30°C in an orbital shaker. The following day, cultures were scaled up to 250 ml volume of LB Broth containing kanamycin, grown in conical flasks in orbital shaker at 30°C for another 16-24 hours and finally centrifuged at 5000 rpm (~4600 x *g*) in a Beckman Coulter Avanti J-E centrifuge for 10 minutes at 4°C.

All further plasmid DNA isolation steps were performed as per manufacturer's instructions. DNA was precipitated with isopropanol and washed with 70% ethanol. DNA pellets were air dried under laminar flow sterile conditions, then re-suspended in 200 μl of TE buffer and stored at 4°C.

Glycerol stocks of positive clones were prepared by mixing 820 μl of culture from the first inoculum with 180 μl of sterile 80% glycerol. All glycerol stocks were catalogued and immediately stored at -80°C.

2.1.7 DNA sequencing

PCR products and plasmid DNA were sequenced using Sanger's chain terminator sequencing method, either in-house or by a commercial sequencing company using a 96 well plate format (Source BioScience, Nottingham, UK).

Sequencing reactions prepared in-house were set up using Big Dye Terminator v1.1 kit (Applied Biosystems, Warrington, UK). Sequencing primers at 3.2 pmol per reaction were: VR1012_fwd, VR1012_rev, Memphis780_fwd and Memphis2090_rev for sequencing of cloned FIV *envs*. Primers 2F2 and 1R4 were

used for sequencing of Phusion Blood II Direct amplified PCR products. Thermo-cycling conditions were set up as per manufacturer's instructions and are listed in Appendix 2. Purification of sequencing reactions was performed either by precipitation with 70% alcohol or, on a larger scale, by using the 96 well DyeEx purification kit (Qiagen) following the manufacturer's protocol to separate molecules based on their molecular weight by gel-filtration chromatography. During the clean-up of sequencing reactions, dye terminators are retained in gel filtration material while the DNA fragments are recovered from the flow through.

Purified reactions were re-suspended in 20 µl of highly-deionised formamide (HiDi) (Applied Biosystems) and transferred to 96 well sequencing plates. Sequencing was performed on an in-house ABI Prism 3700 Genetic Analyser (Applied Biosystems).

An external commercial sequencing service was also used (Source BioScience) to aid sequencing of the large number of plasmid DNA products generated in the study. Each Plasmid DNA concentration was equilibrated to 100 µg/µl and sent by post in 96 well plate format to Source BioScience, Nottingham. Sequencing results were available to download from the company's server within 24-48 hours upon samples arrival. This approach proved to be extremely time and cost efficient in comparison with in-house sequencing.

2.2 Cell culture

2.2.1 Plasma and PBMCs collection

Blood samples were obtained either in Chicago, IL or Memphis, TN, USA from cats enrolled in the study (described in detail in Chapter 3) by jugular venipuncture, collected into lithium heparin tubes and immediately shipped at 4ºC. Blood samples were in transit for 3 to 5 days before arrival at the Retrovirus Research Laboratory at the University of Glasgow. Upon arrival, plasma was separated by centrifugation at 2000 rpm (~ 370 x g) for 10 minutes, and stored in 200 µl aliquots at -80°C until required. The remaining blood pellets were used for blood direct amplification with Phusion Blood II Direct PCR Kit (described in section 2.1.1.1) and collection of peripheral blood mononuclear cells (PBMCs).

PBMCs were collected in order to set up virus isolations and confirm the FIV status of cats enrolled in the study. PBMC collection was performed by flash lysis of erythrocytes. Briefly, blood pellets were diluted in 1 ml of PBS, transferred to a universal container containing 20 ml of pre-warmed red blood cell lysis buffer and incubated at room temperature for up to 10 minutes until the solution became clear. Lysed erythrocytes and intact PBMCs were centrifuged for 5 minutes at 1000 rpm (~200 x g) in the Eppendorf 5810R centrifuge (Rotor A-4-81), supernatants discarded and the PBMCs were washed twice with PBS-BSA buffer and collected in 1.5 ml eppendorf tubes before being immediately used for virus isolation.

2.2.2 Virus isolation

To exclude the possibility of false positive diagnosis by SNAP FIV/FeLV Combo Test (IDEXX) used in clinical setting, all cats enrolled in the study had their FIV status confirmed by virus isolation, considered the gold standard in FIV infection diagnosis (Hosie et al., 2009). Approximately 10^7 fresh PBMCs were co-cultivated with 2 x 10^6 of Mya-1 cells (section 2.2.3.1.1) in 5 ml of complete RPMI 1640 in T25 cell culture dishes (Corning, Warrington, UK) and cells were observed under an inverted cell culture microscope daily. Cytopathic effect such as syncytia formation, cell swelling and cell death visible under the microscope were indicative of viral replication, which was further confirmed by screening co-

cultivation supernatants for reverse transcriptase activity or FIV p24 capsid protein (section 2.3.1 and 2.3.2). Positive cultures were harvested by centrifugation at 1000 rpm (~200 x g) for 5 minutes and supernatants were collected in 5 ml containers for long term storage at -80°C. Cell pellets were washed in PBS, centrifuged again at 1000 rpm (~200 x g) for 5 minutes and stored at -80°C.

2.2.3 Cells

Frozen aliquots of each cell line were recovered from liquid nitrogen by rapid thawing in a 37°C water bath. Cells were washed in 10 ml of medium, centrifuged at 1000 rpm (~200 x g) for 5 minutes and re-suspended in 5 ml of complete RPMI 1640 or DMEM before being moved to T25 tissue culture dishes (Corning). All cells were grown in humidified incubators in an atmosphere of 5% of CO_2 at 37°C. Recovered cells were examined daily and, once confluent, were passaged and scaled up as required.

2.2.3.1 Suspension cells and their maintenance

Suspension cells were maintained in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% heat inactivated fetal bovine serum, 50 µM 2-mercaptoethanol, 2 mM glutamine, 0.11 mg/ml sodium pyruvate, 100 IU/ml penicillin and 100 mg/ml streptomycin, subsequently referred to as complete RPMI 1640. Centrifugation of suspension cells was performed at 1000 rpm (~200 x g) for 5 minutes, unless stated otherwise.

2.2.3.1.1 Primary feline T lymphoblastoid cell line

The feline primary T lymphoblastoid cell line, Mya-1, was established by prolonged cultivation of PBMCs isolated from a 5 month old specific pathogen free (SPF) cat previously inoculated with blood collected from a 3 year old cat seronegative for FeLV, FeCoV and seropositive for FIV and FeSFV (Miyazawa et al., 1989). Mya-1 cells were shown to be free from endogenous retroviruses and exogenous FIV (Miyazawa et al., 1989) and proved useful for the propagation of FIV. Mya-1 cells are interleukin-2 (IL-2) dependent and were maintained in complete RPMI 1640 medium supplemented with 100 IU/ml of IL-2 (provided by T. Miyazawa). Cells were maintained at a density of 1 x 10^6 cells per ml before

being passaged. To minimise cell damage during passage, centrifugation was performed at 800 rpm (~128 x *g*) for 5 minutes with no braking.

2.2.3.1.2 CLL-CD134 cells

The canine chronic lymphocytic leukaemia cell line CLL was established by extended *in vitro* culture of PBMC isolated from a leukaemic dog; CLL cell line is $CD3^+$ $CD4^-$ $CD8^-$ $CD134^-$. Stable transduction of the CLL cell line with feline CD134 (primary receptor for FIV) rendered cells susceptible to productive FIV infection (Willett et al., 2006b). CLL-CD134 cells grow well in suspension and were maintained by subculturing into fresh medium every 3-4 days at 1:10 dilution. Cells were maintained in complete RPMI 1640 medium as described above and selected with 400 μg/ml of Geneticin 418 (Invitrogen).

2.2.3.2 MCC cells

The MCC cell line was derived from an abdominal mass from a 13 year old castrated cat and has the phenotype of a large granular lymphocyte (LGL) (Cheney et al., 1990). MCC cells are CD134 negative but, when stably transduced with different chimeric constructs of CD134 can be used to elucidate the role of CD134 in FIV entry and the complexity of the virus-receptor interaction (Willett et al., 2006b), (Willett et al., 2006a). MCC-CD134 lines expressing three chimeric CD134 molecules (MCC-FFF, MCC-FFHH and MCC-HHH) were used to reveal different utilization of CD-134 by diverse strains of FIV (Willett et al., 2006b). MCC cells and MCC-CD134 were cultured in complete RPMI 1640 medium and maintained by addition of the selection antibiotic Geneticin 418 (Invitrogen).

2.2.3.3 Adherent HEK-293 cells and their maintenance

The HEK 293 cell line was generated following transformation of human embryonic kidney cells by a DNA fragment of human Adenovirus 5 (Graham et al., 1977) and since has been widely used in molecular biology for transfections. HEK 293 cells were cultured in disposable tissue culture dishes (Corning) and maintained in high glucose (4.5 g/l) Dulbecco's modification of Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 0.11 mg/ml sodium pyruvate, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells were selected with 400 μg/ml of Geneticin 418

(Invitrogen) and passaged into fresh medium at 1:10 ratio, usually every 3-4 days. Medium was removed, the confluent cell monolayer was washed with PBS and then treated with 0.25% trypsin prior to incubation at 37°C for 5 minutes or until the cell monolayer detached. Detached cells were washed with complete DMEM to stop trypsin activity, centrifuged at 1000 rpm for 5 minutes and resuspended in fresh DMEM before being subcultured in preparation for transfection the next day.

2.2.4 Cryogenic preservation and storage

For long term storage, cells were pelleted at 1000 rpm (~200 x g) for 5 minutes and re-suspended to a density of $5x10^6$ cells/ml in cryo-preservation medium containing 70% complete medium, 20% heat inactivated foetal bovine serum and 10% dimethyl sulphoxide (DMSO), transferred into 2 ml cryo-preservation tubes (Nunc, Denmark) and taken at a constant rate of -1°C/minute to -80°C in a cryo-freezing container (Nalgen, USA) filled with isopropanol, before long term storage in the vapour phase of liquid nitrogen.

2.2.5 Transient transfection of adherent cells and pseudotype production

HIV-luc (FIV) pseudotypes were prepared by transfecting 293T cells with plasmid VR1012 (Vical Inc., San Diego, USA) expressing a specific wild type FIV env gene and the HIV-1 NL4-3 based vector with deleted env and vpr genes and bearing the luciferase reporter gene (Figure 2-1) (Connor et al., 1995). Such constructs are capable of a single round of infection only and deliver the luciferase reporter gene to target cells, permitting quantification of infection efficiency.
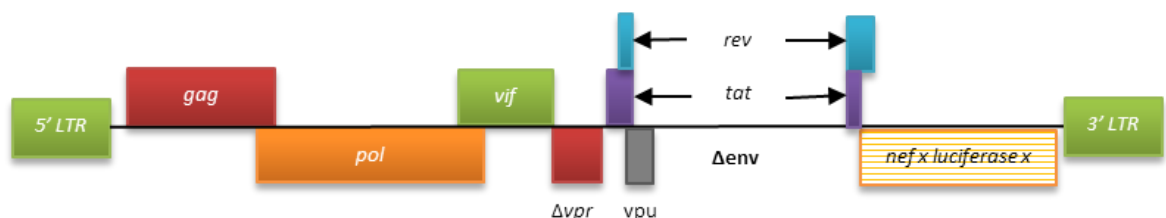
Figure 2-1 Schematic representation of HIV NL 4-3 strain based vector expressing firefly luciferase reprorter gene inserted into *nef* gene. pNL4-3-Luc-E⁻R⁻luc contains defective *vpr* gene (Δ*vpr*) and 5' frame shift within the *env* gene (Δenv), preventing the expression of the envelope glycoprotein. Adopted from (Akkina et al., 1996).

293T cells were transiently transfected using Superfect transfection reagent (Qiagen) following the manufacturer's instructions. Superfect assembles DNA together in compact structures and binds it to the cell surface before the complex is taken inside the cell via endocytosis. One day prior to transfection, 0.5×10^6 293T cells were plated into each well of a 6 well culture cluster (Corning) in 2ml of complete DMEM and incubated overnight until 70-80% confluent. The following day, 2 µg of HIV pNL4-3-Luc-E⁻R⁻luc and 2 µg of each FIV *env* expressing VR1012 were mixed with 10 µl of Superfect Transfection Reagent (Qiagen), added to 100 µl of serum free DMEM and incubated for 15 minutes at room temperature to allow formation of transfection complexes. Transfection complexes were then mixed with 600 µl of complete DMEM, immediately transferred drop-wise to each well and incubated for 3 hours at 37°C, 5% CO_2. Following incubation, the cells were washed with 1 ml PBS, 2 ml of fresh complete DMEM was added and the cells were incubated in 6 well cell culture clusters at 37°C, 5% CO_2 for 72 hours. Culture fluids containing pseudotypes were harvested, centrifuged at 1000 rpm (~200 x *g*) for 5 minutes, passed through 0.45 µm filters, aliquoted and stored at -80°C until required.

To monitor transfection efficiency, titres were assessed by incubating 50 µl of each pseudotype with 50 µl of 0.5×10^6 CLL-CD134 cells in a CulturPlate™-96 assay plate (Perkin Elmer, Beaconsfield, UK) at 37°C, 5% CO_2. Following 72 hours incubation, 100 µl HTS steadylite substrate for luciferase (Perkin Elmer) was added to each well and the luciferase activity was quantified by single photon counting on 1450 MicroBeta luminometer (Perkin Elmer).

2.2.6 Receptor usage assay

MCC FFF, MCC F(FH)H, MCC HHH and CLL-CD134 cells (described above) were seeded (1×10^4 cells per well) in triplicate in a CulturPlate™-96 assay plate (Perkin Elmer). The cells were infected with 50µl of each HIV-luc (wild type FIV) pseudotype alongside reference controls of HIV-luc (FIV GL-8) and HIV-luc (FIV B2542). Following 72 hours incubation at 37°C, 5% CO_2, luciferase activity was quantified following the addition of 100 µl of Steadylite HTS™ (Perkin Elmer) substrate for luciferase and single photon counting using a MicroBeta luminometer (Perkin Elmer).

2.2.7 Virus neutralisation assay

Plasma samples (detailed in Chapter 3) were tested for virus neutralising antibodies (VNAb) using the HIV-luc (FIV) pseudotypes described above. Plasma samples were diluted 10, 100, 1000 and 10000 fold in complete RPMI 1640 medium. 25 µl of each dilution were incubated in triplicate for one hour at 37˚C with 25 µl of HIV-luc (FIV) luciferase pseudotype and then 50 µl (5×10^4 cells) of CLL-CD134 (Willett et al., 2006b) were added. The cells were cultured in CulturPlate™-96 assay plates (Perkin Elmer) for 72 hours. Luciferase activity was quantified as described above and fold neutralisation was calculated by dividing the mean luciferase counts of no plasma control wells with the mean luciferase counts for each plasma dilution.

2.3 Protein based quantitative techniques

Mya-1/PBMC cell culture fluids (described in section 2.2.2) were examined each day from day 5 of co-cultivation for reverse transcriptase (RT) activity by Lenti RT Activity Kit (Cavidi, Uppsala, Sweden) or for p24 capsid protein by FIV Pet Check ELISA (IDEXX Laboratories, Netherlands). Selection of screening technique was based on the availability of reagents from their manufacturers.

2.3.1 Reverse transcriptase activity detection assay

200 µl of co-cultivation cell culture fluid was centrifuged at 1000 rpm (~92 x *g*) for 5 minutes and supernatant collected. 10 µl of supernatant was used to screen for reverse transcriptase (RT) activity using the Lenti RT Activity kit which detects or quantifies lentiviral reverse transcriptase activity. It is based on the principle of DNA-strand synthesis on template RNA coated plates by the reverse transcriptase present in each sample tested. The synthesised double stranded DNA/RNA molecule is targeted by alkaline phosphatase conjugated α-BrdU antibody. Quantification of the product is facilitated by addition of a colorimetric alkaline phosphatase substrate, the activity being proportional to the reverse transcriptase activity in the sample. Reaction mixture and Poly A plates coated with RNA template were prepared as per manufacturer's instructions. All steps were carried out following the semi quantitative protocol.

2.3.2 p24 ELISA

Culture fluids were collected as described above and 100 μl of cell culture supernatant was used to screen co-cultivations for FIV p24 capsid protein employing the commercial ELISA PetCheck FIV test kit (IDEXX Laboratories, Netherlands), an enzyme-linked immunosorbent assay that detects viral p24 capsid protein either in plasma samples or cell culture supernatants. Screening was performed following the manufacturer's protocol and absorbance values were measured using 405 nm filter. Samples were classified as positive or negative by comparing spectrophotometry values to positive and negative controls provided by the manufacturer.

2.4 *In silico* techniques

2.4.1 Sequence assembly and alignments

Sequence data generated in house from ABI 3700 Genetic Analyser were base called and initially analysed using Sequencing Analysis Software v5.4 (Applied Biosystems, UK). All sequence data, whether generated in-house or by a commercial sequencing company, were further assembled in DNA Dynamo (Blue Tractor Software, UK). The full length FIV *env* sequence (approx. 2500 bp) from each clone was assembled by using 3 sequencing reads overlapping by approximately 200 bp and manually checked for mismatches. Assembled sequences were saved in FASTA format for further analysis.

2.4.2 Phylogenetic analysis

Phylogenetic analyses were performed using various software packages, depending on specific research questions and are described in detail in Chapter 4. In summary, nucleotide and peptide sequence alignment was performed using Muscle algorithm (Edgar, 2004) in MEGA5 software package (Tamura et al., 2011). DNA sequences were translated to corresponding sequences using Virtual Ribosme (Wernersson, 2006). Final alignments were curated manually following codon optimization by submission of MUSCLE aligned peptide sequences and corresponding not aligned nucleic acid sequences to RevTrans 1.4 Server (Wernersson and Pedersen, 2003). Phylogenetic trees were constructed using the Maximum Likelihood method under HKY nucleotide substitution model (Tamura et al., 2011) in MEGA5. Evolutionary divergence between sequences was calculated using Maximum Composite Likelihood model (Tamura et al., 2004) in MEGA5. Recombination testing and estimate of selection were performed with the HyPhy software package (Pond et al., 2005). Evolutionary rates were estimated using Bayesian Evolutionary Analysis Sampling Trees (BEAST) v 1.4.6 software (Drummond and Rambaut, 2007). N-linked glycosylation sites were predicted using N-GlycoSite online tool available at Los Alamos, National laboratory server (Zhang et al., 2004). Highlighter analysis was performed by submitting aligned protein or nucleic acid sequences into highlighter tool available at the same Los Alamos server.

2.4.1 Graphs and statistical analyses

All graphs and statistical analyses were performed in GraphPad Prism v 5.00 (GraphPad Software, San Diego, California, USA).

Chapter 3. Study group

Studying a naturally acquired infection in the natural host species presents many challenges. The major problem is not at the level of recruiting but rather it is difficult to retain the involvement of study participants over a sufficiently long period of time to collect sufficient useful data. This may explain why, despite over 25 years of FIV research, relatively little is known about the longitudinal course of FIV infection in its natural host.

It became apparent during the first phase of this study that it may be extremely difficult (if not impossible) to retain the privately owned cats belonging to single households across the United Kingdom that were initially recruited for this project. The distress caused to cats and their owners that were associated with phlebotomy during the relatively frequent visits to the veterinarians were cited as the main reason for failing to arrange the follow-up appointments and subsequently opting out from the study. To address this problem, which would have biased the project and its research questions significantly, we recruited naturally infected cats from two separate locations in the United States. We believe that the unique characteristics of the selected populations, in particular the highly dedicated, well informed owners, were key to the success of this study.

In this chapter, the study population comprising 44 American cats naturally infected with FIV that were recruited to the Glasgow FIV Project is described. The complete interpretation of the molecular properties of the isolated viral envelopes described later in this thesis, as well as the virus-host interactions at the level of viral entry and the humoral immune responses to the virus would not have been possible without linking it to clinical data.

In this chapter the timeline of the project is described, giving an overview of the study population, the distribution of parameters such as age, length of infection, health status, CD4:CD8 ratio, and other clinical and pathological observations in the context of a diverse cat population infected with naturally acquired FIV. An attempt has been also made to evaluate and identify potential prognostic indicators amongst the parameters which are accessible for veterinarians in a clinical setting.

3.1 Materials and methods

Recruitment, physical examinations, bleeding, clinical data collection and retention of all the cats recruited for the study were performed by our collaborator, Dr Annette Litster, Purdue University, West Lafayette, IN, USA and her colleagues. Post mortem examinations of deceased cats and necropsy reports were provided by the Animal Disease Diagnostic Laboratory of Purdue University. Clinical pathology results and lymphocyte flow cytometer analyses were delivered by IDEXX laboratories, Westbrook, ME, USA.

3.1.1 Animals

Forty four FIV infected cats were enrolled in the Glasgow FIV Project, comprising our "study group". Within the study group, 27 cats were homed in the Memphis Crew's FIV sanctuary, referred to as "Memphis group", and 17 cats had been rehomed from PAWS Chicago, referred to as "PAWS Chicago group". Details of the study group are described mainly on a population level in each section of this chapter, highlighting individuals only when significant information can be learned from specific cases. Detailed clinical data concerning the individual cats are provided on the CD that accompanies this thesis.

3.1.2 Statistical analysis and graphs

Graphing and statistical data analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Descriptive data were shown as means and interquartile range (IQR) (median, 5th, 95th quartile). Data were expressed as numbers and percentages. The paired and unpaired t-test was used to detect differences between laboratory parameters. Significance was set at a p value < 0.05.

3.2 Ethical approval and owners' consent

The study and its aims were reviewed and approved by the University of Glasgow and the Purdue University Ethics Committees.

Cats from Memphis Crew's FIV shelter had been accepted in the past into the sanctuary that had been established for FIV infected cats. Therefore, the cats' owner and carer, being very much aware of the implications of FIV infection, gave her consent for her cats to participate in the Glasgow-Purdue-IDEXX study without further education, understanding that the proposed (free of charge) veterinary health check-ups, haematology and biochemistry analyses would have a positive impact on the long term health of her cats.

PAWS Chicago cats were either still living in the PAWS adoption centre or had been rehomed into single cat households at various times before the study commenced. These cats were well cared for by PAWS staff and fully dedicated owners, who had been made aware of their FIV status and health implications at the time of adoption. Prior to recruitment to the study, cat owners were contacted again, informed about the aims of the project and the importance of regular check-ups and follow up blood sampling during the length of the study. Owners who were willing for their cats to participate in the project were provided with written information sheets and consent forms which were signed and returned to the study co-ordinator.

3.3 Eligibility criteria for selection and participation

All cats from the two populations were initially selected into the study based solely on the basis of a history of FIV infection. At the time of enrolment, the FIV infection status was confirmed using the SNAP FIV/FeLV Combo Test (IDEXX), regardless of breed, sex, age and health status.

To exclude the possibility of false positive and negative SNAP FIV/FeLV Combo testing, initially the infection status of all enrolled cats was further confirmed in our laboratory by virus isolation (described in detail in Chapter 2), considered to be the "gold standard" for FIV diagnosis (Hosie et al., 2009).

3.3.1 Study group size

The University of Glasgow FIV Prognosis Project (referred to as Glasgow FIV Study) forms part of a larger collaborative Glasgow-Purdue-IDEXX FIV study. It is important to emphasise that cats were enrolled by Dr Litster for the collaborative study over the period of 2 years (enrolment was completed in February 2012, at the time of writing this thesis). In total, 91 naturally infected FIV-positive cats and 93 FIV-negative matching control cats were enrolled for this study.

For the Glasgow FIV study, 44 FIV infected cats from two distinct locations in the USA (Memphis, TN and Chicago, IL) were included. We were aware that by selecting and including cats from two populations (each with different geographic locations, housing conditions as well as health care access) minimised bias of the study. Indeed, it became evident that analysis of the two populations allowed a useful comparison of the effects of long-term FIV infection in shelter cats compared to single-household cat populations. The total number of 44 cats included in the study was chosen for logistic reasons.

3.3.1.1 Memphis study group size

All cats from the Memphis study population lived together in a single household, referred to as "FIV sanctuary", and were cared for by one family. At the time of enrolment, 50 FIV positive cats were housed together in the Memphis Crew's FIV sanctuary, from which for logistic reasons, 27 were randomly selected and included in this study.

3.3.1.2 PAWS Chicago study group size

The Chicago population cats were obtained (usually but not exclusively) following feral cat trapping in various parts of Chicago as part of the PAWS Chicago rehoming and adoption initiative. PAWS cats were well cared for by dedicated owners who were aware of their FIV status, had access to professional health care and all of these cats were living in single cat households, with the exception of two cats who had been rehomed together and were living in the same household. Another exception was two cats that had been rehomed in pairs

with two other FIV positive cats not enrolled in the project. There were 17 cats in total from the Chicago population that were included in this study.

3.3.2 Coding and identifying system

A standardised scheme to code and identify the study participants was established to facilitate data collection and analysis. The coding system that was developed is described in detail below and was used throughout the entire project and this thesis. This scheme was useful for data collection and to allow all results to be related back to each study participant.

3.3.2.1 Memphis cats

Each cat from the Memphis FIV Sanctuary was assigned the letter "M" followed by a number from 1 to 50 (assigned randomly to each cat's name). The names of the Memphis cats and their corresponding codes are listed in Table 3-1.

Table 3-1 Coding system for Memphis Crew's FIV Sanctuary cats. Letter "M" followed by number from 1 to 50 were randomly assigned to each cat's name. There are 27 cats coded according to this scheme.

No.	Code	Cat Name	No.	Code	Cat Name
1	M1	Truman	15	M28	Franklin
2	M2	Jasmine	16	M29	Stormy
3	M3	Sidney	17	M30	Buddy
4	M5	Mrs Wootrie	18	M31	Mae
5	M8	Ted	19	M32	Buzz
6	M10	Rocket	20	M33	Slater
7	M11	Indy	21	M41	Charlie
8	M12	Tank	22	M44	Layla
9	M14	Cinnamon	23	M46	Mandy
10	M15	Domino Crews	24	M47	Wrigley
11	M16	Tiger	25	M48	Edgar
12	M20	Freddie Mercury	26	M49	Buster
13	M25	Shelby	27	M50	Seamus
14	M26	Brian May			

3.3.2.2 PAWS Chicago cats

Each cat from PAWS Chicago was assigned a code with the letter "P" followed by a number from 1 to 22 (randomly assigned to each cat's name). The names of the Chicago cats and their corresponding codes are listed in Table 3-2.

Table 3-2 Coding system for cats from PAWS Chicago. Letter "P" followed by number from 1 to 22 randomly assigned to each cat's name. There are 17 cats coded according to this scheme.

No.	Code	Cat Name	No.	Code	Cat Name
1	P1	York Cat	10	P11	Boots
2	P2	Smudge	11	P13	Athena Ramona
3	P4	Obiwan	12	P14	Jinx
4	P5	Frisco Hamilton	13	P15	Franco
5	P6	Ingrid	14	P17	Blondie
6	P7	Luther	15	P18	Stosh/Jack
7	P8	Orangello Mac	16	P21	Iko
8	P9	Rocky	17	P22	Big Tuna
9	P10	Buddy Sammy			

3.3.3 Study timeline and collection dates

FIV prognosis project began on the 1st of October 2009 and the first blood samples from 27 Memphis cats were collected 4 months later on the 1st of February 2010. Subsequent follow up samples from the Memphis cats were collected at 6 monthly intervals until the end date of 9th of July 2011, such that a total of 4 samples were collected from each cat over a period of 18 months.

The first blood samples from the PAWS Chicago cats were obtained on the 10th of May 2010, with follow up samples being collected at 6 monthly intervals until the last samples were obtained on the 6th of November 2011. Similarly to the Memphis group, 4 samples from each PAWS Chicago cat were collected over a period of 18 months.

To facilitate data management letters "A", "B", "C" and "D" were assigned to each collection date as described in details in Table 3-3.

Table 3-3 Blood collection dates with assigned corresponding codes ("A","B","C","D") for Memphis and PAWS cat populations.

	Collection date			
Code	A	B	C	D
Memphis	01/02/2010	18/07/2010	22/01/2011	09/07/2011
PAWS	10/05/2010	15/11/2010	16/05/2011	06/11/2011

Blood sample collections were synchronized in order to facilitate the study, not only logistically but also allowing the synchronisation of sample processing with on-going experiments in the laboratory.

3.3.4 Assignment of follow up results

Throughout this thesis, the blood/plasma samples and the results generated from these samples are denoted thus: the code for each cat followed by the code for each collection time point. For example M1A indicates a plasma sample collected from Memphis cat M1 (Truman) at time point A (01/02/2010) and P1A indicates a plasma sample from PAWS Chicago cat P1 (York cat) at time point A (10/05/2010).

Samples collected at terminal stage disease are denoted with letter "T" in brackets placed after the code for each cat's name and the sample collection date. For example M11C(T) indicates a terminal blood sample collected from Memphis cat M11 (Indy) at time point C (22/01/2011) where (T) signals that the cat had died before the subsequent sampling time.

3.4 Study group detailed characteristics

An overview and the study group characteristics are described separately for the Memphis and PAWS Chicago populations, with summaries for each study group at the end of each paragraph.

Detailed signalement, clinical history, physical examination data, weights and lymphocyte subset data obtained by flow cytometry were assessed every 6 months and are listed on the CD that accompanies this thesis.

3.4.1 Breed composition

Cats recruited in the study mainly belonged to the domestic short haired (DSH) breed. This was particularly reflected in the PAWS Chicago group where 16 cats were DSH (94%) and only one belonged to the domestic long haired (DLH) breed (6%). In the Memphis group there were 20 DSH (74%), 4 DLH (15%) and 3 Siamese cross (11%) cats.

Breed composition within the study group was heterogeneous, where DSH constituted 82%, DLH 11% and Siamese cross 7% of the enrolled cats.

3.4.2 Age distribution

Accurate dates of birth for each cat were rarely available. The age of the cats enrolled in the study was estimated by Dr Litster (the range +/- 1 year), based on a physical examination and the clinical history provided by the cats' owners.

As demonstrated in Figure 3-1, in the Memphis population, the median age at the time of recruitment was 5 years, ranging from 2 to 10 years. In the Chicago group, the median cat age was estimated to be 4 years, ranging from 1 to 9 years. For the combined study group (Memphis and Chicago), the median age was estimated as 4.5 years (range 1-10 years).
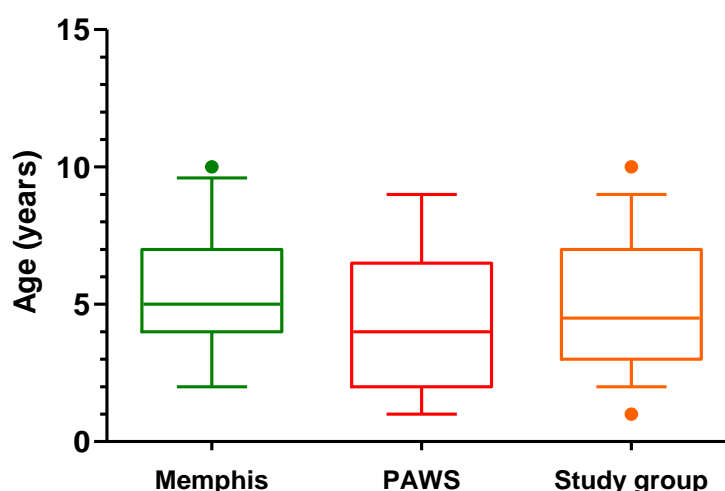
Figure 3-1 Age distribution at the time of recruitment. From left to right: First box and whisker plot showing age distribution within Memphis population (median 5 years), second and third plots show age distribution within PAWS (median 4 years) and whole study (median 4.5 years) populations. Data presented as interquartile range (IQR), (median, , 5th, 25th, 75th and 95th quartile).

3.4.3 Duration of infection prior to enrolment

FIV first diagnosis dates were available from each cats' clinical history. In most cases, for obvious reasons, it was not known exactly when the cats acquired FIV and therefore the dates of infection have been estimated by calculating the midpoint between the date of birth and the first diagnosis date. This rather crude, but only available, approach was applied to most of the cases with the exception of cat M10 which was known to be positive at the age of 6 months and cat M1 where there was clear evidence for vertical transmission of the virus.

The average length of FIV infection at the time of recruitment in the Memphis group was estimated to be 3.9 years (median 3.3 years) ranging from 1.3 to 8.8 years. PAWS Chicago cats were estimated to be infected with FIV on average for 2.8 years (median 2.9 years) ranging from 0.8 years to 5.4 years. The estimated duration of infection for the whole study group was calculated to be 3.5 years on average (median 3.3 years), ranging from 0.8 years to 8.8 years (Figure 3-2).
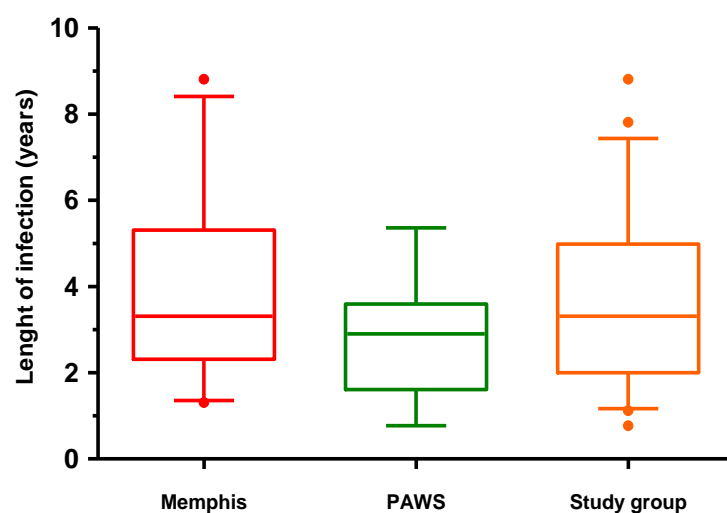
Figure 3-2 Estimated length of infection at the time of enrolment. From left to the right: Memphis population (median 3.3 years), PAWS population (median 2.9 years), study group (median 3.5 years). Data presented as interquartile range (IQR), (median, , 5th, 25th, 75th and 95th quartile).

3.4.4 Age at time of FIV infection

It was difficult to estimate the cats' age at the time of FIV exposure for the study population. However, it is very important to attempt to do so, in order to understand the epidemiology of infection and the age-related immune status of infected cats at the time of exposure to the virus. Hence, estimates of the ages of the cats at the time of first infection were calculated as the midpoint between the dates of birth and dates at which FIV was first diagnosed.

It was estimated that cats from the Memphis group had been infected with FIV at the age of 1 (median 0.8 years), on average (Figure 3-3). Vertical transmission from mother to kitten had been documented in the Memphis population and the range extended to 2.3 years as a result of a 9 year old cat.

Cats from the PAWS Chicago population were estimated to have been exposed to the virus at the age of 1.7 years (median 1.1 years) on average, ranging from 0.2 years in a 1 year old cat to 3.9 years in 8 years old cat.

For the whole study group, the average age at the time of infection was estimated to be 1.3 years, ranging from 0 to 3.9 years.
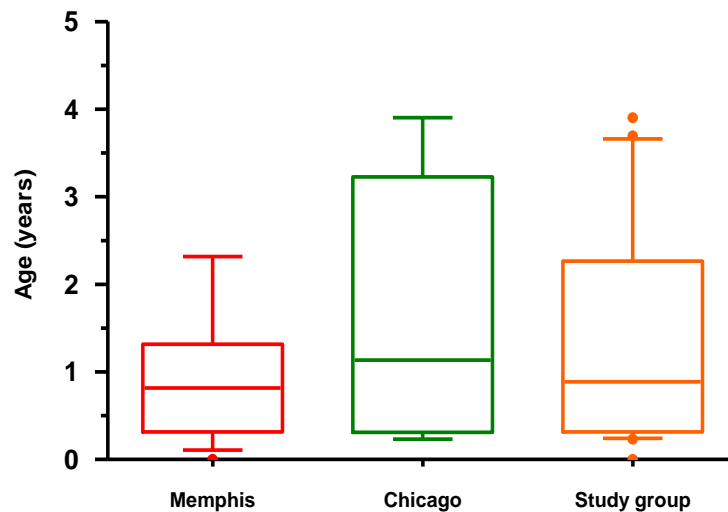
Figure 3-3 Estimated age at FIV infection. Memphis (median 0.8), Chicago (median: 1.1), study group (median: 0.9).

The age at the time of first exposure to the virus can influence the course of FIV infection (George et al., 1993) and may determine the outcome of disease (Akhtardanesh et al., 2010). Previous epidemiological observations suggest that first natural exposure to the virus occurs at the age of 1 year approximately (Grindem et al., 1989), (Ishida et al., 1989), (Yamamoto et al., 1989), similar to the findings reported here.

3.4.5 Sex distribution and reproductive status

All cats enrolled in the study had been neutered prior to adoption and rehoming. In the PAWS group there were 3 female (3/17, 18%) and 14 male (14/17, 82%) cats. The Memphis group consisted of 9 females (9/27, 33.3%) and 18 males (18/27, 66.6%). As shown in Figure 3-4, in the whole study group there were 12 female spayed (12/44, 27%) and 32 male neutered (32/44, 73%) cats. This finding is consistent with previously published data (Gruffydd-Jones et al., 1988), (Hosie et al., 1989), (O'Connor et al., 1991), (Yilmaz et al., 2000), (Spada et al., 2012) which demonstrated that FIV infection is more prevalent in male than in female cats.
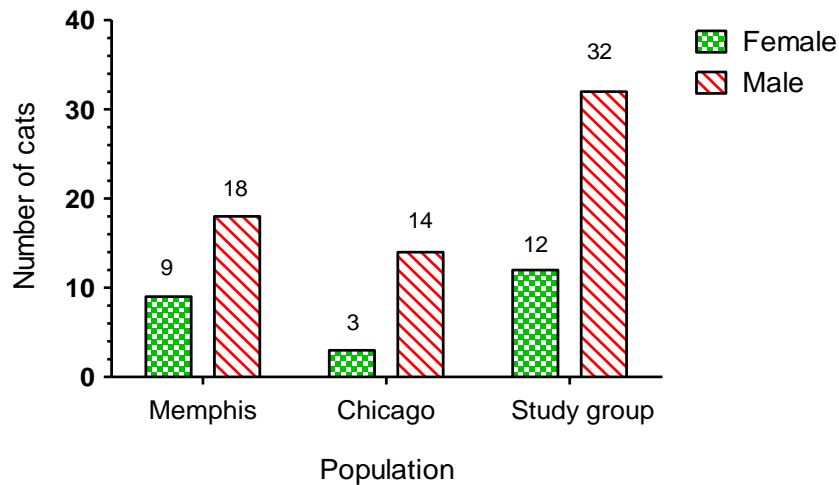
Figure 3-4 Sex distribution amongst cats recruited to the study. Memphis group (n=27) consisted of 18 males and 9 females, Chicago (n=17) of 14 males and 3 females. There were 32 males and 12 females in the whole study group (n=44).

3.4.6 Body weight

Body weight, and its alteration over the course of disease, is a commonly used parameter for the assessment of the overall health status of the patient. For the Memphis group, weights were recorded by the cats' owner each month, with the last weight data being collected in September 2011. Body weights for the PAWS Chicago cats were recorded by Dr Lister every 6 months during check-up visits at the time of blood sampling, with the last weights being recorded in November 2011.

To analyse alterations in body mass we compared weights at enrolment with the last available body weight from each cat (terminal in case of deceased cats). Figure 3-5 shows alterations of body weight comparing live and deceased Memphis cats.

In the Memphis population, 10 of 27 cats remained alive during the study period. 3 cats gained weight, 2 cats retained their weight and 5 cats lost weight (up to 34% of initial body mass). Marked weight loss between recruitment and terminal measurement was pronounced amongst the group of deceased cats. On average, the cats that died lost 59.9% of their body mass between enrolment (median 3.9 kg) and terminal stage disease (median 1.9 kg); this weight loss was statistically significant (paired t-test, $p < 0.0001$) (Figure 3-5).
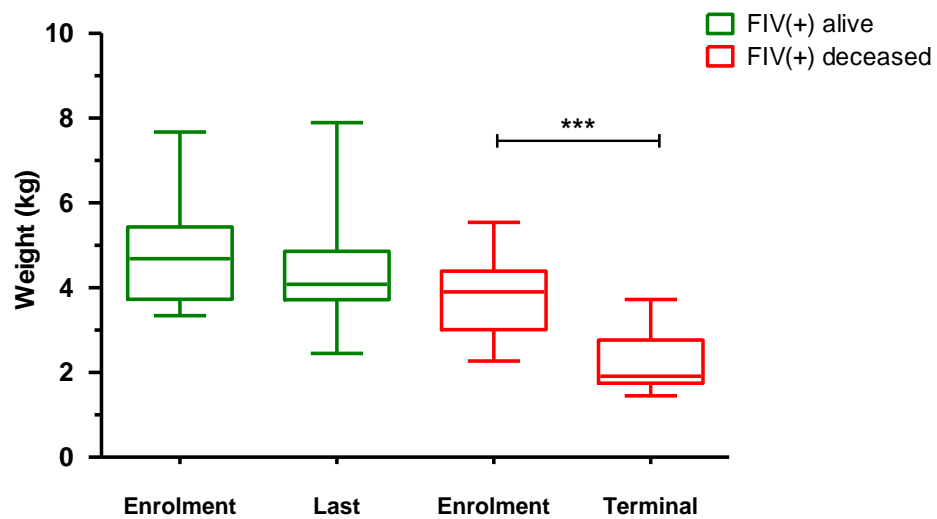
Figure 3-5 Body weights of deceased (red) and alive (green) Memphis cats as of September 2011. Enrolment weights are compared with the last available weight from each cat.

The Chicago cats (Figure 3-6) gained 0.6 kg in their body mass, on average, over a period of 18 months (median 5.8 kg vs. 6.4 kg, paired t-test, p=0.35). Only one cat (P2) from this group died. The increased body weight of cat P2 at the necropsy was most likely attributed to the fluid retention caused by the congestive heart failure (CHF) as revealed by post mortem examination. Therefore the CHF confounds the body weight estimates in this case.
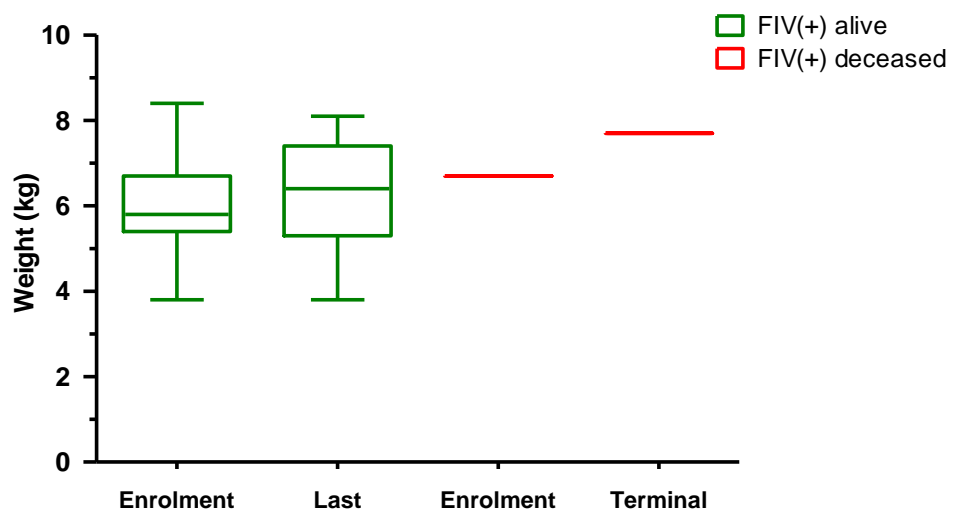
Figure 3-6 Body weights of alive (green) and one deceased (red) Chicago cat as for December 2011. Enrolment weights are compared with last available weight from each cat.

In conclusion, body weight can be, and very often is, used as a parameter to assess general health in veterinary medicine. It is not unusual, however, to observe weight loss during the progression of various diseases. Indeed it is very nonspecific parameter and, although body weights can be assessed easily both by owners and clinicians to monitor the health status of FIV infected cats, its prognostic value is limited.

3.4.7 Lymphocyte subsets and CD4:CD8 ratio

Changes in lymphocyte population have been widely reported in HIV infection, with inversion of the CD4:CD8 ratio being indicative of disease progression to AIDS (Margolick et al., 2006). In cats, decreased CD4+ helper T lymphocyte counts, increased or stable CD8+ cytotoxic T lymphocyte counts and subsequently inversion of the CD4:CD8 ratio has been described in both naturally acquired (Novotney et al., 1990), (Tompkins et al., 1991), (Hoffmann-Fezer et al., 1992), and experimental FIV infections (Ackley et al., 1990), (Barlough et al., 1991), (Tompkins et al., 1991), (Torten et al., 1991), (Bishop et al., 1992), (Lehmann et al., 1992), (Hanlon et al., 1993).

In order to assess the prognostic value of lymphocyte subset assessments in our study population, we monitored these parameters in blood samples collected over period of 18 months. Reference ranges for these values were established from 44 sex- and age-matched control cats from similar geographical locations.

Bearing in mind the differences between the two populations of cats enrolled in the study, a three-way comparison of lymphocyte subsets and CD4:CD8 ratios were conducted: one for the Memphis group, a second for the Chicago group and third for the entire study group. Figure 3-7 compares the CD4:CD8 ratios obtained over 18 months for the FIV infected Memphis cats with reference values (median, 5th, 95th quartile) acquired from uninfected, healthy control cats from a similar geographic location. The CD4:CD8 ratio values for the FIV infected cats were profoundly reduced compared to the values for the seronegative control cats. The median value (from four separate time points) for FIV infected cats (0.8) is below the 5th quartile for the control value (1.03).
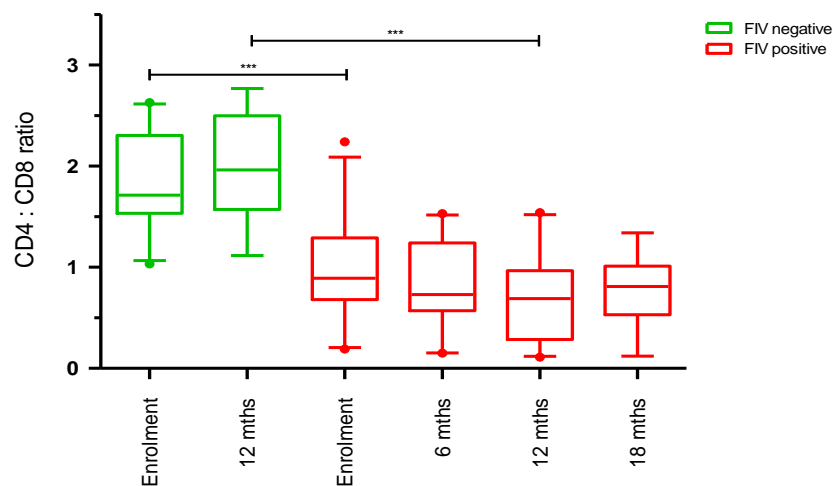
Figure 3-7 CD4:CD8 ratio of FIV positive Memphis cats (in red) and matched FIV negative control cats from the Memphis area (in green). At the time of enrolment, data were collected from 27 FIV positive cats and 27 FIV negative controls; 6 months later from 23 surviving FIV positives; 12 months later from 21 surviving positive cats and 14 negative controls; 18 months later from 16 surviving FIV positive cats.

A highly significant (unpaired t-test, p< 0.0001) difference in CD4:CD8 ratios was observed between FIV positive cats from the Memphis population and seronegative controls. This observation was consistent at the time of enrolment and 12 months later (Figure 3-7). Similarly, the CD4:CD8 ratio in surviving FIV infected cats was maintained over a period of 18 months at a consistently low level (median values: 0.89, 0.73, 0.69, 0.81 for each time point), characteristic for FIV infection.

The CD4:CD8 ratios for the PAWS Chicago FIV infected cats are compared with reference values acquired from healthy seronegative controls from the same city in Figure 3-8. As observed in the shelter Memphis population, there was a significant difference in the CD4:CD8 lymphocyte ratio (unpaired t-test, p< 0.0001) between FIV positive and control cats; the range (0.08-1.37) was below the median value (1.88) for the control cats. The CD4:CD8 ratios in the Chicago FIV positive cats were maintained over a 12 month period at a relatively low level, with median values of 0.78, 0.75 and 0.65 for each time point, in agreement with previous observations in the Memphis population and as published previously. Despite the generally better health status of the Chicago cats, their CD4:CD8 ratios were lower (median 0.73) than those of the Memphis shelter population (median 0.8).
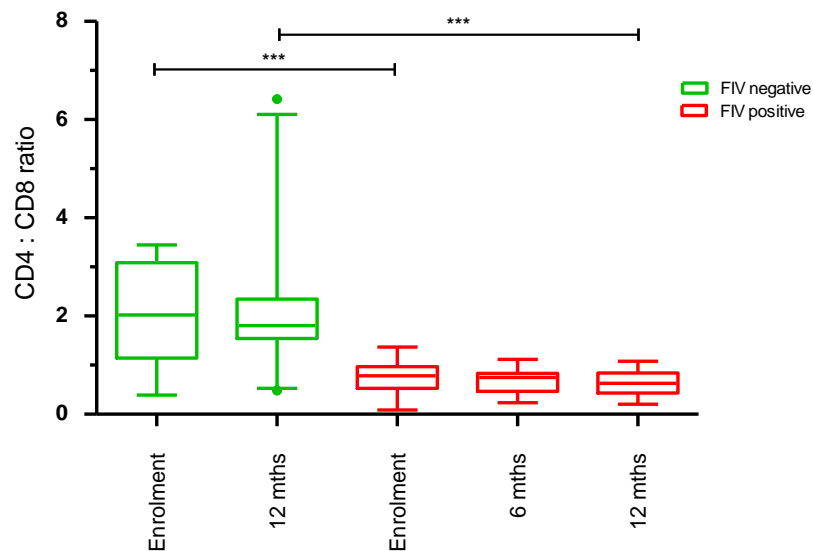
Figure 3-8 CD4:CD8 lymphocyte ratios of FIV infected PAWS Chicago cats (red) and matched uninfected control cats from Chicago (green). At enrolment, data represent 17 FIV positive cats and 11 FIV negative controls; at 6 and 12 months, data represent 16 surviving FIV positive cats and 21 uninfected control cats.

This observation led us to question whether differences in CD4:CD8 ratios would be observed between FIV-infected cats with and without clinical signs in the two populations.
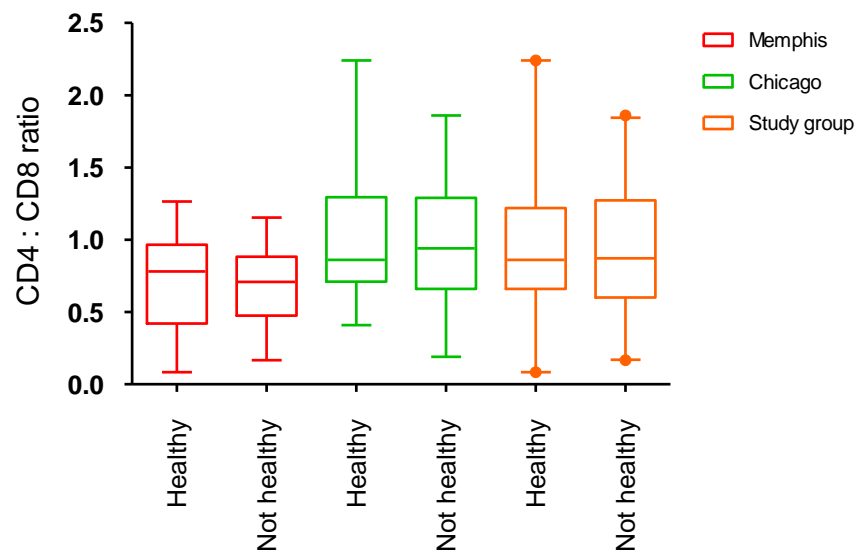
Figure 3-9 CD4:CD8 ratio of FIV positive Memphis cats (in red), Chicago cats (in green) and the whole study group (in orange) separating cats classified as healthy and not healthy at the time of recruitment. Data collected at enrolment.

Figure 3-9 shows a comparison of the ratios in cats that were classified as "healthy" or "not healthy" for each group and for the combined study population. No statistically significant divergence was observed (unpaired t-test, p=0.84 for Memphis, p=0.97 for Chicago and p=0.99 for the study group) between the CD4:CD8 lymphocyte ratios in unhealthy (ranging from 0.16 to 1.86) compared to healthy cats (ranging from 0.08 to 2.24).

As in HIV infection, the observed differences in CD4:CD8 ratios could be the result of either a loss of CD4+ cells or an increase of CD8+ cells (or both). To determine the kinetics of the CD4:CD8 ratio inversion over the course of infection, we compared absolute CD4+ and CD8+ T lymphocyte numbers from 17 cats that died during the study.

In Figure 3-10, the lymphocyte subset values of the 17 cats that died are shown from the time of enrolment and death.
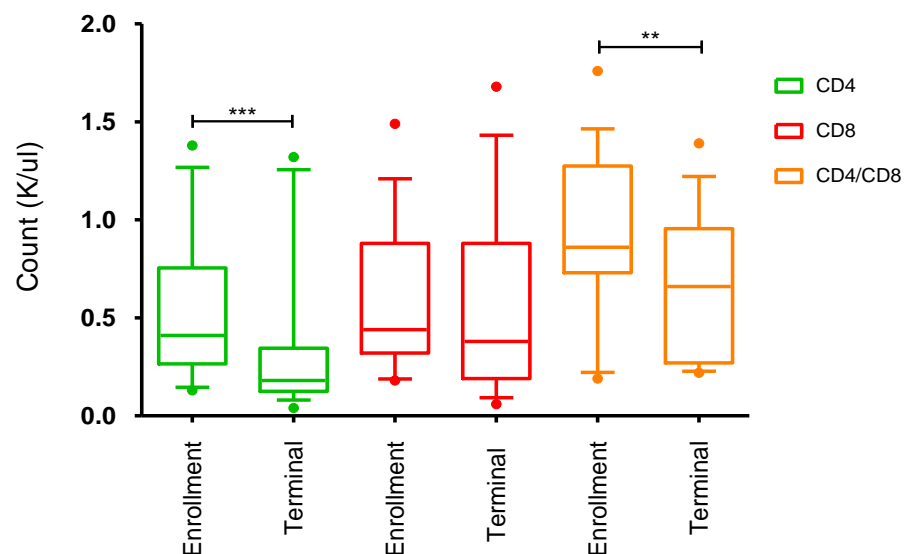
Figure 3-10 The number of CD4+ and CD8+ lymphocytes and CD4:CD8 ratio at enrolment and terminally from 17 FIV infected Memphis cats that died during the study. The different CD4:CD8 ratios between the two time points reflect decreases in circulating CD4+ T helper lymphocytes (green) and is shown in the two last box and whisker plots.

The observed decrease in CD4+ helper T cell numbers (medians 0.41 K/µl and 0.18 K/µl respectively) was statistically significant (paired t-test, p=0.0009). There was no difference however in the number of circulating CD8+ cytotoxic T cells (medians 0.44 K/µl and 0.38 K/µl respectively). Therefore, we concluded

that in the FIV infected cats, the observed decreased CD4:CD8 ratio (where median terminal value fell below the 25% percentile of the value at enrolment) could mainly be attributed to the loss of CD4+ T helper lymphocytes.

Further analysis of the total lymphocyte counts in the deceased cats showed that the decrease in absolute lymphocyte numbers between enrolment (median 1.73K/µl) and death (median 1.25 K/µl) was the result not only of a loss of CD4+T cells, but also by depletion of CD21+ B cells (median counts at enrolment 0.26 k/µl versus 0.17 K/µl in terminal samples; paired t test, p=0.01).

The hallmark of FIV infection is an inverted CD4:CD8 ratio which, as demonstrated above and in previous studies, is the consequence of a loss of CD4+ T cells. In our study we provide further evidence that FIV infected cats, regardless of their living conditions and health status, have altered proportions of CD4+ and CD8+ lymphocyte subsets, with the range falling below the median value for seronegative control cats. We observed a statistically significant difference in CD4:CD8 ratio between the time of enrolment and the terminal sampling of deceased cats; there was no significant difference between generally healthy, "asymptomatic" single household Chicago cats and deprived, more symptomatic Memphis shelter cats (median values 0.8 and 0.73 respectively). This finding questions the prognostic value of CD4:CD8 ratio in staging FIV infection and predicting the onset of immunodeficiency. By comparing absolute numbers of lymphocyte subsets, it is possible to distinguish FIV positive and negative individuals. However, healthy and unhealthy FIV infected cats could not be distinguished on the basis of their CD4:CD8 cell ratios. No correlation was seen between FIV associated immunodeficiency and inverted CD4:CD8 ratio. Both groups of cats had low CD4:CD8 ratios with median values less than 1, regardless of their health status. In fact, cat P4 which had the lowest CD4+ lymphocyte count from all (0.09 K/µl) and the lowest CD4:CD8 ratio (0.08) at the time of enrolment was classified as healthy and remains alive twenty four months later. Similar observations have been reported in FIV infected cats previously (Novotney et al., 1990) and HIV infected humans (Levy, 1988) where seropositive individuals remain asymptomatic despite significant loss of CD4+ T lymphocytes.

Hence the prognostic value of monitoring CD4+ T lymphocyte numbers or CD4:CD8 ratios in FIV infection appears to be limited in the light of these findings. There may be marked deviation amongst lymphocyte subset numbers in cats as well as variation in virulence amongst circulating wild type FIVs. The variability of CD4:CD8 ratio values amongst healthy and not healthy cats limits their use for predicting the progression of disease.

3.4.8 Health status

Assessment of the health status and general condition of an FIV infected cat is essential in order to stage the phase of infection and to monitor disease progression and, according to ABCD guidelines (Hosie et al., 2009), should be performed every 6 months. To monitor disease progression, at least two scoring systems have been adopted from those used in human medicine. One protocol, proposed by Ishida (Ishida and Tomoda, 1990) and described in (Shelton et al., 1990), is based on the staging system used in HIV infection (Asymptomatic carrier (AC), AIDS-related complex (ARC) and AIDS). The second is Karnofsky's scoring system, which is used mainly in cancer patients and has been modified for use in feline medicine (Hartmann and Kuffer, 1998).

After an initial assessment of our study population and consultations with other feline medicine specialists, we realised that while those protocols may be useful in well-controlled, experimental settings, neither is practical in staging FIV-infected cats in our study. Instead we classified cats by their general health condition. Cats suffering only minor clinical abnormalities were classified as healthy. When more than two abnormalities were detected on physical examination, cats were classified as not healthy.

The health status of each cat has been evaluated at 6 monthly intervals by the experienced feline medicine specialist, Dr Litster. At each clinical examination heart and respiration rates were measured, general appearance evaluated and lymph nodes, musculoskeletal, cardiovascular, respiratory, digestive, urogenital systems were examined, together with a neurological examination. During the examination of the oral cavity, the degree of faucitis/gingivostomatitis characteristic of FIV infection was graded using a scale of 0 to 3. At the time of

enrolment there were 12 healthy (44%) and 15 not healthy (56%) cats in the Memphis population.
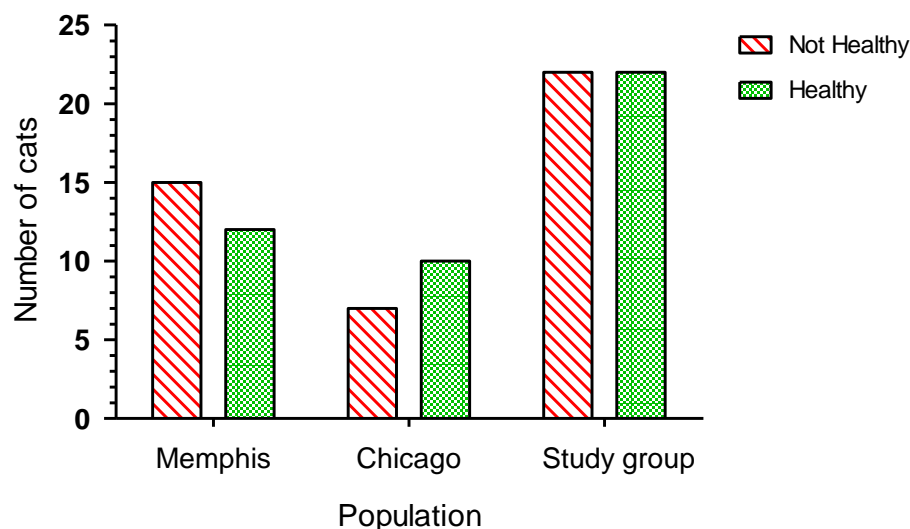
Figure 3-11 Health status at enrolment. Memphis (n=27): healthy (n=12), not healthy (n=15); Chicago (n=17): healthy (n=10), not healthy (n=7); Study group (n=44): healthy (n=22), not healthy (n=22).

The proportion of healthy cats in the PAWS Chicago population was greater than in the Memphis group, with 10 healthy (59%) and 7 not healthy (41%) cats. In the combined study group there were equal numbers of healthy and not healthy animals at the time of enrolment (Figure 3-11).

Within the Memphis population, 12 cats initially classified as healthy and 15 cats classified as unhealthy remained in their assigned groups throughout the study. However, 17 cats from the Memphis cohort become anorexic, showed significant weight loss (Section 3.4.6) and eventually died at various time points (Table 3-5).

It is important to emphasize that 6 of 12 cats initially classified as healthy (50%) and 11 of 15 unhealthy cats (73%) died during the study (Fisher's exact test, p=0.2566). 4 of 9 cats (44%) which were diagnosed post mortem with lymphoma were classified as healthy and, surprisingly, in none of the cases were any abnormalities detected during clinical examination which included palpation of peripheral lymph nodes. This raises the question of when a basic clinical

examination will detect such abnormalities and when lymphoma presents clinically.

Table 3-4 Abnormalities detected during clinical examinations of 44 FIV (+ve) cats over period of 18 months.

	Memphis (27)	Chicago (17)	Study group (44)
Description	Cats with abnormalities (number), (%)		
Dental/oral cavity (faucitis)	19 (70%)	12 (71%)	31 (70%)
Skin (alopecia, dermatitis)	12 (44%)	10 (59%)	22 (50%)
Ears	4 (15%)	3 (18%)	7 (16%)
Ocular disease (mucopurulent discharge)	5 (19%)	2 (12%)	7 (16%)
Cardio-vascular	2 (7%)	3 (18%)	5 (11%)
Digestive tract	4 (15%)	0 (0%)	4 (9%)
Respiratory (URI, nasal discharge)	2 (7%)	0 (0%)	2 (5%)
Lymph nodes	0 (0%)	0 (0%)	0 (0%)
Uro-genital	0 (0%)	0 (0%)	0 (0%)
Neurologic	0 (0%)	0 (0%)	0 (0%)
Musculoskeletal	0 (0%)	0 (0%)	0 (0%)
Weight loss/anorexia	22 (81%)	0 (0%)	22 (50%)

Within the Chicago population, 10 cats (59%) were classified as healthy at the time of enrolment and the remaining 7 cats (41%) were unhealthy. All but one cat remained in this classification over the period of study. A single cat died and had not shown any abnormalities on physical examination, despite being diagnosed post mortem with hypertrophic cardiomyopathy (HCM).

The clinical abnormalities detected during the examinations are listed in Table 3-4. The most common presentation across both populations was gingivo-stomatitis/faucitis, detected in 70% of cats. However, although the remaining 30% of cats were classified as normal on oral examination, each cat had at least one minor abnormality detected in their mouth during one examination. The second most common clinical sign (50% of cases) was a skin abnormality, not only limited to various types of dermatitis but notably including non-healing fight wounds and the presence of fleas (*Ctenocephalides felis)* (3 Memphis cats). Ocular disease with mucopurulent discharge was recorded in 16% of cases. Other abnormalities included digestive system signs that presented as mild bowel thickening on palpation in 9% of cases, while upper respiratory infections with nasal discharge were seen in 5% of cases. No abnormalities were detected on examination of musculoskeletal, urogenital or neurologic systems. Surprisingly,

none of cats displayed lymphadenopathy, nor even slightly enlarged peripheral lymph nodes on any of the four occasions when they were physically examined.

Additional health problems not detected during examination, but reported by owners and/or the cats' regular veterinarians, included: urinary tract infections (8 cats, 18%), skin allergy (1 cat, 2%), diabetes (1 cat, 2%) and diarrhoea (1 cat, 2%).

FIV infection subsequently leads to immunosuppression, which in many cases can progress to feline AIDS. Infected cats are at greater risk of opportunistic infections, neoplasia and neurological disorders. Time to disease progression and death of 17 out of 27 cats (63%) in the Memphis cohort within 18 months of observation was unexpectedly high in comparison to 18% drop off rate reported by Barr during two years of observation (Barr, 2000). The health status of cats in the Chicago cohort was similar to a household reported previously (Addie et al., 2000), where FIV infection did not affect the cats' life expectancy, at least over a period of 2 years. There are several clinical abnormalities associated with FIV infection, with gingivostomatitis being the most common. Despite the fact that one study (Quimby et al., 2008) did not observe an association between FIV infection and gingivostomatitis, the high prevalence of this abnormality in our study group was in agreement with previous reports (Bandecchi et al., 1992), (Hofmann-Lehmann et al., 1995), (Knotek et al., 1999), (Hosie et al., 2009). Ulcero-proliferative lesions are often painful and can eventually lead to tooth loss. Because gingivostomatitis is usually not seen in experimentally infected SPF cats, exposure to other infective agents (such as co-infection with feline calicivirus (FCV)) may be responsible for the manifestation of this syndrome in natural infections (Tenorio et al., 1991), (Reubel et al., 1994). Other abnormalities attributed to FIV-induced dysregulation and impairment of immune surveillance (Barlough et al., 1991), (Reche Jr et al., 2010) included dermatitis, ocular disease, renal insufficiency, lower urinary tract and other opportunistic infections. Although neurologic abnormalities have been reported to be very common in natural and experimental, acute and chronic FIV infections (Dow et al., 1990), (Abramo et al., 1995), (Podell et al., 1997), (Podell et al., 1999) we observed neither behavioural nor motor abnormalities in our study group.

The relationship between FIV infection and disease is unclear, with some studies reporting that virus infected cats are at increased risk of morbidity and mortality (Muirden, 2002) while others observed no such association (Norris et al., 2007), (Akhtardanesh et al., 2010). It is difficult to associate mortality and morbidity with FIV infection when examining cats within the whole study group, but an association became apparent when the two populations were examined separately. Our observations in the Memphis population indicated that poor living conditions, exposure to opportunistic pathogens and limited healthcare access play a major role in more rapid disease onset. In contrast, the Chicago cats living in generally much better conditions in single households remained alive and in relatively good health compared to the Memphis population. In spite of the difficulties elucidating an association with disease, and given the short observation period of this study, nevertheless disease associated with FIV infection appears more pronounced in shelter cats, living in more demanding conditions. Cats living in single households and not exposed to opportunistic pathogens were more likely to remain free of clinical signs, in accord with the observations of many clinicians whose expert opinion is that FIV in such cases does not cause severe clinical abnormalities.

3.4.9 Cat deaths during the study

During the two years of study, 1 of 17 (5.9%) cats from the Chicago cohort and 17 of 27 cats (63%) from the Memphis group died. The mortality rate of 63% in the Memphis population over a relatively short period of time was unexpected. Figure 3-12 illustrates the percentage survival of Chicago versus Memphis cats, highlighting a marked difference in survival between the two populations. The causes of death and possible associations with FIV infection were established following post mortem examinations (Table 3-5). Hypertrophic cardiomyopathy and congestive heart failure were associated with death of the single Chicago cat. For the Memphis group, necropsy data were not available for two cats (M33 and M14), no diagnoses were made post mortem for another two cats (M44 and M50) and in 4 other cases various degrees of respiratory tract infection and subsequent pneumonia were associated with mortality.

Post mortem examinations revealed that the most prominent cause of death in the Memphis group was lymphoma; various anatomical types were recorded in 9 out of 15 Memphis cats (60%) that were examined post mortem.
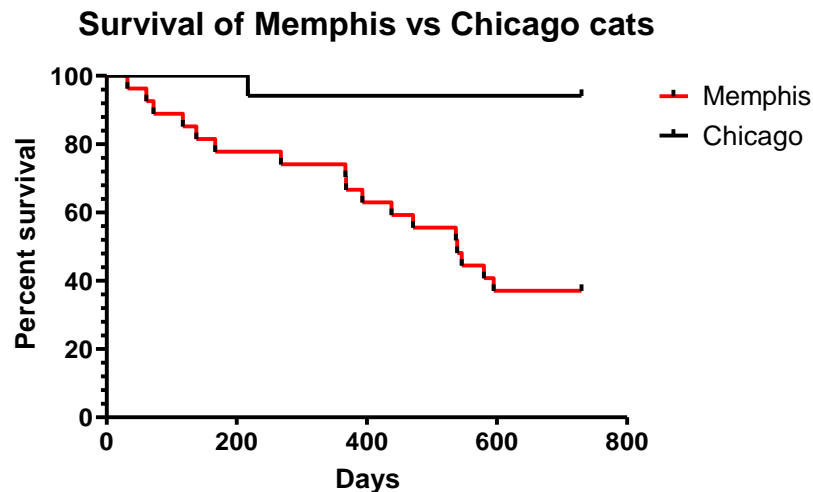
Figure 3-12 Kaplan Mayer survival curves for Memphis (red) and Chicago (black) groups. Difference in survival rate is statistically significant (Gehan-Breslow-Wilcoxon Test p=0.0006). Survival rates in Memphis and PAWS populations were 37% and 94% respectively.

Other pathological changes not directly associated with mortality but most likely linked with FIV infection included lymphoid hyperplasia and lymphoid depletion, noted in 13 of 16 cats (81%) that were examined post mortem. These were pronounced in various tissues, mainly in spleen and lymph nodes but in 4 cats Peyer's patch atrophy was also observed. The second most notable abnormalities were erythroid and myeloid bone marrow hyperplasia, seen in 9 out of 16 cats (56%). Multifocal lymphocytic interstitial nephritis, possibly associated with FIV infection as reported previously (Ishida et al., 1992), was detected in 6 cats (37.5%) and was the third most common abnormality detected post mortem.

FIV infection is generally associated with immune suppression but, paradoxically, immune hyper-activation is equally important in the pathogenesis of disease and the development of the AIDS-like syndrome. B cell activation, manifest as polyclonal hypergammaglobulinemia, expansion of CD8+ T cells with an activated phenotype and subsequent CD8+ T cell lymphocytosis are both directly

and indirectly associated with abnormalities detected post mortem. The lymph node follicular hyperplasia and dysplasia seen amongst the cats in this study have been reported previously in acute and terminal stage disease (Yamamoto et al., 1988), (Shelton et al., 1989a), (Matsumura et al., 1993). Reactive changes within bone marrow seen in 56% of cats have been documented in HIV and FIV infections as well as various other disorders (Geller et al., 1985), (Breuer et al., 1998), (Bain et al., 2009) and are regarded as non-pathognomonic.

Upper respiratory tract (URT) signs presented in 4 cats; these were initially mild but became more severe over a relatively short period of time, perhaps associated with impairment of cell mediated immunity leading to opportunistic infections. URT infections are very common in FIV infected cats and have been previously reported in the literature (Pedersen et al., 1989), (Yamamoto et al., 1989).

The renal damage observed in 33% of cases may have been associated with deposition of viral-antibody immune complexes or may have resulted from direct viral infection of renal epithelium and thrombotic micro-angiopathy (TMA). Direct infection of epithelial cells within several parts of the nephron has been documented in HIV-associated nephropathy, predominantly in Africans (Ross et al., 2001), while deposition of virus-antibody complexes accounts for the majority of nephropathies in Caucasians (Nochy et al., 1993), with immune complex-mediated glomerulonephritis being the most common manifestation. The mechanism of TMA kidney damage is poorly understood, but it seems that epithelial cell damage caused by the virus may cause platelet activation and deposition in the renal microvasculature (Röling et al., 2006). Although it is difficult, based only on anatomical pathology data, to elucidate the mechanism of interstitial nephropathies in our study population, extrapolating from HIV (Melica et al., 2011), it appears that prolonged immune stimulation and massive, non-specific, FIV-driven B cell activation and subsequent interstitial lymphoplasmacytic infiltration may be highly associated with the type of nephropathy seen amongst the FIV study cats.

Table 3-5 Cats deceased during the study. Health status, date and cause of death.

Cat	Date of death	Status	Cause of death
M26	15/04/2011	Not Healthy	Chronic focal granulomatous nematode larval pneumonia; Bone marrow hyperplasia
M30	19/09/2011 Euthanized	Not Healthy	Lymphoid depletion; *Aelurostrongylus abstrusus* pneumonia; Left front leg suppurative dermocellulitis; Bone marrow hyperplasia, with erythroid and myeloid hyperplasia
M49	18/06/2011	Healthy	**Lymphoma - Bone marrow**
M41	03/02/2011	Not Healthy	**Lymphoma - Bone marrow and lymph nodes**
M14	02/08/2011	Not Healthy	No necropsy performed
M48	05/03/2010	Not Healthy	**Lymphoma - Bone marrow and lymph nodes**
M11	04/02/2011	Healthy	**Lymphoma – multicentric**
M44	14/04/2010	Not Healthy	Undetermined/Emaciation/Nephritis/Pancreatitis
M31	01/03/2011	Not Healthy	**Lymphoma - Bone marrow**
M46	23/07/2011	Healthy	**Lymphoma - Bone marrow**
M5	18/05/2011	Not Healthy	**Lymphoma - Bone marrow**
M10	25/07/2011	Healthy	**Lymphoma - Bone marrow and lymph nodes**
M50	27/10/2010	Healthy	Undetermined/Emaciation
M25	04/09/2011	Healthy	Upper and lower bacterial respiratory infection, probably secondary to viral infection
M3	04/03/2010 Euthanized	Not Healthy	**Lymphoma – Retrobulbar**
M33	29/05/2010	Not Healthy	No necropsy performed
P2	13/12/2010	Healthy	Cardiac failure – HCM
M16	21/06/2011	Not Healthy	Pulmonary congestion and oedema with focal bacterial bronchopneumonia

3.4.10 FIV associated lymphoma

FIV-associated neoplastic transformations, predominantly lymphomas, have been documented in both natural and experimental FIV infections (Pedersen et al., 1989), (Poli et al., 1994), (Terry et al., 1995), (Gabor et al., 2001b) (Shelton et al., 1989b) ranging from 1 to 27% of cases (Feder and 1990), (Zenger, 1990), (Hutson et al., 1991), (Ravi et al., 2010) with the highest rate of 50% reported in an Australian retrospective study (Gabor et al., 2001b). In our study the rate was strikingly higher; 53% of all cases in Memphis cluster diagnosed with various anatomical types of lymphoma as shown in Table 3-6.

Table 3-6 Organs with neoplastic lymphocyte infiltrates among cats post mortem diagnosed with lymphoma.

Cat	Organ involved									
	Bone marrow	Lymph node	Spleen	Kidney	Liver	Jejunum	Tongue	Trachea	Heart	Eye
M11	+	+	+	+	+	+	+	+	+	
M41	+	+	+							
M5	+	+	+							
M48	+	+								
M10	+	+								
M31	+									
M49	+									
M46	+									
M3										+

Post mortem examinations revealed that four cats had lymphoma limited to a single site (retro-bulbar site in 1 case and bone marrow in 3 cases), while in five other cats varying degrees of dissemination were seen. Bone marrow involvement was noted in all but one case. The second most common site for lymphomas was nodal, seen in 5 cats (56%), accompanied by splenic lymphoma in 3 cases (33%). The youngest cat (M11) presented with a disseminated form involving kidney, liver, jejunum, heart, trachea and tongue (glossal lymphoma). Lymphoma cats from the Memphis cohort had a median age of 6 years (ranging from 3 to 10 years). Differentiation of T-cell and B-cell antigen expression in the lymphoma tissue was not performed and the phenotype of those lymphomas is unknown. In light of previous studies, however (Poli et al., 1994), (Terry et al.,

1995), (Gabor et al., 2001b) it is predicted that the majority, if not all, were of B-cell origin.

The high prevalence of lymphoma raised questions of environmental, host, viral and other factors that may be associated with lymphoma development in the Memphis cohort. The first candidate causative agent would appear to be FIV. One likely mechanism of cancer development, proposed both in FIV (Barr et al., 1993), (Callanan et al., 1996) and in HIV (Safai et al., 1987), (O'Hara, 1989) infections, may be attributed to virally induced immune suppression and lack of anti-neoplastic immune control capacity (Penn, 1986). Another mechanism could be attributed to a high viral mutation rate and infection of dividing cells, rendering the cells more prone to neoplastic transformation. Most importantly however, viral integration into host genome and subsequent alteration of cellular transcription profiles, either by activation of cellular oncogenes or interrupting tumour suppressor genes, could explain neoplastic transformations following retroviral infection. This mechanism has been linked with neoplasia following infection with FeLV (Jackson et al., 1993), MuLV (Suchman et al., 1995) and MMTV (Nusse and Varmus, 1982). Viruses from other families also have been shown to provoke carcinogenesis via a similar mechanism, for example human papilloma virus (Baker et al., 1987). Although clonal integration and possible subsequent dysregulation of the cellular transcription profile was demonstrated in two studies (Beatty et al., 1998), (Wang et al., 2001), the majority of studies did not observe integration of FIV proviral DNA in lymphomas (Terry et al., 1995), (Callanan et al., 1996), (Endo et al., 1997). Therefore it appears that while insertional mutagenesis may be directly associated with carcinogenesis in rare cases of FIV and possibly one case of HIV infection (Shiramizu et al., 1994), in the majority is induced by a different, indirect mechanism (Levine, 1992). It is possible that lentivirus-induced dysregulation of the immune system may reveal the oncogenic potential associated with other viruses, as occurs with human herpes virus 8 (HHV8) which leads to development of Kaposi's sarcoma (Rabkin et al., 1991) in HIV-infected patients. Kaposi's sarcoma (KS) is recognized as an AIDS defining cancer and its manifestation is highly associated with HIV infection; the likelihood of KS development is 300 fold higher in HIV-infected individuals compared to patients receiving immunosuppressive therapies (Beral et al., 1990), clearly indicating a synergistic

effect between the two viruses. On the other hand, lymphomas associated with HIV infection have been linked also with Epstein-Barr virus (EBV) coinfection, proposed to occur through prolonged B-cell activation (Shibata et al., 1993). Prolonged B cell antigenic stimulation is a potential mechanism for lymphoma development in FIV infected cats, as suggested previously (Hutson et al., 1991). Recent studies demonstrated serological responses to EBV (Chiou et al., 2005), (Milman et al., 2011) and the presence of EBV-like viral DNA and protein (Huang et al., 2012b) in malignant lymphocytes of domestic dogs diagnosed with lymphoma. It was suggested that canine EBV-like virus may be linked with B-cell lymphomagenesis, as antibody titres against the viral antigens p18-VCA and p23-VCA were significantly higher in dogs with lymphoma compared to healthy dogs (Huang et al., 2012b).

The high number of lymphoma cases arising in the Memphis cluster compared to the Chicago cohort was striking and merits further investigation. Firstly, carcinogenesis may be attributed to environmental factors as the living conditions were very different for the Memphis cats compared to the Chicago cats. The presence of carcinogens in the environment cannot be ruled out, but the Memphis cats lived in more demanding conditions, with poorer healthcare which may have led to weakened anti-neoplastic immune surveillance, resulting in neoplasia in this cohort. We examined FIV strain variability and the possibility that the Memphis FIV strains had greater oncogenic potential than the strains infecting the Chicago cohort but a comparison of long terminal repeat sequences revealed no significant differences (data not shown). Host factors, such as breed were unlikely to play a role, as the cats affected with lymphomas, as well as the cats in the Chicago cohort were DSH. Based on the epidemiological situation, it seems likely that the occurrence of lymphoma in the Memphis cluster may be associated with an infectious agent. A role for FeLV infection was excluded based on FIV/FeLV Snap test (IDEXX) results which were negative for FeLV antigen. However, we cannot exclude the possibility of latent FeLV infection, with FeLV provirus remaining integrated in the host genome within the bone marrow cells in the absence of detectable antigenaemia. Cats that are latently infected with FeLV test negative on routine antigen testing (Jackson et al., 1993), (Gabor et al., 2001a) and hence some cats in our study group may have been latently infected with FeLV. However, a recent report employing two

different PCR assays failed to detect FeLV proviral DNA in blood, tumour or bone marrow samples from FeLV antigen negative cats that developed lymphoma (Stutzer et al., 2011). Hence it is unlikely that latent FeLV infection is the cause of this malignancy in antigen negative cats. Furthermore, FeLV involvement in lymphoma may have been overestimated, particularly in the last 20 years where effective vaccine and eradication programs have marginalised the impact of FeLV infection. In our study population, it is likely that the FeLV antigenemia corresponds to a lack of proviral FeLV DNA integration, implying that there is no significant FeLV involvement. Although this hypothesis will be tested in our laboratory, there is likely to be a factor other than FeLV triggering carcinogenesis in these cats.

In light of early findings in HIV cancer patients, it was suggested that lymphoma could be triggered by the recently discovered HHV-6 isolated from HIV patients by the same group (Salahuddin et al., 1986). More recently it was shown that HHV-6 can cause persistent infection by inserting its DNA into chromosomes (Pellett et al., 2011); the region of insertion was subsequently mapped to telomeres (Arbuckle and Medveczky, 2011). This recent finding and the identification of the site of insertion in chromosomal fragments responsible for aging and carcinogenesis, sheds light on the mechanisms by which HHV-6 can lead to neoplastic transformation. Mechanism of persistent, latent infection with HHV-6 is unique amongst human herpesviruses; all other known to date human herpes viruses establish latency as nuclear circular episome (Poffenberger and Roizman, 1985). This phenomenon is not only limited to HHV-6 as another herpesvirus, Marek's disease virus (MDV) that infects chicken and induces T cell lymphoma, has also been shown to integrate its DNA into chromosomes (Kaufer et al., 2011). Strikingly, the chromosomal integration sites of MDV harbour telomeric repeat sequence, identical to that of the host telomeres and the site of MDV integration has been shown to be similar to that of HHV-6 (Kaufer et al., 2011). It is tempting to speculate that in cats from the Memphis cluster (and possibly in others including wild felids), carcinogenesis may be triggered by an as yet unidentified feline virus belonging to the herpesvirus family and integrated on host telomeres. It is possible that, similar to chromosomally integrated HHV-6 (Arbuckle and Medveczky, 2011), such a virus could be transmitted vertically through germinal cells and may be fixed in the cat population worldwide. This

hypothesis is supported by recent research on MDV and HHV-6. At the same time, cats are the species most prone to developing lymphoma and leukaemia, which are responsible for over 30% of all feline tumours, compared to any other animal species. In many cases neither FIV nor FeLV can be linked with those malignancies, indeed one study reported lymphoma in specific pathogen free (SPF) cats that had never been exposed to either FeLV or FIV (Jarrett et al., 1984). Lymphoma development in the immunocompromised cats within the Memphis cohort requires further investigation and will be addressed in our laboratory with the aim of identifying viruses which have the potential to be linked with feline lymphomas.

3.5 Discussion

In this chapter we discussed detailed characteristics of cats recruited to our study, to describe the clinical status of the enrolled cats as well as factors to which the study population had been exposed and which are relevant to the interpretation of the results presented later in this thesis. We emphasized the diversity of the study population and the significant differences between the Memphis and Chicago cohorts. Indeed, it become apparent during the observation period that completely different clinical outcomes of FIV infection arose in the two populations. Despite some initial doubts about enrolling cats from two separate cohorts (one of which contained cats living together and potentially infecting each other), our study of two cohorts has proved to be very useful in elucidating the different outcomes of natural infection in diverse populations.

Studying natural infection can be very challenging and as highlighted above it was necessary to make estimations when defining the ages of cats, the time when virus was first encountered and duration of infection. Although it would have been possible to define these parameters as unknown, estimates were made based on clinical examination and the histories that were available from the adoption centre or owners. It is important to recognize, however, that these estimates are crude and not necessarily accurate. But in spite of these problems, the estimates appeared to be consistent with previous studies (Grindem et al., 1989), (Ishida et al., 1989), (Yamamoto et al., 1989) and provided a basis for the interpretation of the results presented in this thesis.

One of the aims of this chapter was to identify potential prognostic markers that could be used by clinician to stage disease and estimate the likelihood of progression to AIDS. Unlike HIV infection, there are no well-established prognostic indicators for FIV disease progression. Analysing all haematological and biochemical parameters and their potential prognostic value were beyond the scope of this study. Instead, this chapter focused on analysing lymphocyte subsets as well as alterations in body weight during the course of infection. Body weight changes between alive and deceased cats were significant, but this is non-specific; weight loss may be observed in any wasting disease and therefore is not necessarily attributable to FIV infection. Despite significant differences

between CD4 and CD8 lymphocyte subset numbers between FIV-infected and uninfected cats, it is difficult to distinguish "asymptomatic" cats with no clinical signs from "symptomatic" cats displaying clinical signs by comparing lymphocyte subset numbers. Although, with some limitations, CD4:CD8 lymphocyte ratio values can be used in monitoring HIV infected patients (Margolick et al., 2006), these ratios are of limited use for staging and estimating the likelihood of disease progression in cats.

Unfortunately it was not possible to include plasma viral RNA load (PVL) data in this chapter, since the estimation of PVL among the cats enrolled in this study is still on going. Bursts in viral replication and correlation between increased PVL and immunodeficiency have been reported in HIV infection (Katzenstein, 2003) and in one study examining kittens experimentally infected with two diverse strains of FIV (Diehl et al., 1996). The usefulness of PVL as a prognostic surrogate marker in naturally infected cats is under investigation and such data will further broaden our understanding natural FIV infection.

Accurate determination of the health status, and therefore classification of cats in the study group as either "symptomatic" or "asymptomatic" has been problematic, in spite of the examinations being conducted by an experienced, board certified feline medicine specialist. Some cases were initially classified as "healthy" but subsequently died during the study and some cases initially classified as "not healthy" are still alive at the time of writing this thesis. It has become apparent, while monitoring the Memphis population, that the health status of FIV infected cats can deteriorate over a few days. This especially applies to cases of lymphoma which can develop within few weeks without any abnormalities having been detected on prior clinical examination, particularly in cases where lymph node involvement revealed post mortem was not detected during physical examination.

Striking differences in survival rate were observed between the cats in the Memphis and Chicago cohorts. The differences in the housing conditions of the two cohorts have been described above and might have impacted upon the different clinical outcomes of FIV infection observed between the two groups. Despite the relatively short observation period, it has become clear that housing conditions, nutrition and access health care can dramatically influence

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the survival time of an FIV-infected cat. It seems that cats, even with compromised immune systems (defined by comparing lymphocyte subset values) can remain relatively healthy when living in single cat households and receiving supportive treatment when necessary. On the other hand, as observed in the Memphis cohort, overcrowded shelter conditions and lack of regular healthcare can significant increase morbidity and mortality. Indeed some studies (Addie et al., 2000), (Ravi et al., 2010) and the impression of veterinary practitioners suggest that the outcome of natural FIV infection is not as dramatic as some of the cases described under experimental conditions (Diehl et al., 1995b). Whereas the cats in the Chicago cohort remained relatively healthy during the monitoring period, the cats in the Memphis cohort represented the other extreme; in this cohort disease progression, and particularly oncogenesis, was dramatic.

The most common cause of the morbidity observed in the Memphis cohort was neoplasia, as discussed above. Although virus-induced immunodysregulation can be linked with neoplastic transformation of B-cells, a direct carcinogenic role for FIV seems unlikely (Magden et al., 2011). Potentially, another unknown infectious agent that could become reactivated in immunocompromised cats could be associated with the high incidence of lymphoma among our study group. This hypothesis remains to be tested and as a first step, samples from these cats will be subjected to next generation sequencing to identify potential novel agents related to the lymphomas.

The results presented in this chapter were limited by the 18 month follow-up period of the study. Furthermore, the inclusion of cats from more diverse geographic locations would have increased the variability in virulence of wild type FIVs examined and their possible contribution to the onset of disease. Nevertheless, based on the relatively large number of cases examined and the differences between the two geographically distinct cat populations in the study population, it can be concluded that while FIV infection may not be associated with severe immunodeficiency in many cases, poor or overcrowded living conditions, especially in shelters, have a significant impact on the FIV-infected cat's immune system and subsequently lead to the more rapid onset of disease and death.

Based on these observations, FIV infected cats can remain in relatively good health when living in stable, single cat households. No signs of disease were observed in the cats within the Chicago cohort that were housed in single cat households over a 24 month period. The average duration of the "asymptomatic" phase of FIV infection remains unknown and this question will be addressed by observing study participants over the next 5 years.

It is crucial not to underestimate the importance of careful selection of study participants as well as the critical interpretation of clinical studies where the selection of study subjects (even when based on power calculations) can dramatically influence the outcome and conclusions of the research study. The selection of study participants from different locations and housing conditions minimized potential results bias. It is apparent that the conclusions of this research would have been very different indeed, had efforts been focused on only one of the cohorts presented in this study.

Chapter 4. Molecular epidemiology of FIV

Deciphering FIV evolution is essential in understanding the virus interaction with the host immune system. In this chapter, viral diversity and evolution are quantified in order to examine the influence on the outcome of natural infection. This knowledge can aid in the development of more effective diagnostic tests, control strategies and, ultimately, may inform the design of a fully protective vaccine since the limited efficacy of the FIV vaccine that is currently available is largely attributed to the high genetic diversity of wild type viruses.

Serial samples were collected from 44 cats naturally infected with FIV and multiple full-length *env* sequences from each subject were subjected to evolutionary analyses. A relatively low overall within-host *env* sequence diversity was observed, indicating the presence of largely homogenous viral populations within most cats, regardless of their health status. We observed, however, relatively high within-host diversity and evolutionary rate of the leader/signal fragment of the *env* gene, suggesting that this fragment may play an important role in regulating viral replication and infectivity. Employing rigorous recombination testing, we report for the first time the major role of recombination in generating *env* gene diversity; our findings highlight profound limitations in the current phylogenetic classification of FIV. By contrasting the evolution of a.) non- recombinant and recombinant sequences and b.) *env* sequences of viruses isolated from healthy and sick animals, we observed considerable alterations in their evolutionary kinetics. Lastly, examining the global pattern of potential N-linked glycosylation sites between and within cats over the study period, we described a potential dual role in maintaining glycoprotein integrity and contributing to immune evasion.

The results of this study shed new light on the evolution of FIV, illustrating the relatively high genetic stability of the virus in natural infection. Recombination, rather than nucleotide misincorporation by the viral reverse transcriptase, appears to generate viral diversity on the population level. Furthermore, the altered evolutionary dynamics observed in the terminal stage of disease are more likely to be a result rather than a cause of immunodeficiency.

4.1 Introduction

FIV is currently assigned to five distinct subtypes, denoted A, B, C, D and E, based on the diversity of the V3-V5 region of the *env* gene (Nishimura et al., 1998). Subtype A is prevalent in the west coast of USA (Sodora et al., 1994) and Australia (Kann et al., 2006) and is found exclusively in the UK (Samman et al., 2011), while subtype B is most prevalent on the east coast of USA (Sodora et al., 1994) and central (Steinrigl and Klein, 2003) and southern (Duarte and Tavares, 2006) Europe. Subtype C has been identified in California (Sodora et al., 1994) and Canada (Bachmann et al., 1997), (Reggeti and Bienzle, 2004) and subtype D in Japan (Hohdatsu et al., 1996), (Nishimura et al., 1998), while the putative subtype E was reported in Argentina (Pecoraro et al., 1996). Hence, despite extensive movement of humans and their cats, geographical clustering of FIV (based on the current classification) is still evident, although subtypes A, B and C have been found in more than one continent. The majority of published sequences represent only the short 700bp V3-V5 region of FIV *env* and therefore likely contain too little information to represent an accurate phylogenetic classification; full-length gene sequence is required to enable the precise detection of recombination and to permit an accurate phylogenetic classification.

Recombination, together with point mutations introduced by the error prone reverse transcriptase (Preston and Dougherty, 1996) and the activity of host restriction factors (Chiu and Greene, 2006), is regarded as the most important mechanism of generating genetic diversity among retroviruses on both the population and the intra-host level (Charpentier et al., 2006), (Onafuwa-Nuga and Telesnitsky, 2009). Two features of the retroviral life cycle increase the likelihood of recombination: 1) the presence of two RNA genomes within each viral particle and 2) the feature of reverse transcriptase (RT) which tends to switch between those RNA molecules during provirus synthesis (Zhuang et al., 2002), (Levy et al., 2004a). Specific features of RT may result in the synthesis of recombinant DNA with a mixed ancestry, originating from both RNA molecules (Basu et al., 2008). Although in HIV-1 infection recombination occurs between homologous viral variants of similar fitness at an exceptionally high rate (Kuwata et al., 1997), (Jetzt et al., 2000), its significance in generating genetic diversity increases dramatically when a cell becomes infected with two or more

genetically distinct virions (Blackard et al., 2002). Recombination may occur between virions of the same or different subtypes, resulting in the generation of intra- or inter-subtype recombinants respectively (Blackard et al., 2002). Recombination can have profound implications on the fitness of the generated viral quasispecies and subsequently on pathogenesis and the clinical outcome of infection (Tebit et al., 2007). Intra- and inter-subtype recombinants have been identified in HIV infection (Robertson et al., 2000), with some being present as unique recombinant forms (URF) with limited transmission and commonly circulating recombinant forms (CRF) accounting for over 20% of infections in several countries (Peeters and Sharp, 2000).

Although FIV recombinants have been identified (Bachmann et al., 1997), (Carpenter et al., 1998), (Reggeti and Bienzle, 2004), (Hayward and Rodrigo, 2008), the frequency of recombination and the prevalence of CRF amongst naturally infected cats is unknown. Given the limitations of the majority of previous studies, which focused upon relatively small fragments of DNA sequences, it is apparent that the occurrence and role of recombination in natural FIV infection may have been underestimated. There is a significant lack of knowledge in FIV molecular epidemiology; how prevalent are recombinant sequences? What is their composition amongst field isolates and what role does recombination play in generating intra-host viral diversity?

The majority of FIV phylogeny research has focused upon sequence variation at the population level (Olmsted et al., 1992), (Sodora et al., 1994), (Pistello et al., 1997), (Carpenter et al., 1998), (Teixeira et al., 2010), (Samman et al., 2011) and has highlighted substantial diversity. In contrast, less is known about the mutation rate of FIV and within-host viral diversity in natural infection (Motokawa et al., 2005), (Huisman et al., 2008b). In HIV-1 infection quasispecies composition varies greatly, with some studies reporting diversity of 10% (Delwart et al., 1997). It has also been demonstrated that intra-host diversity may influence the outcome of disease and viral diversity correlated with the stage of infection (Delwart et al., 1997), (Shankarappa et al., 1999). Similar data is not available for FIV; how does the quasispecies composition contribute to the clinical outcome of infection? Is there a correlation between viral diversity and disease progression?

Natural selection acting on viral sequences shapes their genetic variation and creates viral diversity. Selection measures have been applied previously to assess forces acting upon the evolution of different genes and different regions within the same gene (Choisy et al., 2004). For example in HIV-1 infection, the *gag* gene is under negative selection (Kils-Hutten et al., 2001), while *env* gene evolution is generally shaped by positive selection (Wolfs et al., 1990). The *gag* gene encodes structural proteins and its conserved nature is essential to maintain viral integrity; inversely the HIV-1 *env* gene is constantly under immune selection pressure and sequence variation permits immune evasion (Yamaguchi and Gojobori, 1997). Extrapolating from HIV research, it would be predicted that FIV *env* should be under the same positive selection pressure as its human counterpart, but relatively little is known of selection forces acting upon FIV *env* in naturally infected cats. Is FIV *env* evolution in natural infection driven by positive selection? Is the selection pressure distributed uniformly across the gene or exerted at particular regions?

Furthermore, little research has been conducted to elucidate rates of FIV evolution (Greene et al., 1993), (Hayward and Rodrigo, 2010). No data are available concerning rates of evolution of the entire FIV *env* gene in the domestic cat. Evolutionary studies of HIV-1 infection (Lukashov et al., 1995), (Zhang et al., 1997), (Leitner and Albert, 1999), (Shankarappa et al., 1999), (Korber et al., 2000) suggest that within-host evolution is influenced by both viral and host-dependent factors. Some studies reported increased evolutionary rates during the early stages of infection, declining towards terminal stage disease (Delwart et al., 1997), (Shankarappa et al., 1999), while others did not observe a similar correlation (Mikhail et al., 2005). Nevertheless the assumption that, in immunocompetent individuals, virus is under constant pressure and evolves at a high rate to adapt to a changing environment provides a plausible explanation for the differences in evolutionary rates at different stages of disease (Lukashov et al., 1995). It is apparent that rates of evolution are equally dependent on the host as viral factors, as demonstrated by comparing the evolutionary tempo of different HIV-1 subtypes (Abecasis et al., 2009) as well as differences in replication rates of HIV-1 and HIV-2 (MacNeil et al., 2007b). It has been hypothesized that FIV clade B viruses are evolutionary older and more host-adapted, hence less pathogenic, than those of clade A (Sodora et al., 1994). Are

there differences in the evolutionary rates of different strains of FIV identified as non-recombinant and recombinant? Are recombinant sequences less host-adapted and do they evolve at a higher rate? Are different regions of the gene evolving at the same or different rates? Finally, can we predict the outcome of infection by analysing rates of viral evolution?

Highly variable, glycosylated surface proteins of enveloped retroviruses play an important role in the immune evasion (Zhang et al., 2004). N-linked glycosylation is the process of post-translational modification in which carbohydrate is attached to the nitrogen atom of asparagine on viral envelope precursor (Marshall, 1974); alterations in glycosylation pattern may influence the conformation of the viral glycoprotein and render variants bearing mutations more (or less) susceptible to host immune responses. Hence, it is important to analyse the global glycosylation patterns amongst viruses from our study population in order to understand viral immune evasion and its possible role in disease progression. FIV Env (Pancino et al., 1993b), similarly to HIV Env (Myers and Lenroot, 1992), is heavily glycosylated and is referred to as having an immunologically silent glycan shield (Moore and Sodroski, 1996). The number of potential N-linked glycosylation sites (PNGS) in HIV and SIV is highly variable (Wolinsky et al., 1992) and for HIV-1 gp120 has been estimated to range from 18 to 33 (Korber et al., 2001). To our knowledge, due to the relatively low number of full length FIV *env* sequences available in GenBank, no analyses of global FIV cross-clade N-glycosylation sequons has been performed to date. What is the global distribution of PNGS on the FIV envelope glycoprotein? How can alterations in PNGS patterns facilitate immune evasion and do these correlate with the emergence of escape mutants and subsequent disease progression?

4.2 Materials and methods

4.2.1 FIV ORF *env* sequences

Multiple full-length FIV *env* sequences (~2500 bp) were isolated from three serial blood samples collected over a period of 12 months from cats recruited in the study and described in detail in Chapter 3. Multiple FIV *env* genes were amplified directly from blood, cloned and sequenced according to the protocol detailed in Chapter 2.

There were 355 serial *env* sequences from 44 cats available for analysis from the two cohorts, as detailed below. Analyses were performed using the entire *env* DNA sequences (with the exception of N-linked glycosylation analyses where amino acid sequences were used) and, when appropriate, also the three fragments: 1) leader/signal region (approx. 509 bp in length), 2) V3-V5 region (approx. 630bp) and 3) remaining concatenated fragments of the entire *env* after exclusion of the V3-V5 region, denoted NV (approx. 1900bp).

4.2.1.1 Memphis cohort

There were 228 *envs* available from cats in the Memphis cohort, with 2 to 27 sequences being obtained from each cat (Table 4-1).

Table 4-1 Number of sequences isolated from each time point from each Memphis cat.

No	Cat	Time point A	B	C	Available sequences	No	Cat	Time point A	B	C	Available sequences
1	M5	4	3	3	10	15	M41	3	0	10	13
2	M11	3	3 (*6)	18	24 (*27)	16	M49	3	0	3	6
3	M14	3	1(*3)	5	9(*11)	17	M2	0	0	2(*3)	2(*3)
4	M15	3	2(*3)	3	8(*9)	18	M3	2(*3)	0	0	2(*3)
5	M28	3	5	3	11	19	M8	0	0	2	2
6	M30	5	15(*16)	5	25(*26)	20	M10	2	0	0	2
7	M46	3	3	3	9	21	M20	1(*2)	0	0	1(*2)
8	M47	3	2(*3)	2(*3)	7(*9)	22	M29	0	0	4	4
9	M1	2(*3)	0	3	5(*6)	23	M44	2	0	0	2
10	M16	4	0	2	6	24	M32	3	0	0	3
11	M25	3	0	3	6	25	M50	4(*5)	0	0	4(*5)
12	M26	3	0	3	6	26	M12	0(*1)	0	0(*2)	0(*3)
13	M31	1(*2)	0(*2)	13	14(*17)	27	M48	0(*3)	0	0	0(*3)
14	M33	7(*8)	14(*16)	0	21(*24)						
										Total	202(*228)

* including sequences containing a premature stop codon.

The number of sequences available for analysis varied according to the availability of follow up samples, since 67% of Memphis cats died during the study. Accordingly, sequences from 3 time points (A, B, C) were available only from 8 cats. For 9 cats, sequences were available from the first and the last time point (A, C) and for the remaining 11 cats, sequences were accessible only from one time point and were therefore excluded from the majority of evolutionary analyses. From the total number of 228 sequences, 26 contained premature stop codons (Table 4-1). It is important to acknowledge that sequences with premature stop codons (although cannot take part in downstream evolution) bear the signature of evolutionary process and for this reason their inclusion in the analyses is justified.

4.2.1.2 Chicago cohort

There were 127 *env* gene sequences available for analysis from 16 Chicago cats, with 2 to 18 sequences obtained from each animal over 12 months (Table 4-2). The seventeenth cat (P1) was excluded from the analyses, as this cat was not brought back for blood sampling after the initial sampling. Accordingly, sequences from 3 time points were obtained from 6 cats and sequences from 2 time points were available from an additional 6 animals. For the remaining 4 cats, data were available only from one time point. From the total of 127 sequences, 23 contained premature stop codons.

Table 4-2 Number of sequences generated for each time point for each Chicago cat.

No	Cats	Time point			Available sequences	No	Cats	Time point			Available sequences
		A	B	C				A	B	C	
1	P4	3	4	3(*4)	10(*11)	9	P7	0(*3)	1	1(*4)	2(*8)
2	P5	2(*3)	3(*4)	3	8(*10)	10	P9	3	0	3	6
3	P6	3	2	5	10	11	P10	3	1	0	4
4	P13	3	3	3	9	12	P11	0	2	4	6
5	P14	2	4(*5)	3	9(*10)	13	P15	0(*2)	0(*3)	0(*3)	0(*8)
6	P17	2	4	2	8	14	P18	0	1	0	1
7	P21	0	3	2(*3)	5(*6)	15	P8	5	0	0(*3)	5(*8)
8	P2	3	15	0	18	16	P22	0	0	3	3
										Total	104 (*127)

* including sequences containing a premature stop codon.

4.2.1.3 Reference sequences

A limited number of complete *env* sequences was available in GeneBank; fifteen reference sequences were included in this analysis: FIV Aomori 1; D37814.1, FIV Aomori 2; D37811.1, FIV C; AF474246.1, FIV Dixon; L00608.1, FIV Dutch; X60725, FIV Fukuoka; D37817.1, FIV Sendai 1; D37816, FIV Shizuoka; AY139094.1, FIV UK2; X57001, FIV UK8; X69494, FIV USIL2489; U11820.1, FIV Yokohama; D37815.1, FIV Petaluma M25381.1, FIV PPR X69496.1, FIV Leviano FJ374696.1 and four V3-V5 region sequences representing Clade E: FIV Argentina E D84496, FIV Argentina E D84498, FIV Argentina E D84500, RUS14 EF447297. The selected sequences represented a good geographical, intra- and inter-clade distribution, with overall *env* diversity exceeding 26%. The *env* sequence M49C C76 (2583 base pairs in length) was selected as a reference sequence in this study.

4.2.2 Multiple sequence alignment

Multiple sequence alignments are a crucial prerequisite for phylogenetic analysis and were conducted using the Muscle algorithm (Edgar, 2004) in the MEGA 5 software package (Tamura et al., 2011). Before Muscle alignment was performed, all DNA sequences were translated to the corresponding peptide sequences using Virtual Ribosome (Wernersson, 2006). Final alignments were curated manually following codon optimization by submission of MUSCLE aligned peptide sequences and reverse translation to nucleic acid sequences using the RevTrans 1.4 Server (Wernersson and Pedersen, 2003). Particular attention was paid to ensure re-positioning of incorrect gapping in sequences of various lengths. Alignments were numbered according to positions in the M49C C76 sequence and were saved in FASTA and NEXUS formats for downstream analyses. The data set included one master alignment of all sequences and specific alignments based on the master alignment were prepared for the purpose of various analyses (available on the accompanying CD).

4.2.3 Phylogenetic trees

Maximum likelihood (ML) trees were constructed under the HKY nucleotide substitution model in MEGA5 (Tamura et al., 2011). Statistical support for ML trees was estimated using bootstrapping analysis (Efron et al., 1996) with 1000 replicates. The HKY model selection was based on jMODELTEST analysis (Posada,

2008), since this model has been shown to perform best for the majority of retrovirus data sets (Shapiro et al., 2006). All trees were examined and prepared for presentation in FigTree v 1.3.1 (http://tree.bio.ed.ac.uk/). The initial ML tree was constructed using the entire data set (355 wild type and 19 reference sequences) and examined thoroughly for non-monophyletic clustering of intra-host viral variants. Due to size restrictions and for clarity, the tree presented here generally includes one sequence per individual. In cases of non-monophyletic clustering of variants from some cats, all inconsistently assigned sequences were included and highlighted in the tree shown in this chapter.

4.2.4 Recombination testing

All sequences, including the reference *env* sequences obtained from GeneBank were subjected to rigorous three fold recombination testing. The initial recombination analysis was performed with the Datamonkey webserver (Delport et al., 2010), employing Single Breakpoint (SBP) and Genetic Algorithm Recombination Detection (GARD) methods (Kosakovsky Pond et al., 2006). Neighbour joining trees for each recombination segment identified by GARD and assessed by Akaike Information Criterion (AIC) (Sugiura, 1978) were constructed within Datamonkey webserver and prepared for presentation in FigTree v 1.3.1. Additional testing was performed in RDP4 v4.16 (Martin et al., 2010) employing a range of various detection methods such as RDP (Martin and Rybicki, 2000), BOOTSCAN (Martin et al., 2005), Maximum Chi Square (Smith, 1992), GENECONV (Padidam et al., 1999), CHIMAERA (Posada and Crandall, 2001) and Sister Scanning (SISCAN) (Gibbs et al., 2000). Representative figures visualizing recombination breakpoints were generated in SimPlot software v 3.5.1 (Lole et al., 1999). SimPlot computes and plots the similarity of each sequence to the set of reference sequences in a window of adjustable size (which was set to 200bp for this study).

4.2.5 Intra-host diversity

Intra-host sequence variation amongst the entire *env* sequences and three fragments of the gene (V3-V5, NV and the leader/signal) isolated from each cat were calculated as average and highest Kimura-2-Parameter (K2P) pairwise distances (Kimura, 1980), employing the Maximum Composite Likelihood model

(Tamura et al., 2004) in the MEGA 5 software package (Tamura et al., 2011). The codon positions included were 1st, 2nd, 3rd and non-coding. The K2P parameter is estimated as following a nucleotide by nucleotide comparison of the set of sequences under analysis (Kimura, 1980).

4.2.6 Selection (dN/dS ratio)

Nucleotide sites under diversifying/purifying selection at $p<0.05$ were identified and dN/dS ratios for every codon in the alignment were estimated using the Single Likelihood Ancestor Counting (SLAC) method incorporated in the Datamonkey webserver (Delport et al., 2010). The measure of selection is determined by the omega value (ω), the ratio of non-synonymous (dN) to synonymous mutations (dS) at each nucleotide site. A higher proportion of non-synonymous mutations at a given site ($\omega>1$) indicates evidence for positive (diversifying) selection. Inversely, the dominance of synonymous mutations at a particular site ($\omega<1$) indicates negative (purifying) selection. An equal rate of synonymous and non-synonymous mutations ($\omega=1$) indicates no selection (Yang et al., 2000). Synonymous substitutions reveal mutation rates, while non-synonymous substitutions may be influenced by selective pressure exerted by the immune system (Yang et al., 2000).

4.2.7 Rates of molecular evolution

Rates of evolution of full length and fragments (V3-V5, NV and the leader/signal fragment) of FIV *env* sequences for each cohort and the entire study group were estimated using Bayesian Evolutionary Analysis Sampling Trees (BEAST) version v.1.7.1 (Drummond and Rambaut, 2007). Additional data sets incorporated into the BEAST analysis included non-recombinant and recombinant sequences and sequences from alive and deceased cats.

The BEAST software package employs the Bayesian Markov Chain Monte Carlo (MCMC) method to estimate the rates of molecular evolution, offering the advantage of incorporating specific prior information into the analysis. The output of BEAST is presented as posterior probability distributions for all estimated parameters (Drummond et al., 2002).

Sequence alignments from animals from which data were available from more than 1 time point: 17 Memphis cats (197 taxa) and from 12 Chicago cats (108 taxa) were saved in NEXUS format prior to loading into BEAUti. The calibration information was included in the taxa names as the fraction of the year corresponding to the dates of blood sampling. Subsequently, the dates of each taxum were estimated by BEAUti and incorporated into the analysis. The HKY evolutionary model of substitution with four category gamma distribution selecting (1+2), 3 partitions into codon positions (Shapiro et al., 2006) was used. The BEAUti-generated XML file was used to run a Bayesian evolutionary analysis in the BEAST program using unconstrained root prior. The analyses were performed estimating independent trees for each cat, with a single clock rate across all trees, under strict and relaxed lognormal clock models with uniform distribution clock rate priors. The length of MCMC chain was set for 200 000 000 iterations with 20 000 000 burn-in but in some instances was run shorter or longer, until convergence was obtained.

The BEAST generated output log file was analysed in TRACER v1.5. Estimates of mean clock rate, 95% HPD (highest posterior density) lower and 95% HPD higher intervals were collected following the analysis of the posterior distribution for each parameter, ensuring that the Effective Sample Score (ESS) was greater than 100 since this indicates sufficient sampling of the posterior distribution (Drummond et al., 2002).

4.2.8 Identification of potential N-linked glycosylation sites

Potential N-linked glycosylation sites, and their positions in the protein alignment, were identified using the N-GlycoSite tool available at Los Alamos National Laboratory web server http://www.hiv.lanl.gov/. Potential N-linked glycosylation sites comprise an asparagine residue presented in the context of another amino acid succeeded by serine or threonine in following sequence: asparagine-X-serine or asparagine-X-threonine, where X can be any amino acid (Marshall, 1974) except proline (Gavel and von Heijne, 1990). Figures representing the sequence wide number of N-linked glycosylation sites were prepared in GraphPad Prism v 5.00 (GraphPad Software, San Diego California USA) using data output from N-GlycoSite tool.

4.3 Results

4.3.1 Phylogenetic inference

The Maximum Likelihood (ML) tree based on the HKY model and with bootstrap support of 1000 replications was constructed using the entire data set (not shown due to the size). The initial tree was carefully examined for evidence of non-monophyletic clustering of multiple sequences isolated from each individual. Intra-host sequences isolated from the majority of individuals (n=40, 93%) clustered together forming monophyletic groups. Sequences from three animals (7%) isolated from different time points showed inconsistent phylogenetic assignment. Sequences from cat P8 from time point A clustered together with clade A viruses, while sequences from the same animal from time point C were assigned to clade B. A similar pattern was noted with sequences from cat P21; sequences from time point B and time point C were classified as clade B and clade A respectively. The *env* genes from cat M5 were all assigned to clade B, but sequences from the last time point did not cluster together with sequences from the previous two time points.

The knowledge gained following the examination of the initial tree described above was used to construct a further ML tree based on the same model, generally containing one sequence per individual but including all non-monophyletic sequences isolated from the animals described above and rooted on the reference clade C *env* sequence (accession number AF474246.1) (Figure 4-1). Analysis of the tree that was generated after excluding data from cats with inconsistent clade assignment revealed that sequences from 25 animals (61%) clustered together with previously published clade B reference sequences, while sequences from 16 individuals (39%) were assigned to clade A. The number of *env* genes classified as clade A (7, 50%) and B (7, 50%) were equally distributed in the Chicago cats, while this distribution was skewed in favour of clade B (67%) in the Memphis cohort. Sequences with incongruent assignment from cats P8, P21 and M5 are highlighted in blue, orange and with a red star respectively (Figure 4-1).
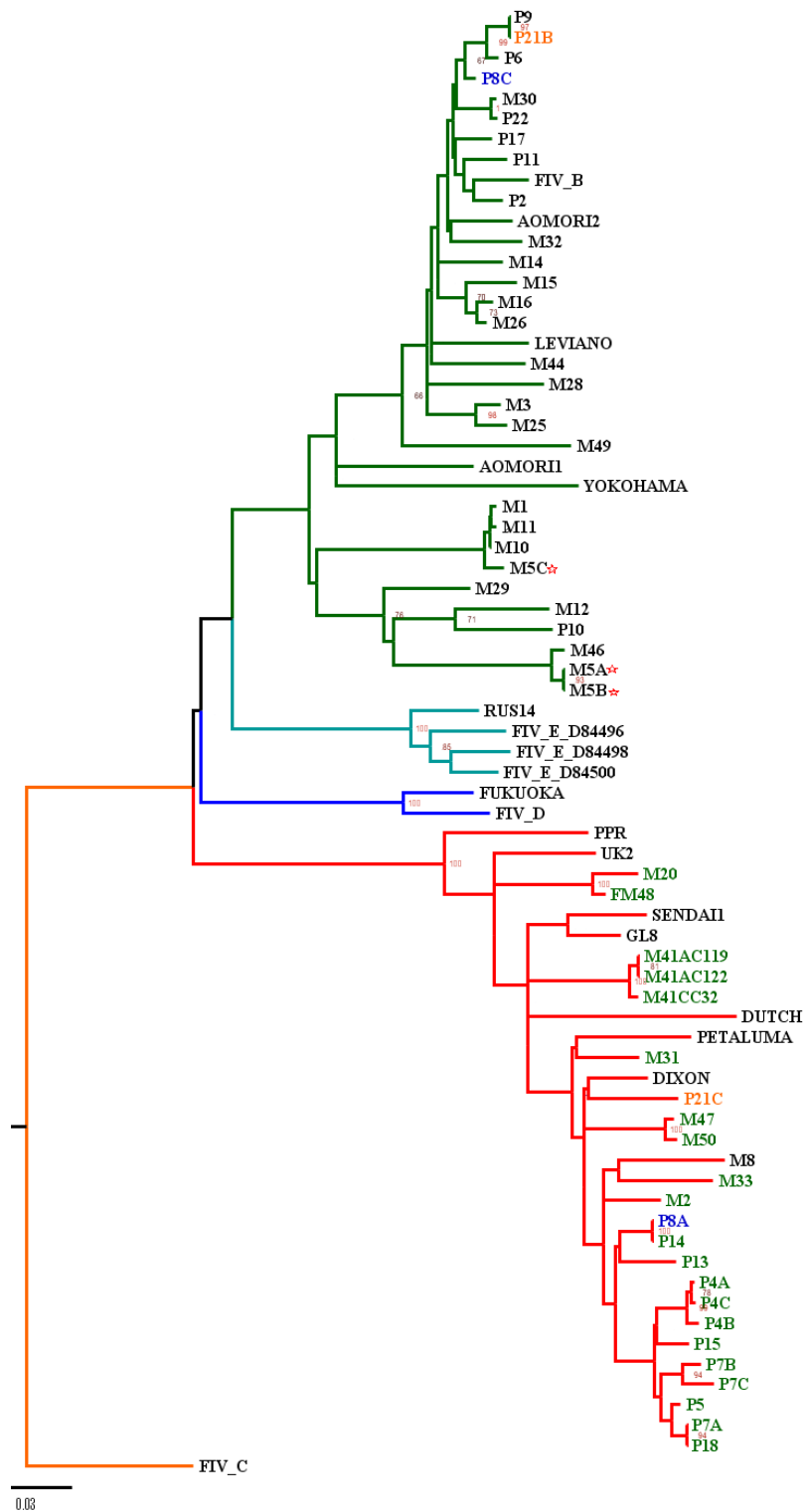
Figure 4-1 Maximum likelihood tree based on the HKY model, rooted on a clade C reference FIV *env*. Percentage bootstrap values are shown next to branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis included 54 nucleotide sequences (representative of a total of 355 sequences) and 19 reference sequences. Tree branches representing clade A, clade B, clade C, clade D and clade E viruses are highlighted in red, green, orange, blue and turquoise respectively. Taxa with inconsistent clade assignments from cats P21 and P8 are highlighted in orange and blue respectively. Non-monophyletic taxa from cat M5 are marked with red stars. Reference sequences accession numbers as in materials and methods section. Leaves of taxa classified by ML as clade A, but subsequently identified as A/B recombinants are highlighted in green.

It was notable that sequences from cats M1, M10 and M11 clustered together in the ML tree (Figure 4-1); further examination of the nucleotide alignment revealed that the sequences were highly similar, with overall K2P mean distance of 0.3%. The ML tree of sequences from those individuals based on the HKY model and bootstrap support of 1000 replications rooted on the clade B *env* reference sequence (accession number U11820.1) is presented in Figure 4-2 (root removed for clarity). On this tree, one sequence from the male cat M10 (M10A C285) appeared at the root of the whole group. Examination of the clinical records of these cats revealed that they are closely related to each other, with cat M1 being the offspring of cat M11. Given the housing conditions, clinical history and date of first FIV diagnosis of these 3 cats, it is likely that cat M1 acquired the virus vertically (cat M11). It is also likely that M10 sired cat M1. A description of the ages, duration of infection and clinical history for these cats that provides support for the above hypothesis is presented in detail in Chapter 3 and a CD that accompanies this thesis.
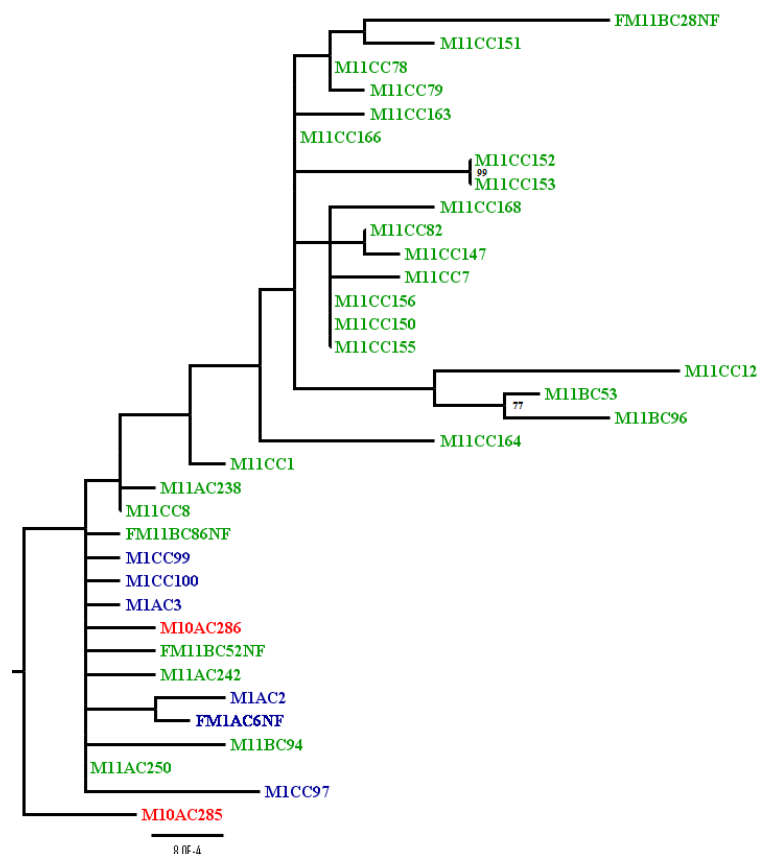
Figure 4-2 Maximum likelihood tree of 35 *env* sequences isolated from 3 closely related cats (M1, M10 and M11 highlighted in blue, red and green respectively) rooted on reference clade B *env* sequence (root removed for clarity). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values greater than 70% are shown next to branches.

4.3.2 Recombination

Initial recombination analysis involved Single Breakpoint (SBP) testing of the entire data set within the Datamonkey web server and provided evidence for recombination, giving grounds for further recombination testing using more sophisticated methods.

Examining the Memphis sequences, Genetic Algorithms for Recombination Detection (GARD) provided evidence of four breakpoints (positions in nucleotide alignment: 354, 576, 1575, 1947) with significant topological incongruence (p=0.01) (Table 4-3). The submitted alignment was further spliced by GARD at the breakpoints into five segments: 1) 1-562; 2) 563-989; 3) 990-1752; 4) 1753-2286 and 5) 2287-2625, which were used to create five NJ trees, one for each segment (Figure 4-5). Examination of these GARD-determined trees revealed that sequence segments from 18/27 (67%) Memphis animals showed consistent clade assignment and represent non-recombinant clade B *envs*, while only one individual (M8) (1/27; 4%) yielded non-recombinant clade A sequences. Clade A/B recombinant sequences were identified in 8/27 (29%) cats. Recombinant sequences had different mosaic compositions and were classified in three groups: 1) sequences from cats M2, M33, M47 and M50 in which only the first segment (1-562) belonging to clade B, while the remaining four segments consistently clustered as clade A, 2) sequences from cats M20 and M41 in which only the last segment (2287-2625) was assigned to clade B, while all other prior segments were assigned to clade A, and 3) sequences from M31 and M41 in which the first (1-562) and the last (2287-2625) segments clustered with clade B, while the 3 central segments (563-2286) were assigned to clade A (Table 4-3). Next, RDP software was used to examine putative parents contributing to the mosaic structure of recombinant sequences. RDP consistently identified the clade A, FIV Dixon strain (L00608.1) as a putative major parent and determined clade B Chicago strains P10BC64 and P2BC24 as putative minor parents for M2, M31, M33, M47, M50 and M20, M48 respectively. The analyses and generation of figures representing recombination breakpoints were performed for all recombinant Memphis sequences in SimPlot (data summarised in Table 4-3). A representative SimPlot analysis is shown in Figure 4-3, illustrating recombination breakpoints determined by the similarity of the Memphis M31 query sequence to two putative clade A and B parental sequences.
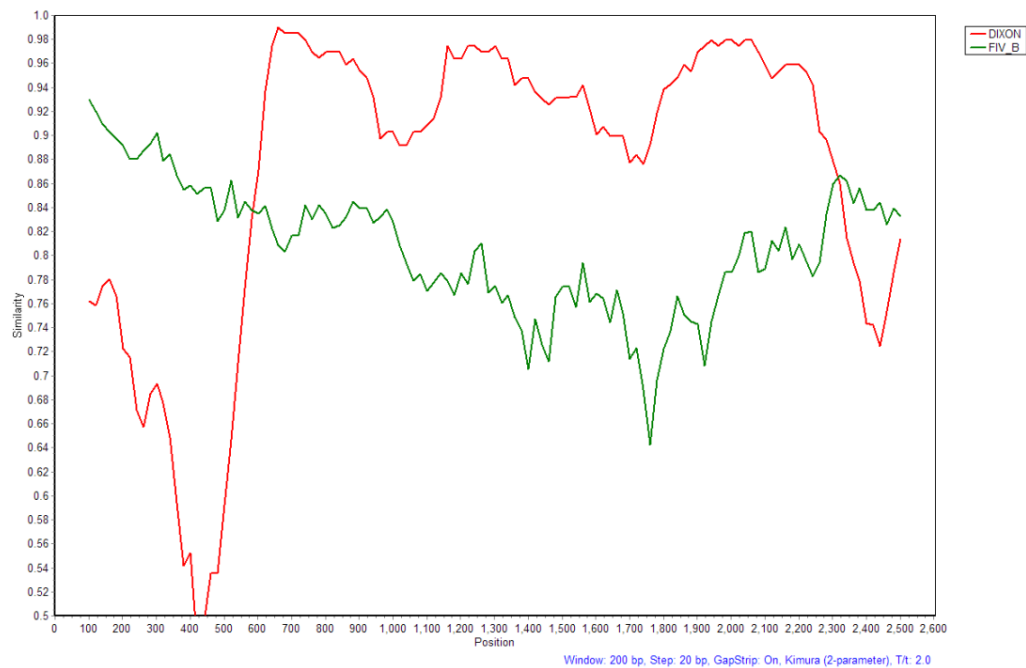
Figure 4-3 Similarity analysis of Memphis A/B recombinant sequence (M31) and two reference sequences (clade A, DIXON (L00608.1) in red and clade B, FIV_B (U11820.1) in green) performed in SimPlot. Each line represents the percentage similarity of the query M31 sequence to the reference sequences. Recombination breakpoints are placed at the intersections of the red and green curves (positions 562 and 2287 on the X axis).

For the Chicago sequences, GARD also provided evidence for four recombination breakpoints (p=0.01) and splice aligned sequences into five segments: 1) 1-354; 2) 355-576; 3) 577-1575; 4) 1576-1947 and 5) 1948-2589 (Table 4-4). The locations of the breakpoints in this cohort were different compared to the Memphis sequences; nevertheless recombination hotspots between the leader and the stem of the V1/V2 region (approx. position 565) and at the V5 (approx. position 1600) loop of the SU region of Env were common among recombinants from both cohorts. Recombination analysis revealed that sequences from 7 cats (50%) were non-recombinant and consistently clustered with the reference clade B *env* sequence. In contrast to the Memphis cohort, there were no pure clade A *env* sequences amongst the Chicago cats; Sequences from the remaining 7 animals (50%) were identified as A/B recombinants. The mosaic composition of the recombinant sequences fell into two groups: 1) sequences from cats P4, P5, P7 and P13 in which segment 2 (355-576) belonged to clade B while the rest of the sequence (1-354 and 577-2589) clustered with clade A and 2) sequences from cats P14 and P18 in which segments 1 and 2 (1-576) were assigned to clade B and the remaining part of the sequence (577-2589) clustered with clade A (Table

4-4). A similarity plot representative for the Chicago recombinants in relation to reference A and B *env* sequences is shown in Figure 4-4.
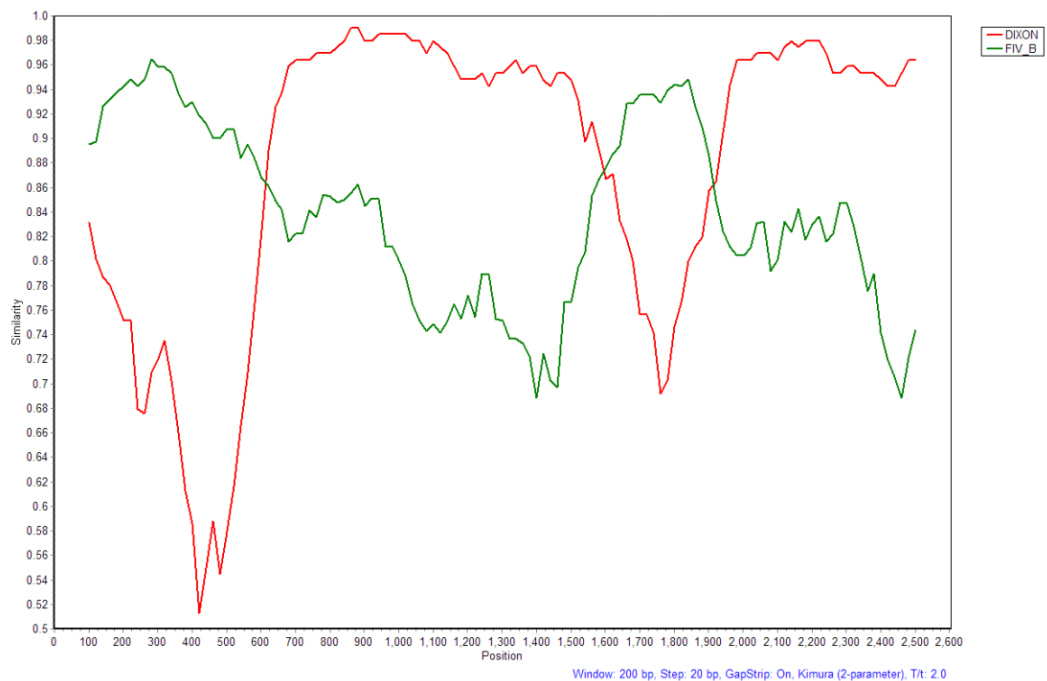
Figure 4-4 Similarity analysis of PAWS A/B recombinant sequence (P21C) and two reference sequences (clade A, DIXON (L00608.1) in red and clade B, FIV_B (U11820.1) in green) performed in SimPlot. Each line represents the percentage similarity of the query M31 sequence to the reference sequences. Recombination breakpoints are placed at the intersections of the red and green curves (positions 576, 1576 and 1947 on the X axis).

The Chicago data set also included *env* sequences from the remaining cats P8 and P21, identified previously as forming non monophyletic groups (section 4.3.1). Recombination analysis revealed that P8 sequences from time point C were non-recombinant clade B, while time point A sequences were classified as A/B recombinants. For cat P21, time point B sequences appeared to be entirely clade B, while time point C sequences showed an incongruent assignment and were identified as clade A/B mosaics (Table 4-4).

Table 4-3 GARD and RDP recombination testing results of Memphis sequences. Segments clustering with clade A and clade B viruses are denoted in red and green respectively. The final clade assignment following recombination testing is shown in the column "CLADE". Non recombinant and recombinant sequences are denoted NR and R respectively. RDP determined major and minor parents for mosaic recombinant sequences are shown in the final two columns.

VIRUS	GARD DETERMINED BREAKPOINTS					RDP DETERMINED RECOMBINANTS			
	SEGMENT 1 (1-562)	SEGMENT 2 (563-989)	SEGMENT 3 (990-1752)	SEGMENT 4 (1753-2286)	SEGMENT 5 (2287-2625)	EVIDENCE OF RECCOMBINATION	CLADE	MINOR PARENT	MAJOR PARENT
M1	B	B	B	B	B	NR	B	NR	NR
M2	B	A	A	A	A	R	A/B	P10BC64	DIXON
M3	B	B	B	B	B	NR	B	NR	NR
M5A†	B	B	B	B	B	NR	B	NR	NR
M5C†	B	B	B	B	B	NR	B	NR	NR
M8	A	A	A	A	A	NR	A	NR	NR
M10	B	B	B	B	B	NR	B	NR	NR
M11	B	B	B	B	B	NR	B	NR	NR
M12	B	B	B	B	B	NR	B	NR	NR
M14	B	B	B	B	B	NR	B	NR	NR
M15	B	B	B	B	B	NR	B	NR	NR
M16	B	B	B	B	B	NR	B	NR	NR
M20	A	A	A	A	B	R	A/B	P2BC24	DIXON
M25	B	B	B	B	B	NR	B	NR	NR
M26	B	B	B	B	B	NR	B	NR	NR
M28	B	B	B	B	B	NR	B	NR	NR
M29	B	B	B	B	B	NR	B	NR	NR
M30	B	B	B	B	B	NR	B	NR	NR
M31	B	A	A	A	B	R	A/B	P10BC64	DIXON
M32	B	B	B	B	B	NR	B	NR	NR
M33	B	A	A	A	A	R	A/B	P10BC64	DIXON
M41	B	A	A	A	B	R	A/B	P10BC64	DIXON
M44	B	B	B	B	B	NR	B	NR	NR
M46	B	B	B	B	B	NR	B	NR	NR
M47	B	A	A	A	A	R	A/B	P10BC64	DIXON
M48	A	A	A	A	B	R	A/B	P2BC24	DIXON
M49	B	B	B	B	B	NR	B	NR	NR
M50	B	A	A	A	A	R	A/B	P10BC64	DIXON

† sequences from the same individual but different time points with previously identified non-monophyletic clustering.

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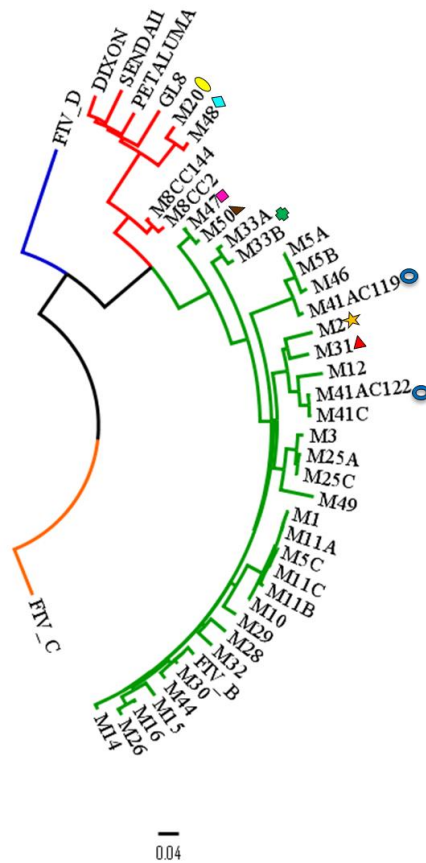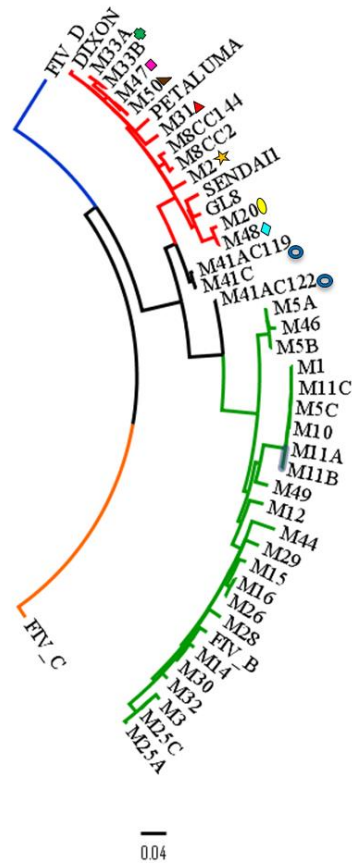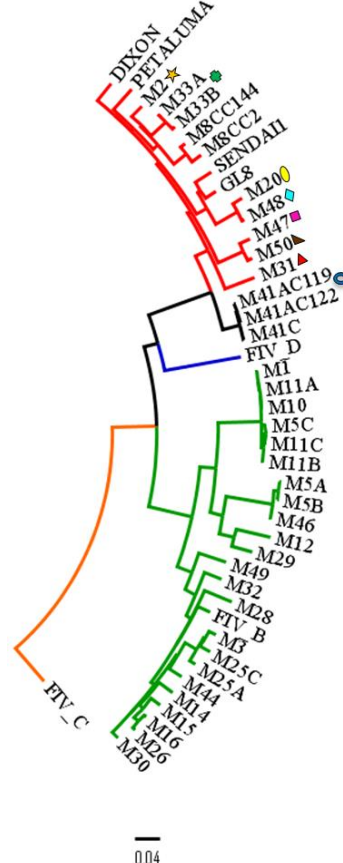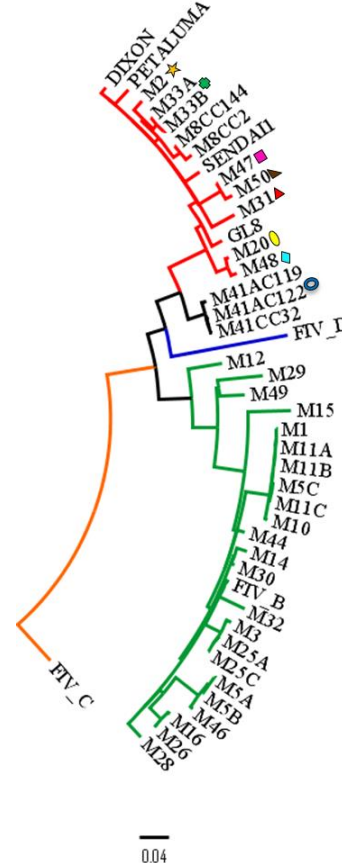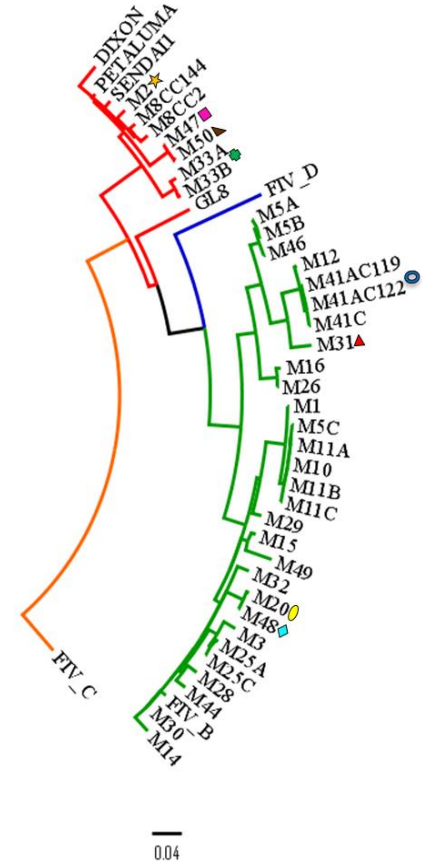
Figure 4-5 NJ trees for each of five GARD determined segments of Memphis sequences. Trees A, B, C, D and E represent phylogenetic inference of segments 1 (1-562), 2 (563-989), 3 (990-1752), 4 (1753-2286) and 5 (2287-2625) respectively. Recombinant sequences are indicated by various shapes placed next to sequence names - note various clade assignment for each sequence segment from the same individual at each of the trees (e.g. follow red triangle for M31). Reference clade A sequences (DIXON, SENDAI, PETALUMA, GL-8) are highlighted in red, clade B (FIV-B) in green, clade C (FIV_C) in orange and clade D (FIV_D) in blue and the clades of tree branches are identified accordingly. All trees are rooted on the FIV_C reference *env* sequence.

Table 4-4 GARD and RDP recombination testing results of Chicago sequences. Segments clustering with clade A and B viruses are highlighted in red and green respectively. The final clade assignment following recombination testing is shown in the column "CLADE". Note that there were no pure non-recombinant clade A *envs* in this cohort. Non recombinant and recombinant sequences are denoted NR and R respectively. RDP determined major and minor parents for mosaic recombinant sequences are shown in the final two columns.

	GARD DETERMINED BREAKPOINTS					RDP DETERMINED RECOMBINANTS			
VIRUS	SEGMENT 1 (1-354)	SEGMENT2 (355-576)	SEGMENT3 (577-1575)	SEGMENT4 (1576-1947)	SEGMENT5 (1948-2589)	EVIDENCE OF RECCOMBINATION	CLADE	MINOR PARENT	MAJOR PARENT
P2	B	B	B	B	B	-	B	NR	NR
P4	A	B	A	A	A	+	A/B	P10BC64	DIXON
P5	A	B	A	A	A	+	A/B	P10BC64	DIXON
P6	B	B	B	B	B	-	B	NR	NR
P7	A	B	A	A	A	+	A/B	P10BC64	DIXON
P8A†	B	B	A	A	A	+	A/B	P10BC64	DIXON†
P8C†	B	B	B	B	B	-	B	NR	NR
P9	B	B	B	B	B	-	B	NR	NR
P10	B	B	B	B	B	-	B	NR	NR
P11	B	B	B	B	B	-	B	NR	NR
P13	A	B	A	A	A	+	A/B	P10BC64	DIXON
P14	B	B	A	A	A	+	A/B	P10BC64	DIXON
P15	B	B	A	A	A	+	A/B	P10BC64	DIXON
P17	B	B	B	B	B	-	B	NR	NR
P18	B	B	A	A	A	+	A/B	P10BC64	DIXON
P21B†	B	B	B	B	B	-	B	NR	NR
P21C†	B	B	A	B	A	+	B/A	P10BC64	DIXON†
P22	B	B	B	B	B	-	B	NR	NR

† sequences from the same individual but different time points with previously identified non-monophyletic clustering.

Figure 4-6 NJ trees for each of five GARD determined segments of Chicago sequences. Trees A, B, C, D and E represent phylogenetic inference of segments 1 (1-354), 2 (355-576), 3 (577-1575), 4 (1576-1947) and 5 (1948-2589) respectively. Recombinant sequences are indicated by various shapes placed next to sequence names - note various clade assignment for each sequence segment from the same individual at each of the trees (e.g. follow red triangle for P14). Reference clade A sequences (DIXON, SENDAI, PETALUMA, GL-8) are highlighted in red, clade B (FIV-B) in green, clade C (FIV_C) in orange and clade D (FIV_D) in blue and the clades of tree branches are identified accordingly. All trees are rooted on the FIV_C reference *env*.

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4.3.3 Intra-host viral diversity

The K2P overall mean and highest pairwise distances representing the intra-host diversity of the entire *env* sequences and the three fragments (leader, V3V5 and NV) from the Memphis cohort are presented in Table 4-5. The average mean pairwise distance for the entire *env* was calculated as 0.57% (median 0.3%; range from 0% to 5.5%), while the mean highest pairwise distance was estimated as 1.1% (median 0.5%; range from 0% to 11.7%). The average mean pairwise distances for the leader, V3V5 and NV regions were estimated to be 0.77% (median 0.3%; range from 0% to 6.3%), 0.79% (median 0.2%; range from 0% to 8.9%) and 0.44% (median 0.3%; range 0% to 2.2%) respectively.

Table 4-5 Average and highest K2P distances (as percentages) for the leader, V3V5, NV fragments as well as the entire *env* sequences from each Memphis cat obtained over 12 months.

Cat	OVERALL MEAN AND HIGHEST PAIRWISE DISTANCE (%)							
	entire *env*		leader		V3V5		NV	
	mean	highest	mean	highest	mean	highest	mean	highest
M1	0.2	0.4	0.2	0.4	0.2	0.5	0.2	0.3
M2†	0.8	0.9	1.6	1.8	0.9	1.3	0.8	0.8
M3†	0.9	1	1.3	1.6	0.9	1.1	0.9	1
M5‡	5.5	11.7	6.3	13.7	8.9	19	2.2	4.7
M8†	1.9	1.9	2	2	2.5	2.5	1.7	1.7
M10†	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.3
M11	0.3	0.7	0.5	1.8	0.5	1.1	0.3	0.7
M12	0.5	0.7	0.3	0.4	1.4	1.8	0.3	0.2
M14	0	0.2	0	0.2	0	0	0.1	0.2
M15	0.1	0.2	0.2	0.6	0	0.2	0.1	0.2
M16	0.2	0.4	0.5	1	0.1	0.2	0.2	0.4
M20†	0.2	0.2	0.8	0.8	0	0	0.4	0.4
M25	0.7	1.1	0.5	0.8	2	3.2	0.4	0.5
M26	0.1	0.2	0.1	0.4	0.2	0.5	0.1	0.1
M28	0.2	0.6	0	0.2	0.3	1.1	0.1	0.4
M29†	0	0.1	0	0	0	0	0.1	0.1
M30	0.1	0.5	0.2	0.7	0	0.3	0.1	0.5
M31	0.4	0.7	0.4	0.8	0.9	1.8	0.3	0.5
M32†	0.1	0.1	0.1	0.2	0	0	0.1	0.1
M33	0.5	1.1	0.9	2.9	0.5	1.3	0.5	1.2
M41	0.9	4.1	2.7	14.6	0.3	0.8	1.2	5.6
M44†	0	0	0.2	0.2	0	0	0.1	0.1
M46	0	0.2	0.1	0.4	0	0.2	0	0.2
M47	0.4	0.9	0.4	1.2	0.6	1.3	0.3	0.7
M48†	0.4	0.5	0.3	0.4	0.2	0.3	0.3	0.5
M49	0.7	1.3	0.7	1.2	0.9	1.7	0.7	1.2
M50†	0.1	0.2	0.2	0.4	0	0	0.1	0.3

† Sequences obtained from one time point only, ‡ outlier data excluded from mean calculations. Values in red highlight outlier pairwise distance values (excluded from mean calculations).

The average highest pairwise distances were 1.81% for the leader (median 0.7%; range from 0% to 14.6%), 1.5% for the V3-V5 region (median 0.5%; range from 0% to 19%) and 0.84% for the NV region (median 0.4%, range from 0.1% to 5.6%).

It was apparent that sequences from cat M5, as already highlighted by the ML tree (section 4.3.1), showed the highest degree of diversity, with values regarded as outliers and therefore excluded from further analysis. Similarly, data obtained from only one time point (highlighted with "†" in Table 4-5) were limited and were excluded from adjusted intra-host diversity calculations. After excluding the outlier data, the overall mean distance for the entire *env* was 0.33% (median 0.25%; range 0% - 0.9%) and the average highest pairwise distance was 0.83% (median 0.65%; range 0.2% - 4.1%). For the leader, V3-V5 and NV fragments, the average mean distances were calculated as 0.48% (median 0.35%; ranging from 0% to 2.7%), 0.49% (median 0.3%; ranging from 0% to 2%) and 0.3% (median 0.25%; ranging from 0% to 1.2%). The average highest pairwise distances were estimated as 1.73% for the leader (median 0.75%; ranging from 0.2% to 14.6%), 1% for the V3-V5 region (median 0.95%; ranging from 0% to 3.2%) and 0.8% for the NV region (median 0.45%; ranging from 0.1% to 5.6%).

The mean and highest pairwise distances calculated for sequences isolated from the Chicago cohort are shown in Table 4-6. As highlighted previously (section 4.3.1) cats P8 and P21 showed highest pairwise distances greater than 10% and so these cats were excluded from mean calculations. The average overall mean distance for the entire *env* sequences from the Chicago cats was calculated as 0.42% (median 0.2%; range from 0% to 1.7%) and the average highest pairwise distance was 0.87% (median 0.4%; range from 0.1% to 2.9%). The average mean pairwise distances for the leader, V3-V5 and NV fragments were 0.48% (median 0.35%; range from 0% to 1.4%), 0.43% (median 0.15%; range from 0% to 1.8%), and 0.42% (median 0.2%; range from 0% to 1.6%). The highest pairwise distances for the leader, V3-V5 and NV fragments were 1.07% (median 0.8%; range from 0.2% to 2.4%), 1.13% (median 0.4%; range from 0.2% to 3.9%) and 0.88% (median 0.55%; range from 0.1 to 2.7%).

For the whole study group, the overall mean distances for the entire *env* ranged from 0% to 1.7% (mean 0.37%; median 0.2%) and highest pairwise distances ranged from 0.1% to 4.1% (mean 0.85%; median 0.5%). The overall mean

distances for the fragments of *env* ranged from 0% to 2.7% for the leader (mean 0.48%; median 0.35 %), from 0 to 2% for the V3-V5 region (mean 0.47%, median 0.25%,) and from 0% to 1.6% for the NV region (mean 0.35%, median 0.2%,). Highest pairwise distances ranged from 0.2% to 14.6% (mean 1.45%, median 0.8%), from 0% to 3.9% (mean 1.05, median 0.65%) and from 0.1% to 5.6% (mean 0.84%, median 0.5%) for the leader, V3-V5 region and NV region respectively.

Table 4-6 Average and highest K2P distances (percentages) for the leader, V3V5, NV regions and the entire *env* sequences from each Chicago cat obtained over 12 months.

Cat	OVERALL MEAN AND HIGHEST PAIRWISE DISTANCE (%)							
	entire *env*		leader		V3V5		NV	
	mean	highest	mean	highest	mean	highest	mean	highest
P2	0.5	1.5	0.4	0.8	0.5	1.1	0.5	1.7
P4	0.9	1.8	0.8	2.1	1	3.9	0.8	1.5
P5	0.2	0.5	0.3	1.2	0.1	0.3	0.2	0.6
P6	0.2	0.4	0.3	0.8	0	0.2	0.2	0.6
P7	1.7	2.9	1.4	2.4	1.8	3.3	1.6	2.7
P8 ζ	11	20.8	4.2	8.4	8.9	19	12.1	23
P9	0.2	0.4	0.4	0.8	0.1	0.2	0.2	0.5
P10	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2
P11	0	0.1	0.1	0.4	0.1	0.2	0	0.1
P13	0.1	0.4	0.2	0.4	0.5	2	0.1	0.2
P14	0.1	0.2	0	0.2	0.1	0.3	0.1	0.2
P15	0.9	1.6	1.2	2.4	0.7	1.3	1	1.9
P17	0.2	0.4	0.5	1.2	0.2	0.5	0.2	0.4
P18*†	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
P21ζ	7.1	11.9	3.2	5.8	21.8	36.5	10.5	17.7
P22†	0.3	0.4	1.5	2.1	0.3	0.5	0.3	0.4

† sequences available from one time point only and excluded from mean calculations
* only one sequence available
ζ outlier data (in red) excluded from mean calculations

An overall comparison of intra-host diversity amongst the entire *env* sequences as well as fragments from both cohorts, represented as highest pairwise distances, is shown in Figure 4-7. It is evident that at the level of the study group that the highest mean intra-host diversity occurs in the leader/signal region of *env* (0.15% greater than for the V3-V5 fragment). A similar pattern of high intra-host diversity within the leader/signal region was evident in the Chicago cohort but not in the Memphis cohort.
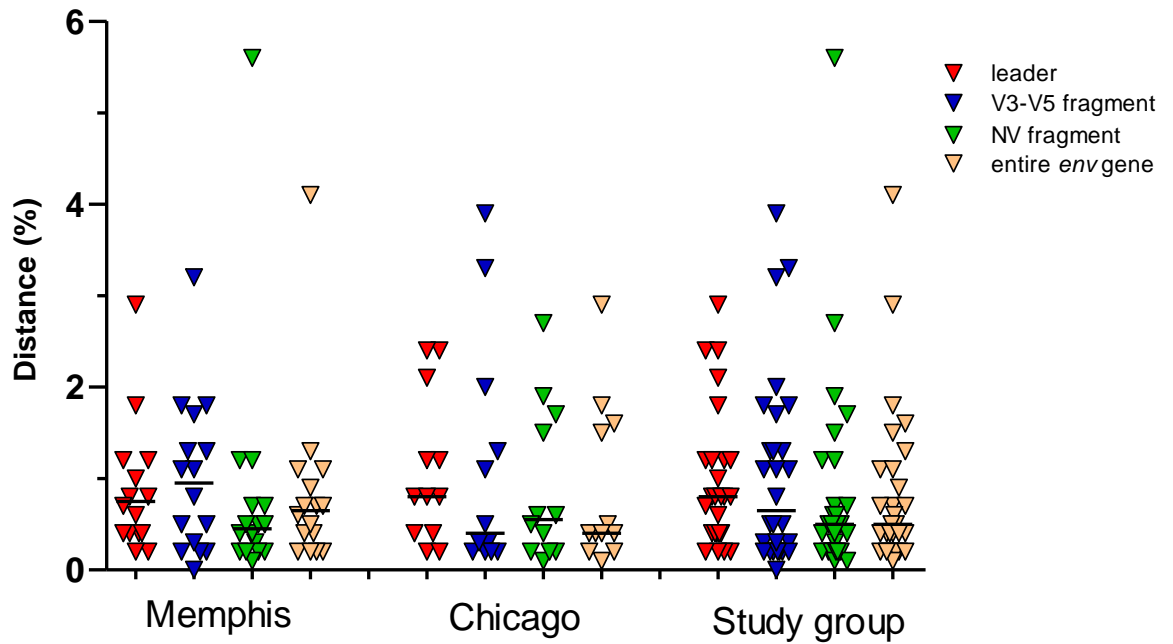
Figure 4-7 K2P highest pairwise distances between fragments and the entire *env* sequences isolated over 12 months from 12 Chicago, 16 Memphis cats and the entire study group. Sequences obtained from cats from only one time point and outlier data from cats M5, P8 and P21 were excluded from the analysis. Median values (horizontal bars) for the leader, V3-V5, NV and *env*: Memphis (0.75%, 0.95%, 0.45% and 0.65%), Chicago (0.8%, 0.4%, 0.55% and 0.4%), study group (0.8%, 0.65%, 0.5% and 0.5%) respectively. Values for the leader of *env* M41 (14.6) are not included for clarity.

4.3.4 Selection (dN/dS ratio)

The mean ω value (dN/dS ratio) for *env* sequences obtained from the study group was 0.34. Examining the entire data set, at p=0.05 significance level, SLAC indicate 14 (1.6%) positively and 201 (23%) negatively selected sites (Table 4-7). For the Memphis and Chicago cohorts, ω values were 0.29 and 0.36 respectively, with 7 and 2 positively selected sites respectively. However, a marked difference in the number of negatively selected sites was observed between the two cohorts; 169 and 37 negatively selected sites were identified for the Memphis and Chicago *env* sequences respectively. A similar trend in the number of negatively selected sites was evident when comparing selection forces acting upon recombinant and non-recombinant sequences (Table 4-7).

A comparison of selection forces acting upon sequences from cats of different health status revealed that 5 and 3 sites were positively selected while 125 and

97 sites were under negative selection in alive and deceased cats respectively (p=1, Fisher's exact test).

Table 4-7 SLAC determined dN/dS ratios and tally of positively and negatively selected sites within each cohort examined. Positions of positively selected sites are highlighted in the final column.

Cohort	dN/dS	Number of selected sites		Positively selected sites
		positively	negatively	
Study group (305†)	0.34	14	201	8, 62, 96, 156, 157, 364, 394, 400, 456, 487, 565, 750, 853, 865
Memphis(191†)	0.29	7	169	62, 364, 392, 400, 565, 567, 750
PAWS (114†)	0.36	2	37	8, 364
Non recombinants (172†)	0.28	5	121	62, 176, 364, 567, 750
Recombinants (133†)	0.41	6	51	8, 96, 156, 392, 400, 726
Alive (141†)	0.32	5	125	8, 168, 364, 455, 760
Deceased (164†)	0.28	3	97	62, 103, 394

† tally of sequences (identical, duplicate sequences were excluded prior to analysis)

The sites identified as being under positive selection (at p=0.05) are listed in Table 4-7 and are restricted to specific regions of the viral Env: sites 8, 62, 96, 103, 156, 168, 175,176 lie within the leader region, sites 364, 394, 400 in the V3 region and sites 455, 456, and 487 in the V4 region while sites 565, 567 lie within the V5 region. The remaining positively selected sites, 726, 750, 760, 853 and 865, were located within the TM region of Env (Figure 4-14). The selection hot spots at positions 394, 400, 487, 750 and 853 were located within B cell epitopes identified in previous studies (Pancino et al., 1993a), (Lombardi et al., 1993), as shown in Figure 4-8, suggesting that non-synonymous mutations at those positions may have arisen as a result of immune selection pressure.
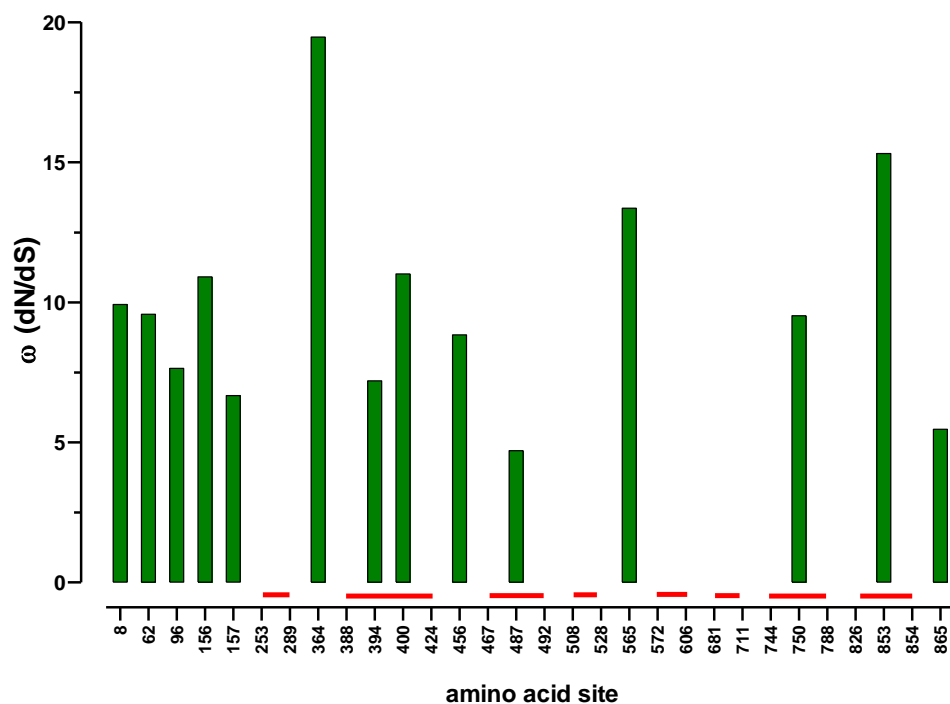
Figure 4-8 Sites under positive (p<0.01) selection identified by SLAC in the complete data set, with corresponding ω values. Red lines along the X axis indicate the locations of B cell epitopes (Pancino et al., 1993a), (Lombardi et al., 1993). It was notable that positively selected sites at positions 394, 400, 487, 750 and 853 lay within previously identified B cell epitopes.

4.3.5 Rates of molecular evolution

Estimated evolutionary rates for the leader, V3-V5, NV regions as well as the entire *env* gene isolated from Memphis (M), Chicago (C) and whole study group (SG) were calculated under strict and relaxed lognormal clock models with unconstrained root prior and are shown in Table 4-8, together with HPD (higher posterior density) and effective sample size (ESS) values.

As demonstrated in the Table 4-8, the entire *env* gene from the Memphis population evolved at the mean rate of 4.58×10^{-4} substitutions per site per year, corresponding to 0.46% per decade, under a strict clock model. Relaxing the clock model to the lognormal adjusted the evolutionary rate estimate to 9.94×10^{-4} substitutions per site per year (0.99% per decade). To determine whether specific regions of the *env* gene in the Memphis population evolved at different rates, MCMC chain was used to estimate clock rates for the leader (approx. 509 bp), V3-V5 region (approx. 630 bp) and remaining concatenated fragments of the entire *env* gene after exclusion of the V3-V5 region, denoted

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NV (1900 bp). As shown in Table 4-8, the leader region evolved at a rate of 1.2% per decade under strict clock model and 3.1% per decade under relaxed clock model. The V3-V5 region evolved at a significantly slower rate of 0.7% per decade (strict) and 0.8% per decade (relaxed). The remaining 1900 bp NV fragment was estimated to evolve, under strict and relaxed clock models respectively, at the mean rate of 0.52% and 0.95% per decade (Table 4-8).

Table 4-8 Evolutionary rates for the entire *env* gene, the leader, V3-V5 region and concatenated entire *env* excluding the V3-V5 region (NV), estimated under strict and relaxed clock models for genes amplified from the Memphis and Chicago cohorts and the entire study group (Memphis and Chicago). Additional comparisons of evolutionary rates include: 1) entire *env* of recombinant and non-recombinant viruses and 2) the entire and fragments of *env* sequences from alive and deceased cats. Evolutionary rates are presented as the number of substitutions per site per year. Effective sample size (ESS) values and lower (95% HPD lower) and upper (95% HPD upper) posterior densities are denoted for each cohort and clock model.

Cohort (†,*)	Clock model	Evolutionary rate ($*10^{-4}$ substitutions/site/year)				95% HPD lower ($*10^{-4}$)				95% HPD upper ($*10^{-4}$)				ESS			
		leaderζ	V3-V5‡	NV Ŧ	*env*Ŧ	leaderζ	V3-V5‡	NV Ŧ	*env*Ŧ	leaderζ	V3-V5‡	NV Ŧ	*env*Ŧ	leaderζ	V3-V5‡	NV Ŧ	*env*Ŧ
Memphis (17†,197*)	Strict	11.8	7.05	5.29	4.58	4.49	2.56	2.19	1.7	19.5	11.6	8.76	7.7	449	262	323	225
	Relaxed lognormal	31.4	7.82	9.52	9.94	9.36	2.58	4.02	1.6	59.4	14.1	16.3	16.2	328	337	157	303
Chicago (12†,108*)	Strict	21.2	11.5	6.39	7.85	9.56	3.48	1.46	2	32.7	19.9	11.6	13.3	1118	1208	307	640
	Relaxed lognormal	33.4	21.1	17.7	17.3	15.27	5.25	5.76	5.7	56.4	39.9	31.9	30.3	1125	1452	530	584
Study group (29†, 305*)	Strict	15.25	7.92	5.49	5.11	8.59	3.83	2.78	2.25	22.3	12.2	8.2	7.9	246	370	216	137
	Relaxed lognormal	34.3	10.8	12	11.6	15.52	4.99	5.94	6.99	56	17.4	18.7	16.7	383	350	123	243
Non recombinants (18†,184*)	Strict	-	-	-	2.44	-	-	-	1	-	-	-	4.5	-	-	-	208
	Relaxed lognormal	-	-	-	6.6	-	-	-	1.9	-	-	-	11.8	-	-	-	224
Recombinants (11†,123*)	Strict	-	-	-	8.85	-	-	-	4.25	-	-	-	13.6	-	-	-	584
	Relaxed lognormal	-	-	-	21.4	-	-	-	9	-	-	-	34.5	-	-	-	373
ψALIVE (6†,46*)	Strict	17.69	23.8	8.24	6.72	1	6.57	2.51	1.3	34.9	42.5	14.68	12	2800	4129	5157	3105
	Relaxed lognormal	20.5	27.38	8.61	8.7	1.3	6.28	2.39	1.0	40.8	50.9	15.26	17.3	979	3620	4204	1479
ψDECEASED (11†,151*)	Strict	13.1	5.91	5.11	4.58	4.4	1.29	1.39	1.7	23.15	10.3	8.67	7.8	601	695	363	260
	Relaxed lognormal	33.7	6.49	10.53	10.5	9.7	2	3.39	4.2	66.6	11.7	18.51	17.6	232	398	376	315

†tally of partitions; * tally of taxa; ζ leader – rates estimated for 509 bp fragment including leader/signal region; ‡ V3-V5 – rates estimated for highly variable region of viral *env* (630bp); Ŧ NV – rates estimated for "non-variable" regions of *env* comprising concatenated sequence after exclusion of V3-V5 region (~1900bp), Ŧ *env* – rates estimated for entire gene (~2500 bp); ψ – Memphis. Rates for recombinant/no recombinant sequences are calculated for the entire gene only due to various mosaic compositions of recombinant sequences.

Evolutionary rate estimates for the Chicago sequences were markedly higher than for the Memphis sequences (Figure 4-9). The mean evolutionary rates under the strict clock model for the entire, leader, V3-V5 and NV fragments of the *env* were estimated as 0.78%, 2.1%, 1.2% and 0.6% per decade respectively. Relaxing the clock model returned estimates of 1.7%, 3.3%, 2.1% and 1.8% per decade respectively (Table 4-8).

Evolutionary rates under strict clock model for the whole study group (SG) were 1.5%, 0.8%, 0.55% and 0.5% per decade for the leader, V3-V5, NV and the entire *env* respectively. Relaxing clock model returned estimates equivalent to 3.4%, 1.1 %, 1.2 % and 1.16 % per decade per respective fragments (Table 4-8).

Further, as demonstrated in Figure 4-9 A and B, we compared the rates of evolution under both clock models between and within the cohorts. The median rates of evolution of the entire gene and all three fragments under both clock models were markedly higher for the Chicago sequences compared to the Memphis sequences. Next we asked which region of the *env* gene evolved at the highest pace and which was most conserved. The rates calculated for the respective fragments across three data sets (Chicago and Memphis cohorts and the entire study group), under the strict clock model, consistently showed that the leader region evolved at a markedly higher rate compared to the V3-V5 or NV regions (Figure 4-9 A). This pattern across the three data sets was confirmed under the relaxed clock model for the leader region, which was consistently seen to evolve at a higher rate than the V3-V5 or NV regions (Figure 4-9 B). In contrast, the rate differences between the V3-V5 and NV regions under the relaxed clock model did not show the same pattern as the respective estimates under the strict model. Although the same pattern was visible within the Chicago cohort, the inverse was observed compared to the strict clock model estimates for the Memphis V3-V5 region which was found to evolve at slightly lower rates than the NV fragment.
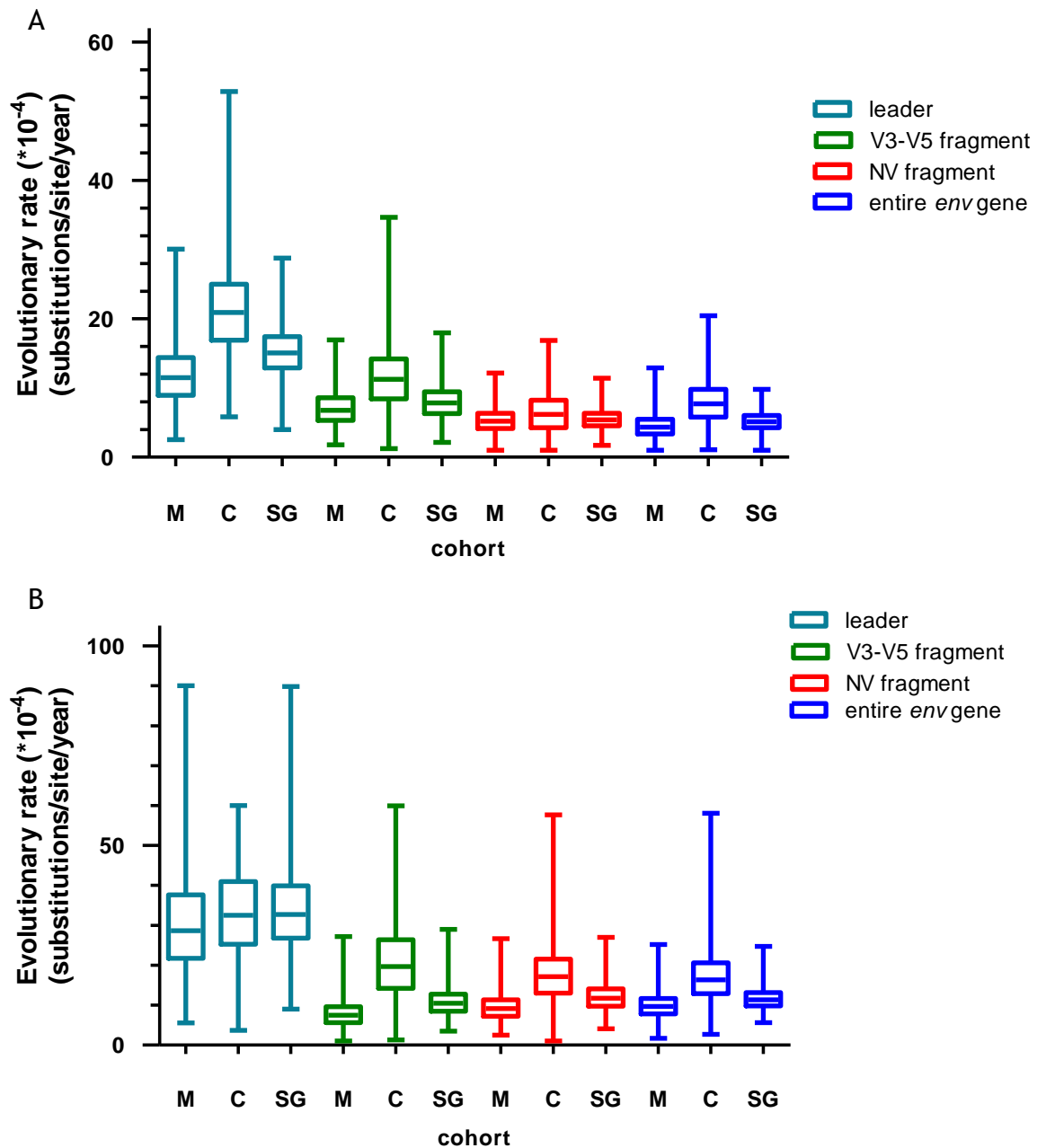
Figure 4-9 Distribution (median, 5, 25, 75 and 95 percentiles shown) of the evolutionary rates calculated under A) strict and B) relaxed lognormal clock model for the: 1) leader (grey), 2) V3V5 fragment (green), 3) NV fragment (red) and 4) entire *env* gene (blue). Evolutionary rates are compared between sequences isolated from Memphis (M), Chicago (C) and entire study group (SG). Rates are represented as x10^{-4} substitutions per site per year.

To test the hypothesis that the recombinant sequences (n=123) were evolutionary younger than the pure non-recombinant sequences (n=184), the distribution of their evolutionary rates were compared (Table 4-8). As demonstrated in Figure 4-10, a clear difference in the rates between the two groups was observed. Non recombinant *envs* were estimated to evolve at the rates of 2.44 and 6.6 x 10^{-4} substitutions per site per year (0.24% and 0.66% per

decade) under strict and relaxed clock models respectively, whereas the recombinant sequences evolved faster, at the rates of 8.85 and 24.4 x 10^{-4} substitutions per site per year (0.89% and 2.4% per decade) under strict and relaxed clock models respectively.
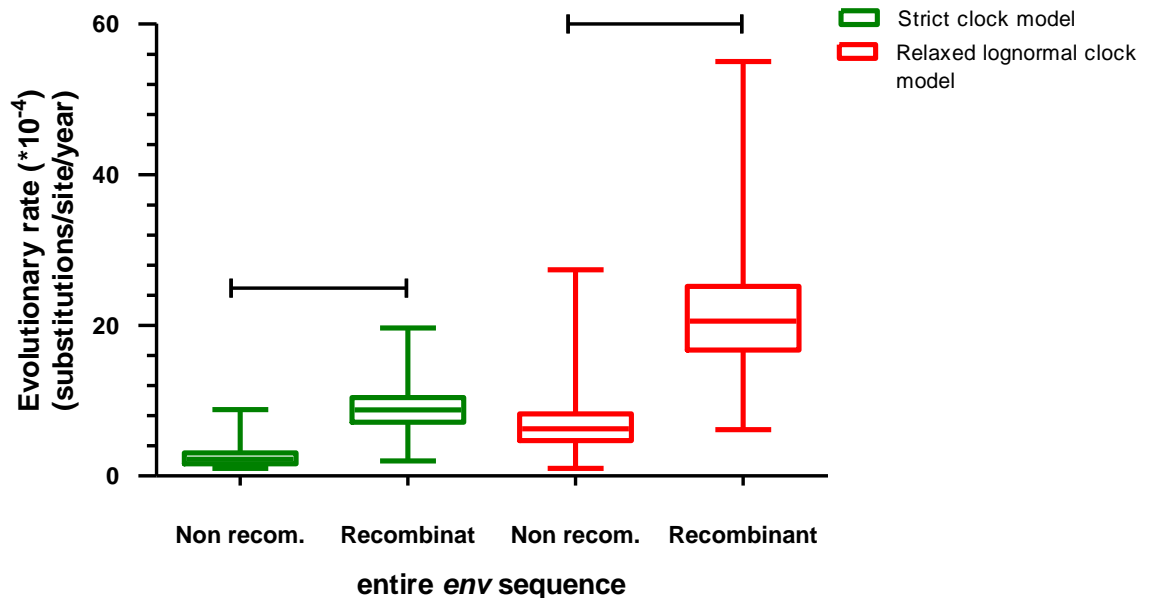
Figure 4-10 Distribution of evolutionary rates of recombinant (n=123) and non-recombinant *env* sequences (n=184) calculated under strict (green) and relaxed (red) clock models. Compared groups are highlighted with horizontal bars.

Finally we hypothesised that rates of evolution in healthy cats would be higher than in terminally ill (deceased) cats. Figure 4-11 A and B illustrate a comparison of the evolutionary rates under the two clock models for *env* sequences, and fragments, from 11 deceased and 6 animals which remained healthy and free from clinical signs during the study period. Under the strict clock model the entire *env*, the leader, V3-V5 and NV fragments from healthy cats evolved at markedly higher rates than those from deceased animals (6.72 and 4.58, 17.69 and 13.1, 23.18 and 5.91, 8.24 and 5.11 x 10^{-4} substitutions per site per year respectively) (Figure 4-11 A). This relationship, however, was reversed under the relaxed clock model for all of the fragments (*env*, leader and NV) except for the V3-V5 region (Figure 4-11 B) which consistently, under both clock models, was shown to evolve at a higher rate in healthy cats.
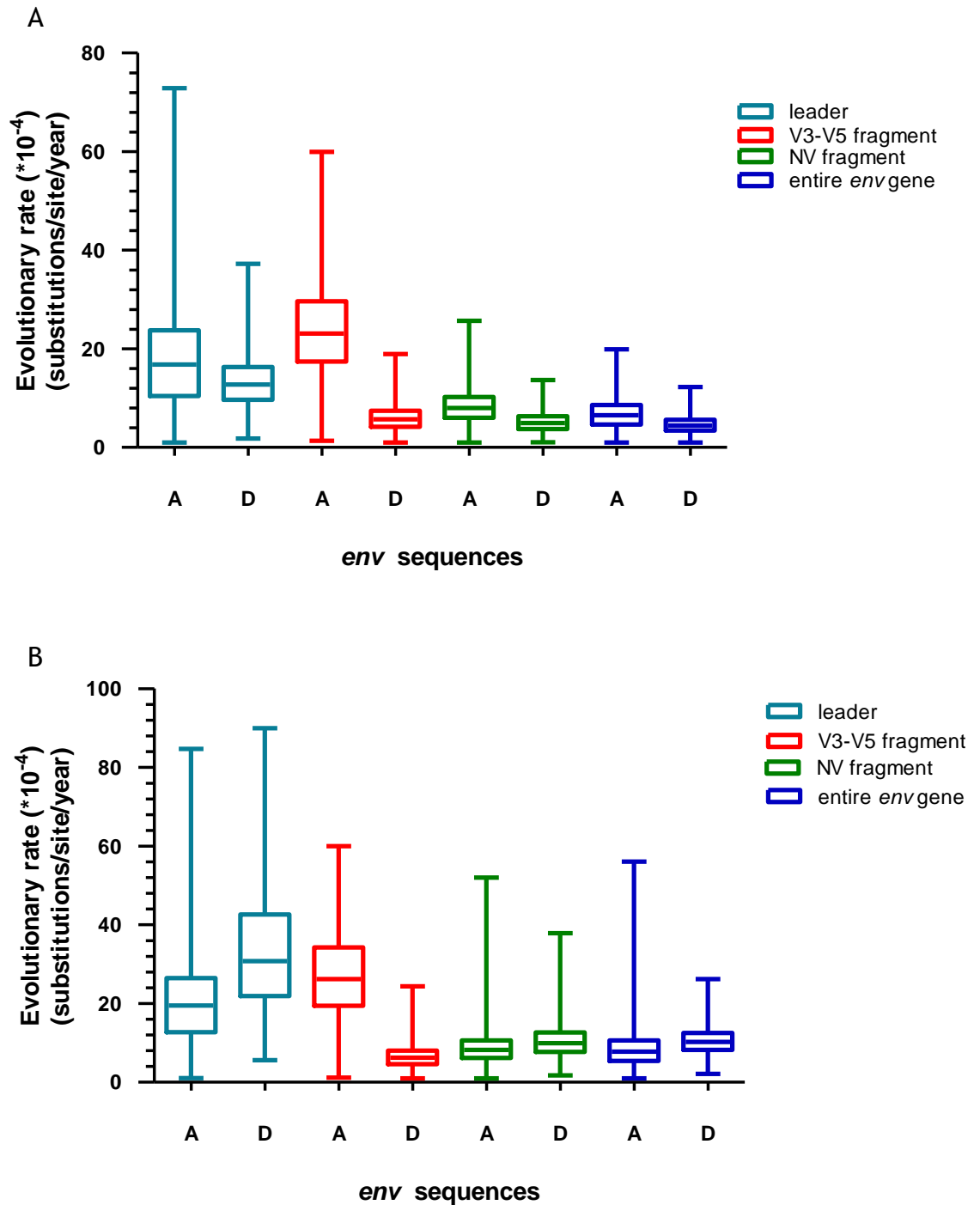
Figure 4-11 Comparison of evolutionary rates under: A) strict and B) relaxed clock models of the leader, V3-V5, NV fragments and the entire *env* genes isolated from 6 alive (healthy, A) and 11 deceased (sick, D) cats during the 18 month observation period.

4.3.6 Potential N-linked glycosylation sites

Analysis of 329 FIV Env sequences revealed variation in length between 848 (M30C C88) and 861 (M49C C76) amino acids. As shown in Figure 4-12, the tally of estimated N-linked glycosylation sites varied from 20 to 26 across the Env

glycoprotein. The majority of analysed sequences (32.5%) contained 25 N-linked glycosylation sites. 92 Envs (27.9%) contained 23 such sequons, while 79 (24%) Envs were predicted to be glycosylated at 24 positions. The lowest number, 20 glycosylation sites, was observed in 6 Envs (1.8%), namely M2C C1, M2C C5, M47A C135, Shizuoka, Petaluma and Dixon. At the other extreme, 7 novel Envs (2.1%) contained 26 potential sequons: P5A C7, P5B C118, M27A C104, M27A C194, M27A C200, M27C C63 and M27C C65.
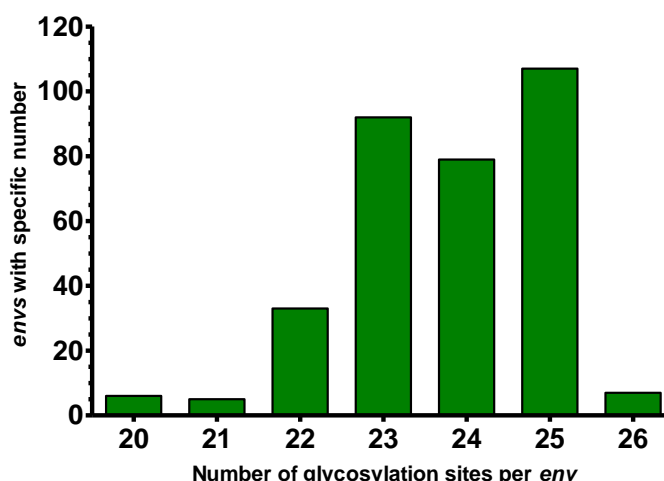
Figure 4-12 Frequency of potential N-linked glycosylation sites (sequons) on the surface of the SU and TM region of Env (n=315) with inclusion of reference sequences (n=14) representing clades A, B, C and D: FIV Aomori 1; D37814.1, FIV Aomori 2; D37811.1, FIV C; AF474246.1, FIV Dixon; L00608.1, FIV Dutch; X60725, FIV Fukuoka; D37817.1, FIV Sendai 1; D37816, FIV Shizuoka; AY139094.1, FIV UK2; X57001, FIV UK8; X69494, FIV USIL2489; U11820.1, FIV Yokohama; D37815.1, FIV Petaluma M25381.1, FIV PPR X69496.1.

An investigation of the global distribution of predicted N-linked glycosylation sites revealed that, regardless of the high genetic variability of the analysed sequences (which differed from each other by over 26%, calculated as K2P highest distance in peptide alignment), conserved sequon sites do indeed exist across all the analysed sequences. As shown in Figure 4-13, six sequons at positions 303, 335, 347, 536, 553 and 738 were fixed amongst all the Envs examined (n=329, 100%). Sequons at positions 263, 279, 423, 427, 504 and 734 were fixed in 328 (99.6%) Envs, while positions 341, 523 and 746 were glycosylated in 327 (99.3%) sequences. Three other positions (namely 754, 274 and 474) were frequently glycosylated and were present in 99%, 98.7% and 97.2% of sequences, respectively. Additional analyses revealed the existence of a second pattern of N-linked glycosylation sites for which the frequency at the

population level was inconsistent. These shifting sequons, shown in Figure 4-13, were mainly located between sites 120-225, 473-494 and 556-569 and at positions 377, 831 and 853 of the Env. To investigate whether those sites were common amongst diverse isolates of FIV we examined their variability at the intra-host level. For the purpose of this analysis we included sequences from 21/44 (47.7%) cats in which intra-host variation of N-linked glycosylation sites was evident. Sequences from the remaining 23 (52.3%) cats were excluded because in those individuals there was no evidence of variation (data not shown).
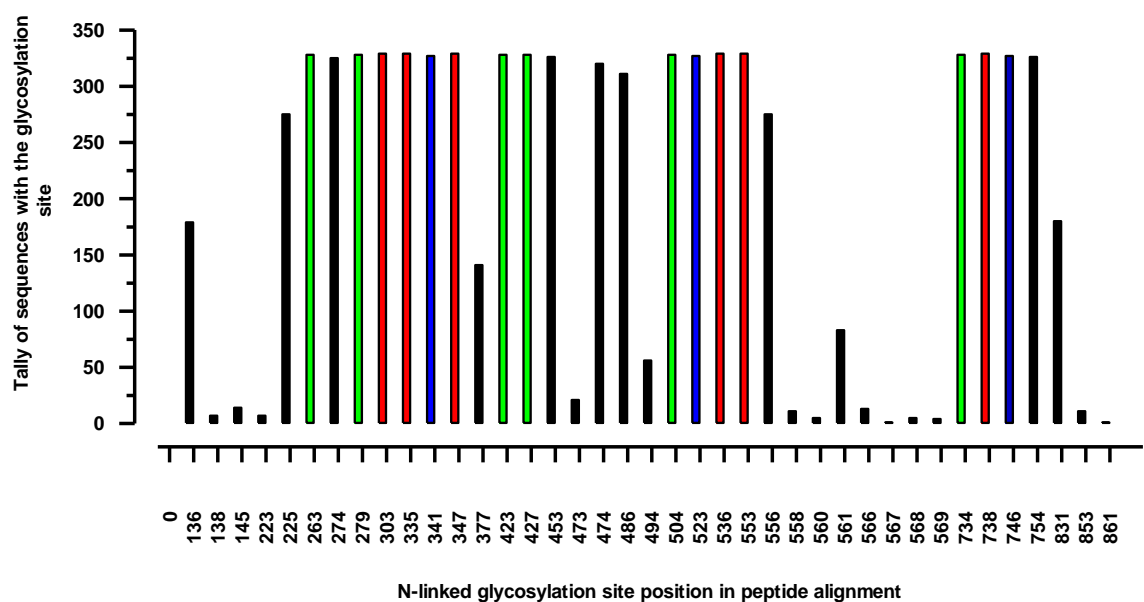
Figure 4-13 Frequency of predicted N-linked glycosylation sites on the FIV Env. Analysis was performed on Env sequences isolated in this study and reference sequences representing clades A, B, C and D (n=319). Positions 303, 335, 347, 536, 553 and 738 (red) represent fixed sequons among all clades (n=329) from different geographic origins. Sequons at positions 263, 279, 423, 427, 504, 734 (green) and 341, 523, 746 (blue) were also fixed and were present in 328 and 327 sequences respectively. The remaining glycosylation sites were fixed in less than 97% of Env sequences (black). The positions of sequons in the peptide alignment are based on the reference isolate M49CC76.

In Table 4-9 the positions in the alignment and intra-host range of N-linked glycosylation sites are highlighted. An analysis of shifting sequons revealed the existence of N-linked glycosylation sites, which, as a result of substitutions, appear and disappear and differ between Env variants from the same individual. Four shifting sites, at positions 486, 556, 561 and 831, were shared between 3 (14%), 4 (19%), 4 (19%) and 5 (24%) cats respectively, suggesting that variations at those sites may play a role in immune evasion. The locations of the putative

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sequons identified in this study are shown on the model of FIV SU and TM (Figure 4-14) and coincide with previously identified B-cell epitopes (Lombardi et al., 1993), (Pancino et al., 1993a).

Table 4-9 Frequency patterns and locations of predicted shifting N-linked glycosylation sites on Env. The comparison is based on the pattern of N-linked glycosylation sites from viral variants isolated from each cat over 3 time points. Note the shifting number of sequons within homologous viral variants isolated from each individual (column 3). There were 4 "hot spots" for shifting sequons identified (positions 486, 556, 561 and 831, highlighted in red) which consistently appeared and disappeared within Env variants isolated from 3, 4, 4 and 5 cats respectively. For clarity, this comparison includes Env sequences isolated from 21/44 (47.7%) cats in which variation of N-linked glycosylation sites was identified. Position of sequons in peptide alignment is based on the reference sequence of isolate M49CC76. The sequon locations are shown relative to the V1-V9 regions of Env.

Cat	Tally of isolated functional *envs*	Range of sequons on gp160	leader 136	138	V1-V2 225	263	279	341	V3 377	423	453	V4 474	486	494	523	V5 553	556	560	561	566	567	568	569	734	V7-V9 831	861
P1	3	24-25																	🔴							
P2	15	24-25						●											🔴							
P4	10	23-25				●				●						●	🔴	●	🔴	●	●					●
P5	8	25-26																							🔴	
P7	2	22-23									●	●		●												
P9	6	23-24																							🔴	
P10	4	24-25											🔴													
P17	8	23-24																							🔴	
P21	5	22-24							●																🔴	
M3	2	24-25	●																							
M5*	10	21-23					●												🔴						🔴	
M8	2	21-23							●				🔴													
M11	24	22-23													●											
M14	9	24-25																						●		
M25	6	24-25			●																					
M26	6	23-24															🔴									
M31	14	22-24				●						●														
M33	21	23-24															🔴									
M44	2	24-25	●																							
M47	8	20-22											🔴						🔴							
M49	6	23-25																				●	●			

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Figure 4-14 Model of SU and TM proteins of FIV (adapted from (Pancino et al., 1993b) with modifications by (Willett et al., 2008)) illustrating location of predicted N-linked glycosylation sites identified in this study as fixed and shifting. Fixed sequons are highlighted in red, green and blue (see Figure 4-13). Sequons identified as shifting are highlighted in orange. Di-sulphate bonds are shown as red lines between Cysteines. Predicted α-helices are denoted with grey lines. Positions in peptide alignment are based on the reference isolate of M49CC76.

4.4 Discussion

Analyses of the FIV *env* gene sequences isolated in this study offered a unique opportunity to examine the molecular epidemiology and dynamics of FIV in naturally infected cats.

The phylogenetic relationship of the sequences isolated from both cohorts, as estimated using the ML method, was in agreement with previously published data demonstrating clade A and clade B viruses in the USA (Sodora et al., 1994). It should be emphasized, however, that the ML method initially employed, did not account for potential recombination and, indeed, erroneously classified isolated sequences. However subsequent, more rigorous, recombination testing demonstrated that Envs with shared recombination points and with inconsistent clade assignment were widely circulating in Memphis and Chicago; such results confound the current phylogenetic classification of FIV isolates.

Recombination is an important event in retroviral evolution, which, in cases of super-infection, may lead to the emergence of novel viral variants. Newly created recombinants may exhibit novel pathogenicity compared to the parental strains, for example manifesting more severe clinical outcomes or spreading more easily within and amongst hosts (Onafuwa-Nuga and Telesnitsky, 2009). Cats living in free roaming populations, where the abundance of FIV is high, are likely to share viruses during multiple transmission events. This phenomenon has been documented in experimental FIV infection (Okada et al., 1994), with one study describing recombination following super-infection (Kyaw-Tanner et al., 1994). Recombinant sequences in the present study were consistently identified in subsequent follow up samples, suggesting that such genotypes did not arise as a result of either PCR errors or contaminants. The high prevalence of clade A/B recombinants with common break points in our study group may suggest that these represent enzootic recombinant viruses with a significant fitness advantage that may be widely distributed in the field. A similar pattern of recombination, with a shared break point within *gag,* was reported in a Canadian study, suggesting the existence of enzootic circulating recombinant forms in Ontario (Reggeti and Bienzle, 2004). In HIV infection, recombinant viruses sharing identical mosaic structures play an important role in the HIV pandemic

and are referred to as circulating recombinant forms (CRF) (Robertson et al., 2000). However, the term CRF can only be applied to viruses from distinct epidemiological areas. Therefore, taking into account the history of the Chicago and Memphis cohorts, we propose that the mosaic viruses identified in this study represent putative CRF of FIV.

The results of this study highlight the advantage of examining entire gene sequences. The detection of recombination increases with the nucleotide length screened and therefore the majority of previous studies (which examined only short 500-700bp fragments of the *env* gene) underestimated the role of recombination using a simplified phylogenetic classification. It is apparent that applying the common approach of examining the most variable V3-V5 loop of the *env* gene may result in possible misidentification of intra- and inter-subtype recombinants and erroneous subtype assignment. For example, viruses from this study such as M2, M33 and M48 would be wrongly classified as clade A if only 700 bp fragments of the *env* were examined. Indeed, there was no recognition of recombination in the study of Texas isolates of FIV that focused on the V3-V4 sequence of *env* (Weaver et al., 2004). Similarly, a study by (Duarte and Tavares, 2006) focusing upon the V3-V5 region of *env* failed to consider recombination and, as proven by (Hayward and Rodrigo, 2008), erroneously classified isolated sequences as a result.

The lack of information about CRF of FIV in the field has wider implications than FIV classification (Nishimura et al., 1998); most importantly it poses a significant question surrounding the efficacy of the current FIV vaccine. It is not known what degree of protection against clade A/B recombinant viruses is achieved following Fel-O-Vax FIV vaccination. Furthermore, the existence of recombinant sequences can also confound the molecular diagnosis of FIV infection as recombinant viruses may not be detected by the PCR-based diagnostic tests that are used to distinguish FIV vaccinated and FIV infected cats in countries where the vaccine is widely available (Little et al., 2011).

Our analyses revealed the existence of at least two common recombination break points, located at the stems of the V1/V2 and V5 loops of the SU of FIV Env. Common recombination breakpoints, also referred to as hotspots (Onafuwa-

Nuga and Telesnitsky, 2009) have been reported in both FIV (Reggeti and Bienzle, 2004) and HIV (Anderson et al., 1998), (Wooley et al., 1998), (Jetzt et al., 2000), (Zhuang et al., 2002), (Levy et al., 2004a), (Dykes et al., 2004) infections. Although recombination occurs frequently *in vitro* (An and Telesnitsky, 2002), (Rhodes et al., 2003), (Chen et al., 2006), it has been suggested that recombination hot spots depend on selection rather than reflecting RT template switching hotspots (Onafuwa-Nuga and Telesnitsky, 2009). The vast majority of recombinant viruses display low fitness, do not survive within the host (Archer et al., 2008) and therefore the recombinant Envs identified in our study may possess a significant fitness advantage in comparison with parental sequences.

Of particular interest is the first recombination breakpoint separating the leader region from the remainder of the *env* gene. Indeed several recombinant *env* sequences were identified in which the leader/signal region clustered with clade A while the other segments clustered with clade B. Given the location of this first recombination breakpoint, together with the relatively high number of positively selected sites and the highest evolutionary rate of this segment, it may be speculated that the leader region has a significant function in the virus life cycle. The N-terminal signal peptide of FIV plays an important role in post-translational targeting of the Env precursor and its translocation through the endoplasmic reticulum (ER) (Verschoor et al., 1993). Recent studies of viral leader sequences highlighted their enormous complexity, suggesting a role not only in post-translational but also in post cleavage events (Lemberg and Martoglio, 2002), (Hegde and Bernstein, 2006). Leader encoded peptides can play a role in self-antigen presentation (Borrego et al., 1998), a property exploited by human cytomegalovirus that has a signal peptide with nine residues identical to the MHC I signal peptides (Tomasec, 2000) and facilitates evasion of NK cells detection by presenting a virus-encoded MHC I molecule (Ulbrecht et al., 2000). Furthermore, sequence variation amongst the signalling peptides of acute and chronic isolates of HIV has been demonstrated (Gnanakaran et al., 2011). The present study highlights for the first time that the highest intra-host diversity and highest evolutionary rate occurs within the FIV leader/signal sequence when all *env* regions were examined. These findings suggest that the

leader/signal region is under positive selection and may play an important role in regulating Env expression on the viral surface (Hegde and Bernstein, 2006) and therefore viral infectivity. This hypothesis however requires validation in functional studies and warrants further investigation.

Sequences isolated from the majority of animals (93%) formed highly monophyletic groups, suggesting that viruses were not transmitted between cats in spite of them being either continuously or at least at some point in contact with each other. In the Memphis cohort, where all cats live in the same household, sequences from a single cat (M5), isolated from the last sample collected prior to its death, did not cluster together with sequences from two previous time points; rather there was a high similarity to sequences from cats M1, M10 and M11. Given the clinical history of M5, a female cat that was not involved in territorial fights and lived a solitary lifestyle, the classical virus transmission via biting appears unlikely in this case. However, cat M5 did experience a severe cat flea (*Ctenocephalides felis*) infestation during the last months of her life, prior to the last blood sampling. Although FIV transmission by cat flea has not been documented, this blood sucking ectoparasite is a potential vector for transmission of FeLV (Vobis et al., 2003). If cat M5 had become superinfected between time points B and C via any route, we would have expected to isolate at least two groups of viral sequences (M5AB and M10 like), as well as possibly mosaic sequences arising following recombination between superinfecting and already existing viruses. But three sequences isolated from time point C did not have those features; rather there was high similarity to sequences from animals M1, M10 and M11. Given the small number of sequences isolated from this time point and sampling bias, it remains possible that M5A and M5B sequences remained but were less abundant than freshly acquired M10 like viruses. It is also possible that the degenerate primers used for amplification bound more effectively to the latter group of viruses. Sequencing more viral variants from the last sampling from cat M5 could possibly resolve this issue.

In the Chicago group, sequences from two different time points from each of two cats (P8A, P8C and P21B, P21C) similarly did not form monophyletic groups and were even assigned, using the ML method, to two different clades. Given the sequencing and recombination testing data from other cats in the Chicago

cohort, it was apparent that cat P8 shared viral sequences from time point A with cat P14. Taking into account the shared accommodation and fight wounds reported on clinical examination of those two cats, one-way transmission might have occurred. However, time point A virus was not isolated from cat P8 at time point C, a finding that could be explained by primer sampling bias, as discussed above. Sequence data from cat P21 showed a similar pattern; in this case however, sequences from time point B were highly similar to that of cat P9 and classified as clade B, while sequences from time point C clustered within clade A. Although there was no reported history of fighting between those cats, super-infection may still have occurred.

Finally, it cannot be completely excluded that sequences from those three animals represented contaminants rather than viruses circulating in those cats. Special measures were undertaken to avoid contamination, both in the clinical and laboratory settings. Cats were double identified prior to blood sampling, PCR reactions were prepared in a designated UV treated room and fresh, unopened reagents were used at each separate time points throughout the 18 months of study to avoid any possibility of cross-contamination.

Analyses of sequences from three closely related animals (M1, M10 and M11) and their clinical history, age and date of first FIV diagnosis led to speculation that the oldest male cat M10 may have infected female M11 during mating, followed by vertical or horizontal transmission to its offspring M1. The overall mean distance between all sequences obtained from these three cats following at least two postulated transmission events is remarkably low. However, a study examining V3-V5 *env* sequence diversity of the Aomori-2 strain of FIV following vertical transmission demonstrated 100% homology between viruses isolated from the mother and kitten 48 weeks post transmission, suggesting that a high degree of homology is maintained for at least 2 years after infection (Motokawa et al., 2005). A similar study examining V3-V5 *env* sequence diversity in 3 cats over a period of 9 years found a single amino acid change compared to the initial inoculum, indicating the high genetic stability of FIV (Ikeda et al., 2004). Hence, in agreement with two previous studies (Ikeda et al., 2004), (Motokawa et al., 2005) the results presented here suggest that the *env* sequences of clade B, M10

like viruses are stably maintained during vertical transmission and display little diversity following transmission to the new host.

Although the leader signal sequence displayed the highest intra-host variation, overall the intra-host diversity of *env* in the majority of cases examined was surprisingly low in light of the high mutation rate of viruses carrying the error prone RT enzyme. However, differences in intra-host diversity and evolutionary rates were observed between different cats and can be attributed to replication tempo differences between wild type strains, as described for HIV-1 and the less pathogenic HIV-2. Although both viruses achieve similar proviral loads in infected individuals, HIV-2 replicates to lower titres in the plasma (Popper et al., 2000), (MacNeil et al., 2007b), displays a lower rate of sequence evolution (MacNeil et al., 2007a), lower pathogenicity reflected by a slower decline in CD4+ T cell numbers and slower disease progression (Marlink et al., 1988), (Marlink et al., 1994), (Whittle et al., 1994), (Popper et al., 1999). Similarly, the low pathogenic bovine immunodeficiency virus (BIV) which is closer to FIV than primate lentiviruses in evolutionary terms, also exhibits very little sequence variation (Carpenter et al., 2000). The results presented here demonstrate a similar pattern to HIV-2 infection and are in agreement with previous studies indicating a relatively high genetic stability of FIV (Ikeda et al., 2004), (Motokawa et al., 2005), (Huisman et al., 2008b). Similarly, low intra-host diversity was reported in a study of clade A GL-8 experimental infection employing end point dilution sequencing of the provirus (Kraase et al., 2010). The results obtained here from naturally infected cats and employing amplification direct from blood samples are in agreement with previous reports, despite the different methodologies. The range of the entire *env* intra-host diversity observed here is likely attributable to differences in the replication rates of wild type FIV isolates, as suggested for SIV infection (Eastman et al., 2008). Similarly it has been postulated that differences in intra-host sequence variation between HIV-1 and HIV-2 could be attributed to different rates of replication of those two closely related viruses (MacNeil et al., 2007a), (MacNeil et al., 2007b).

The high genetic stability of some lentiviruses may be associated with the fidelity of their reverse transcriptase (Lewis et al., 1999) as well as with the replication rate. Mutations in the catalytic YMDD motif of HIV-1 RT have been

implicated in increasing fidelity (Olmsted et al., 1989), (Operario et al., 2005). However mutations in this particular motif were not evident in FIV RT sequences; instead, the V148S mutation associated with an increased fidelity of the SIV RT was apparent (Huisman et al., 2008b). This may lead to speculation that FIV RT has better proofreading capacity compared to the RT of HIV-1. The presence of identical or almost identical sequences, in the present study may indicate that a high proportion of FIV is present in a latent form within the cellular compartment. It is possible that virus evolution is constrained due to the high fitness cost associated with divergence from the parental virus.

The slow rate of evolution documented in terminal HIV-1 infection has been suggested to be linked to a weakening immune system (Shankarappa et al., 1999), (Delwart et al., 1997). In the present study, a comparison of the evolutionary rates between healthy and terminally sick cats of the leader, NV and the entire *env* gene did not show a consistent pattern under two clock models. However, the V3-V5 fragment which contains neutralisation epitopes and is under immune system surveillance (Pancino et al., 1993a), (Lombardi et al., 1993) was consistently shown, under both clock models, to evolve at a markedly higher rate in healthy cats. In addition, the rates of evolution across all data sets from the Chicago cats (which were generally in good health) were consistently higher that those estimated for sequences from the Memphis cats (which tended to have more clinical signs). Similarly we observed significantly higher evolutionary rates of recombinant compared to non-recombinant sequences. Those results support the hypothesis that as a result of strong selection pressure, the virus evolves at a higher rate in healthy immune-competent animals and that the rate of viral evolution is increased following the recombination event.

By comparing amino acid sequences and global patterns of potential N-linked glycosylation sites (PNGS), 19 sites were identified that were conserved among over 97% of the examined sequences, with six PNGS displaying 100% fixed cross-clade pattern. This striking consistency at the population level suggests that glycosylation at these sites is likely to play an essential role in the folding and integrity of the viral glycoprotein. Patterns of fixed N-linked glycosylation sites have also been noted in infections with SIV (Chackerian et al., 1997) and HIV-1

group M subtypes A through G (Gao et al., 1996). A comparison of all FIV *env* sequences revealed that the number of N-linked glycosylation sites varies from 20 to 26 between various isolates and a similar trend has been documented in HIV gp120, with numbers of PNGS ranging from 18 to 33 (Korber et al., 2001). In comparison to the range of N-linked glycosylation sites in HIV (Zhang et al., 2004), the range reported here is lower.

At the intra-host level, the existence of a second shifting in glycosylation pattern was evident. Shifting PNGS appeared and disappeared on the glycoprotein and differed between variants isolated from the same individual over a period of time. Although they are distributed throughout Env, we identified 4 shifting sites that were consistently present in 14 % of the analysed sequences, two of which were located in the V5 loop of SU. The role of these variable sequons, which as the result of mutation appear and disappear, might be associated with immune evasion, for example by masking epitopes for neutralising antibodies (Samman et al., 2010) or CTLs, as has been demonstrated for HIV (Chen et al., 2009). Indeed, glycosylation sites identified in this study overlapped with previously identified B cell epitopes (Pancino et al., 1993a), (Lombardi et al., 1993). Moreover, the alteration of a single N-linked glycosylation site in the FIV Env has been shown to modulate the virus-receptor interaction (Willett et al., 2008), highlighting the potential importance of shifting N-linked glycosylation sites in immune evasion and viral adaptation. Whether alterations in the glycan shield on the individual level play a role in the virus-host interaction and are responsible for escape from immune surveillance will be addressed in Chapter 5 of this thesis.

Finally, the short (12 month) sampling period provided limited information about the intra-host diversity and rate of evolution compared to a longer study (Seo et al., 2002); additional longitudinal samples were not examined due to time restraints. The methodology used in this study also has potential limitations, attributed mainly to PCR sampling and cloning bias (Simmonds et al., 1990), (Liu et al., 1996b). However, in contrast to previous studies, the high fidelity Phusion enzyme was used rather than the error prone *Taq* polymerase. Furthermore, in order to minimize template switching during the PCR, three independent amplifications were set up from each blood sample. All procedures were

performed separately at various time points over a period of 18 months to exclude the possibility of cross-contamination. Indeed, the finding that this methodology yielded identical sequences, particularly those with shared recombination points over a period of 12 months, as well as the precautionary cross-contamination measures, provides evidence that the amplicons that were identified did reflect real circulating sequences and were neither the result of PCR induced errors nor associated with polymerase template switching. Moreover the results of the present study are in agreement with previous FIV studies employing "bulk" PCR (Ikeda et al., 2004), (Motokawa et al., 2005), end-point dilution proviral DNA PCR (Kraase et al., 2010) as well as a study examining sequences from plasma viral RNA (Huisman et al., 2008b). These studies using different methodologies consistently suggested that FIV sequence variation is relatively low, highlighting its relatively high genetic stability.

In conclusion, a high abundance of recombinant *env* sequences was found in naturally infected cats, with relatively low intra host diversity and a low rate of evolution of the entire FIV *env*. It was demonstrated that various fragments of the *env* gene evolve at significantly different rates, with the leader sequence displaying the largest number of substitutions over time. The results of this study suggest that the potential of the FIV reverse transcriptase to generate variation is likely offset by functional limitations and the fitness of the resulting virions. The relatively low rate of viral evolution and greater genetic stability of FIV compared to HIV-1 can perhaps be attributed to different replication kinetics, as has been suggested for HIV-1 and HIV-2. Based on the data presented here as well as clinical observations, it appears that FIV in naturally infected cats behaves more like HIV-2, being less pathogenic and exhibiting less dramatic clinical signs than have been reported in some experimental infections (de Rozières et al., 2004).

Chapter 5. Neutralising antibody response to FIV infection

Little is known about the role of neutralising antibodies (NAbs) in controlling FIV infection and subsequent disease progression. Here we present studies examining the neutralisation of homologous and heterologous FIV *env* (HIV-luc) pseudoviruses by sequential plasma samples collected from naturally infected cats. We evaluated the breadth of the NAb response against non-recombinant homologous and heterologous clade A and clade B viral variants and recombinants and assessed the results, looking for evidence of an association with duration of infection, CD4 T lymphocyte numbers, the health status and survival times of the infected cats.

Our results demonstrated that neutralisation profiles varied significantly between FIV infected cats and that strong autologous neutralisation, assessed using *in vitro* assays, was not related to the clinical outcome. No association was observed between strong NAb responses and either improved health status or survival time of infected animals, implying that other protective mechanisms are likely to be involved. Similarly, there was no correlation between the development of autologous NAbs and the duration of infection. Furthermore, we identified cross-neutralising antibodies in only a small proportion of animals. This may suggest, given the relatively long duration of infection prior to enrolment and the genetic stability of FIV *env* (as demonstrated in the previous chapter), that there was a low level of antigenic stimulation following natural FIV infection. Alternatively, the lack of cross-reactive antibodies may be related to the non-specific hypergamma-globulinemia that is induced following infection with FIV.

5.1 Introduction

Neutralising antibodies (NAbs) are immunoglobulins (Ig) produced by plasma cells that comprise a crucial component of humoral immunity against various pathogens including viruses (Amanna et al., 2008), (Plotkin, 2008). NAbs are believed to comprise an essential component of the protective immune response induced by vaccines against FIV and HIV infections (Hosie et al., 2011), (Kwong et al., 2012). However, relatively little is known about the role of humoral immunity in controlling retroviral infections and subsequent disease progression, particularly for FIV infection (Hogan and Hammer, 2001a), (Hogan and Hammer, 2001b), (Xiao et al., 2002), (Piantadosi et al., 2009), (Gray et al., 2011b), (Hosie et al., 2011).

HIV infection results in the rapid production of binding and neutralising antibodies (Baum, 2010). Binding antibodies recognise viral particles but are not always capable of neutralisation; nevertheless such antibodies may still offer a modest degree of protection, as has been demonstrated in SIV (Demberg et al., 2007) and HIV (Rerks-Ngarm et al., 2009b) vaccine trials. Neutralising antibodies specifically target epitopes on SU and TM, including receptor and co-receptor binding sites (Binley et al., 2008). However, their efficacy is subject to significant challenges. Env is covered with host glycans that shield neutralisation epitopes, often rendering them inaccessible to NAbs (Myers and Lenroot, 1992). Furthermore, HIV and FIV Envs may display significant length polymorphisms (Kraase et al., 2010), (Euler and Schuitemaker, 2012) that may result in conformational changes and concealment of neutralisation epitopes (Hoxie, 2010).

Mechanisms of blocking retroviral infection other than direct neutralisation of free viral particles include antibody dependent cell mediated cytotoxicity (ADCC) and antibody dependent cell mediated viral inhibition (ADCVI) (Baum, 2010). Cells infected with HIV express viral epitopes on their surface which may be targeted by specific antibodies (Isitman et al., 2012). Following the initial binding to infected cells, the Fc region of such antibodies binds FcγIII receptors on the surface of effector cells, such as natural killer cells (NK) or monocytes, which subsequently kill virus-infected cells (Forthal and Moog, 2009).

Antibodies recognising HIV-1 Env appear approximately two weeks after infection but lack neutralising activity (Tomaras and Haynes, 2009). Autologous, highly strain-specific, polyclonal NAbs appear within 3 months after infection, exert selection pressure and lead to the emergence of escape mutants (Richman et al., 2003) (Li et al., 2006), (Li et al., 2007), (Moore et al., 2008), (Li et al., 2009). It has been documented in HIV-1 infection that autologous NAbs have little or no protective effect on disease progression (Richman et al., 2003), (Bunnik et al., 2008), (Mahalanabis et al., 2009), (van Gils et al., 2010), (Gray et al., 2011b). The lack of correlation between autologous NAbs and disease progression is mainly attributed to the rapid emergence of escape mutants at low fitness cost (van Gils et al., 2010), (Bunnik et al., 2010).

It has been suggested that NAbs appear too late following infection with HIV-1 in order to be effective in controlling disease progression, leading to the emergence of autologous neutralisation escape mutants (Richman et al., 2003), (Rong et al., 2009), (Moore et al., 2009). However, NAbs have been shown to have a potential role in controlling SHIV infection in macaques depleted of cytotoxic T lymphocytes (CTLs) (Rasmussen et al., 2002). Furthermore, pre-exposure passive transfer of broadly neutralising monoclonal antibodies has been shown to confer protection against SIV and SHIV-1 in the rhesus macaque model (Mascola et al., 1999), (Mascola et al., 2000), (Baba et al., 2000), (Veazey et al., 2003), (Ferrantelli et al., 2004), (Hessell et al., 2009a), (Hessell et al., 2009b), providing evidence that NAbs do indeed play a protective role and may be an essential component of a protective vaccine response (Hoxie, 2010).

The emergence of neutralisation escape mutants with altered glycosylation patterns has been demonstrated both in HIV (Burton et al., 2005), (van Gils et al., 2010), and FIV (Samman et al., 2010) infections. Neutralisation escape, accompanied by the subsequent evolution of the antibody response following a delay, adapts during the course of infection in response to the evolving viral shield, until the eventual exhaustion of the immune system (Euler and Schuitemaker, 2012). This explains why NAbs from a specific time point can neutralise viruses isolated from earlier time points but fail to neutralise contemporaneous viral variants (Geffin et al., 2003), (Richman et al., 2003), (Mascola and Montefiori, 2010), (Overbaugh and Morris, 2012).

HIV infection, in the majority of patients, leads to a robust production of antibodies that often possess the ability to neutralise autologous (Zolla-Pazner et al., 2004) but not heterologous viral variants. Broadly cross-reactive neutralising antibodies (Cr-NAbs) are relatively rare; elicited in some individuals, Cr-NAbs neutralise not only autologous viral variants but also neutralise other viral subtypes (Walker et al., 2011). Several broadly neutralising monoclonal antibodies (mAbs) such as b12, 2G12, 2F5, 4E10 have been isolated, their binding epitopes characterized (Muster et al., 1993), (Gorny et al., 1994), (Trkola et al., 1996b), (Burton et al., 1994), (Zwick et al., 2001) and protective roles in animal models demonstrated (Mascola et al., 1999), (Mascola et al., 2000), (Binley et al., 2004). Recent evidence suggests that Cr-NAbs are more common than previously estimated (Walker et al., 2011), (Bonsignori et al., 2011), (Medina-Ramirez et al., 2011), (Mikell et al., 2011), (Euler and Schuitemaker, 2012), emerging in approximately one third of HIV-1 infected individuals (Stamatatos et al., 2009). However, neutralisation breadth does not develop until approximately 3 years post infection (Mikell et al., 2011), (Gray et al., 2011b). It remains unknown why, and by which mechanism, such antibodies develop in some individuals or why the broadly neutralising response is significantly delayed in response to infection (Gray et al., 2011b). Furthermore, it is unclear whether neutralisation cross-reactivity can be attributed to a single, highly potent antibody, or perhaps a combination of different antibodies that act in synergy (Scheid et al., 2009).

It was observed that the strength and breadth of the NAb response was greater in progressors compared to aviraemic or long-term non-progressors (LTNP) (Doria-Rose et al., 2009). Studies of elite controllers (EC) revealed that individuals who controlled viral replication such that their viraemia was below detectable levels, had the lowest levels of NAbs (Pereyra et al., 2009), (Lambotte et al., 2009). Consistent with this observation, the breadth of Cr-NAbs was positively correlated with higher plasma viral loads, lower CD4 lymphocyte counts and disease progression (van Gils et al., 2009), (Piantadosi et al., 2009), (Sather et al., 2009), (Euler et al., 2010). These studies suggest that the development of Cr-NAbs is influenced by strong antigenic stimulation (Gray et al., 2011b). On the other hand, individuals who did not develop Cr-NAbs, fail to

do so as a result of insufficient antigenic stimulation and possibly non-specific hypergamma-globulinemia (Euler and Schuitemaker, 2012). Despite the breadth and potency of Cr-NAbs *in vitro*, such antibodies do not appear to influence HIV-1 disease progression; rather their incremental development is associated with increased viral loads and declining numbers of CD4+ T lymphocytes (Piantadosi et al., 2009), (van Gils et al., 2009), (Euler et al., 2010), (Gray et al., 2011a).

Very little is known about the role of NAbs in controlling natural FIV infection and subsequent disease progression (Hosie et al., 2011). What is the relationship between the duration of infection, health status, survival time and NAb response in FIV infected cats? Can a strong NAb response delay disease progression? Is there evidence for broadly Cr-NAbs in plasma samples from naturally infected cats? In this chapter we address these questions by measuring the NAb responses in cats naturally infected with FIV, testing samples collected at 6 monthly intervals over an 18 month period.

5.2 Materials and methods

5.2.1 FIV pseudotypes

Full length wild type *env* genes amplified from 44 participating cats from various time points as well as reference *envs* from clade A (GL-8) and reference clade B (B2542) were cloned into the eukaryotic expression vector VR1012 (Hartikka et al., 1996) and transformed into Escherichia Coli MAX Efficiency® DH5α™ Competent Cells (Invitrogen), using methods described in Chapter 2. Next, the VR1012 plasmids expressing FIV *env* were transiently co-transfected with HIV pNL4-3-Luc-E⁻R⁻luc plasmid (an *env*-deleted HIV provirus containing the luciferase reporter gene) (Connor et al., 1995) into HEK 293T cells (Graham et al., 1977) using Superfect Transfection Reagent (Invitrogen) as per the manufacturer's instructions. Following a 72 hour incubation in 6 well culture clusters (Corning), culture fluids containing pseudoviruses were harvested, centrifuged at 1000 rpm (~200 x *g*)for 5 minutes, passed through 0.45 µm filters and stored at -80°C until required.

The resulting pseudotypes, bearing FIV Envs on an HIV backbone and denoted HIV(FIV Env)luc are single round, replication competent pseudoviruses that can be used to assess the neutralisation properties of test plasma samples.

Pseudotypes bearing naturally occurring FIV Envs (1 to 23 per cat, subject to availability) and 2 reference HIV(GL-8) and HIV(B2542) pseudotypes were assessed in neutralisation assays. Pseudotypes were prepared that bore Envs from 38 cats (38/44, 86.4%); we were unable to produce viable pseudoviruses bearing Envs from 5 cats (5/44, 11.4%), (predominantly because of premature stop codons in the *env* sequences) and one cat dropped out of the study.

The nomenclature for the pseudotypes bearing the naturally occurring FIV Env proteins that were isolated from three sequential sampling times (A, B and C) is detailed in Chapter 3.

5.2.2 Plasma samples

Plasma samples were collected from 38 FIV infected cats from Memphis and Chicago at 6 monthly intervals over a period of 18 months, unless cats had died during the interim period (Chapter 3). Plasma samples denoted A, B, C and D relate to each time point respectively (Chapter 3).

5.2.3 Neutralisation assay

Plasma samples were tested for NAb using HIV(FIV)luc pseudotypes. Plasma samples were diluted 1 in 10, 100, 1000 and 10000 in complete RPMI 1640 medium (Invitrogen). 25µl of each plasma dilution were incubated in triplicate for one hour at 37°C with 25µl of HIV(FIV)luc pseudotype and then 5×10^4 of CLL-CD134 cells (Willett et al., 2006b) were added in 50µl. The cells were cultured in CulturPlate™-96 assay plates (Perkin Elmer) for 72 hours. Luciferase activity was quantified following the addition of 100µl of Steadylite HTS™ (Perkin Elmer) substrate and single photon counting was conducted using a MicroBeta luminometer (Perkin Elmer).

The neutralisation activity of the tested plasma samples is presented here as "fold neutralisation". Fold neutralisation was calculated by dividing the mean luciferase counts of control wells containing no plasma (NP luc) with the mean luciferase counts for wells containing 1 in 10 plasma dilutions (P luc) according to the following equation:

$$fold\ neutralisation = \frac{\text{NP luc}}{\text{P luc}}$$

Fold neutralisation is comparable to percentage neutralisation (Table 5-1) calculated according to the following equation:

$$\%\ neutralisation = \frac{(\text{NP luc} - \text{P luc})}{\text{NP luc}} * 100\%$$

Plasma samples were classified as not neutralising, weakly, moderately or strongly neutralising according to empirical cut-off values as indicated in Table 5-1.

Table 5-1 Classification of neutralisation potency of plasma samples.

	Neutralisation potency								
	absent		weak		moderate		strong		
fold neutralisation	1.0	1.6	1.7	2.4	2.5	5.0	5.6	10	100000
% neutralisation	0 %	39 %	40 %	59 %	60 %	80 %	81 %	90 %	100%

5.2.4 Graphs and statistical analyses

Graphs and statistical analyses were performed in GraphPad Prism v 5.00 (GraphPad Software, San Diego, California, USA). For clarity, values for fold neutralisation at 1 in 10 plasma dilutions are shown in the summary table presented in this chapter.

Data for individual cats are presented in Appendix 4. Pseudotypes bearing Envs isolated at time points A, B and C are highlighted in red, green and blue respectively in all graphs. The values for the reference pseudotypes HIV(GL-8) and HIV(B2542) are shown in orange and purple respectively. Plasma samples from time points A, B, C and D are shown on the x axis in red, green, blue and black respectively.

5.3 Results

5.3.1 Autologous and heterologous neutralisation patterns in FIV infected cats

In order to determine the potency and pattern of neutralisation, one to four sequential plasma samples (subject to availability) from each cat (n=38) were tested for neutralisation against a panel of pseudotypes bearing Envs of heterologous reference isolates of FIV (clade A, GL-8 and clade B, B2542) as well as Envs from autologous isolates (numbers varied from 1-23 autologous Envs from each cat tested). Plasma samples displayed variable autologous and heterologous neutralisation patterns, ranging from strong, through moderate to no neutralisation.

As shown in Table 5-2, sixteen cats from the study group (16/38, 42.1%) displayed potent neutralisation against at least one of the pseudotypes bearing an autologous Env. This pattern was observed in eight (8/16, 50%) of the cats which remained alive for the duration of the study and eight (8/16, 50%) of the cats that died during the study. Six cats (6/38, 15.8%) displayed moderate neutralisation of pseudotypes bearing autologous Env variants; all but one of these cats remained alive during the observation period. Plasma samples from sixteen cats (16/38, 42.1%) failed to neutralise pseudotypes bearing autologous Env variants; nine of these cats (9/16, 56%) remained relatively healthy whereas seven (7/16, 44%) of the cats with no detectable NAbs died during the study period. Only five cats (5/38, 13%) demonstrated strong heterologous neutralisation of at least one pseudotype (Table 5-2). Graphs representing the neutralisation patterns of the plasma samples from each cat are presented in Appendix 4.

Table 5-2 Summary of autologous and heterologous NAb responses of plasma samples, expressed as fold neutralisation. Sequential plasma samples from 38 cats were assessed against pseudotypes bearing autologous Envs and two reference pseudotypes, bearing Envs of GL-8 and B2542 (shown below each set of autologous pseudotypes for each respective cat). Plasma samples collected at 6 monthly intervals (A, B, C and D) expressing weak, moderate and strong neutralisation are highlighted in yellow, orange and red respectively. Plasma samples that failed to neutralise are shown in background colours. The number of PNGS is shown next to the specific FIV Env variant assessed for its susceptibility to neutralisation. (NA-not available, PNGS-potential N-linked glycosylation site).

FIV Env	PNGS	Plasma collected at sequential time points				FIV Env	PNGS	Plasma collected at sequential time points			
		A	B	C	D			A	B	C	D
M15A C13	25	1	1	1	1	P14A C25	24	303	381	357	356
M15A C42	25	2483	2594	1744	2693	P14A C26	24	3475	3498	3497	2953
M15A C45	25	183	748	963	689	P14A C27	24	80	99	54	109
M15A C48	25	3017	4197	4134	774	P14B C48	24	66	63	616	404
M15B C53	25	2917	479	2927	4058	P14B C28	24	552	927	1065	225
M15B C57	25	704	2581	409	818	P14B C52	24	552	927	1065	225
M15C C13	25	2230	5908	5512	4114	P14C C1	24	159	259	407	887
M15C C14	25	3453	471	782	430	P14C C2	24	158	261	755	493
M15C C15	25	582	2306	2437	1859	P14C C5	24	415	5500	532	1114
GL-8		1	1	1	1	GL-8		3	3	2	1
B2542		1	1	1	1	B2542		1	1	1	1
	PNGS	A	B	C	D		PNGS	A	B	C	D
M46A C73	23	2	3	4	3	P13A C71	25	3	10	19	28
M46A C74	23	7	6	7	10	P13B C82	25	4	202	182	82
M46A C75	23	9	7	8	9	P13C C25	25	4	202	182	82
M46B C82	23	6	10	5	11	P13C C26	25	4	202	182	82
M46B C83	23	8	10	9	9	P13A C72	25	4	202	182	82
M46C C48	23	9	5	10	7	P13A C74	25	6	15	22	30
M46C C49	23	6	5	9	7	P13B C81	25	1	8	11	15
M46C C50	23	9	8	7	8	P13B C84	25	1	7	12	14
GL-8		1	1	1	1	P13C C27	25	1	1	1	1
B2542		1	1	1	1	GL-8		1	3	4	4
	PNGS	A	B	C	D	B2542		1	1	2	2
M25A C100	25	2	92	787	782		PNGS	A	B	C	D
M25A C98	25	579	890	3892	3321	M14A C254	25	1	1	15	1258
M25A C99	25	682	2390	3205	1610	M14A C262	25	1	0	12	1813
M25C C37	25	127	164	674	934	M14A C267	25	1	1	16	887
M25C C38	25	6	4	4	5	M14B C20	25	0	1	25	3405
M25C C39	24	6971	5261	4074	4602	M14C C181	25	1	1	12	2320
GL-8		1	1	1	1	M14C C183	25	1	1	14	891
B2542		2	1	1	1	M14C C184	25	1	1	35	592
	PNGS	A	B	C	D	M14C C64	25	1	1	16	1186
M31A C253	22	64	72	332	NA	M14C C65	25	1	1	10	1801
M31C C1	22	1	1	1	NA	M14C C7	25	1	1	19	990
M31C C10	21	1	1	1	NA	GL-8		1	1	1	1
M31C C11	22	1	1	1	NA	B2542		1	1	1	1
M31C C12	22	43	50	137	NA		PNGS	A	B	C	D
M31C C13	22	1	1	1	NA	M30A C39	25	1	1	2002	118
M31C C14	22	60	48	213	NA	M30B C29	25	1	1	622	109
M31C C15	22	51	47	176	NA	M30B C13	25	1	1	622	109
M31C C16	22	12	14	37	NA	M30B C8	25	1	1	622	109
M31C C167	22	1	1	1	NA	M30B C1	25	1	1	622	109
M31C C17	22	1	1	1	NA	M30A C40	25	1	1	622	109
M31C C2	22	1	1	1	NA	M30A C41	25	1	1	502	183
M31C C4	23	17	17	60	NA	M30A C42	25	1	1	1893	75
M31C C5	22	1	1	1	NA	M30A C45	25	1	1	3921	148
M31C C7	22	1	1	2	NA	M30T C12	25	1	1	2168	85
M31C C8	22	18	18	28	NA	M30T C27	25	1	2	174	146
M31C C9	22	1	1	1	NA	M30T C30	25	1	1	927	260

Sample	PNGS	A	B	C	D
GL-8		1	1	2	NA
B2542		1	1	1	NA
	PNGS	A	B	C	D
P2B C17	24	1	2	NA	NA
P2B C18	24	32756	23812	NA	NA
P2B C21	24	2	3	NA	NA
P2B C24	24	530	3192	NA	NA
P2B C4	24	1	3	NA	NA
P2B C43	24	1	4	NA	NA
P2B C44	24	2	3	NA	NA
P2B C52	24	2432	985	NA	NA
P2B C55	24	4	2	NA	NA
P2B C59	24	6752	6031	NA	NA
P2B C6	24	1573	4508	NA	NA
P2B C64	24	2	3	NA	NA
P2B C65	24	1015	4877	NA	NA
P2B C3	24	2	3	NA	NA
P2B C10	24	2	3	NA	NA
P2B C72	24	2	3	NA	NA
GL-8		1	1	NA	NA
B2542		1	2	NA	NA
	PNGS	A	B	C	D
P4A C31	25	102	16	24	17
P4A C32	25	111	19	31	22
P4B C109	23	1	1	3	7
P4B C2	25	1	1	2	3
P4B C3	24	1	1	3	6
P4C C20	24	2	1	1	1
GL-8		1	1	2	2
B2542		1	1	1	1
	PNGS	A	B	C	D
P5A C2	25	1	4	5	6
P5B C118	26	0	2	4	3
P5B C3	25	1	4	5	5
P5C C25	25	1	4	7	6
P5C C26	25	1	3	4	4
P5B C4	25	1	3	4	4
P5A C5	25	1	3	4	4
P5C C27	25	1	3	4	4
GL-8		1	1	1	1
B2542		1	1	1	1
	PNGS	A	B	C	D
P7B C50	23	2	3	3	5
P7C C61	22	2	2	2	8
GL-8		2	2	2	2
B2542		2	2	2	2
	PNGS	A	B	C	D
P8A C10	24	3	4	3	NA
GL-8		3	5	4	NA
B2542		8	15	14	NA
	PNGS	A	B	C	D
P22C C73	23	6217	14833	5678	NA
P22C C80	24	54080	31011	9650	NA
GL-8		5	7	5	NA
B2542		3	4	4	NA
	PNGS	A	B	C	D
M2C C1	20	4	4	277	69
M2C C5	20	2	1	6	4
GL-8		1	1	1	1
B2542		1	1	1	1
	PNGS	A	B	C	D
M3A C168	24	3	NA	NA	NA

Sample	PNGS	A	B	C	D
M30T C31	25	1	1	2513	85
M30T C4	25	1	1	2223	63
M30T C7	25	1	2	759	147
M30C C15	25	1	1	374	88
M30C C16	25	1	1	1911	95
M30C C88	25	1	1	1348	112
M30C C91	25	1	1	3769	103
M30C C92	25	1	1	2555	75
GL-8		1	1	1	1
B2542		1	2	2	3
	PNGS	A	B	C	D
M26A C109	23	12	17	6	NA
M26A C110	23	21	65	12	NA
M26A C112	23	46	101	30	NA
M26C C49	24	1	1	1	NA
M26C C50	23	24	17	12	NA
M26C C58	24	1	2	1	NA
GL-8		1	1	1	NA
B2542		1	1	1	NA
	PNGS	A	B	C	D
P9A C19	24	4	7	8	5
P9A C20	23	6	9	11	5
P9A C22	24	8	12	12	7
P9C C49	24	8	11	12	6
P9C C52	24	5	7	8	6
GL-8		1	1	2	1
B2542		1	1	2	2
	PNGS	A	B	C	D
M47A C135	19	1	2	1	11
M47A C139	22	1	1	5	987
M47A C144	22	1	1	6	926
M47B C90	22	1	1	10	1153
M47B C91	22	1	2	163	813
M47B C94	21	1	1	27	1634
M47C C65	22	1	1	8	994
M47C C68	22	1	1	7	957
GL-8		1	1	1	1
B2542		1	1	1	1
	PNGS	A	B	C	D
M49A C85	25	1	1	0	0
M49A C86	25	1	1	1	1
M49A C87	23	88	167	152	70
M49C C74	25	1	1	1	1
M49C C76	24	1	1	1	1
M49C C77	25	1	1	1	1
GL-8		0.7	0.9	0.8	0.7
B2542		0.8	1.1	1	1.1
	PNGS	A	B	C	D
P11B C35	25	2	3	3	2
P11B C40	25	2	3	3	2
P11B C46	25	2	3	3	2
P11C C87	25	2	3	3	2
P11C C89	25	2	3	3	2
P11C C90	25	2	3	3	2
P11C C88	25	5	6	7	6
GL-8		5	9	9	5
B2542		4	7	8	5
	PNGS	A	B	C	D
P18B C13	25	3028	3084	2562	NA
GL-8		7	10	6	NA
B2542		2	2	2	NA
	PNGS	A	B	C	D
P21B C75	24	1	2	2	NA

Sample	PNGS	A	B	C	D
GL-8		1.6	NA	NA	NA
B2542		4	NA	NA	NA
	PNGS	A	B	C	D
M33A C146	23	1	1	NA	NA
M33A C147	24	2	1	NA	NA
M33A C150	23	2	1	NA	NA
M33A C152	23	2	1	NA	NA
M33A C153	23	2	1	NA	NA
M33A C155	23	2	1	NA	NA
M33B C36	23	2	2	NA	NA
M33B C37	23	2	2	NA	NA
M33B C39	23	1	2	NA	NA
M33B C41	23	2	2	NA	NA
M33B C42	23	1	2	NA	NA
M33B C43	23	2	1	NA	NA
M33B C44	23	2	1	NA	NA
M33B C45	23	1	1	NA	NA
M33B C46	23	2	1	NA	NA
M33B C52	24	1	2	NA	NA
M33B C54	23	2	1	NA	NA
M33B C62	23	2	1	NA	NA
M33B C64	23	1	1	NA	NA
GL-8		1	1	NA	NA
B2542		1	1	NA	NA
	PNGS	A	B	C	D
M11A C250	23	1	1	1	NA
M11A C242	22	1	1	1	NA
M11A C238	23	0	1	1	NA
M11B C53	23	1	1	1	NA
M11B C94	23	1	1	1	NA
M11B C96	23	0	0	0	NA
M11C C150	23	1	1	1	NA
M11C C82	23	1	1	1	NA
M11C C7	23	1	1	1	NA
M11C C168	23	1	1	1	NA
M11C C151	23	1	1	1	NA
M11C C152	23	1	1	1	NA
M11C C78	23	1	1	1	NA
M11C C164	23	1	1	1	NA
M11C C1	23	1	1	1	NA
GL-8		1	1	1	NA
B2542		1	1	1	NA
	PNGS	A	B	C	D
M16AC130	24	1	1	1	NA
M16AC220	24	1	1	1	NA
M16AC215	24	1	1	1	NA
M16AC209	24	1	1	1	NA
M16C C30	24	1	1	1	NA
M16C C32	24	1	1	1	NA
M16C C80	24	1	1	1	NA
GL-8		1	1	1	NA
B2542		1	1	1	NA
	PNGS	A	B	C	D
M20A C284	23	1	1	NA	1
GL-8		1	1	NA	1
B2542		1	1	NA	1
	PNGS	A	B	C	D
P6B C3	24	1	1	1	1
P6A C68	24	1	1	1	1
PA C69	24	1	1	1	1
P6A C70	24	1	1	1	1
P6B C1	24	1	1	1	1
P6B C2	24	1	1	1	1

Sample	PNGS	A	B	C	D
P21B C76	24	1	2	2	NA
P21B C77	24	1	2	2	NA
P21B C79	24	1	2	2	NA
P21C C51	22	1	2	2	NA
P21C C52	22	2	2	3	NA
GL-8		2	3	2	NA
B2542		2	2	2	NA
	PNGS	A	B	C	D
M28A C70	25	1	2	1	2
M28A C74	24	1	1	1	1
M28A C79	25	1	2	1	2
M28B C62	25	1	1	1	1
M28B C63	25	1	1	1	2
M28B C65	25	1	1	1	1
M28B C66	25	1	2	1	2
M28B C67	25	1	1	1	1
M28C C75	25	1	1	1	1
M28C C76	25	1	1	1	1
M28C C77	25	1	1	1	1
GL-8		1	1	1	1
B2542		1	1	1	1
	PNGS	A	B	C	D
M8C C144	21	1	1	1	1
GL-8		1	2	2	2
B2542		1	1	1	1
	PNGS	A	B	C	D
M29C C1	25	1	1	1	1
M29C C2	25	1	1	1	1
M29C C52	25	1	1	1	1
GL-8		1	1	1	1
B2542		1	1	1	1
	PNGS	A	B	C	D
M32A C159	25	1	1	1	1
M32A C160	25	1	1	1	1
M32A C161	25	1	1	1	1
GL-8		1	1	1	1
B2542		1	1	1	1
	PNGS	A	B	C	D
P17B C94	24	1	1	1	NA
P17A C100	24	1	1	1	NA
P17A C99	23	1	1	1	NA
P17B C1	24	1	1	1	NA
P17B C3	24	1	1	1	NA
P17B C93	24	1	1	1	NA
P17B C95	24	1	1	1	NA
P17C C43	24	1	1	1	NA
P17C C48	24	1	1	1	NA
GL-8		1	1	1	NA
B2542		1	1	1	NA
	PNGS	A	B	C	D
M5A C14	22	1	1	1	NA
M5A C15	22	1	1	1	NA
M5A C17	22	1	1	1	NA
M5B C62	23	1	1	1	NA
M5B C68	22	1	1	1	NA
M5C C21	23	1	1	1	NA
M5C C22	23	1	1	1	NA
M5C C28	23	1	1	1	NA
	PNGS	A	B	C	D
M41A C120	22	1	1	1	NA
M41A C122	22	1	1	1	NA
M41C C32	22	1	1	1	NA
M41C C34	22	1	1	1	NA

	PNGS	A	B	C	D		PNGS	A	B	C	D
P6C C22	24	1	1	1	1	M41C C39	22	1	1	1	NA
P6C C63	24	1	1	1	1	M41C C66	22	1	1	1	NA
GL-8		1	1	1	1	M41C C67	22	1	1	1	NA
B2542		1	1	1	1	M41C C72	22	1	1	1	NA
	PNGS	A	B	C	D	M41C C73	22	1	1	1	NA
M1A C3	23	1	1	1	1	M41C C82	22	1	1	1	NA
M1C C100	23	1	1	1	1	M41C C87	22	1	1	1	NA
M1C C97	23	1	1	1	1	GL-8		1	1	2	NA
GL-8		1	1	1	1	B2542		1	1	1	NA
B2542		1	1	1	1						

5.3.2 Association between development of autologous NAbs and duration of infection

We tested the hypothesis that the development of autologous neutralisation is positively correlated with the age of the cats and the duration of infection.
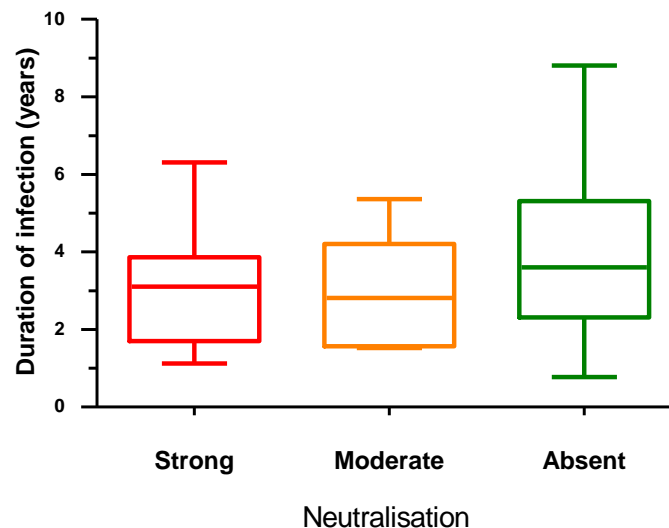
Figure 5-1 Association between the duration of infection and the development of autologous NAbs. Cats with strong (n=16), moderate (n=6) and absent (n=16) autologous neutralisation responses are highlighted in red, orange and green respectively.

Figure 5-1 depicts the relationship between the potency of autologous neutralising antibody response and the duration of infection for cats from the entire study group. The median duration of infection for cats with strong, moderate and absent autologous NAb responses was: 3.1 (range: 1.1 to 6.3) years, 2.8 (range: 1.5 to 5.4) years and 3.6 (range: 0.8 to 8.8) years respectively. No statistically significant differences between each of the groups (unpaired two tailed t test) were observed.

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5.3.3 NAb responses in cats infected with recombinant and non-recombinant viruses.

We hypothesised that cats infected with recombinant viruses would have more potent autologous and heterologous NAb responses than cats infected with non-recombinant viruses. However, as demonstrated in Figure 5-2, there were no statistically significant differences between the strength of autologous neutralisation in cats infected with recombinant and non-recombinant viruses (Fisher's exact test, p=0.45).
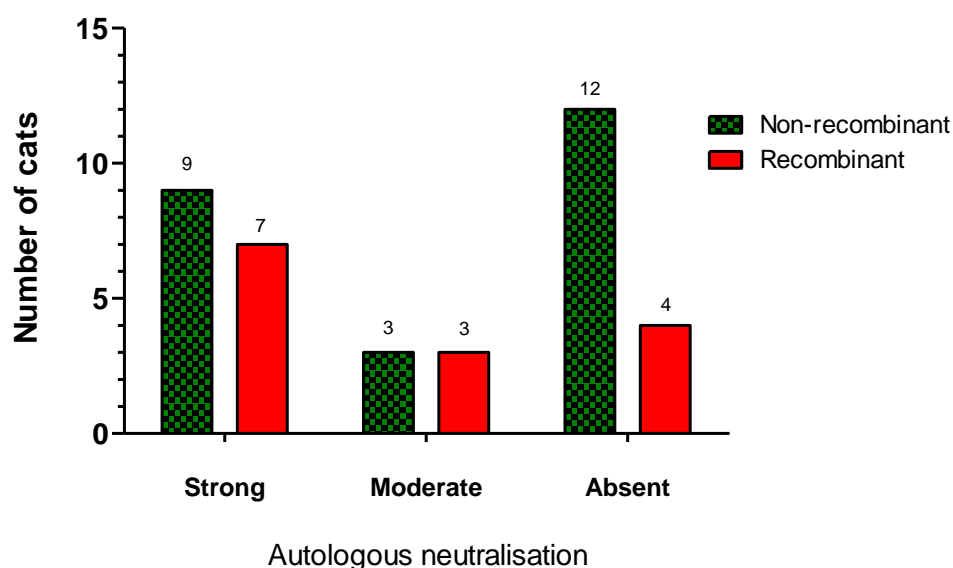
Figure 5-2 Autologous neutralisation responses in cats infected with non-recombinant (n=24; green) and recombinant (n=14; red) viruses.

Next, we asked whether cross reactive NAb responses were more common in cats infected with recombinant clade A/B viruses compared to cats infected with non-recombinant viruses. There was no statistically significant difference between the two groups; of 5 cats that demonstrated heterologous neutralisation (against at least one pseudotype), 3 were infected with non-recombinant viruses while 2 were infected with recombinant viruses (Fisher's exact t test, p=1) (Figure 5-3).
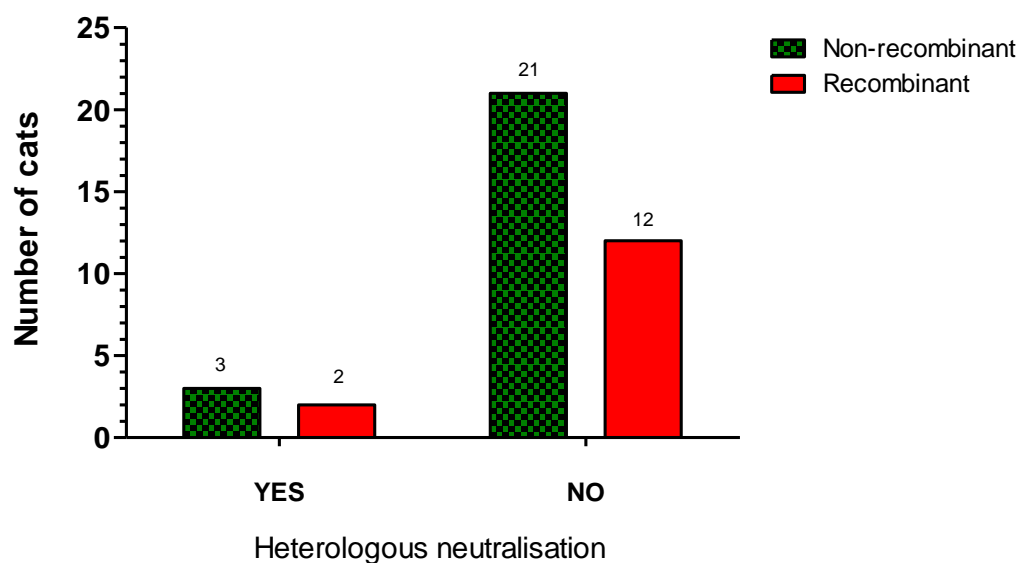
Figure 5-3 Heterologous neutralisation in cats infected with recombinant and non-recombinant viruses. Of the 38 cats tested, only 5 displayed heterologous neutralisation: 2 were infected with recombinant and 3 with non-recombinant viruses.

5.3.4 Association between NAb response and health status of infected animals

In addition, we tested for an association between the presence of an autologous NAb response and the health status of infected cats.
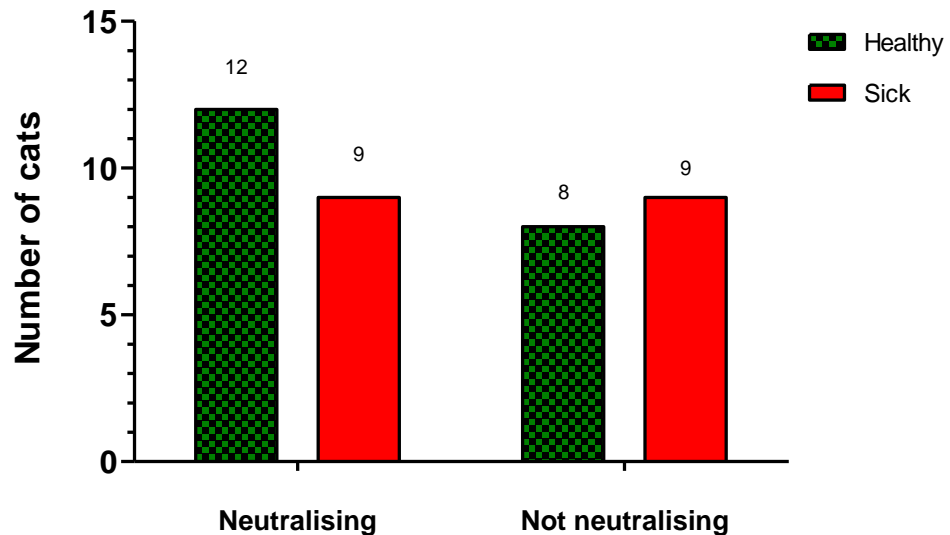
Figure 5-4 Relationship between the health status and neutralising antibody response. Within the group of cats expressing autologous NAb responses (n=21) there were 12 healthy and 9 sick cats. Within the group with no autologous neutralisation (n=17), there were 8 healthy and 9 sick cats. The differences between the groups were not statistically significant (Fisher's exact test, p=0.53).

Because health status assessments are subjective, we also examined the data to determine whether autologous NAbs might provide protection against a progressive decline in CD4 lymphocytes. As demonstrated in Table 5-3, all but two of the cats within the Memphis cohort (n=24) demonstrated a progressive decline in CD4+ T lymphocytes. The median ΔCD4 over the 18 month observation period was - 340 cells/ μl (ranging from -1120 to + 30 cells/μl).

Table 5-3 CD4 lymphocyte counts (K/μl) for cats in Memphis cohort for each time point (A, B, C and D) unless cat was deceased (D) or sample was not available (NA). ΔCD4 in the final column represents the difference between the first (A) and the last available sampling. All but two cats (highlighted in red) displayed a progressive decline in CD4 lymphocyte numbers over the 18 month observation period.

Cat	CD4 count (K/μl)				ΔCD4 (K/μl)
	Time point				
	A	B	C	D	
M2	1.74	0.36	0.76	0.62	-1.12
M29	1.48	0.62	0.93	0.62	-0.86
M1	0.87	0.36	0.34	0.26	-0.61
M15	0.87	1.03	1.09	0.47	-0.4
M8	0.55	0.21	0.2	0.15	-0.4
M49	0.41	0.31	0.27	0.04	-0.38
M28	1.23	0.89	1.77	0.9	-0.34
M14	0.45	0.29	0.15	0.14	-0.3
M25	0.36	0.39	0.68	0.09	-0.27
M20	1.5	0.81	NA	1.25	-0.25

M47	0.29	0.14	0.14	0.1	-0.19
M32	0.38	0.32	0.35	0.21	-0.17
M30	0.13	0.15	0.26	0.1	-0.04
M46	0.15	0.19	0.18	0.18	+0.03
M11	0.98	0.14	0.48	D	-0.5
M16	0.35	0.37	0.36	D	+0.01
M26	0.54	0.14	0.13	D	-0.41
M31	0.8	0.88	0.26	D	-0.55
M41	0.34	0.35	0.12	D	-0.22
M5	0.56	0.15	0.16	D	-0.4
M50	1.38	1.32	D	D	-0.07
M33	0.2	D	D	D	N/A
M3	0.33	D	D	D	N/A
M44	1.24	D	D	D	N/A

D – Deceased, NA – not available.

Within the Chicago group (n=14), 7 cats displayed progressive declines in CD4 T lymphocyte numbers, 5 displayed increased numbers, while 2 cats maintained their CD4 T lymphocyte numbers over a period of 12 months (Table 5-4). The median ΔCD4 was calculated as -15 cells/ µl (ranging from - 760 to + 240 cells/µl).

Table 5-4 CD4 lymphocyte counts (K/µl) for Chicago cats for each time point (A, B and C) unless the cat died during the study (D). ΔCD4 in the final column represents the difference between first (A) and the last available sampling. 5 cats (highlighted in red) displayed increasing CD4 lymphocyte numbers over the 12 month observation period).

Cat	CD4 count (K/µl)			ΔCD4 (K/µl)
	Time point			
	A	**B**	**C**	
P4	0.09	0.34	0.33	+0.24
P14	0.97	1.16	1.09	+0.12
P8	0.50	0.86	0.60	+0.10
P6	0.48	0.35	0.57	+0.09
P7	0.40	0.26	0.40	0.00
P11	0.45	0.35	0.45	0.00
P13	0.46	0.28	0.27	-0.18
P17	0.49	0.55	0.28	-0.21
P9	0.63	0.35	0.18	-0.45
P5	0.79	0.72	0.30	-0.49
P2	0.40	0.36	D	-0.03
P21	NA	0.93	0.57	-0.36
P22	NA	1.55	0.79	-0.76
P18	NA	0.73	0.75	+0.02

NA – not available

We then asked whether a strong autologous NAb response can protect cats against a progressive decline in CD4 T lymphocytes. We compared three groups of cats: 1) with absent (n=15) 2) moderate (n=5) and 3) strong (n=15) NAb

responses for which ΔCD4 values were available (n=35). As demonstrated in Figure 5-5, there was no evidence that cats with strong NAb responses were less likely to display progressively declining CD4 T lymphocyte numbers (median ΔCD4= -0.27 K/µl) as a similar range of ΔCD4 values was observed within the group of cats which failed to mount autologous NAb responses (median ΔCD4= -0.25 K/µl). There were no statistically significant differences between the two groups (unpaired 2-tailed t test, p=0.97). Three animals with moderate levels of NAb maintained their CD4 counts, while two animals showed declining CD4 lymphocyte numbers.
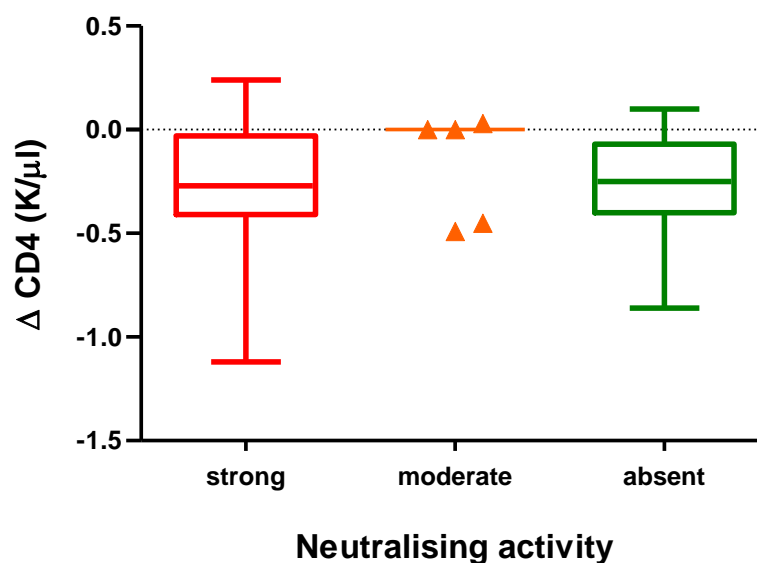
Figure 5-5 Changes in CD4 lymphocyte count (K/µl) over the course of infection in three groups of cats: 1) strong (n=15) (median -0.27, range -1.12 to +0.24)) 2) moderate (n=5) (median 0.0, range -0.49 to +0.03) and 3) with absent (n=15) (median -0.25, range -0.86 to +0.1) NAb responses.

5.3.5 Association between NAb response and survival time of infected animals

We examined the relationship between the autologous neutralisation responses and survival times of infected cats since the (estimated) time of infection. Kaplan-Meyer survival curves were constructed for 3 groups of cats expressing: 1) strong (n=16), 2) moderate (n=6) or 3) no (n=16) autologous neutralisation (Figure 5-6).

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The estimated median survival time for cats with strong autologous NAb responses was 2840 (range 1061 – 2840) days and for the group with no NAb was 2476 (range 1384 – 3387) days. There was insufficient data within the moderate neutralisation group to estimate median survival. A comparison of the Kaplan-Meyer curves (Log-rank (Mantel-Cox) test) revealed that survival times were not significantly different amongst the three groups (p=0.48). Further testing (Log-rank test for trend) revealed no significant trend between the three survival curves (p=0.36).
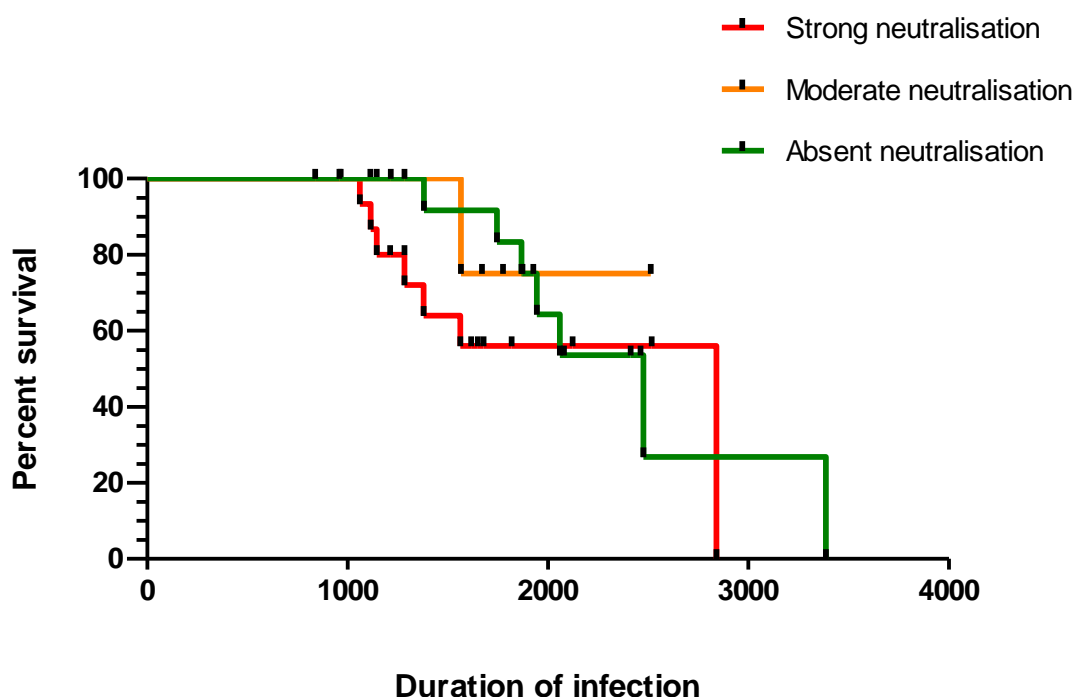
Figure 5-6 Kaplan-Meyer survival curves for cats with strong (n=16) (red), moderate (n=6) (orange) or absent (n=16) (green) autologous neutralisation.

5.3.6 NAbs in the terminal stages of disease

Finally, we asked whether the NAb response was preserved in the terminal stages of disease when the immune system was weakened. There were 13 cats in the study group with CD4 lymphocyte counts below 200 cells/µl recorded at the final sampling (Table 5-3 and Table 5-4), indicative of terminal infection. Plasma samples from 6 (46%) of these cats in the terminal stage of disease did not neutralise pseudotypes bearing homologous Envs, while plasmas from 7 (54%) of

the cats showed NAb responses against pseudotypes bearing autologous Envs, in spite of being in the terminal stages of disease.

5.4 Discussion

Here, we demonstrated that cats naturally infected with FIV have variable NAb responses against pseudotypes bearing autologous and heterologous Envs. When neutralisation assays were conducted against pseudotypes bearing Envs from autologous and heterologous viruses, we observed no correlation between either the health status or survival time following infection and the NAb response. Similar neutralisation profiles were observed for plasmas from healthy and unhealthy cats as well as cats that survived or died during the study period. Such findings argue against a role for NAb in controlling disease progression. Furthermore, our results demonstrate that FIV infected cats, regardless of strength of NAb response, showed progressive declines in CD4+ T lymphocyte numbers; NAb responses, even when potent, did not appear to protect against the loss of CD4 lymphocytes. A similar trend has been observed in HIV-1 infection, where the presence of autologous (Cecilia et al., 1999), (Geffin et al., 2003), (Schmitz et al., 2003) and cross-reactive NAbs was not associated with a prolonged AIDS free, asymptomatic period (Piantadosi et al., 2009)Piantadosi et al., 2009), (van Gils et al., 2010), (Euler et al., 2010). Several studies suggested that neutralisation breadth and potency depends on the duration of infection (Moog et al., 1997), (Deeks et al., 2006), (Sather et al., 2009). However, the results presented here do not support this proposal; rather, no association was observed between the duration of infection and the potency of autologous neutralisation.

Almost half of the cats examined showed strong autologous NAb responses, and in 24% of those animals a steady increase in neutralisation potency was observed during the course of infection. A similar increase in the strength of autologous NAb response has been reported for HIV-1 infection (Arendrup et al., 1992), (Richman et al., 2003), (Geffin et al., 2003). Although NAbs failed to protect against contemporaneous viruses, it was suggested that such antibodies might exert selection pressure on the emergence of viral variants of lower fitness, for example with decreased replicative capacity, and thus might indirectly delay HIV-1 associated disease progression (Friedrich et al., 2004), (Leslie et al., 2004). However, in this study no correlation was observed between the strength of NAb response and survival time in cats naturally infected with FIV.

The remaining half of the cats that were examined did not develop NAbs. A high level of antigenic stimulation is crucial for the development of broad and potent NAb responses (Deeks et al., 2006), (Rodriguez et al., 2007), (Doria-Rose et al., 2009), (Sather et al., 2009). It is possible that the immune systems of those cats that did not neutralise pseudotypes bearing autologous Envs had not had sufficient antigenic stimulation following infection to induce NAbs. This is most likely the case with cat M1, which acquired the virus vertically and failed to mount a NAb response. Given that a high viral load and high viral diversity following infection influences the development of potent and broad NAbs (Sather et al., 2009), (Piantadosi et al., 2009), (Euler et al., 2010), (Gray et al., 2011a), (Gray et al., 2011b), it is tempting to speculate that the cats in our study group which failed to develop NAb response may have had relatively low viral load set points compared to the cats which developed NAbs. An analysis of the viral and proviral loads in these cats would allow this speculation to be tested.

In addition, the marked, highly non-specific CD4+ T lymphocyte-dependent polyclonal hyper-gammaglobulinaemia (Recher et al., 2004), (Lang et al., 2007) that arises as an initial response to FIV infection might also contribute to the lack of effective neutralisation observed in this group. It is possible that high CD4+ T lymphocyte counts at the time of virus acquisition might be responsible for a non-specific, overwhelming hyper-gammaglobulinaemia and subsequent failure of NAb responses to develop (Euler et al., 2010), (Gray et al., 2011a). This scenario could also explain why only a small fraction of cats in our study demonstrated cross neutralisation of the heterologous GL-8 and B2542 pseudotypes, regardless of whether they were infected with recombinant or non-recombinant viruses.

A study examining a similar number of HIV-1 infected individuals (n=40) for the breadth of neutralisation found that 17.5% of patients developed broadly NAbs (Gray et al., 2011a). Other studies reported higher numbers of individuals with broadly NAb, up to 30% of samples examined (Doria-Rose et al., 2009), (Gray et al., 2009), (Piantadosi et al., 2009), (Sather et al., 2009), (Euler et al., 2010). In contrast, only 13% of plasma samples collected from our study group displayed cross reactivity. This may suggest that Cr-NAb are rarer in FIV infected cats than in HIV-1 infected individuals. However, in this study plasma samples were tested

for neutralisation against only two reference pseudotypes bearing FIV Envs. It is possible that testing a greater number of pseudotypes bearing Envs from more strains of FIV could have revealed a higher prevalence of Cr-NAbs. Had we found broadly and strongly neutralising plasma, we could have included it in our assays as a control to tackle the possible differences in infectivity of pseudotypes bearing wild type *envs.*

It has been suggested that neutralisation breadth develops slowly over a period of two to four years post seroconversion (Mikell et al., 2011), (Gray et al., 2011a). Given the duration of infection in our study group, it would be predicted, by analogy, that more cats would have developed Cr-NAbs, but this scenario was not supported by the data presented here.

Finally, differences in neutralisation profiles might also be related to different kinetics of viral replication between various strains of FIV infecting cats in the study group. It is possible that more virulent, and more replication competent, strains are more likely to induce effective humoral responses compared to isolates with a lower replicative capacity.

The results presented here demonstrate that humoral immunity was preserved in cats that subsequently developed AIDS (CD4 counts below 200 cells/μl), consistent with the observation that the rate of viral evolution slows during the terminal stage of disease (Chapter 4). Thus, autologous antibodies elicited during the earlier stages of infection remain capable of neutralisation, owing to the relatively high genetic stability of the virus terminally. However, these NAb, although capable of neutralisation *in vitro* and while preserved in terminal disease, fail to prevent disease progression.

In this study we tested pseudotypes bearing 1 to 18 Env variants from each time point to assess sensitivity to neutralisation by autologous plasma. It is difficult to assess how representative the cloned Envs were, compared to the pool of Env variants within the cats. Nevertheless, the alternative approach of testing a single representative clonal Env variant would have led to an underestimation of viral diversity within the host. The sensitivities to neutralisation amongst pseudotypes bearing Env variants isolated from each cat tended to be similar;

this suggests that, where changes were observed, these were likely a true indication of the range of neutralisation sensitivity and resistance amongst the pool of variants in individual cats.

Taken together, the results presented here demonstrated that humoral immunity did not significantly alter the clinical course of natural FIV infection. One explanation for the lack of correlation is that any factor promoting a strong NAb response may negatively influence other immune responses, leading to exhaustion of polyfunctional CD4 and CD8 lymphocytes (Harari et al., 2004), (Betts et al., 2006), (Streeck et al., 2008). In light of recent evidence from studies with HIV-1, it is plausible that cell mediated immunity, as well as host genetic factors, are more likely to influence the clinical course of infection than NAbs (Nomura and Matano, 2012), (Huang et al., 2012a).

Chapter 6. Cell tropism of FIV

Data from both *in vitro* culture experiments and *in vivo* experimental FIV infections suggest that the FIV-receptor interaction changes as disease progresses, similar to the switch of co-receptor usage from CCR5 to CXCR4 observed during the course of HIV-1 infection.

In order to validate experimental observations and assess whether similar findings extend to naturally acquired infection, we analysed the cell tropism of sequential FIV Env variants isolated from 38 cats. In this study we employed the previously proposed model (Willett and Hosie, 2008) wherein "early" viral variants which demonstrate a strong affinity interaction with CD134 evolve during the course of infection towards CD134 independence.

It was demonstrated that sick cats were more likely to harbour "late" viruses than healthy animals, suggesting that variants with the "late" phenotype are likely to contribute to the decline in CD4 lymphocyte counts and disease progression. Depending on the grouping criteria used, we found that putative "late" viruses were present in 61.5-75% of cats presenting with clinical signs. In contrast, variants with a similar "late" phenotype were present in only 22.7-36% of healthy cats. There was no correlation between the strength of the autologous neutralisation response and the existence of viruses with "early" or "late" phenotypes, suggesting that other mechanisms may drive the emergence of the "late" phenotype during the course of infection.

These results are consistent with a proposed model in which FIV isolates requiring complex determinants on the host cell receptor for infection evolve towards requiring a less stringent interaction during the course of infection. Such a shift may contribute to more widespread viral dissemination, extending to other cellular compartments and subsequently leading to disease progression.

6.1 Introduction

The interaction between the virus and its receptor on the susceptible cell is the first event of lentiviral infection. The specificity of this interaction influences viral cell tropism and pathogenicity (Willett et al., 2008).

Primate immunodeficiency retroviruses predominantly target helper T cells using the CD4 molecule as an entry receptor (Sattentau and Weiss, 1988) and subsequently induce a progressive decline in CD4 expressing cells during the course of infection (Weiss, 1993), (Douek et al., 2009). For productive infection to occur, the initial binding with CD4 is followed by a viral Env interaction with chemokine co-receptors, including CXCR4 (Bleul et al., 1997), (Feng et al., 1996), CCR5 (Alkhatib et al., 1996), (Trkola et al., 1996a). The utilisation of the two major co-receptors partially explains the differential cell tropism of primate lentiviruses. Generally, CXCR4-dependent strains are T-cell tropic (Correa and Munoz-Fernandez, 2001), while CCR5-dependent strains preferentially target memory CD45RA+ T cells and macrophages (Collman et al., 1989). Nevertheless, macrophage tropic X4 strains (Simmons et al., 1998) and dual tropic R5X4 strains, which utilize both co-receptors (Yi et al., 1999), (Whitcomb et al., 2007) have been identified. Furthermore, co-receptors other than CXCR and CCR5 such as CCR8 (Rucker et al., 1997), CCR3 (Doranz et al., 1996), GPR1 (Shimizu et al., 2000), GPR15 (Farzan et al., 1997), CXCR6 (Alkhatib et al., 1997) and RDC1 (Willey et al., 2003) and six other chemokine receptors (Meehan et al., 2010) also can be used by HIV and SIV to establish productive infection *in vitro*, highlighting the complex role of the viral Env in cell tropism.

It has been demonstrated for HIV-1 that an assessment of cell tropism can be useful to monitor disease progression (Waters et al., 2008), (Shepherd et al., 2008). For example, R5 strains predominate during the early stages of infection and there is a subsequent evolution towards CXCR4 usage as disease progresses (Shankarappa et al., 2001). The emergence of X4 variants is usually accompanied by a rapid decline in CD4 lymphocyte numbers and the onset of immunodeficiency (Jekle et al., 2003). It has been reported that this cell tropism pattern is observed in only ~50% of infected individuals (Regoes and Bonhoeffer, 2005); however, recent studies employing more sensitive detection

techniques reported this proportion as higher (Wilkin et al., 2011). It is not well understood why this co-receptor switch occurs in only a fraction of infected individuals (Regoes and Bonhoeffer, 2005). The switch from CCR5 to CXCR4 usage has been associated with the loss of potential N-linked glycosylation sites (PNGS) within the V3 region of the SU domain of Env (Pollakis et al., 2001) as well as with mutations that increase the positive charge of the V3 region (Cardozo et al., 2007). Furthermore, mutations within other domains of SU and TM, such as V1/V2 and C4 respectively, have also been linked with altered co-receptor usage by various strains of HIV-1 (Hoffman et al., 2002), (Otto et al., 2003), (Nabatov et al., 2004).

FIV, like its human and simian counterparts, induces a progressive decline of CD4+ T lymphocytes, subsequently leading to immunodeficiency and death (Yamamoto et al., 1989), (Novotney et al., 1990). FIV shares a similar cell tropism to HIV and productively infects T lymphocytes (Novotney et al., 1990), B cells (English et al., 1994), macrophages (Brunner and Pedersen, 1989), monocytes (Dow et al., 1999), megakaryocytes (Beebe et al., 1992), dendritic cells (Reggeti et al., 2008), microglia and astrocytes (Nakagaki et al., 2001), (Johnston et al., 2002), (Hein et al., 2003).

Despite sharing a similar cell tropism and pathogenicity, and in contrast to its human counterpart, FIV utilizes feline CD134 as the primary entry receptor (Shimojima et al., 2004), (de Parseval et al., 2004a) and CXCR4 as a co-receptor (Willett et al., 1997b), (Richardson et al., 1999). No other co-receptors for FIV have been identified to date (Willett et al., 2010), (Hu et al., 2010). A determinant of FIV tropism is located within the V3 region of Env (Hohdatsu et al., 1996), (Lerner and Elder, 2000), (Hu et al., 2010).

CD134, the primary entry receptor for FIV, is a 43 kDa glycoprotein (Hong et al., 2010) which belongs to the tumour necrosis factor/nerve growth factor receptor superfamily (TNFR/NGFR) (Locksley et al., 2001) and is expressed on feline CD4+ T cells *in vitro* (Shimojima et al., 2004) and *in vivo* (Joshi et al., 2005), consistent with the cell tropism of FIV and the progressive depletion of the CD4+ lymphocyte subset that occurs during the course of FIV infection. FIV binds specifically to cells expressing CD134 but, when pre-treated with soluble CD134,

can productively infect CD134(-) CXCR4(+) cells (de Parseval et al., 2005). The structure of CD134 resembles that of classical TNFR molecules, with an extracellular binding domain comprising three cysteine rich domains (CRD1, CRD2, CRD3) and an additional C-terminal domain (Bodmer et al., 2002).

Various strains of FIV differ in the affinity of their interaction with the primary binding receptor (Willett et al., 2006a). The PPR and B2542 strains (thought to be late, chronic isolates) of FIV achieve productive infection following an interaction with residues within the cysteine rich domain 1 (CRD1) of CD134 (de Parseval et al., 2005), (Willett et al., 2006b) while primary strains such as GL-8 and CPG41 (defined as early, acute isolates) require a more stringent interaction involving not only CRD1 but also determinants within the cysteine rich domain 2 (CRD2) of CD134 (Willett et al., 2006a), (Willett et al., 2006b), (Willett et al., 2010). It has been proposed that viruses which require only CRD1 of CD134 (or are able to infect *via* CXCR4 alone) emerge during the terminal stage of disease (Willett and Hosie, 2008). Therefore, it is likely that FIV may evolve *in vivo* towards a less CD134-dependent or CD134-independent mechanism of infection, facilitating viral spread to different cellular compartments (Willett et al., 2006b) consistent with the expanded cell tropism observed during the course of infection (English et al., 1993), (Dean et al., 1996).

Experimental data support this hypothesis; infection with the PPR strain of FIV can occur in CD134 (-) cells simply by overexpression of CXCR4 (de Parseval et al., 2004b), which is not the case with the primary isolate GL-8 (Willett et al., 2002). It has been speculated that FIV variants that emerge in sick cats differ from those isolated during the acute phase of infection (Willett and Hosie, 2008), similar to the switch from CCR5 to CXCR4 co-receptor usage observed during the course of HIV-1 infection. Furthermore, late, chronic FIV isolates require a lower affinity interaction with CD134; such isolates have a more exposed CXCR4 binding site, rendering them more susceptible to neutralising antibodies (Willett et al., 2010). It has been proposed that the emergence of more readily neutralised variants with broader cell tropism is a result of a weakened immune system, which in sick animals mounts an insufficient autologous humoral response (Willett et al., 2006b).

The switch of coreceptor usage in HIV-1 infection is a predictive indicator of disease progression (Weiser et al., 2008), (Shepherd et al., 2008), (Lin and Kuritzkes, 2009), (Wilkin et al., 2011). The emergence of X4 viruses coincides with the accelerated decline in CD4+ lymphocyte numbers and the subsequent onset of AIDS (Connor et al., 1997), (Wilkin et al., 2011). Taken together with the results of studies on experimental FIV infection and proposed hypotheses (Willett et al., 2006a), (Willett and Hosie, 2008), (Willett et al., 2009), (Willett et al., 2010), we asked whether a similar pattern of FIV evolution (from a complex to a more simple interaction with CD134) is indicative of disease progression in cats naturally infected with FIV. Do the experimental data extend to natural infection? Can the onset of immunodeficiency in FIV infected cats be predicted by defining the receptor usage of their autologous viral variants?

6.2 Materials and methods

6.2.1 FIV pseudotypes

HIV(FIV) pseudotypes bearing wild type and reference FIV Envs (GL-8 as the representative early, acute isolate and B2542 as the representative late, chronic isolate) were prepared according to the protocol described in Chapter 2. Pseudotypes bearing wild type FIV Envs which were tested for neutralisation (Chapter 5) were subsequently examined in receptor usage assays. Accordingly the cell tropism was assessed for 1 to 23 pseudotypes bearing wild type Envs isolated from each of 38 cats.

6.2.2 Receptor usage assay

MCC FFF, MCC F(FH)H, MCC HHH and CLL-CD134 cells (Willett et al., 2006b) (Figure 6-1) were seeded (1×10^4 cells per well) in triplicate in a CulturPlate™-96 assay plate (Perkin Elmer). The cells were infected with 50 µl of each HIV-luc (FIV wild type Env) pseudotype, alongside reference controls of HIV-luc (GL-8) and HIV-luc (B2542).
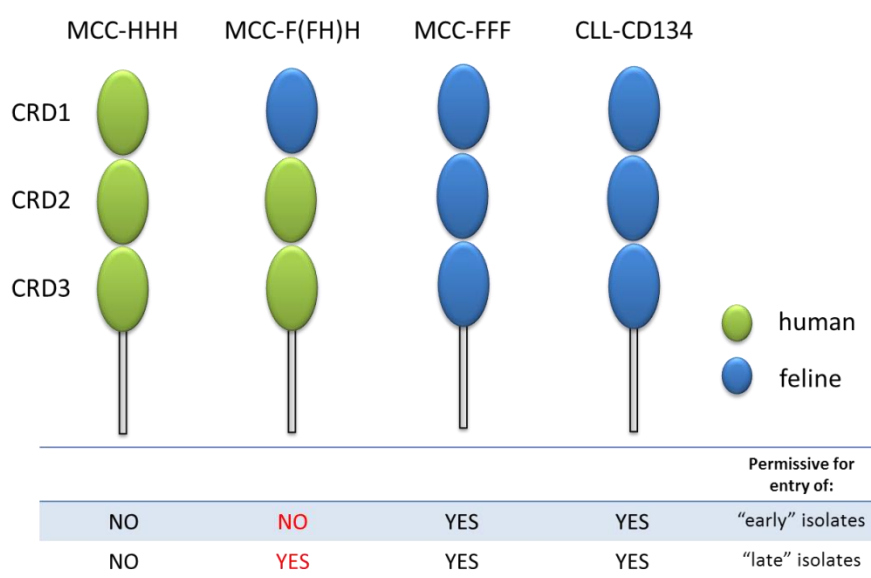
Figure 6-1 Schematic representation of the panel of cell lines bearing chimaeric CD134 molecules, used to assess receptor usage by isolates of FIV. CD134 consists of 3 CRDs; MCC-HHH comprises the entire human CD134, while CLL-CD134 comprises the entire feline CD134. These constructs served as negative and positive controls respectively. MCC-FFF stably expresses feline CD134 while MCC-F(FH)H bears a chimeric feline/human CD134 and is permissive for the entry of prototypic "late" isolates of FIV such as B2542 (Willett et al., 2006a). Adapted from (Willett et al., 2006a).

After 72 hours incubation at 37°C in an atmosphere of 5% CO_2, the luciferase activity was quantified following the addition of 100 μl of Steadylite HTS™ (Perkin Elmer) luciferase substrate and single photon counting using a MicroBeta luminometer (Perkin Elmer).

The MCC cells used in the receptor usage assays had been stably transduced with different chimeric constructs of feline/human CD134 (Willett et al., 2006b). As shown in Figure 6-1, each of three MCC cell lines expressed three CD134 molecules: 1) MCC-FFF, 2) MCC-F(FH)H and 3) MCC-HHH, where F and H denotes feline and human cysteine reach domains (CRDs) of CD134 respectively. CLL-CD134 cells expressed the complete feline CD134 molecule and served as a positive control in the assay. This panel of cell lines allowed a comparison of CD134 utilization by diverse strains of FIV (Willett et al., 2006a), (Willett et al., 2006b). The control pseudotype bearing the GL-8 Env (an early, acute isolate) required a strong affinity interaction with CD134 and was only able to infect CLL-CD134 and MCC-FFF cells. The control B2542 pseudotype (a late, chronic isolate) required a less stringent interaction with CD134, displayed broader cell tropism and could infect cells bearing feline CRD1 only in the context of feline/human CRD2 and human CRD3 (cell line MCC- F(FH)H).

The affinities of the receptor interactions with wild type FIV Envs isolated in this study were compared to the interactions with the GL-8 and B2542 Envs, in order to assess receptor usage by Env variants isolated at different stages of natural FIV infection.

6.2.3 Site directed mutagenesis

A PCR based mutagenesis strategy, employing The Quick Change II site-directed mutagenesis kit (Agilent Technologies, UK), was used to introduce the desired mutations into the *env* gene. PCR reactions and thermo cycling conditions were set up as per the manufacturer's instructions. Primers and their description are detailed in Table 6-1.

Following PCR amplification, the methylated parental DNA template was digested with DpnI endonuclease as per the manufacturer's instructions. Treated

DNA was subsequently transformed into XL1-Blue Supercompetent® Cells (Agilent Technologies, UK) and the introduced mutations were confirmed by sequencing on an ABI 3700 genetic analyser (Applied Biosystems).

Table 6-1 Oligonucleotides used for the site directed mutagenesis of clones isolated from cat M11 with description of sequence targets within the *env.*

Primer sequence (5' to 3')	Used for:
ACCTTATTGTACATTTCAATATGACAAGAGCTGTGGAAATGT	Fwd primer to reinstate Ala520 to Thr in C242
ACATTTCCACAGCTCTTGTCATATTGAAATGTACAATAAGGT	Rev primer to reinstate Ala520 to Thr in C242
TGAAGAAGAATACTTATCAATGGAATTGAGGAGAAATGGCAGGC	Fwd primer to reinstate Lys838 to Glu in C242
GCCTGCCATTTCTCCTCAATTCCAATTTCATTGATAAGTATTCTTCTTCA	Rev primer to reinstate Lys838 to Glu in C242
ACCTTATTGTACATTTCAATATGGCAAGAGCTGTGGAAATGT	Fwd primer to reinstate Thr520 to Ala in C164
ACATTTCCACAGCTCTTGCCATATTGAAATGTACAATAAGGT	Rev primer to reinstate Thr520 to Ala in C164

Site directed mutagenesis studies on viral variants isolated from cat M11C were designed by myself but were performed by Nicola Logan and Kuan Liang Liu as part of the latter's MRes degree project placement in our laboratory.

6.3 Results

Pseudotypes bearing FIV Envs isolated from 38 infected cats displayed a broad spectrum of cell tropism, as determined by the receptor usage assay based on cells expressing human/feline chimaeric CD134 molecules, with the results as summarized in Table 6-2. Of the 284 pseudotypes tested bearing wild type *envs*, 219 (77.1%) utilised CD134 in the same manner as the representative "early" GL-8 pseudotype. Forty four (15.5%) pseudotypes displayed CD134 utilisation more similar to that of the representative "late" B2542 pseudotype. Sixteen (5.6%) displayed an intermediate pattern of receptor usage, with results between those of the reference GL-8 and B2542 pseudotypes. Interestingly, five pseudotypes (1.8%) achieved higher titres on MCC F(FH)H cells than the B2542 pseudotype, suggesting that these pseudotypes may have the capacity to infect CD134 (-) target cells, binding CXCR4 directly. As a representative example, the receptor usage of pseudotypes bearing selected Env variants from cat M31 are shown in Figure 6-2.

Table 6-2 Mode of CD134 utilisation displayed by 284 pseudotypes bearing Envs from 38 cats. Cats were classified according to their health status (H=healthy, S=sick), CD4 count (< and > 200 cells/µl) and survival during the study period (A=alive, D=deceased). Mode of CD134 interaction: 1) GL-8 like-stringent, high affinity utilization of CRD-1 and requiring determinants on CRD-2 of CD134; 2) B2542 like-requiring only CRD1 of CD134; 3) >B2542-very low interaction with CD134, isolates potentially can utilise CXCR4 alone; 4) undetermined-intermediate requirement for determinants within the CRD2 of CD134 (neither GL-8 nor B2542-like). Numbers and percentages (in brackets) of pseudotypes classified in each group are shown.

Cat	Alive/ Dead	Health status	CD4 count	Mode of CD134 interaction				No. of pseudotypes tested
				GL-8 like	B2542 like	>B2542	undetermined	
M28	A	H	< 200	11 (100%)	0	0	0	11
M47	A	H	< 200	8 (100%)	0	0	0	8
M8	A	S	< 200	2 (100%)	0	0	0	2
P9	A	S	< 200	6 (100%)	0	0	0	6
M1	A	H	> 200	4 (100%)	0	0	0	4
M2	A	H	> 200	2 (100%)	0	0	0	2
M29	A	H	> 200	3 (100%)	0	0	0	3
P1	A	H	> 200	3 (100%)	0	0	0	3
P11	A	H	> 200	7 (100%)	0	0	0	7
P14	A	H	> 200	9 (100%)	0	0	0	9
P18	A	H	> 200	1 (100%)	0	0	0	1
P21	A	H	> 200	6 (100%)	0	0	0	6
P22	A	H	> 200	2 (100%)	0	0	0	2
P4	A	H	> 200	4 (44.4%)	2 (22.2%)	0	3 (33.3%)	9
P5	A	H	> 200	7(87.5%)	1 (12.5%)	0	0	8

| P6	A	H	> 200	8 (100%)	0	0	0	8
M15	A	S	> 200	6 (66.6%)	3 (33.3%)	0	0	9
M20	A	S	> 200	1 (100%)	0	0	0	1
M32	A	S	> 200	3 (75%)	1 (25%)	0	0	4
P13	A	S	> 200	8 (88.9%)	1 (11.1%)	0	0	9
P17	A	S	> 200	9 (100%)	0	0	0	9
P7	A	S	> 200	1 (50%)	0	0	1 (50%)	2
P8	A	S	> 200	5 (100%)	0	0	0	5
M25	D	H	< 200	3 (50%)	2 (33.3%)	0	1 (16.7%)	6
M46	D	H	< 200	7 (87.5%)	1 (12.5%)	0	0	8
M49	D	H	< 200	6 (100%)	0	0	0	6
M14	D	S	< 200	9 (90%)	1 (10%)	0	0	10
M26	D	S	< 200	4 (66.7%)	2 (33.3%)	0	0	6
M30	D	S	< 200	15 (94%)	1 (6%)	0	0	16
M33	D	S	< 200	2 (11%)	16 (84%)	1 (5%)	0	19
M41	D	S	< 200	9 (75%)	1 (8.3%)	1 (8.3%)	1 (8.3%)	12
M5	D	S	< 200	5 (55.6%)	3 (33.3%)	0	1 (11.1%)	9
M11	D	H	> 200	17 (81%)	2 (10%)	1 (5%)	1 (5%)	21
M50	D	H	> 200	2 (100%)	0	0	0	2
P2	D	H	> 200	16 (100%)	0	0	0	16
M16	D	S	> 200	0	1 (14.3%)	0	6 (85.7%)	7
M3	D	S	> 200	0	1 (50%)	0	1 (50%)	2
M31	D	S	> 200	9 (53%)	5 (29%)	2 (12%)	1 (6%)	17
M44	D	S	> 200	2 (100%)	0	0	0	2
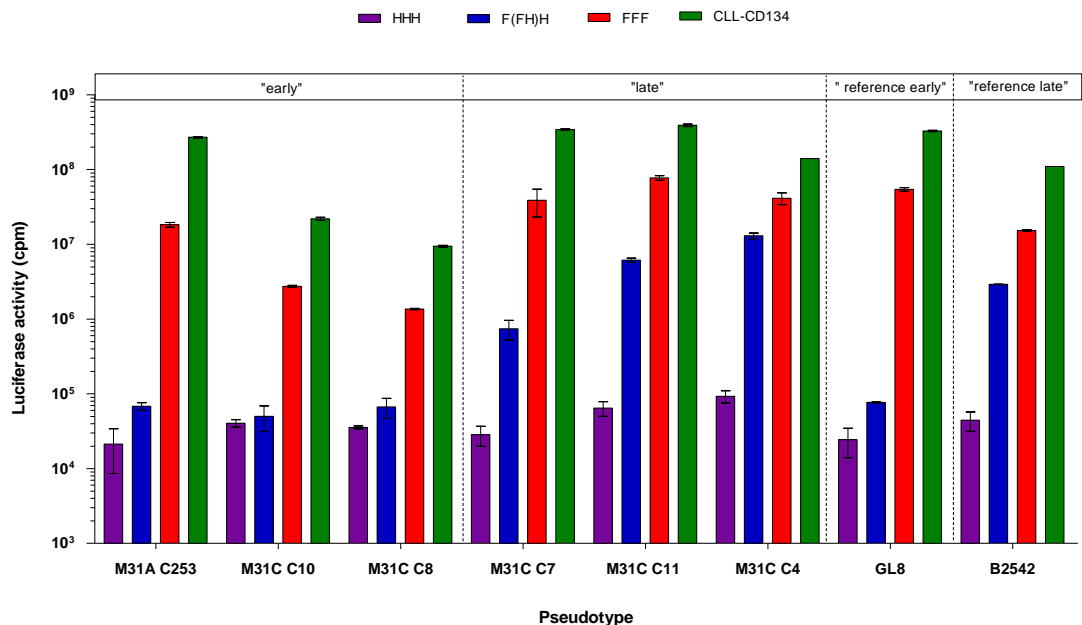
Figure 6-2 Representative example of results from receptor utilisation test of 6 selected autologous viral variants isolated from cat M31 and 2 reference pseudotypes, GL-8 (prototypic "early") and B2542 (prototypic "late"). Note the differences in titres on MCC F(FH)H cells (blue) and subsequent pseudotype classification as "early" and "late". The

mean luciferase counts (cpm) for each cell line with standard errors (n=3) are presented for MCC HHH, MCC F(FH)H, MCC FFF and CLL-CD134 cells, shown in purple, blue, red and green respectively.

6.3.1 "Late" Env phenotype prevalent amongst terminally infected cats

During the study period 16/38 cats (42.1%) died, while 22/38 cats remained alive (57.9%). Amongst the deceased cats, 12/16 (75%) gave rise to at least one FIV Env variant that utilised CD134 in a similar manner to the representative "late" B2542 Env. In the "alive" group, 5/22 cats (22.7%) gave rise to at least one Env variant demonstrating similar receptor usage to B2542 Env. The remaining 17/22 cats, (77.3%) in the "alive" group had only GL-8-like Envs, with the exception of cat P7 which harboured both GL-8-like Env variants as well as a variant of intermediate, undetermined receptor utilisation. The median number of pseudotypes bearing Env variants from each individual and assessed for receptor usage in this analysis was 6 and 8.5, for alive and deceased animals respectively. By comparing receptor utilisation between the "alive" and "deceased" cats (Figure 6-3), we observed that "late" B2542-like Env variants were more common in cats that died during the study; in comparison, healthy cats were more likely to harbour "early" GL-8-like Envs (Fisher's exact test, p=0.0026).

6.3.2 Receptor usage of viruses isolated from healthy and sick cats

Employing different grouping criteria to analyse the data, we compared the receptor usage of Env variants between healthy and sick cats (Figure 6-3). Of the 38 cats examined, 19 (50%) were classified as healthy and the remaining 19 cats (50%) displayed clinical signs and were therefore classified as sick. The median number of pseudotypes assessed for receptor usage from each healthy and sick cat was 7, minimising any bias in the analysis. Amongst the group of nineteen sick cats, twelve cats (63.2%) had at least one Env variant that utilized CD134 in a similar manner to the representative "late" B2542 Env. However, this pattern was not observed amongst the healthy group; in contrast, 5/19 healthy cats (26.3%) gave rise to at least one B2542-like Env variant. A statistically significant difference was observed between the two groups; sick cats were more likely to

harbour B2542 like "late" Env variants than healthy cats (p=0.0489, Fisher's exact test).

Since an assessment of health status may be subjective, we further compared receptor utilisation by pseudotypes with CD4 lymphocyte values collected at the last sampling time point from each cat. Thirteen of 38 cats (34.2%) were considered to be in the AIDS stage of disease (CD4 lymphocyte counts below 200 cells/µl). Eight of these 13 cats (61.5%) gave rise to at least one B2542-like Env variant. Amongst the remaining 25 cats (65.8%) with CD4 lymphocyte numbers greater than 200 cells/µl, nine cats (36%) gave rise to at least one Env variant with similar receptor usage to B2542 Env. Although those nine animals were arbitrarily classified in the non-AIDS group according to their CD4 values, it was apparent that they had relatively low CD4 lymphocyte counts (median value of 330 cells/µl) compared to the remaining 16 cats (median CD4 lymphocyte count was 610 cells/µl), suggesting that the existence of B2542-like Envs is associated with lower CD4 lymphocyte values. By comparing these two groups (CD4> 200 cells/µl and CD4< 200 cells/µl, median number of pseudotypes assessed for each group being 7 and 8 respectively), we observed a similar trend as previously; Env variants with the "late" phenotype were more common in terminally sick cats. Nevertheless there was no statistically significant correlation between the existence of "late" Env variants and CD4 counts below 200 cells/µl compared to cats with CD4 counts above 200 cells/µl (Fisher's exact test, p=0.1776).

6.3.3 Prognostic value of receptor utilisation for predicting disease progression

As demonstrated above, by employing three different grouping criteria, we identified a trend for the presence of "late" B2542-like Env variants in sick cats compared to healthy ones. Healthy cats with CD4 counts >200 cells/µl were more likely to harbour "early" GL-8-like Env variants.
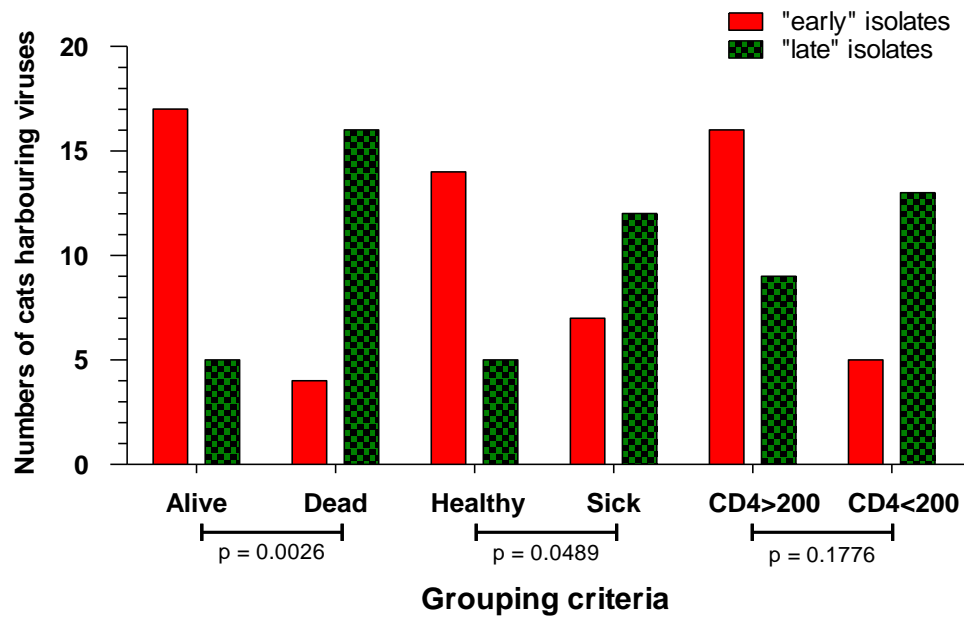
Figure 6-3 Numbers of cats harbouring prototypic "early" and "late" Envs. Cats were classified into 3 groups: 1) alive/deceased 2) healthy/sick 3) CD4 lymphocyte counts above and below 200 cells/µl. The cats which died during the study, displayed clinical signs (sick) or had CD4 counts below 200 cells/µl were more likely to harbour at least one prototypic "late" Env variant (green). Conversely, the majority of alive, healthy cats with CD4 counts >200 cell/µl were more likely to harbour only "early" GL-8-like Env variants (red). P-values (Fisher's exact test) are denoted below x axis.

Taking these results together, depending on grouping criterion (alive/deceased, healthy/sick or absolute CD4 lymphocyte counts above and below 200 cells/µl), at least one "late" Env variant was present in 61.5 to 75% of animals considered as being in the terminal, symptomatic stage of disease. In contrast, such "late" Env variants were only present in 22.7% to 36% of cats considered to be in the "asymptomatic" stage of FIV infection.

6.3.4 Correlation between FIV receptor utilisation and neutralising antibody response

To determine whether the existence of putative "late" Env variants is associated with the presence or absence of neutralising antibodies, neutralisation profiles from cats harbouring at least one "late" Env variant were compared against those that harboured only "early" GL-8-like Envs. Amongst the 17 cats harbouring at least one "late" Env variant, 10 cats neutralised pseudotypes

bearing homologous Envs while the remaining 7 cats did not (Fisher's exact test, p=0.75).

6.3.5 Loss of a single PNGS within the V5 region of Env alters receptor utilisation

When examining the receptor utilisation of Env variants from 38 cats, we observed a complete spectrum of receptor utilisation by Env variants isolated from cat M11. We examined pseudotypes bearing twenty-one Env variants isolated from three time points up until the cats' death. As demonstrated in Figure 6-4, 17/21 (81%) of M11 Env pseudotypes demonstrated an "early" phenotype; these Env variants utilised CD134 in the same way as the representative "early" GL-8 Env. Two Env variants (10%) utilised CD134 in the same manner as the prototypic "late" B2542, indicating a late phenotype. One Env variant (5%) displayed an intermediate requirement for CRD2 on CD134. Strikingly, the remaining pseudotype M11A C242 (5%), infected MCC F(FH)H cells more effectively, achieving higher titres than the representative "late" B2542 Env pseudotype. Analysis of peptide sequence alignments identified two unique residues: 1) T520A and 2) E838K that were present in M11A C242 Env but not in any other Env variant from this cat. Furthermore, by analysing potential glycosylation patterns, we observed that substitution of T520 with A520 resulted in the loss of a PNGS, suggesting that T520A rather than E838K was associated with altered utilisation of CD134.

To confirm this observation, PCR-based site directed mutagenesis was employed to reinstate 1) alanine at position 520 to threonine and 2) lysine at position 838 to glutamic acid in order to create M11A C242 A520 and M11A C242 K838 mutants respectively. By comparing the receptor utilisation of these mutant Envs with the wild type M11A C242 Env, we found that the M11A C242 A520 mutant achieved a 10 fold lower titre on MCC F(FH)H cells compared to either the parental wild type M11A C242 or the M11A C242 K838 mutant Env, confirming that the T520, with the subsequent loss of a PNGS, played an important role in receptor utilisation (Figure 6-5).
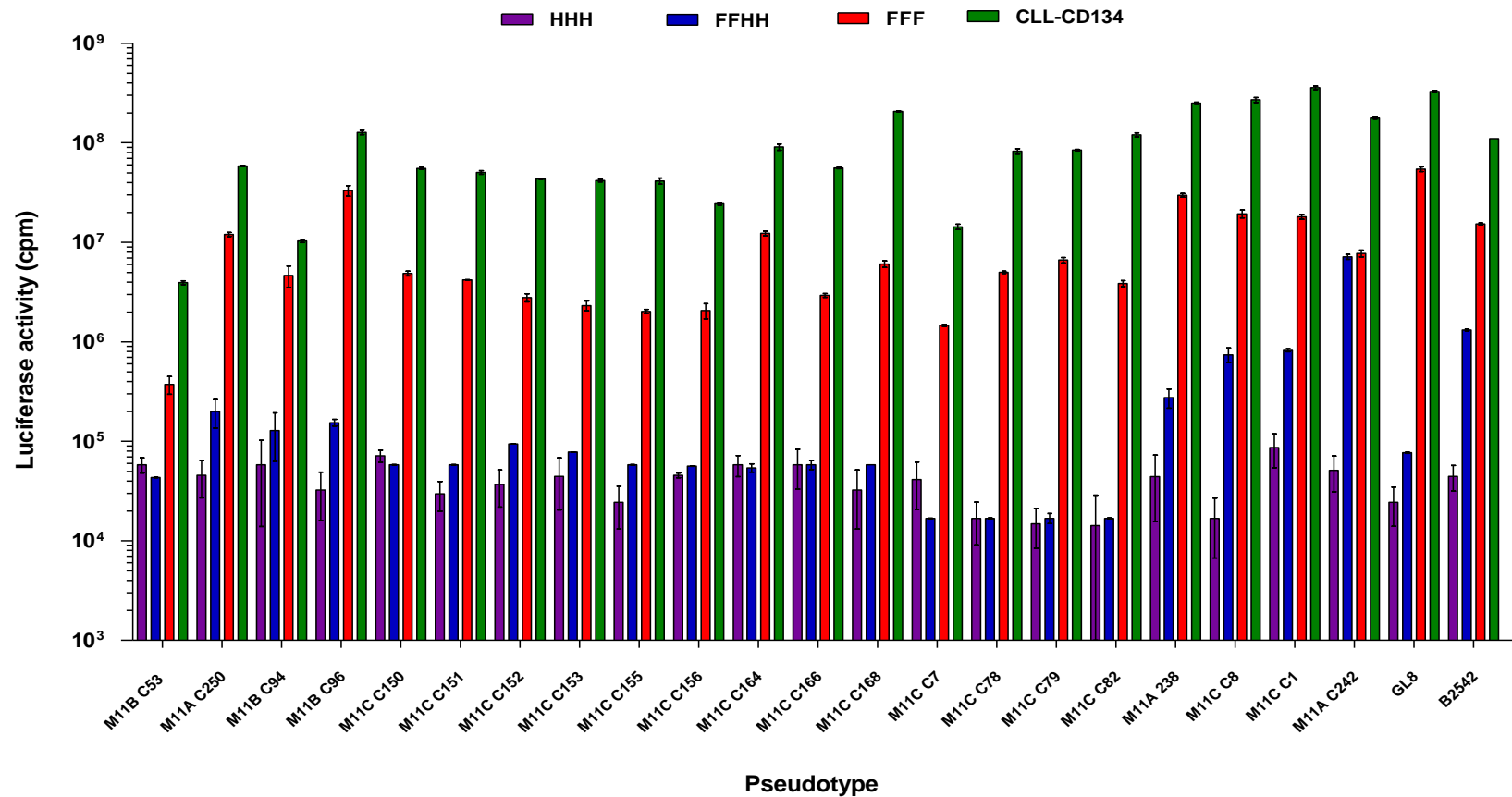
Figure 6-4 Receptor usage of 21 autologous viral variants isolated from cat M11 at 3 time points (A, B and C) and 2 reference pseudotypes bearing the Envs of GL-8 (prototypic "early") and B2542 (prototypic "late") assessed on MCC HHH (purple), MCC F(FH)H (blue), MCC FFF (red) and CLL-CD134 (green). Seventeen (17/21, 81%) pseudotypes expressed the same mode of interaction with CD134 as GL-8, two (2/21, 10%) interacted with CD134 in the same manner as prototypic B2542 and one (1/21, 5%) showed an intermediate requirement for the CRD2 of CD134. M11C C242 (1/21, 5%), infected MCC F(FH)H cells more effectively than B2542. Each bar represents mean luciferase activity (cpm) ± standard error (n=3).
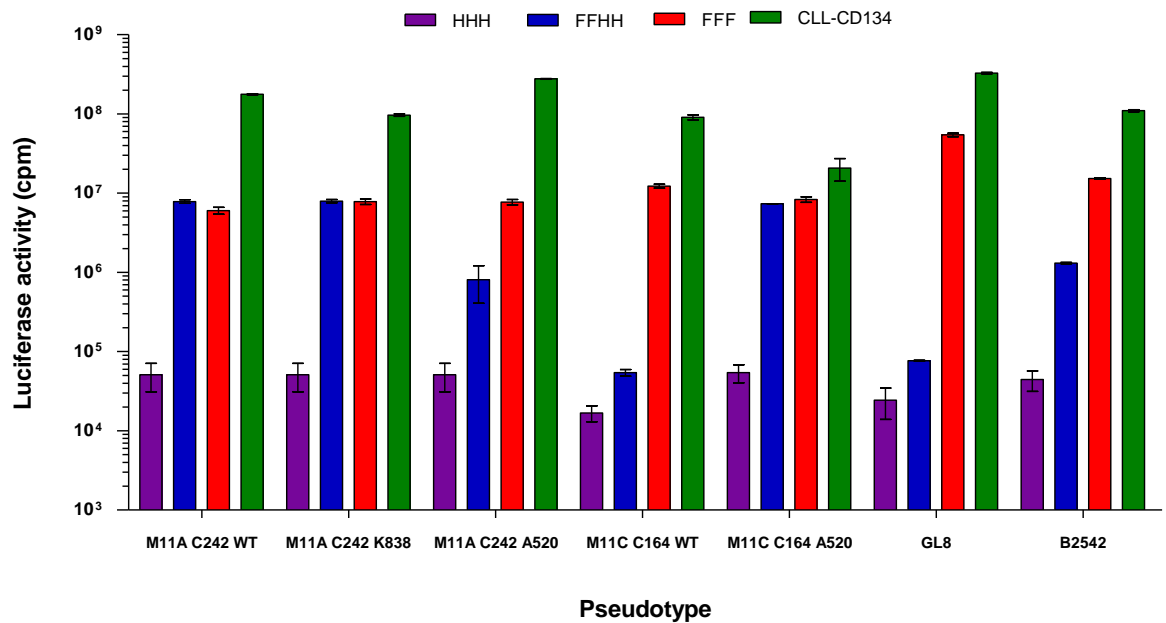
Figure 6-5 Results of site directed mutagenesis; a PNGS within the V5 region was associated with receptor utilisation. Receptor utilisation by M11A C242 WT was compared with the respective K838 and A520 mutants. To confirm the role of the PNGS at position 520, receptor usage of M11A C164 was compared with the respective A520 mutant. GL-8 and B2542 pseudotypes were tested in parallel as representative "early" and "late" pseudotypes respectively. Each bar represents mean luciferase activity (cpm) ± standard error (n=3).

To confirm the role of A520 and the subsequent loss of a PNGS determining receptor utilisation, mutants of the Env clone M11C C164, (which expressed the "early" phenotype of CD134 usage, similar to the representative "early" GL-8 Env) were constructed. Substitution of T520 with A520 resulted in the generation of mutant M11C C164 A520 which productively infected MCC F(FH)H cells. These results confirmed our hypothesis that the PNGS at position 520 within the V5 region of the SU of Env was responsible for modulating the virus-receptor interaction.

We next asked whether Env variants with different phenotypes for CD134 utilisation display different susceptibilities to neutralisation. However, analysis of the neutralisation profile of three plasma samples collected from cat M11 (results presented in Appendix 4) revealed that none of the plasma samples tested neutralised pseudotypes bearing homologous Env variants, regardless of the Env phenotype for CD134 utilisation.

6.4 Discussion

The aim of this study was to examine whether there was a shift in receptor utilisation and cell tropism as infection progressed and to assess the prognostic value of alterations in receptor utilisation observed during natural FIV infection.

In vitro culture and *in vivo* experimental infection data suggested that FIV Env variants isolated during the early, acute stage of infection required a more stringent interaction with the CRD1 and CRD2 of CD134 (Willett et al., 2006a), known as the "early" phenotype. During disease progression, "late" Env variants (which interact only with CRD1 of CD134) emerge. These Envs may further evolve towards CD134 independence, facilitating virus dissemination to CD134-negative cellular compartments, contributing to disease progression (Willett et al., 2006b), (Willett et al., 2007), (Willett et al., 2008), (Willett et al., 2009), (Willett et al., 2010).

The data presented here suggest that sick cats are more likely to harbour putative "late" viral variants than healthy cats, in agreement with the model proposed previously (Willett and Hosie, 2008); "early" viruses shift from a "complex" to a "simple" interaction with CD134 as disease progresses. Employing three different grouping criteria to analyse the data, similar trends were observed; Env variants with the "simple", less stringent interaction with CD134 arose during the advancement of FIV infection, concomitant with declining health status, lower CD4 lymphocyte counts and leading to shorter survival times. It was apparent however, that the shift from "complex" to "simple" interaction with CD134 was not a necessary prerequisite for disease progression, as 25% of the cats that died during the study did not harbour Env variants displaying the "late" phenotype. Similar observations have been recorded in HIV-1 infected individuals (Berger et al., 1999); CXCR4 dependent "late" viruses emerge only in 50% of individuals who progress to AIDS (Regoes and Bonhoeffer, 2005), (Waters et al., 2008), (Shepherd et al., 2008). Nevertheless, taking together these various hypotheses and empirical evidence, it remains difficult to explain why R5 viruses predominate during early HIV infection and why the proportion of R5:X4 viruses is reversed in the terminal disease stages in some individuals (Regoes and Bonhoeffer, 2005).

It is important to emphasize that the trend in receptor usage shift observed here may have been biased by differences in the detection and cloning of *env*s with the "late" phenotype compared to "early" *env*s and to produce viable pseudotypes bearing those Envs. The median number of pseudotypes assessed was matched in the comparison of healthy and sick cats. Nevertheless, the results of this comparison would have been more convincing if we had assessed the tropism of a larger number of Env variants. The detection and cloning of Envs occurring in low abundance (which may have different tropisms compared to the majority of quasispecies circulating in the blood) poses a significant challenge (Lin and Kuritzkes, 2009). This may explain why recent studies, employing more sensitive detection methods, report higher numbers of HIV progressors infected with X4 viruses (Wilkin et al., 2011) than previous reports (Berger et al., 1999).

It is difficult to assess how representative were the Envs analysed in this study of the entire pool of Env variants circulating within infected animals, especially those within immune privileged compartments rather than peripheral blood. It appears that FIV tends to infect cells *in vivo* which do not express CXCR4 (Troth et al., 2008). A similar trend of evolution from classic usage of CXCR4 and CCR5 towards usage of CCR8 (Rucker et al., 1997), CCR3 (Doranz et al., 1996) or GPR1 (Shimizu et al., 2000) has been observed in HIV and SIV infections. Hence it is possible that FIV infects cells via a non-CXCR4, as yet unknown, co-receptor, especially in the bone marrow which appears to be a major reservoir of FIV infected cells and where the majority of cells do not express either B- or T-cell markers (Troth et al., 2008).

In spite of the potential limitations described above in detecting less abundant Envs, it was observed that FIV Env variants with "late" phenotypes occurred more frequently in sick animals in our study group. How do such variants contribute to the onset of immunodeficiency and what is the molecular basis of disease pathogenesis induced by "late" variants of FIV? It has been proposed that, as disease progresses, the viral Env may evolve towards a less stringent interaction with CD134, subsequently leading to CD134-independent infection of cells *via* CXCR4 alone and consequently a broadening of cell tropism (Willett and Hosie, 2008). The "simple" mode of interaction with CD134 was observed for

both pseudotypes bearing the Envs of i.) B2542 (Diehl et al., 1995a) and ii.) PPR (Sparger et al., 1994); both B2452 and PPR are pathogenic *in vivo*, indicating that alterations in receptor usage resulting in the "late" phenotype are not directly related to the ability of such variants to cause disease. The molecular basis of the hypothetical pathogenic properties of "late" isolates of FIV has yet to be validated. Indeed, the emergence of such "late" variants may be a consequence rather than a cause of disease

It has been suggested that the Env of "late" viruses has a more exposed CXCR4 binding site and therefore may be more susceptible to NAbs (Willett et al., 2010). A similar scenario was suggested for CCR5- and CXCR4-dependent viruses in HIV-1 infection (Bou-Habib et al., 1994) but other studies provided evidence that there were no differences in susceptibility to neutralisation between the two phenotypes (Zhang et al., 1999). Data from this study suggested that there were no differences between "early" and "late" Env variants in susceptibility to autologous neutralisation. Whereas pseudotypes bearing Envs with both "early" and "late" phenotypes were equally well neutralised in some cats, no neutralisation was observed in other cats.

So if NAbs are not responsible for driving viral evolution towards CD134 independence, do cytotoxic T lymphocytes (CTL) play a role? Perhaps cells infected with "early" viruses are more efficient at antigen presentation and are more susceptible to CTL-mediated killing? If CTLs were involved in promoting the shift in receptor usage, it would be important to examine selection pressures on proteins other than Env to validate this hypothesis. In this scenario, the diversifying selection would be predicted to be uniform across the entire viral genome.

It is intriguing to ask, why does FIV evolves towards CD134 independence? According to the transmission-mutation hypothesis proposed for HIV-1 (Regoes and Bonhoeffer, 2005), the change in co-receptor usage during the course of infection is a consequence of viral evolution, which by chance may result in the emergence of X4 viruses. Accordingly, co-receptor switching may simply be a chance event and hence occurs in only a fraction of individuals infected with HIV-1 (Berger et al., 1999). Based on the results of site directed mutagenesis presented here, together with previous results (Willett et al., 2008) and since

a single amino acid substitution can modulate the virus-receptor interaction, it is plausible to conclude that the observed switch is a chance event. Furthermore, it appears that a single point mutation across the whole Env glycoprotein, within the V1/V2 (Willett et al., 2008) or the V5 region (as demonstrated here), alters CD134 utilisation. It appears that no intermediate mutants are required for this shift to occur, as demonstrated by cat M11. Nevertheless, in other cats variants were identified that displayed an intermediate dependency on CD134; such variants could potentially revert, following subsequent mutation events, to either full dependency or independency on CD134.

Another intriguing question is why do viruses requiring a stringent interaction with CD134 predominate in early FIV infection? In HIV-1 infection R5 viruses predominate shortly after transmission (van't Wout et al., 1994). The importance of CCR5 for the establishment of infection was demonstrated by studies of individuals with a homozygous defect in CCR5 (Liu et al., 1996a) who were highly resistant to HIV infection (Wilkinson et al., 1998). A likely explanation of the hypothetical abundance of "early" viruses following viral transmission could be that such variants are selected in the infecting or infected animal. Given the major route of transmission of FIV, which occurs via biting and possibly blood sharing, it is tempting to speculate that selection forces during natural transmission of FIV are not as strong as during mucosal transmission of HIV-1 (Schuitemaker, 1994). Indeed, epithelial and dendritic cells (which are responsible for HIV-1 transmission and transfer) predominantly express CCR5 but not CXCR4, which could in part explain why CCR5 viruses predominate following infection (Reece et al., 1998), (Granelli-Piperno et al., 1998), (Meng et al., 2002). However, given the route of transmission it is difficult to assess how a similar mechanism might be involved in the transmission of FIV. More plausible is the explanation that "early" FIV variants are more pathogenic and therefore expand more rapidly following transmission, dominating the viral population. Similarly it has been observed in HIV-1 infection that R5 isolates are more fit than X4 variants early in infection (Meng et al., 2002). Further research to assess putative differences in replication rates between "early" and "late" variants isolated from animals in this study would be required to validate this hypothesis.

It has been suggested that FIV infection can occur via a CXCR4-independent route; FIV DNA has been detected in CXCR4 negative cells, particularly within the bone marrow (Troth et al., 2008). It is therefore possible that FIV can infect cells using an alternative co-receptor. FIV DNA has been demonstrated also in non-lymphoid cells, such as primary erythroid progenitors which do not express CXCR4 (Troth et al., 2008). This observation, together with the evidence that the bicyclam analogue AMD3100 showed equivocal antiviral effects *in vivo* (Hartmann et al., 2012) and the fact that FIV targets cells such as CD8+ T cells and B cells which do not express (or express very low levels) of CD134 (de Parseval et al., 2004a), it is likely that existing *in vitro* models of FIV tropism do not fully reflect the complexity of the virus-host interaction *in vivo*.

In summary, the results presented in this chapter support a model whereby viruses which require less stringent interactions with CD134 emerge during disease progression, facilitating viral dissemination to CD134-negative cellular compartments and accelerating disease progression. However further research is needed to confirm this model, including an assessment of the sensitivity of Env variants to soluble CD134 (sFc-CD134), CD134 ligand (fCD134L) and anti-CD134 antibody (7D6) (Willett et al., 2009). A comparison of the replication rates *in vivo* of isolates identified as "early" and "late" is crucial to elucidate differences in disease-inducing potential. Finally, a bioinformatics approach correlating the phenotype and genotype pairs identified in this study is required in order to establish a prospective genotypic method to predict receptor usage in FIV infection. Such information might provide a useful prognostic indicator for veterinarians following the diagnosis of FIV in cats in their care.

Chapter 7. Efficacy of commercial FIV vaccine in Australia

Since its discovery in 1987, much FIV research activity has focused on the design of a fully efficacious vaccine since, as well as preventing the spread of FIV infection, such a vaccine would inform the design of vaccines FIV's human counterpart, HIV. The idea of using the feline model to understand correlates of immune protection has been embraced by several conventional and molecular vaccine trials over the last two decades. The efforts of FIV researchers have resulted in many significant discoveries, which eventually led to the release in the US of the first dual subtype commercial FIV vaccine in 2002 (Fel-O-Vax FIV, Fort Dodge Animal Health).

Nevertheless, this vaccine attracted criticism for confounding diagnosis of FIV infection and concerns have been raised about its efficacy against heterologous challenge. Moreover, a single independent study assessing its efficacy reported an early enhancement of the viral load in vaccinated cats following experimental challenge with the GL-8 isolate of FIV (Dunham et al., 2006). Most importantly however, despite the vaccine having been marketed for over a decade, no studies have been conducted to evaluate its performance under natural conditions, against field strains.

In the present study we assessed the neutralizing antibody response induced by the Fel-O-Vax vaccine against a representative panel of FIV isolates, using samples collected from a group of privately owned Australian vaccinates. Furthermore, we examined the molecular and phenotypic properties of the virus isolated from one cat which had been vaccinated and which most likely became infected following natural exposure to FIV. We showed that Fel-O-Vax does not induce cross-reactive neutralizing antibodies, suggesting that other mechanisms may be involved in vaccine-induced protection. Although we found no evidence of antibody dependent enhancement of infection in vaccinates, the data presented in this chapter suggest that the commercial vaccine may not protect against virulent clade A field strains of FIV in Australia. However, further independent research is required to evaluate the performance of the Fel-O-Vax FIV vaccine under field conditions. Such studies will broaden our understanding of the mechanisms of protection elicited by the vaccine, as well as informing the

design of more efficacious vaccine immunogens, with the potential to impact upon HIV vaccine research.

7.1 Introduction

The development of a safe and fully efficacious anti-lentiviral vaccine is urgently needed to halt the HIV pandemic. The challenges lying ahead of the development of such a vaccine are, however, immense and very complex. Despite over 25 years of research in the field, vaccine induced protection against the virus remains elusive (Rerks-Ngarm et al., 2009b), (Girard et al., 2011).

A major obstacle is the fact that spontaneous cure from the disease has neither been documented in HIV nor FIV infection (Hosie et al., 2009), (Haase, 2010). Both viruses target CD4+ T cells and subsequently establish persistent, latent infection of memory cells (Pedersen et al., 1989), (Haase, 2010). Infection is followed by chronic immune activation, manifest by increased T-cell turnover and B-cell hyperactivation. During the course of disease, MHC-I antigen presentation on the surface of infected cells is down-regulated, facilitating viral escape from CTL surveillance (Collins et al., 1998). The high genetic diversity of the viral *env* (particularly that of HIV-1, being the result of intra-and inter-clade recombination) leads to the emergence of neutralization and CTL escape mutants, subsequently leading to the exhaustion of the immune system. How then can we design an efficacious and safe vaccine that will protect under such unfavourable conditions?

Numerous vaccine candidates have been developed, with variable outcomes ranging from full protection to enhancement of infection (Girard et al., 2011). From the various regimens tested in non-human primate models, only four vaccine compositions were advanced to phase IIb or III efficacy trials in human volunteers (Robinson, 2007), (Wijesundara et al., 2011). These included two VaxGen gp120 (B/B' and B/E) trials in the USA (Flynn et al., 2005), (Gilbert et al., 2005) and Thailand (Pitisuttithum et al., 2006), Merck's Ad5-HIV-1 STEP trial (Buchbinder et al., 2008), (McElrath et al., 2008) and lastly the ALVAC+gp120 (RV144) study (Rerks-Ngarm et al., 2009a). Only a modest degree of protection was observed in the RV144 study (Kresge, 2009), while the Merck vaccine trial was halted prematurely when it became evident that vaccination increased the risk of HIV acquisition (Duerr et al., 2012). The failure of these trials raised a very valid question: is our current understanding of the viral biology and

immune correlates of protection sufficient to design a safe and fully efficacious lentiviral vaccine?

The discovery of FIV shortly after its human counterpart offered a unique opportunity to study a virus that resembled HIV in morphology, and most importantly in its ability to induce immunodeficiency in its natural host (Bendinelli et al., 1995). Several FIV vaccine candidates were designed and evaluated using the feline model, yielding valuable insights into the virus biology and correlates of protection induced by these formulations (reviewed in Chapter 1). From all compositions, whole inactivated virus and fixed infected-cell vaccines proved to be the most successful (Bendinelli et al., 1995), (Elyar et al., 1997), (Hesselink et al., 1999).

The knowledge gained from these studies led to the production of the whole inactivated virus, dual-subtype FIV vaccine which induced protection *in vivo* against subsequent challenge with homologous and heterologous subtype virus isolates (Pu et al., 2001). The prototypic vaccine underwent safety and efficacy evaluation by USDA (US Department of Agriculture), resulting in it being released on the market in the US in 2002 (Uhl et al., 2002). Within the first four years after its release, over 1.8 million doses of the vaccine had been sold in the US (Zwijnenberg and Yamamoto, 2007). Subsequently, the vaccine has been licensed for veterinary use in Canada, Australia, New Zealand and Japan.

The Fel-O-Vax FIV vaccine consists of inactivated infected cells and whole inactivated viruses of two distinct strains of FIV: Clade A, FIV Petaluma and Clade D, FIV Shizuoka (Pu et al., 2005). These two strains differ from each other by 21% in a comparison of *env* sequences. The vaccine contains a commercial adjuvant and is reported to protect against homologous and heterologous FIV challenge, with adverse reactions being as low as 1.07% following the field evaluation (Huang et al., 2004). According to the current manufacturer's description, the vaccine is engineered to aid, together with other preventive measures, the preclusion of FIV infection (Boehringer Ingelheim Vetmedica).

Several safety and efficacy studies of the vaccine have been conducted (Pu et al., 2001), (Omori et al., 2004), (Huang et al., 2004), (Pu et al., 2005), (Kusuhara et al., 2005b), (Uhl et al., 2008), (Huang et al., 2010), but most of

these were undertaken by researchers collaborating either directly or indirectly with the vaccine manufacturer.

In the first study which assessed the safety and efficacy of the dual subtype prototypic vaccine, vaccinated SPF cats were challenged intravenously with the (*in vivo* obtained) heterologous Bangston strain of FIV, as well as the homologous FIV Shizuoka and Petaluma strains (Pu et al., 2001). Several experimental protocols were used in this study, particularly concerning the selection and dose of the challenge inoculum. Protection against low-dose (10 CID_{50}) of FIV Bangston was observed in 4 of 5 animals. However, increasing the infectious dose of the same inoculum to 100 CID_{50} resulted in protection of only 2 of 5 animals. Further challenge of the 4 vaccinates from the first group that had resisted infection following the first challenge, using 50 CID_{50} of FIV Petaluma, led to full protection, suggesting that the dual subtype vaccine is efficacious. Further evaluation included challenging 6 vaccinates with homologous *in vivo* and *in vitro* derived FIV Petaluma; all cats were protected against homologous challenge (Pu et al., 2001).

A further efficacy study (Huang et al., 2004), reported that 16% (4/25) of vaccinated cats become infected, while 90% (17/19) of animals in the control group became infected following challenge with a heterologous strain of FIV. This again led to the conclusion that the vaccine was safe and efficacious (Huang et al., 2004). However, the challenge virus used in this study had undergone passage in cell culture before the challenge of 11 median infectious doses was inoculated intramuscularly. Unfortunately, the authors did not provide detailed information concerning the challenge strain used, except that it belonged to clade A and differed in the outer *env* sequence by 11% from FIV Petaluma (Huang et al., 2004).

To address concerns about how representative were the challenge strains used in the previous studies, the Fel-O-Vax vaccine was tested against a heterologous Clade B virus challenge, using the FC1 strain of FIV that was isolated from a naturally infected cat in Florida (Pu et al., 2005). The virulence of FIV FC1 was compared against FIV GL-8 and Petaluma; based on the measurement of proviral load following experimental infection of SPF cats with these three strains, it was concluded that FIV FC1 was much more virulent that Petaluma and of

comparable virulence to GL-8. Nevertheless, such a conclusion based only on the assessment of the proviral load is questionable and requires further confirmation of infectivity and replication. Nevertheless, without providing any data, researchers collaborating with the vaccine manufacturer argued that FIV FC1 can induce severe immunodeficiency and induce more consistent loss of CD4+ T cells than a similar dose of GL-8 (Zwijnenberg and Yamamoto, 2007).

Concerns that previous studies may not reflect the route of natural exposure were further addressed in a study in which six vaccinated and eight unvaccinated cats were housed together in one room with five entire (not castrated) male cats that have previously been infected with the subtype B, Aomori-2 strain of FIV for a period of nineteen months (Kusuhara et al., 2005a). The challenge virus infecting the five infected cats differed from FIV Petaluma and FIV Shizuoka by 18.5% and 19.6% respectively at the level of the *env* sequence (Kakinuma et al., 1995). The authors observed territorial fights and mating behaviour throughout the study period and, four of eight unvaccinated control cats became FIV *gag* PCR positive during the study, while all six vaccinated animals remained protected. This led to the conclusion that the Fel-O-Vax FIV vaccine protected cats against heterologous subtype B challenge in conditions resembling natural exposure (Kusuhara et al., 2005a).

A more recent study (Huang et al., 2010) assessed vaccine efficacy once again, using the same challenge strain of FIV FC1 and reporting protection in 10 of 14 (71%) vaccinated cats. Interestingly, the plasma viral loads (PVL) in four unprotected vaccinates ranged from 2620 to 177310 copies/ml in comparison to the range of 4160 to 66170 copies/ml reported for control cats. Although the difference in PVL between the two groups was not statistically significant, it is apparent that some vaccinated, unprotected cats had almost three fold higher levels of viral RNA in the peripheral blood than infected, unvaccinated cats. This may suggest that the Fel-O-Vax vaccine, when it does not protect, may indeed enhance viral infection following exposure and subsequently lead to more rapid disease progression. Indeed, although the observed differences in PVL were reported as not significant, post mortem examination of lymphoid organs collected from 19 cats examined in this study revealed that vaccinated, unprotected cats had higher PVL in thymus, popliteal and mesenteric lymph

nodes compared to viremic controls inoculated with the same dose and strain of FIV (Huang et al., 2010), suggesting that vaccination induced enhancement of infection in the cats that were not protected following challenge.

Similarly, enhancement of the viral load in Fel-O-Vax vaccinated, unprotected cats was demonstrated in an independent study (Dunham et al., 2006). All six SPF vaccinated cats become infected following challenge with the GL-8 strain of FIV. Strikingly, these cats showed enhanced viral loads two weeks post challenge compared to control cats (Dunham et al., 2006), which subsequently declined to levels more similar to those of the controls. This experimental study demonstrated that Fel-O-Vax does not protect against the clade A, GL-8 isolate which is regarded as a representative strain of FIV in the UK (Hosie and Beatty, 2007), (Samman et al., 2011). The results of this study raise a valid question about the efficacy and safety of the vaccine under field conditions, particularly since vaccine induced enhancement of viral infection was described previously for FIV (Huisman et al., 2009).

The lack of protection against a primary isolate of FIV was demonstrated (Dunham et al., 2006) but subsequently no independent research has been undertaken to evaluate the commercial vaccine's efficacy under the field conditions (Doornenbal et al., 2012). As demonstrated in this thesis, FIV recombination is abundant; it is apparent that putative circulating recombinant forms of the virus are very common at least in the US. How efficacious is the Fel-O-Vax FIV against these recombinant and non-recombinant strains circulating in the field? Can we identify cats that have been vaccinated and subsequently become infected with the virus? Is there any evidence for enhancement of infection following vaccination and, ultimately, does the current vaccine bring more benefit or harm, complicating the diagnosis of infection? This and many more unresolved questions concerning not only efficacy but most importantly the mechanisms and correlates of protection induced by the dual subtype vaccine remain to be answered.

To address some of these questions, a group of privately owned Australian cats with known history of FIV vaccination was studied; we evaluated breadth of NAbs induced by the Fel-O-Vax FIV. Furthermore, in order to determine the strains

that overcame vaccine-induced protection, we aimed to identify cats that had been vaccinated and which subsequently became infected with FIV.

7.2 Materials and methods

7.2.1 Cats

Cats were recruited into the study based on a retrospective search of the administrative search system (AIS) of electronic medical records (EMR) of all feline patients that visited the Valentine Charlton Cat Centre (VCCC) at the University of Sydney between January 2005 and September 2010. The search included cats referred by their veterinarians or presented to either the general practice or emergency service of the hospital. Key words used for the search were: "FIV", "feline immunodeficiency virus", "FIV PCR", "Fel-O-Vax FIV" and "FIV vaccine".

Thus cats with a known history of FIV vaccination were identified. There were 8 vaccinates of known ELISA and FIV PCR status (Gribbles Veterinary Laboratories, Bella Vista, NSW, Australia) and another 2 of unknown ELISA and PCR status, from which blood samples were stored in the hospital. As demonstrated in Table 7-1, one cat (S1V) yielded a positive result for FIV proviral DNA.

7.2.2 Plasma and blood samples

Having obtained the consent of the cat owners, blood samples had been collected into heparinized and EDTA collection tubes during diagnostic procedures performed at the VCCC between January 2005 and September 2010. Samples had been centrifuged at 2000 rpm (~370 x g) for 10 minutes and plasma and cell fractions had been separated and frozen at -80°C. For the present study, stored samples were thawed, 200µl aliquots obtained, re-frozen and shipped on dry ice to our laboratory in Glasgow.

7.2.3 Molecular biology techniques and phylogenetic analysis

The entire FIV *env* was amplified directly from blood from S1V (vaccinated, FIV provirus positive cat) using the primers and nested PCR protocol described in Chapter 2 and appendices 1 and 2. The FIV *env* thus obtained was cloned into an eukaryotic expression vector and 24 Env variants were obtained, sequenced using the chain terminator method and transfected in 293T cells, using the methods described previously (Chapter 2).

Table 7-1 Clinical history of Fel-O-Vax FIV vaccinated cats. Age is accurate for the blood samples collection dates. All except two entirely indoor cats were tested both on FIV ELISA and FIV PCR. One vaccinated cat, S1V tested positive for FIV proviral DNA, while all others were negative. Housing: I-indoor, O-outdoor.

Cat	Breed	Sex	Age (y.)	Housing	ELISA	PCR	Date of FIV vaccination	Blood collection	Chief complaint	Diagnosis	Other
S1V	DSH	MN	11	I/O	+	+	Oct 2006, Nov 2007, Oct 2008, Sep 2009	09.12.09	no nasal and facial sensation, dribbling	trigeminal nerve paralysis, cavernous sinus syndrome	renomegaly, fights, unknown FIV status at the first vaccination
S2V	DMH	FS	10	I/O	+	-	Oct 2008.	19.11.08	inappetent and lethargic	anaemia, hepatitis, splenomegaly, inflammatory bone marrow disease	
S3V	Ragdoll	FS	3	I	+	-	regularly since kitten	06.05.10	lethargy, inappetence and weight loss	idiopathic hypercalcemia	renal insufficiency
S4V	Burmese	MN	6	I/O	+	-	unknown	25.06.09	lethargy, depression, weight loss, multiple joint effusion	polyarthritis immune mediated (?)	fight wounds
S5V	Abyssinian	MN	10	I/O	+	-	regularly, last one Jan 2009	23.07.09	sneezing, ocular and nasal discharge	active chronic rhinitis	n/a
S6V	DSH	FS	9	I	+	-	unknown	14.10.09	sudden onset ataxia	meningioma, right occipital lobe	n/a
S7V	DLH	MN	12	I/O	+	-	unknown	02.03.10	presented for radio-iodine treatment	hyperthyroidism	n/a
S8V	DSH	MN	6	I/O	+	-	unknown	26.10.10	swollen left hind leg	non-regenerative anaemia	fight wounds
S9V	DSH	FS	8	I	n/a	n/a	regularly since kitten	30.04.09	acute vomiting	pancreatitis	n/a
S10V	British short hair	F	1	I	n/a	n/a	once as a kitten	20.07.09	pyrexia, lethargy, inappetence	effusive feline infectious peritonitis	euthanized following diagnosis

Twenty-four complete *env* sequences obtained from cat S1V and 17 reference isolates (as listed in Chapter 4 plus sequences of FIV Bangston; AY620002.1 and FIV FC1; AY621093) were included in phylogenetic analyses. These analyses were performed according to methods described previously (Chapter 4) and included: phylogenetic inference of isolated sequences, recombination testing and intra-host diversity calculation.

Neutralisation assays were set up using the protocol described in Chapter 5. Plasma samples collected from 10 vaccinates were tested for NAbs using a panel of reference pseudotypes bearing Envs of 7 heterologous isolates of FIV: 1) clade A, GL-8 (Hosie et al., 1995), 2) clade, B B2542 (Sodora et al., 1995), 3) clade C, CPG41 (Diehl et al., 1995b), 4) clade A, PPR (Phillips et al., 1990), 5) clade A, M2PET (Giannecchini et al., 2007), 6) clade A, NCSU (Yang et al., 1996), and 7) clade A, KKS (Pistello et al., 2010) .

Twelve pseudotypes bearing Envs obtained from the vaccinated, FIV provirus positive cat S1V were prepared and tested for their cell tropism and sensitivity to autologous neutralization, as described previously (Chapters 4 and 5).

7.2.4 Graphs and statistical analyses

Graphs were drawn using GraphPad Prism v 5.00 (GraphPad Software, San Diego, California, USA).

7.3 Results

7.3.1 Breadth of VNAb response in vaccinated cats

In order to assess the breadth and strength of NAbs in cats vaccinated with Fel-O-Vax, we tested plasma samples collected from vaccinated cats for the ability to neutralise pseudotypes bearing the Envs of 7 reference isolates of FIV. Surprisingly, as demonstrated in Figure 7-1, none of the plasma samples displayed cross neutralising activity. A degree of neutralisation breadth was observed for plasma S3V, which strongly neutralised KKS (17.3 fold) and moderately B2542 (3.5 fold), PPR (3.4 fold) and CPG41 (3.9 fold). In addition, a degree of moderate cross reactivity was displayed by plasma S5V, which neutralised KKS (4.3 fold) and PPR (3.4 fold).
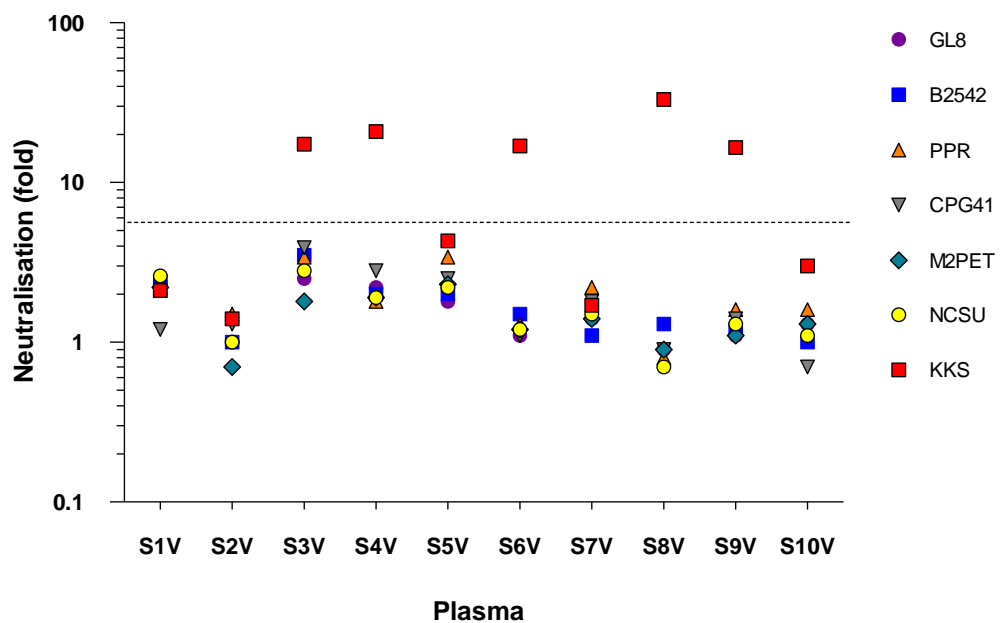

Figure 7-1 Neutralisation profiles of plasmas from 10 vaccinees, shown as fold neutralisation. Plasma samples were tested against a panel of pseudotypes bearing 7 reference Envs (GL-8, B2542, PPR, CPG41, M2PET, NCSU and KKS). Greater than 5.6 fold neutralisation (above dashed horizontal line) indicates strong neutralisation.

Amongst all pseudotypes tested, only the KKS pseudotype (KKS closely resembles FIV Petaluma which is a component of the Fel-O-Vax FIV vaccine), was strongly neutralised by 50% of plasmas (from cats S3V, S4V, S6V, S8V and S9V with 17.3, 20.8, 16.9, 33 and 16.5 fold neutralisation respectively). From the remaining 5

samples, only two moderately neutralised KKS (S5V and S10V with 4.3 and 3 fold neutralisation respectively) while the remaining 3 did not neutralise any of the pseudotypes tested.

7.3.2 Vaccinated, provirus positive cat S1V

7.3.2.1 Phylogenetic inference

The maximum likelihood analysis revealed that the vaccinated, provirus positive cat S1V harboured viruses containing clade A *env* genes. For clarity, only one *env* sequence, representative of the 24 *env* variants isolated from cat S1V (red node) is shown in Figure 7-2 compared against reference sequences.
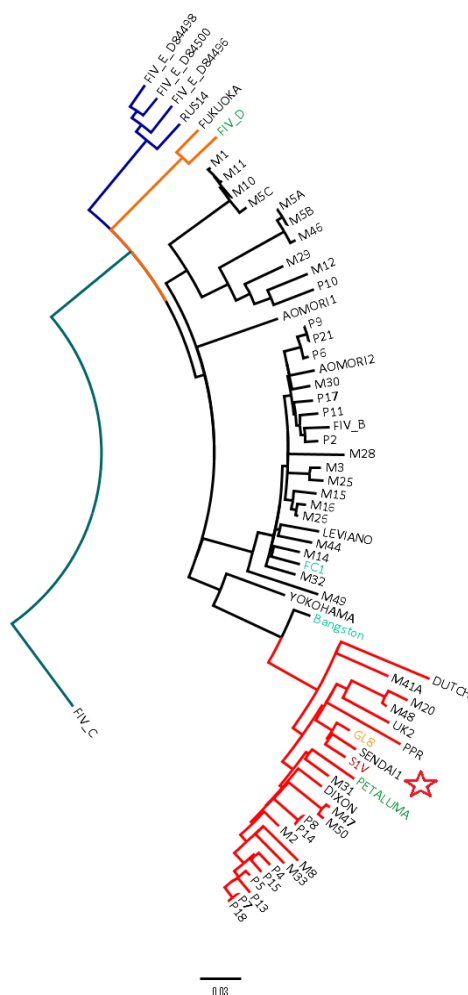

Figure 7-2 Maximum likelihood tree based on the HKY model rooted on the reference *env* of clade C. The tree is drawn to scale, with branch lengths denoting the number of substitutions per site. The analysis included 44 sequences from the Memphis and Chicago cohorts (as described previously in this thesis), 19 reference sequences (detailed in 4.2.1.3) and one sequence representative of 24 *env* gene*s* isolated from cat S1V (red node). Note that sequence S1V clusters closely with GL-8 (red star). Vaccine strains: Petaluma and Shizuoka (FIV Clade D) are highlighted in green. Challenge strains (FC1 and Bangston) used in vaccine manufacturer's efficacy trials are highlighted in turquoise.

The overall mean intra-host diversity (K2P) of the 24 *env* variants isolated from S1V was as low as 0.1% and the highest pairwise distance was 0.2%. By employing rigorous recombination testing (as described in Chapter 4 (4.2.4)), it was noted that all *envs* from cat S1V were non-recombinant clade A sequences.

7.3.2.2 Autologous neutralising antibody response

Plasma S1V did not contain heterologous NAbs since no neutralisation of pseudotypes bearing heterologous Envs was observed. This plasma was also tested against 12 pseudotypes bearing autologous Envs isolated from the same cat.
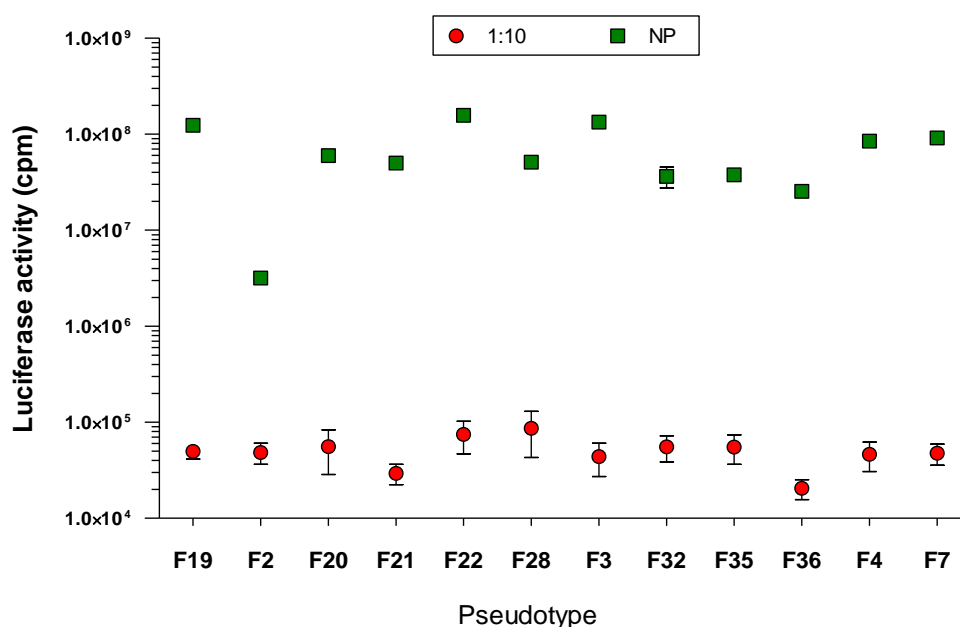
Figure 7-3 Neutralisation of plasma S1V against 12 pseudotypes bearing autologous S1V Envs variants. Graph represents differences between mean (n=3) luciferase activity (cpm) for wells with plasma at 1:10 dilution (red) and wells without plasma (NP, green). Note that all autologous pseudotypes were strongly neutralised by plasma S1V, producing significantly decreasing luciferase counts compared to the no plasma control.

As demonstrated in Figure 7-3, plasma S1V strongly neutralised all pseudotypes bearing homologous Envs (ranging from 65 to 3042 fold neutralisation), in contrast to the lack of neutralisation observed against the reference pseudotypes.

7.3.2.3 Receptor usage

Next, to assess the cell tropism of Envs isolated from S1V, pseudotypes bearing 12 Envs that were representative of the 24 Envs obtained from this cat were tested for their receptor utilisation phenotype. As demonstrated in Figure 7-4, all Envs from cat S1V required a stringent, high affinity interaction with CD134 for productive infection, displaying a phenotype similar to that of the prototypic "early", acute GL-8 isolate.
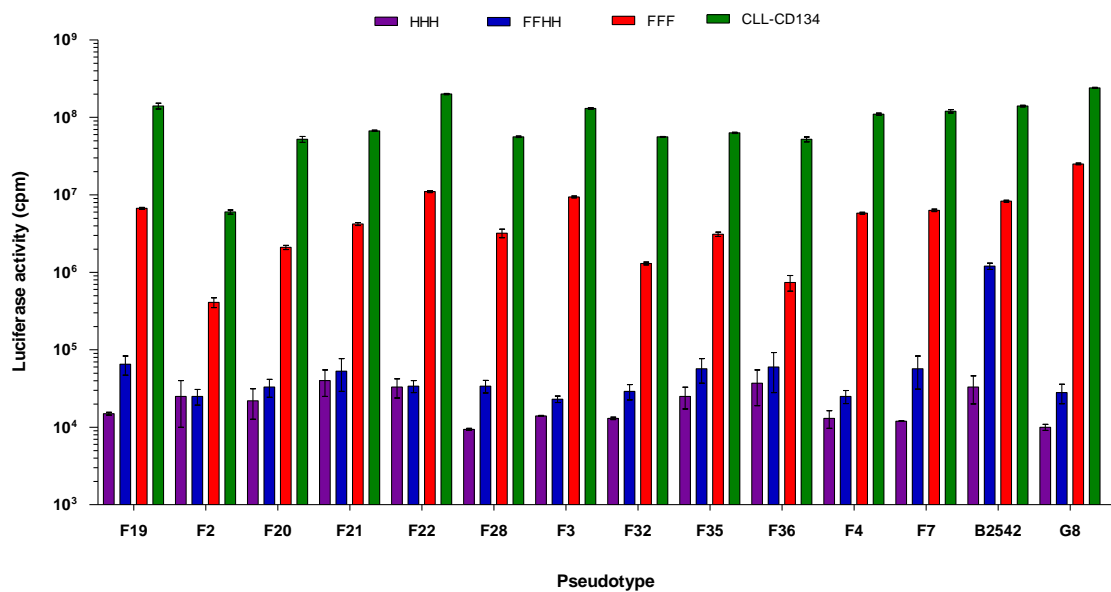

Figure 7-4 Receptor usage of 12 pseudotypes bearing Envs isolated from cat S1V. All pseudotypes displayed a similar phenotype to the prototypic "early", acute GL-8 strain of FIV (for description refer to Chapter 5).

7.4 Discussion

A review of the literature describing studies that have been performed over the last decade concerning safety and efficacy of the Fel-O-Vax FIV vaccine, revealed significant gaps in the current understanding of correlates of protection and it is not clear how the vaccine performs against natural challenge under field conditions.

Given that Fel-O-Vax FIV vaccine did not protect experimental cats against the heterologous challenge with GL-8 (Dunham et al., 2006), the aim of this study was to identify cats which had been vaccinated against FIV and which had subsequently become infected following natural exposure. A search of the electronic medical records of VCCC enabled us to identify one cat (S1V) which had been vaccinated and which tested provirus positive using a diagnostic PCR test; blood samples had been stored and were available for molecular analyses. Although only an incomplete history was available, cat S1V had been vaccinated with Fel-O-Vax FIV annually for at least three years, with the last vaccination having been administered in September 2009, three months prior to its FIV diagnosis and death due to cavernous sinus syndrome.

Phylogenetic analysis revealed that cat S1V was infected with an entirely non-recombinant clade A isolate of FIV, relatively closely related to FIV GL-8. Taking together the receptor utilisation phenotype of S1V Envs, which resembled that of GL-8 and the hypothesis that "early" acute isolates display a distinct receptor utilisation phenotype compared to "late" isolates (Willett et al., 2006a), it is tempting to speculate that the Env variants isolated from S1V represent the putative "early" acute isolates of the virus which are thought to be transmitted in the field (Willett et al., 2006a). This brings to the fore the valid question: is the protection induced by the vaccine sufficiently potent to protect cat S1V against infection with an "acute" virus, entirely belonging to clade A? The incomplete medical history of this cat prevents a definite conclusion in this case. The vaccination and FIV status of cat S1V prior to 2006 was not documented. What is known, however, is the fact that the cat was not tested for FIV prior to vaccination. Therefore it is not possible to rule out the possibility that S1V may have been infected prior to being vaccinated. Nevertheless, given the cat's documented medical history, it is known that it was presented to its

veterinarian on multiple occasions after relocation from Sydney to Canberra in late 2006. Given the territorial behaviour of male cats and a documented history of fight wounds following the relocation at this time, it is likely that cat S1V acquired FIV between 2006 and 2009, when it was thought to be protected against FIV infection as a result of vaccination. Although we cannot be certain, given the information from the cat's owner and its veterinarian, it is likely that the FIV Envs isolated here are indeed representative of Envs from virus isolates that overcame vaccine-induced protection. Furthermore, as demonstrated by the low intra-host diversity of the 24 Env variants isolated from this cat, it is tempting to speculate that vaccination may have contributed to a homogenization of the viral population following infection.

The correlates of protection induced by the Fel-O-Vax FIV vaccine remain unclear (Uhl et al., 2008). Here, the breadth and potency of NAbs elicited against the Env following immunization were measured. To our surprise, none of the ten plasma samples from vaccinated cats that were tested displayed broad cross-reactivity against the panel of seven pseudotypes bearing reference Envs from three different clades of FIV. Strong NAbs were induced only in 5 cats (50%) against the pseudotype bearing KKS Env, which very closely resembles the Petaluma isolate (Pistello et al., 2010) that is contained within the Fel-O-Vax vaccine (Uhl et al., 2002). A strong NAb response has been proposed as an essential correlate of vaccine induced protection (Hosie et al., 2011), (Kwong et al., 2012) and a crucial component of humoral immunity against virus infections (Amanna et al., 2008), (Plotkin, 2008). Initial studies evaluating Fel-O-Vax induced protection reported that NAbs recognising the homologous Petaluma and Shizuoka strains were detected in most of the vaccinated cats (Pu et al., 2001). In the same study, eight of twelve cats displayed NAbs against the heterologous FIV Bangston, leading to the conclusion that the two distinct isolates of FIV contained within the vaccine act synergistically to enhance the development of NAbs against heterologous strains of FIV (Pu et al., 2001). The results of this study, however, demonstrate that strong NAb were elicited only in 50% of the vaccinated cats and were potent only against the Env most closely related to one of the Envs contained within the FIV vaccine (Pu et al., 2001). There was no evidence for strong, cross-reactive NAbs in any of the samples from vaccinated cats that were tested here. It is tempting to speculate that a lack of broadly

cross-reactive NAbs may have resulted in infection of cat S1V following challenge with a field strain of FIV.

Vaccine induced enhancement of lentiviral infection has been described previously (Huisman et al., 2009). Also, antibody-dependent enhancement (ADE) of infection plays an important role in the pathogenesis of Dengue virus (DENV) (Burke and Kliks, 2006), feline coronavirus (FCoV) (Corapi et al., 1992) and several other viral diseases (Takada and Kawaoka, 2003). In the present study there was no evidence of ADE against either heterologous or homologous Envs, concordant with a previous study of the Fel-O-Vax vaccine (Pu et al., 2001). However enhancement by a mechanism other than ADE, such as following activation of cell mediated immunity (Wahl and Orenstein, 1997), (Huisman et al., 2009) may have been involved, but were not examined here.

It was observed that plasma S1V strongly neutralised pseudotypes bearing all of the homologous Envs tested. Together with the results of neutralisation studies of FIV and HIV-1 (Chapter 5), it is likely that the robust NAb response observed in cat S1V was a consequence of antigenic stimulation following infection with an acute, highly replicating strain of FIV. It was demonstrated that experimentally vaccinated cats, following challenge with the virulent GL-8 isolate, displayed significantly enhanced viral loads compared to non-vaccinated controls (Dunham et al., 2006). It is therefore tempting to speculate, given the severe onset of clinical symptoms in cat S1V, that vaccination led to an enhancement of infection and subsequent antigenic hyper-stimulation leading to a robust autologous NAb response by the previously primed immune system. Measuring plasma viral and proviral loads in cat S1V could validate this hypothesis.

It is unclear whether sterilising immunity following FIV vaccination can be achieved without eliciting broadly cross reactive NAbs. It is apparent that virus specific cell mediated immunity plays an important role in restraining retroviral infections (Huisman et al., 2008a); however HIV vaccine candidates which elicited cellular immune response have been shown to be ineffective (Casimiro et al., 2003), (Nature, 2007), (Dubey et al., 2007). Nevertheless it has been demonstrated that immunization with Fel-O-Vax FIV elicits strong cellular immunity (Uhl et al., 2002), namely adaptive T cell immunity, protecting cats against homologous challenge in the absence of NAbs (Omori et al., 2004). Given

the case of cat S1V, however, it can be speculated that any CTL response elicited by the vaccine was not sufficiently potent on its own to protect against a natural challenge with a virulent field isolate of FIV.

In conclusion, the safety and efficacy concerns associated with the release of the Fel-O-Vax FIV vaccine over a decade ago are as valid as ever before (Little et al., 2011). It is apparent that the majority of studies with the dual subtype vaccine have been undertaken by researchers collaborating, either directly or indirectly, with the vaccine manufacturer. As demonstrated in this chapter, vaccination with Fel-O-Vax FIV may not always protect cats against natural field exposure to FIV in Australia, where the vaccine has been licensed since 2004. It was observed that vaccination does not elicit, or at least lead to maintained levels of, broadly NAbs. Given that the correlates of protection induced by the vaccine are not fully understood and the abundance of recombinant strains of FIV (Chapter 4), more research is required to fully assess vaccine efficacy under field conditions. Although it appears that a safe, protective and affordable HIV vaccine remains elusive (Douek et al., 2009), (Mascola and Montefiori, 2010), (Hoxie, 2010), further studies of the current FIV vaccine will likely inform the design of improved formulations against human and feline immunodeficiency viruses and therefore such studies are urgently needed.

Chapter 8. Final discussion

Studying feline viral infections under field conditions can be very challenging and hence relatively little is known about the longitudinal course of FIV infection in the domestic cat (*Felis catus*). The aim of the present study was to investigate FIV infection in cats that had been naturally infected and to assess the efficacy of the commercial FIV vaccine. The observations and insights into natural FIV infection gained during this study were compared with results and hypotheses that arose from studying experimental FIV infections (Willett et al., 2006b), (Willett et al., 2007), (Willett et al., 2008), (Kraase et al., 2010), (Samman et al., 2010), (Willett et al., 2010).

The novel study described in this thesis examined host and viral factors likely to be associated with disease progression over the course of infection in a relatively large study group comprising cats living under two different housing conditions, in two distinct geographical locations. Moreover, in contrast to previous studies, the molecular analyses described here examined the entire *env* gene, not solely the short V3-V5 fragment. This approach provided answers to many intriguing questions surrounding the clinical presentation of FIV infection, dynamics of intra-host evolution, humoral response, cell tropism and lastly the efficacy of the commercial FIV vaccine. However, before drawing final conclusions, it is important to recognize and acknowledge the potential limitations of the study.

Firstly, clinical parameters such as age and time of infection were often estimated on the basis of physical examinations rather than comprehensive clinical histories. Although performed by experienced feline medicine specialist, it is inevitable that these estimates carry some degree of uncertainty.

It can be argued that blood direct PCR amplicons, subsequently cloned into eukaryotic expression vector may not fully represent the complexity of viral variants circulating within the host (Salazar-Gonzalez et al., 2008). It should be emphasized, however, that viral loads in "asymptomatic" FIV infection often fall below the limit of detection (Dieter Klein, personal communication). Use of the single genome amplification (SGA) method to decipher intra-host diversity in acute, peak high HIV-1 infection (Keele et al., 2008), would have biased the

results of the present study by including only those cats with detectable plasma viral loads. Indeed, evolutionary studies of HIV-2, which during asymptomatic phase often achieves undetectable plasma viral loads (Popper et al., 2000), have not employed the SGA method in order to minimise bias (MacNeil et al., 2007a), (MacNeil et al., 2007b).

It has been argued that studies examining amplicons obtained by Taq polymerase may i.) give rise to some products as a result of template switching (Meyerhans et al., 1990), (Shriner et al., 2004), ii.) introduce errors during PCR amplification (Palmer et al., 2005) or iii.) introduce cloning bias (Liu et al., 1996b). Unlike previous studies, the Phusion enzyme with 25 fold higher fidelity than Taq polymerase (Frey, 1995) was used for the studies conducted as part of this thesis. To minimize the possibility of template switching during the PCR, we carried out three independent amplifications from each blood sample. All procedures were performed separately at various time points over a period of 18 months to exclude the possibility of cross-contamination. Since this methodology yielded identical sequences, particularly those with shared recombination points, provides strong evidence that the amplicons that were obtained reflected genuine, *in vivo* circulating sequences and were neither the result of PCR induced errors nor associated with polymerase template switching. Moreover, the results of the present study are in agreement with previous FIV studies employing "bulk" PCR (Ikeda et al., 2004), (Motokawa et al., 2005), end-point dilution proviral DNA PCR (Kraase et al., 2010) as well as a study examining viral RNA sequences in plasma (Huisman et al., 2008b); hence, despite the different methodologies used, it can be concluded that there is relatively low FIV sequence variation, highlighting its relatively high genetic stability.

Finally, the relatively short length of the present study provides only limited information about the longitudinal course of FIV infection and it would be useful to follow up the recruited cats over a longer period of time. Nevertheless, bearing in mind possible limitations, the present study answered several intriguing questions, as discussed below, and broadened our understanding of natural FIV infection.

8.1 Living conditions are likely to influence the outcome of FIV infection

Contrasting clinical outcomes of FIV infection were observed in the two distinct cat populations. Unlike HIV infection, there are no well-established prognostic indicators for FIV and, despite significant differences being observed between CD4 and CD8 lymphocyte subsets between FIV positive and negative cats, it was difficult to distinguish cats with and without clinical signs using these parameters. An examination of CD4 and CD8 lymphocytes in conjunction with PVL could prove valuable and is on-going as part of a collaborative research project.

It has become clear that housing conditions, nutrition and health care access dramatically influence the survival times of FIV infected cats. Supported by studies published previously (Addie et al., 2000), (Ravi et al., 2010) as well as the general impression of veterinarians, the outcome of natural FIV infection is not always as dramatic as has been described following experimental infections. Indeed, the observations of the Chicago cohort were consistent with these reports. However, the outcome for the Memphis cats was dramatically different; the most common cause of morbidity identified in this population was lymphoma. Although virus induced immunodysregulation can be linked with neoplastic transformation of B-cells, a direct carcinogenic role for FIV was unlikely. It is possible that another, unknown infectious agent could have been reactivated in immunocompromised cats, leading to the high incidence of neoplasia. This hypothesis is currently under investigation, using next generation sequencing of tissue samples collected from the cats that developed lymphoma.

8.2 FIV *env* is relatively stable genetically

The overall *env* intra-host diversity in the majority of cases examined here was surprisingly low, given the general expectation of high mutation rates in viruses carrying the error-prone RT. Yet differences in intra-host diversity and evolutionary rates are evident between HIV-1 and the less pathogenic HIV-2. Although both viruses achieve similar proviral load, HIV-2 replicates to lower titres in the plasma (Popper et al., 2000), (MacNeil et al., 2007b), displays lower rates of sequence evolution (MacNeil et al., 2007a) and lower pathogenicity

reflected by a slower decrease in CD4+ T cell counts and subsequently requires a longer time interval for disease progression (Marlink et al., 1988), (Marlink et al., 1994), (Whittle et al., 1994), (Popper et al., 1999). Similarly, the low pathogenic bovine immunodeficiency virus (BIV), which is evolutionarily more closely related to FIV than the primate lentiviruses, also exhibits little sequence variation (Carpenter et al., 2000). The results presented in this thesis demonstrate

a similar pattern to that of HIV-2 infection and are in agreement with previous studies indicating a relatively high genetic stability of FIV (Ikeda et al., 2004), (Motokawa et al., 2005), (Huisman et al., 2008b), (Kraase et al., 2010).

At the population level, a surprisingly high abundance of recombinant viruses circulated amongst cats in the field. The results presented here, therefore, confound the current phylogenetic classification of the virus (Nishimura et al., 1998) and call for its revision.

In this thesis the existence of at least two common recombination break points was demonstrated, located at the stems of the V1/V2 and V5 loops of the SU of the viral Env. Taking the location of the first recombination breakpoint together with the relatively high number of positively selected sites and the highest evolutionary rate of this segment, it is likely that the leader region has a significant function in the virus life cycle. Studies of viral leader sequences highlighted their enormous complexity, suggesting a role, not only in post-translational, but also in post cleavage events (Lemberg and Martoglio, 2002), (Hegde and Bernstein, 2006). Leader encoded peptides can be involved in self-antigen presenting (Borrego et al., 1998) and in regulating Env expression levels on the viral surface (Hegde and Bernstein, 2006), subsequently affecting viral infectivity. This hypothesis, however, requires to be tested in functional studies.

8.3 Humoral immunity does not alter the clinical course of FIV infection

It was demonstrated that cats infected with FIV develop variable NAb responses against pseudotypes bearing autologous and heterologous FIV Envs. No correlation was observed between either health status or survival time following infection; similar neutralisation profiles were observed for plasmas from healthy

and non-healthy cats as well as cats that survived or died during the study. Such findings argue against a role for NAb in influencing disease progression. Furthermore, the results demonstrated that FIV infected cats, regardless of strength of NAb response, experience progressive declines in CD4+ T cell numbers; NAb responses, even when potent, did not protect against the loss of CD4 cells. A similar trend was observed in HIV-1 infection, where the presence of autologous (Cecilia et al., 1999), (Geffin et al., 2003), (Schmitz et al., 2003) and cross-reactive NAbs was not associated with a prolonged AIDS free, asymptomatic period (Piantadosi et al., 2009), (van Gils et al., 2010), (Euler et al., 2010).

The data demonstrated that the humoral immune responses monitored in this study did not impact upon the clinical course of natural FIV infection. One plausible explanation for the apparent lack of correlation is that any factor which promotes a strong NAb response may negatively influence other immune responses, leading to exhaustion of polyfunctional CD4 and CD8 cells (Harari et al., 2004), (Betts et al., 2006), (Streeck et al., 2008). It is likely that cell mediated immunity, as well as host genetic factors, may influence the clinical course of infection rather than NAb (Nomura and Matano, 2012), (Huang et al., 2012a). Further research is needed to decipher these mechanisms.

8.4 FIV variants with altered cell tropism emerge in terminal disease

The data revealed that sick cats were more likely to harbour putative "late" viral variants than healthy cats and were in agreement with the model proposed previously (Willett and Hosie, 2008); there is a switch from "early" viruses that require a "complex" interaction with CD134 to "late": viruses requiring a "simple" interaction, as disease progresses. It was apparent, however, that the shift from a "complex" to a "simple" interaction with CD134 was not a necessary prerequisite for disease progression. Analogous observations were recorded in HIV-1 infected individuals (Berger et al., 1999); CXCR4 dependent, "late" viruses emerged only in 50% of individuals who progressed to AIDS (Regoes and Bonhoeffer, 2005), (Waters et al., 2008), (Shepherd et al., 2008).

A comparison of the replication rates of FIV isolates identified as displaying "early" and "late" phenotypes will be crucial to determine whether there may be differences in their hypothetical disease-inducing potential. From the prognostic point of view, a bioinformatics approach to test for correlations between the phenotype and genotype pairs identified in this study will be required in order to establish a prospective genotypic method for predicting receptor usage in FIV infection which might allow an estimation of the likelihood of disease progression.

8.5 Fel-O-Vax FIV does not induce broadly neutralising antibodies

Broad and strong NAb responses have been proposed as essential correlates of vaccine induced protection (Kwong et al., 2012). Initial studies evaluating Fel-O-Vax FIV-induced protection reported that NAbs recognising the vaccine strains Petaluma and Shizuoka were detected in most vaccinated cats, leading to the conclusion that the two isolates of FIV contained within the vaccine act synergistically to enhance the development of NAbs against heterologous strains of FIV (Pu et al., 2001). Surprisingly, none of the ten plasma samples that were collected from Australian vaccinated cats and tested as part of this thesis displayed broad cross-reactivity; strong NAb were observed only in 50% of the vaccinated cats and were potent against only the pseudotype that bore an Env most closely related to the Petaluma Env contained within the FIV vaccine (Pu et al., 2001). Furthermore, a strain of FIV that had most likely overcome vaccine-induced protection was identified, demonstrating that the Fel-O-Vax FIV vaccine may not always protect against natural exposure to FIV in the field. This apparent lack of protection may have been related to the lack of induction of a strong cross-reactive NAb response in the vaccinated cat that subsequently became infected.

Since the correlates of protection induced by FIV vaccination are not fully characterised and the high abundance of recombinant strains of FIV described in this thesis, further research will be required to properly assess vaccine efficacy under field conditions.

8.6 Concluding remarks

FIV, as well as being an important pathogen of domestic cats, has significant comparative value. Despite almost thirty years of research it is still largely unclear how HIV causes death and what are the correlates of protection. Many competing hypotheses have been proposed, often promoting primate animal models over the non-primate lentiviral models. Perhaps an inevitable anthropocentrism precluded HIV researchers from taking on board the lessons learned from the cat? This question is particularly valid in light of the failure of phase III, HIV vaccine clinical trials in human volunteers (Gilbert et al., 2005), (Flynn et al., 2005), (Pitisuttithum et al., 2006), (McElrath et al., 2008), (Buchbinder et al., 2008) (Rerks-Ngarm et al., 2009a) as well as the fact that in some cases vaccination increased the risk of HIV acquisition (Duerr et al., 2012). Are expensive, high-risk trials on human volunteers justified if we do not even fully understand the correlates of protection induced by the commercial FIV vaccine in the feline model? It is apparent that differences between FIV and HIV-1 are more profound than those between HIV-1 and its simian counterpart. Paradoxically, these differences, and the fact that FIV has coexisted with its host for longer than HIV-1, may prove crucial to better understand the interplay between the lentivirus and its host and to achieve the highly desired fully efficacious vaccine.

Appendix 1: Primers

Name	Sequence (5' to 3')
1F4	TGTAATCAACGYTTTGTKTCTCCTTACAG
1R4	CCAATAMTCWTCCCAGTCCACCCTT
2F2	TATTATTGGCARTTGCAATCTACMTTATC
XR2	CCTCAAAGGGAAGAAATCAGCTCA
G8_Sal1_fwd	GGGTCGACACCATGGCAGAAGGGTTTGCAGCA
M9M12_Sal1_fwd	ACTGGTCGACACCATGGCAGCAGGGAGGATTTACTCAA
M14M25_Sal1_fwd	ACTGGTCGACACCATGGCAGAGGGAGGATTTACTCAA
M8M33M43_Sal1_fwd	ACTGGTCGACACCATGGCAGAAGGATTTGCAGCC
M30M31M49_Sal1_fwd	ACTGGTCGACACCATGGCAGAGGGAGGATTTACTCAA
M47M50_Sal1_fwd	ACTGGTCGACACCATGGCAGAAGGATTTGCAGTC
M6_Sal1_fwd	ACTGGTCGACACCATGGCAGAGGGAGGATTTGCTCAA
M2_Sal1_fwd	ACTGGTCGACACCATGGCAGAAAGATTTGCAGCC
M11_Sal1_fwd	ACTGGTCGACACCATGGCAGAGGGAGGATTTAATCAA
M46_Sal1_fwd	ACTGGTCGACACCATGGCAGAGGGAGGATTTACTCAG
M48_Sal1_fwd	ACTGGTCGACACCATGGCAGAAGGATTCACTCAA
M6_Not1_rev	ACAGCGGCCGCCATCATTTCTCCTCTTTTTCAGAC
M31_Not1_rev	ACAGCGGCCGCGATCATTCCTCCTCTTTTTCAGAC
M9M25M33M43M46_Not1	ACAGCGGCCGCCATCATTCCTCCTCTTTTTCAGAC
M14M30M48_Not1_rev	ACAGCGGCCGCCATCATTCCTCCTCTTTTTCAGAT
M47_Not1_rev	ACAGCGGCCGCCATCATTCCTCCTCTTTTTCAGGT
P4P5P13P15_Sal1_fwd	ACTGGTCGACACCATGGCAGAAGGATTTGCAGCC

Name	Sequence (5' to 3')
P2P6P11P14P17_Sal1	ACTGGTCGACACCATGGCAGAGGGAGGATTTACTCAA
P10_Sal1_fwd	ACTGGTCGACACCATGGCAGAGGGAAGGTTTACTCAA
P2P5P13_Not1_rev	ACAGCGGCCGCCATCATTCCTCCTCTTTTTCAGAT
P14_Not1_rev	ACAGCGGCCGCCATCATTCCTCCTCTTTTTCAGAC
P6P7_Not1_rev	GGGCGGCCGCCATCGCCTCCTCTTTTTCAGA
G8_Not1_rev	GGGCGGCCGCCATCATTCCTCCTCTTTTTCAGAC
VR1012_fwd	CTTTTCTGCAGTCACCGTCG
VR1012_rev	CTGGATCCAGGCGCCTGGTCTA
Memphis780_fwd	TGGAATGAGACTATAACAGG
Memphis2090_rev	GATTACATCCTAATTCTTGCATAG

Appendix 2: PCR cycling conditions

Table A2-1. Thermocycling conditions for Phusion Blood II Direct DNA Polymerase Kit.

	Cycles	Time (minutes)	Temperature
Initial denaturation	1	3 minutes	98°C
Denaturation		10 seconds	98°C
Annealing	35	30 seconds	64.5°C
Elongation		90 seconds	72°C
Final elongation	1	10 minutes	72°C

Table A2-2. Thermocycling conditions for Roche Master DNA Polymerase.

	Cycles	Time	Temperature
Initial denaturation	1	5 minutes	94°C
Denaturation		30 seconds	94°C
Annealing	30	60 seconds	50°C
Elongation		180 seconds	72°C
Final elongation	1	10 minutes	72°C

Table A2-3. Thermocycling conditions for Big Dye Terminator V1.1 sequencing PCR.

	Cycles	Time	Temperature
Initial denaturation	1	1 minute	96°C
Denaturation		10 seconds	96°C
Annealing	25	5 seconds	50°C
Elongation		4 minutes	60°C
Hold	1	∞	4°C

Table A2-4. Thermocycling conditions for GoTaq colony screening PCR.

	Cycles	Time (minutes)	Temperature
Initial denaturation	1	5 minutes	95°C
Denaturation		30 seconds	95°C
Annealing	30	30 seconds	60.5°C
Elongation		150 seconds	72°C
Final elongation	1	5 minutes	72°C

Appendix 3: Buffers and solutions

Name	Contents
HEPES buffered saline	50 mM HEPES, 250 mM NaCl, 1.5 mM NaHPO4
Loading dye (10X)	60% glycerol, 0.25% bromophenol blue
LB agar	1 % bactotryptone, 0.5% yeast extract, 1% NaCl, 7% agar
LB broth	1% bactotryptone, 0.5% yeast extract, 0.5% NaCl; pH 7.0
PBS-BSA	1% bovine serum albumin (BSA) in PBS
PBS-Tween	0.1% Tween® 20 in PBS
PBS	137mM NaCl, 27 mM KCl, 1.5 mM KH_2PO_4,10.2 mM Na_2HPO_4; pH 7.4
SOC medium	LB broth, 0.04% glucose, 10 mM $MgSO_4$, 10 mM MgCl
TBE	1.25 M Tris, 27 mM EDTA, 0.4 M boric acid
TE Buffer	10 mM Tris.HCl pH 8.0, 1 mM EDTA
RBC lysis buffer	0.88% NH_4Cl, 10 mM; pH 7.4

All other buffers were provided with kits detailed in Chapter 2 and their contents are specified by manufacturers in product documentation.

Appendix 4: Autologous and heterologous neutralisation patterns in FIV infected cats

Strong neutralisation

Strong neutralisation displayed by plasma samples collected from cats that remained alive during the study period.
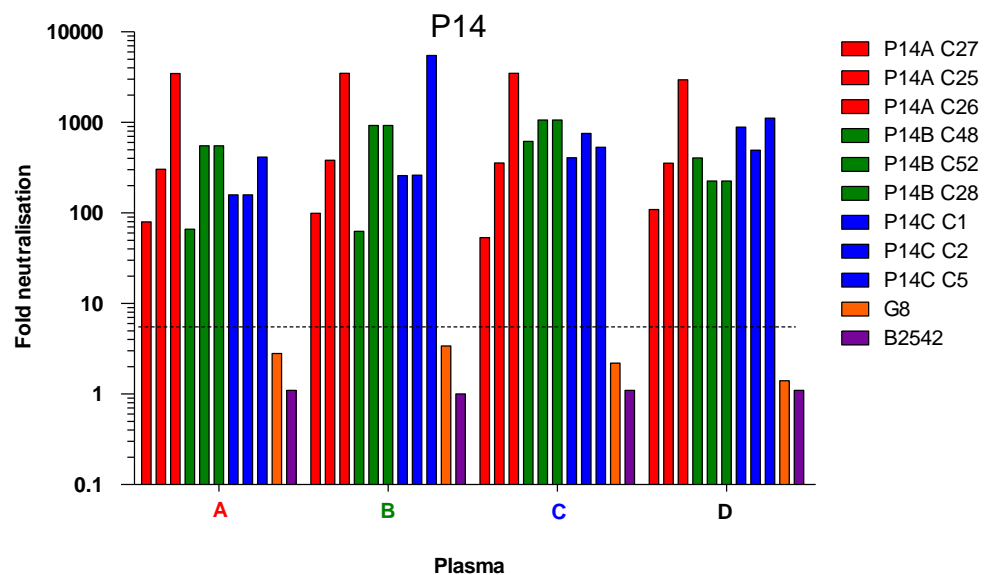

Figure A4-1 Autologous and cross reactive NAb response of plasma from cat P14. Plasma samples from each time point A, B, C and D (letters highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red) 2) 3 Env variants from time point B (highlighted in green) and 3) 3 Env variants from time point C (highlighted in blue). Cross neutralisation activity was assessed against two reference pseudotypes bearing the Envs of GL-8 (orange) and B2542 (purple). Pseudotypes, named according to their Envs (from left to right), are shown in the same order as in the graph legend (top to bottom).

Cat P14. As demonstrated in Figure A4-1, all plasma samples from cat P14 strongly neutralised pseudotypes bearing contemporaneous, earlier and later autologous Env variants (fold neutralisation ranged from 53 to 5499 at a 1 in 10 plasma dilution). The genetic diversity of the Env variants (calculated for the entire *env*) was relatively low (0.2%) (highest K2P pairwise distance) and no changes were observed in the number (24) or the pattern of PNGS (see results in Chapter 4). Cat P14 had been infected for approximately 2 years and maintained CD4 lymphocyte counts around 1000 cells/μl (ΔCD4 over the observation period

= +0.12 K/µl) (Table 5-3). No cross neutralisation of the GL-8 or B2542 pseudotypes was detected.

Cat P13. An increased potency of autologous neutralisation was observed in cat P13 (Figure A4-2). Plasma collected from time point A either did not, or only moderately, neutralised pseudotypes bearing autologous Envs (1.2 to 5.7 fold), but neutralisation potency increased at time point B and remained high against all but one autologous pseudotype during the observation period (10.2 to 201 fold).
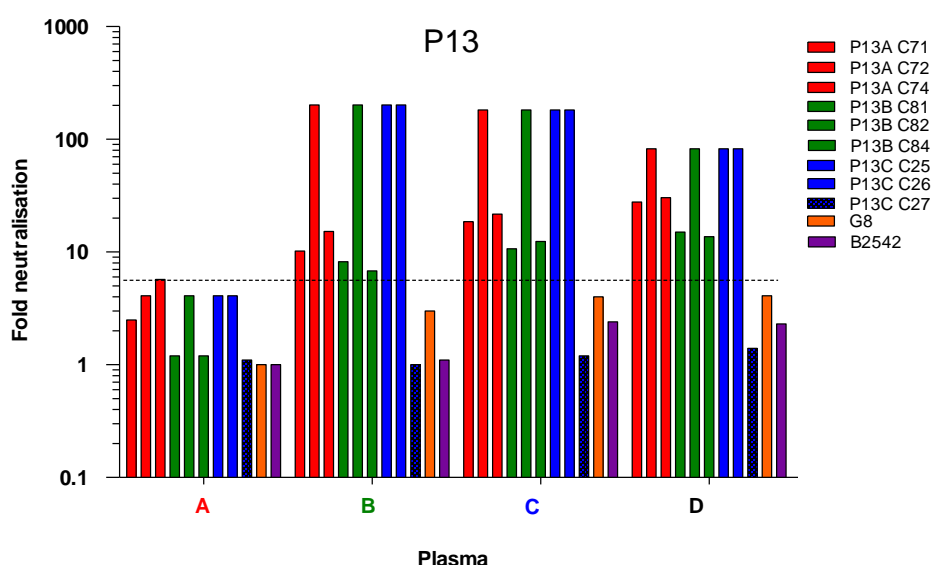

Figure A4-2 Autologous and cross reactive NAb response of plasma P13. Plasma samples from each time point A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red) 2) 3 Env variants from time point B (highlighted in green) and 3) 3 Env variants from time point C (highlighted in blue). The cross neutralisation activity of each plasma sample was assessed against two reference pseudotypes bearing the Envs of GL-8 (orange) and B2542 (purple). Pseudotypes tested are shown (from left to right) in the same order as in the legend (top to bottom). Note that pseudotype P13C C27 was resistant to neutralisation by all plasma samples tested (hatched blue).

A single pseudotype bearing an Env variant from time point C (P13C C27) remained resistant to neutralisation. The genetic diversity amongst the Env variants (calculated for the entire *env* as highest K2P pairwise distance) was 0.4%. We did not observe changes in the number (25) or location of N-linked glycosylation sites (Chapter 4). However, we identified 2 mutations, K450E and G556D unique to isolate P13C C27 that may have rendered it resistant to neutralisation (sequence alignments are provided on the CD that accompanies

this thesis). Cat P13 had been infected for approximately 1 year and a progressive decline in CD4 lymphocyte counts (from 460 to 270 cells/µl) was observed during the follow-up period (ΔCD4 = -180 cells/µl) (Table 5-3). No cross neutralisation of GL-8 or B2542 pseudotypes was detected, although during the course of infection we observed a slight increase in potency against the GL-8 pseudotype (from 1 to 4.1 fold).

Cat M47. Similar to cat P13, an increase in potency of autologous neutralisation was observed for cat M47 (Figure A4-3). Plasma samples from time points A and B did not neutralise autologous pseudotypes (1 to 2.4 fold), but neutralisation potency gradually increased over the course of infection, becoming strong at time point D (10.5 fold for M47A C135 to 1634 fold for M47B C94).
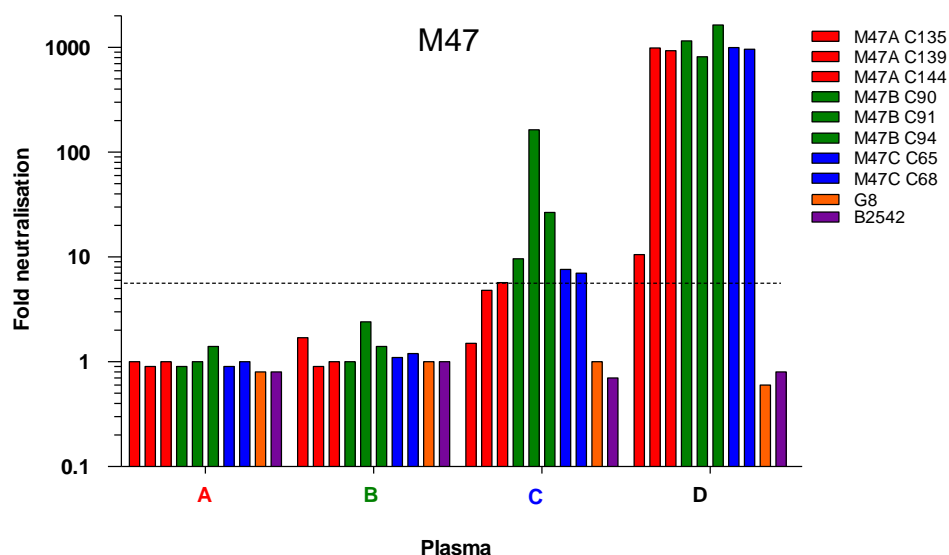

Figure A4-3 Autologous and cross reactive NAb response of plasma samples from cat M47. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red) 2) 3 Env variants from time point B (highlighted in green) and 3) 2 Env variants from time point C (highlighted in blue). Cross neutralisation activity was assessed against two reference pseudotypes bearing GL-8 (orange) and B2542 (purple) Envs. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom). Note the progressive development of neutralisation potency: plasma D neutralised all M47 pseudotypes tested. M47A C135 was least susceptible to neutralisation.

Genetic diversity amongst the M47 *env* clones was 0.9%. The number of N-linked glycosylation sites varied between viral variants from 19 to 22. The least neutralised viral variant M47A C135 (fold neutralisation 10.5) surprisingly, had

the lowest number (19) of N-linked glycosylation sites (discussed in Chapter 4). Cat M47 had been infected for approximately 3 years and, despite the development of a vigorous NAb response, experienced a progressive decline in CD4 T lymphocytes from 290 to only 100 cells/µl at the final sampling time (ΔCD4 = -190 cells/µl) (Table 5-3). No cross neutralisation of the GL-8 or B2542 pseudotypes was detected (Figure A4-3).

Cat M15. Plasma from each time point from cat M15 neutralised all the pseudotypes bearing autologous Env variants that were tested (fold neutralisation ranged from 183 to 5908), with the exception of one pseudotype bearing an Env variant from time point A (M15A C13) which was neutralisation resistant (Figure A4-4). Intra-host genetic diversity calculated for the entire *env* as highest K2P pairwise distance of 0.2% was surprisingly low. There were no changes in the number or positions of N-linked glycosylation sites (25). Cat M15 had been infected for approximately 2 years at the start of the study and demonstrated a progressive decline in CD4 T lymphocytes from 870 to 470 cells/µl during the 18 months of observation (ΔCD4 = -400 cells/µl) (Table 5-3). No cross neutralisation of the GL-8 or B2542 pseudotypes was detected (Figure A4-4).
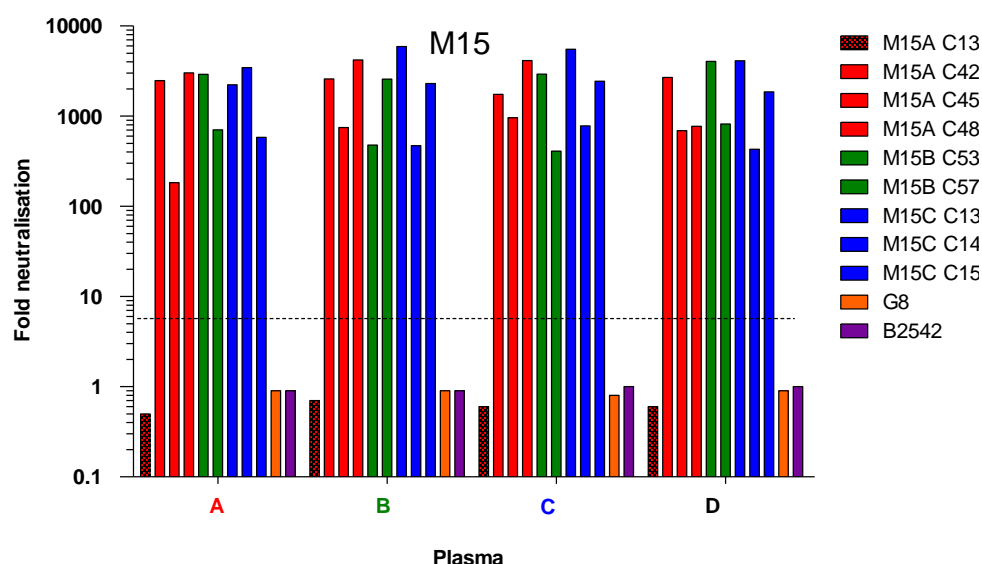

Figure A4-4 Autologous and cross reactive NAb response of plasma M15. Plasma samples from each time point A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 4 Env variants isolated from time point A (highlighted in red) 2) 2 Env variants from time point B (highlighted in green) and 3) 3 Env variants from time point C (highlighted in blue). Cross neutralisation activity of each plasma sample was assessed against two reference pseudotypes bearing GL-8 (orange) and

Cat P4. All P4 plasma samples neutralised autologous pseudotypes from time point A but there was decreased neutralisation potency against pseudotypes bearing time point A Envs during the course of infection (approx. 110 fold for plasma A compared to approx.17 fold neutralisation for plasma D) (Figure A4-5). There was a delayed NAb response against pseudotypes bearing Env variants from time point B, which were moderately to strongly neutralised only by plasma D (2.7 to 6.6 fold).
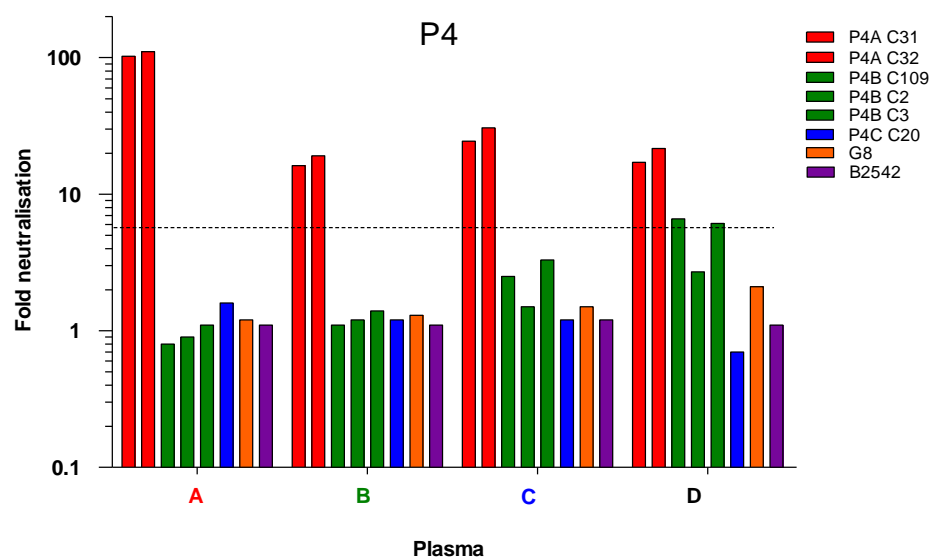

Figure A4-5 Autologous and Cr-NAb responses of plasma P4. Plasma samples from each time point A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 2 Env variants isolated from time point A (highlighted in red) 2) 3 Env variants from time point B (highlighted in green) and 3) 1 Env variant from time point C (highlighted in blue). Cross neutralisation activity of each plasma sample was assessed against two reference pseudotypes bearing GL-8 (orange) and B2542 (purple) Envs. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom). Note delayed neutralisation response against pseudotypes bearing Envs from time point B (P4B, green).

The pseudotype bearing the Env variant P4C C20 resisted neutralisation by the contemporaneous plasma C and the later plasma D. We did not observe Cr-NAb against the GL-8 or B2542 pseudotypes (Figure A4-5). The genetic diversity of the P4 *envs* (highest K2P pairwise distance of 1.8%) was high compared to other cats and there were changes in the number and positions of N-linked

glycosylation sites, ranging from 22 to 25. Cat P4 had been infected for approximately 3.5 years at the start of the study and showed a marked increase in CD4 T lymphocytes, from 90 to 330 cells/µl over the 18 months of observation (ΔCD4 = +240 cells/µl) (Table 5-3).

Cat M2. Only 2 pseudotypes bearing Env variants from cat M2 were available (Figure A4-6). All plasma samples showed different neutralisation potencies against pseudotypes bearing these two variants; the M2C C1 pseudotype was consistently more susceptible to neutralisation than the M2C C5 pseudotype. The strongest neutralisation against M2C C1 (276 fold) and M2C C5 (5.8 fold) was observed with the contemporaneous plasma sample from time point C but decreasing neutralisation was observed at time point D (69.2 and 3.7 fold for C1 and C5 respectively).
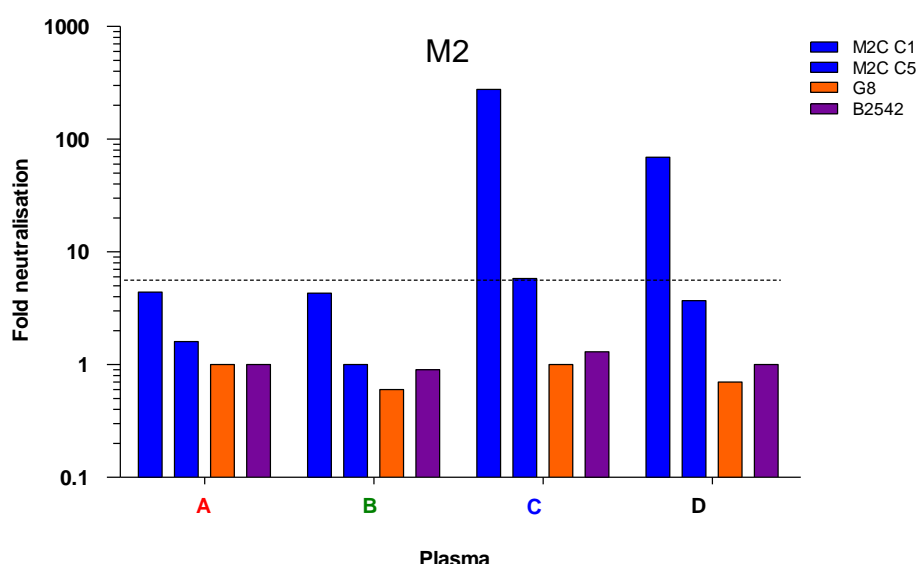

Figure A4-6 Autologous and Cr-NAb response of plasma from cat M2. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against 2 pseudotypes bearing Env variants from time point C (highlighted in blue). The cross neutralising activity of each plasma sample was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) are above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom). Plasma C and D displayed strong neutralisation against M2C C1 and only moderate to strong neutralisation against M2C C5. No cross neutralisation was observed.

The highest K2P pairwise distance between the two *envs* was 0.9 %. Both Env variants had the same number of PNGs but the differences in susceptibility to neutralisation may have been attributed to differences in the N-linked

glycosylation patterns between the two Envs (M2C C1 at position 550, M2C C5 at position 553). Cat M2 had been infected for approximately 6.3 years at the start of the study and displayed a marked decline in the number of circulating CD4 T lymphocytes (from 1740 to 620 cells/µl over 18 months of observation (ΔCD4 = -1120 cells/µl) (Table 5-2). No neutralisation of the GL-8 or B2542 pseudotypes was observed (Figure A4-6).

Cat P22. Plasma samples from cat P22 were available at time points B, C and D and only two Env variants were obtained from this cat (Figure A4-7). All plasma samples strongly neutralised the P22C C73 and P22C C80 pseudotypes (5678 to 54080 fold), the *env*s of which differed from each other by 0.4% (calculated for the entire *env* as the highest K2P pairwise distance).
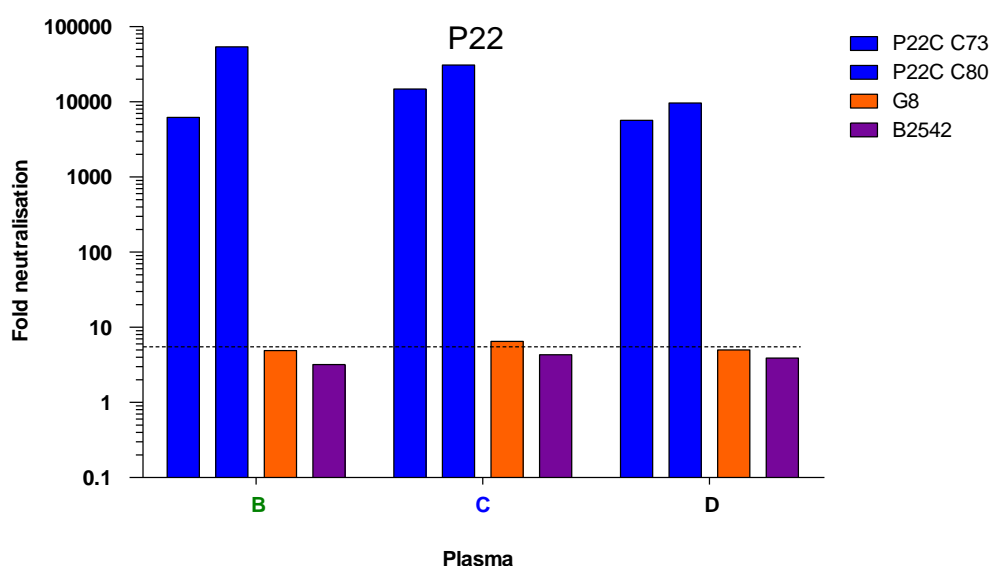

Figure A4-7 Autologous and Cr-NAb response of plasma P22. Plasma samples from time points B, C and D (highlighted on the X axis in green, blue and black respectively) were tested against pseudotypes bearing 2 Env variants from time point C (highlighted in blue). The cross neutralisation activity of each plasma sample was assessed against reference pseudotypes GL-8 (clade A) (orange) and B2542 (clade B) (purple). Values indicative of strong neutralisation (>5.6 fold) are above dashed horizontal line. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom). Moderate cross neutralisation against the B2542 and GL-8 pseudotypes was observed.

Although both pseudotypes were strongly neutralised, the P22C C73 and P22C C80 *env* genes contained 23 and 24 N-linked glycosylation sites respectively. Cat P22 had been infected for approximately 2 years at the start of the study and showed a decline in the number of circulating CD4 T lymphocytes, from 1550 to

790 cells/µl over the 6 month observation period (ΔCD4 = -760 cells/µl) (Table 5-3). Moderate to strong neutralisation was observed against the GL-8 pseudotype (4.9 to 6.5 fold), compared to moderate neutralisation of the B2542 pseudotype (3.2 to 4.3 fold) (Figure A4-7).

Cat P18. Plasma samples from cat P18 were available only from time points B, C and D and only one Env variant was isolated from this cat (Figure A4-8). All plasma samples strongly neutralised pseudotypes bearing P18B C13 (2561 to 3083 fold) which contained 25 PNGs. Cat P18 had been infected for approximately 3 years at the start of the study and showed increasing numbers of circulating CD4 T lymphocytes, from 730 to 750 cells/µl over 6 months of observation (ΔCD4 = +20 cells/µl) (Table 5-3). Moderate to strong cross-reactivity against the GL-8 pseudotype was observed (5.5 to 9.6 fold) (Figure A4-8).
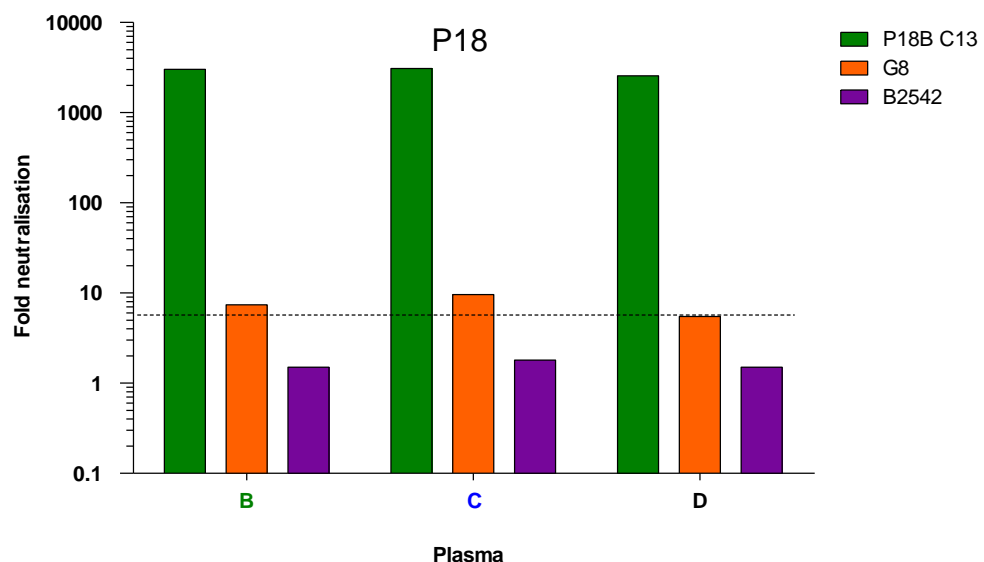

Figure A4-8 Autologous and Cr-NAb response of plasma from cat P18. Plasma samples from time points B, C and D (highlighted on the X axis in green, blue and black respectively) were tested against a pseudotype bearing an Env variant from time point B (highlighted in green). The cross neutralising activity of each plasma sample was assessed against two reference pseudotypes bearing GL-8 (orange) and B2542 (purple) Envs. Values indicative of strong neutralisation (>5.6 fold) are above dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom). The P18B C13 pseudotype was neutralised by all plasma samples. Moderate to strong cross-neutralisation of the GL-8 pseudotype was observed.

Strong neutralisation pattern displayed by plasma from cats which died during the study.

Cat M30. Plasma samples from cat M30 from time points A and B failed to neutralise pseudotypes bearing contemporaneous Env variants (Figure A4-9). Strong neutralisation of all 20 pseudotypes tested was observed by plasma collected at time point C (neutralisation from 173 to 3768 fold) but a decrease in neutralisation potency was observed at time point D (from 63 to 259 fold), shortly before the cat's death (19.09.11). The genetic diversity, calculated for the entire *env* as the highest K2P pairwise distance, was 0.5%. No changes in the number (25) or location of N-linked glycosylation sites were observed (Chapter 4).
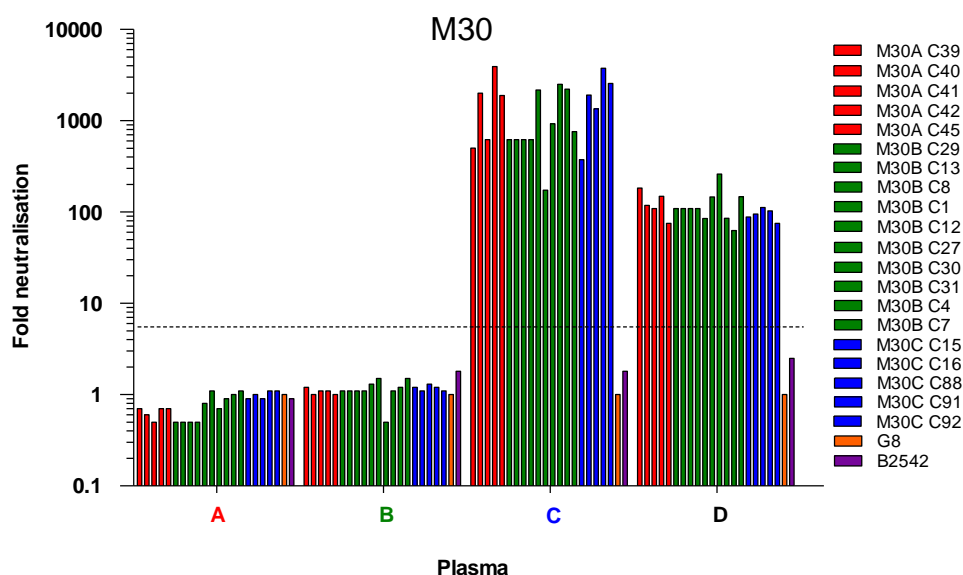

Figure A4-9 Autologous and Cr-NAb response of plasma from cat M30. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 5 Env variants isolated from time point A (highlighted in red) 2) 10 Env variants from time point B (highlighted in green) and 3) 5 Env variants from time point C (highlighted in blue). The cross neutralising activity of each plasma sample was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Pseudotypes are shown (from left to right) in the same order as in the graph legend (top to bottom). Autologous NAbs were measured at time point C but neutralisation was lower at time point D, before the cat's death.

Cat M30 had been infected for approximately 1.5 years at the time of enrolment and, despite vigorous NAb responses, demonstrated a decline in CD4 counts from 130 to 100 cells/µl over 18 months (ΔCD4 = -30 cells/µl). Interestingly, the high neutralisation observed at time point C coincided with the highest CD4

lymphocyte count (260 cells/µl). Subsequently the CD4 lymphocyte count decreased to 100 cells/µl (Table 5-2). No cross neutralisation against the GL-8 or B2542 pseudotypes was observed (Figure A4-9).

Cat M14. Plasma samples from cat M14 collected at time points A and B failed to neutralise pseudotypes bearing contemporaneous Env variants (Figure A4-10) Strong, increasing in potency neutralisation was observed from time point C onwards until the cat's death (20.08.11).
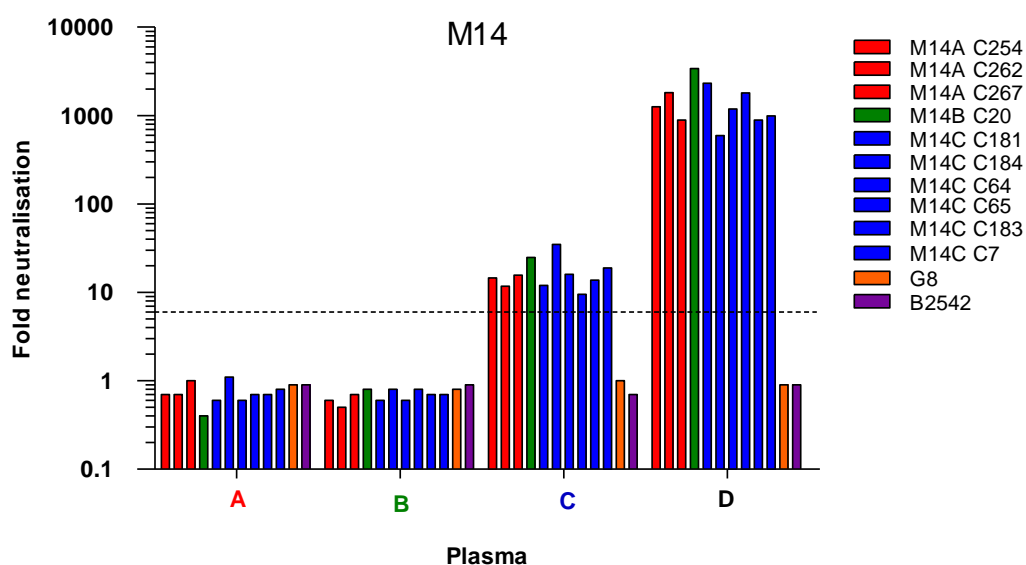

Figure A4-10 Autologous and Cr-NAb response of plasma from cat M14. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red) 2) an Env variant from time point B (highlighted in green) and 3) 6 Env variants from time point C (highlighted in blue). The cross neutralising activity of each plasma sample was assessed against two reference pseudotypes bearing GL-8 (orange) and B2542 (purple) Envs. Pseudotypes are shown (from left to right) in the same order as in the graph legend (top to bottom). Strong autologous NAb responses were present at time point C, with increasing potency observed at time point D, 6 months later.

Plasma D, obtained shortly before the cat's death, strongly neutralised all 10 pseudotypes tested (neutralisation values ranged from 591 to 3404 fold). The genetic diversity amongst the *envs,* calculated for the entire *env* as the highest K2P pairwise distance, was 0.2%. Despite the strong NAb response, there were no changes in the number (25) or location of N-linked glycosylation sites. Cat M14 had been infected for approximately 4.5 years at the time of enrolment and, despite a vigorous NAb response, demonstrated a progressive decline in CD4

lymphocyte counts from 450 to 140 cells/µl over 18 months (ΔCD4 = -310 cells/µl) (Table 5-2). No cross neutralisation of the GL-8 or B2542 pseudotypes was observed (Figure A4-10).

Cat M25. Plasma samples from cat M25 were tested against pseudotypes bearing 3 Env variants from time point A and 3 from time point C (Figure A4-11). Plasma A neutralised the contemporaneous M25A C98 and M25A C99 pseudotypes, but failed to neutralise the M25A C100 pseudotype which became susceptible to neutralisation by later plasma samples. Resistance to neutralisation of the M25A C100 pseudotype by contemporaneous plasma can most likely be attributed to one or more of the several mutations (K452R, H453Y, N495K, D552G, K554D, G555I, N556S, N558T and deletion at position 559) observed in the M25A Env compared to M25A C98 and M25A C99 Env sequences (sequence alignments are provided on the CD that accompanies this thesis).

Figure A4-11 Autologous and Cr-NAb response of plasma from cat M25. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red) and 2) 3 Env variants from time point C (highlighted in blue). Cross neutralisation activity of each plasma sample was assessed against two reference pseudotypes bearing the GL-8 (orange) and clade B, B2542 (purple) Envs. Pseudotypes are shown (left to right) in the same order as in the graph legend (top to bottom). Autologous NAb responses were potent against all pseudotypes except M25A C100 (red with dotted pattern), which was susceptible to neutralisation by sera collected from time point B onwards. Pseudotype M25C C38 (blue chequered pattern) was only moderately to strongly neutralised.

Within the Env variants isolated from blood collected at time point C, we observed that one pseudotype, bearing the M25C C38 Env, was moderately to strongly neutralised by all plasma samples (from 3.6 to 6.1 fold), while the remaining two pseudotypes were strongly neutralised (from 126 to 6971 fold). These differences in resistance to neutralisation can most likely be attributed to several differences in the sequences of the Env variants (N218D, R395K and several mutations at positions from 552 to 559). The genetic diversity calculated for the entire *env*, was 1.1%. All but one Env variant contained 25 PNGS; M25C C39 contained 24 PNGS. Cat M14 had been infected for approximately 1.5 years at the time of enrolment and displayed a progressive decline in CD4 lymphocyte numbers, from 360 to 90 cells/µl recorded shortly before it died (04.09.11) (ΔCD4 = -270 cells/µl) (Table 5-2). There was no cross neutralisation of the GL-8 or B2542 pseudotypes (Figure A4-11**).**

Cat M49. Plasma samples from cat M49 were tested against 3 pseudotypes bearing Envs from time point A and 3 bearing Envs from time point C **(**Figure A4-12**).**
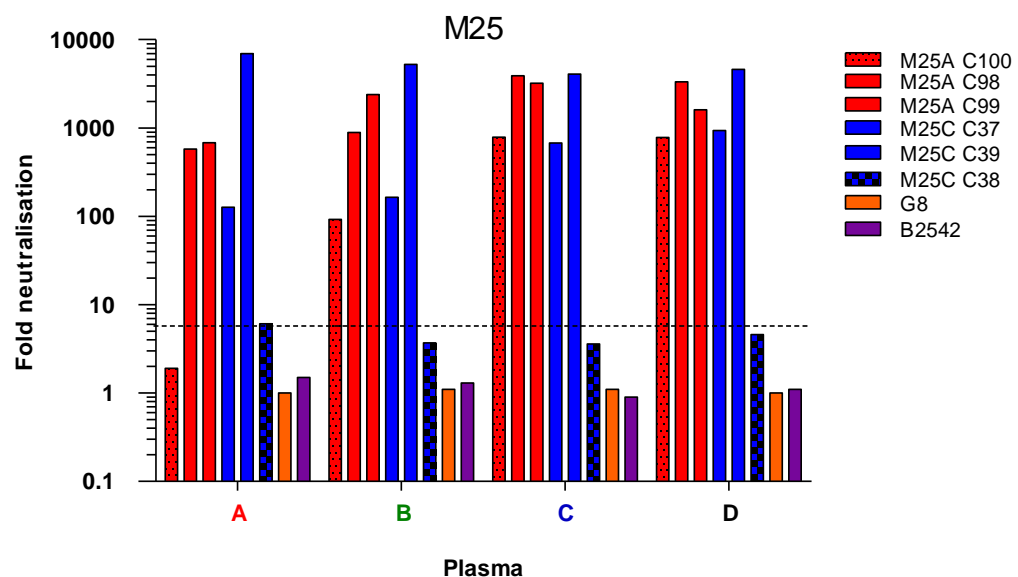

Figure A4-12 Autologous and Cr-NAb response of plasma M49. Plasma samples from time point A, B, C and D highlighted on the X axis in red, green, blue and black respectively were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red) and 2) 3 Env variants from time point C (highlighted in blue). The cross reactivity of each plasma sample was assessed against two reference pseudotypes bearing clade A GL-8 (orange) and clade B B2542 (purple) Envs. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom). Only M49A C87 was susceptible to neutralisation.

Plasma A neutralised one pseudotype bearing a contemporaneous Env (M49A C87) while the remaining two pseudotypes (M49A C85 and M49A C86) were neutralisation resistant.

These two pseudotypes were also resistant to neutralisation by subsequent plasma samples (B, C and D). Similarly 3 pseudotypes bearing Envs from time point C were also neutralisation resistant. All neutralisation resistant pseudotypes bore Envs containing 25 PNGS while the neutralisation susceptible pseudotype bore an Env variant containing only 23 PNGS; this Env was genetically the most distant (highest pairwise distance 1.3%) Cat M49 had been infected for approximately 3.5 years at the time of enrolment and showed a dramatic decline in CD4 lymphocyte counts, from 410 to 40 cells/µl recorded shortly before it died of lymphoma (18.07.11) (ΔCD4 = -370 cells/µl) (Table 5-2). No cross neutralisation was observed against the GL-8 or B2542 pseudotypes (Figure A4-12).

Cat M26. Plasma samples from cat M26 were available from 3 time points and were tested against 3 pseudotypes bearing Envs from time point A and 3 from time point C (Figure A4-13).
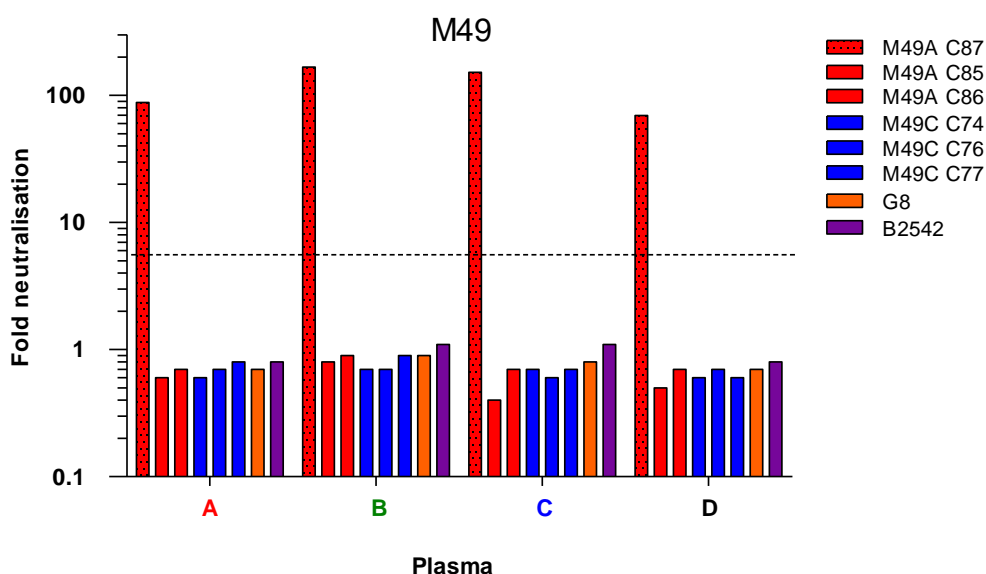

Figure A4-13 Autologous and Cr-NAb response of plasma from cat M26. Plasma samples from time points A, B and C highlighted on the X axis in red, green and blue respectively, were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red) and 2) 3 Env variants from time point C (highlighted in blue). The cross neutralisation activity of each plasma sample was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Pseudotypes are shown (from left to right)

All pseudotypes bearing Envs from time point A (M26 A C109, C110 and C112) and one time point C pseudotype (M26C C50) were strongly neutralised by all plasma samples. Two pseudotypes bearing Env variants from time point C (M26C C49 and C58) remained resistant to neutralisation until the cat's death (15.04.11). Although the genetic distances amongst the Envs was very low (0.2%), resistant Env variants contained additional PNGS at position 551 compared to neutralisation sensitive Envs. Cat M26 had been infected for approximately 3.5 years and showed a dramatic decline in CD4 lymphocyte counts, from 540 to 130 cells/µl recorded shortly before it died (ΔCD4 = -410 cells/µl) (Table 5-2). No cross neutralisation was observed against the GL-8 or B2542 pseudotypes (Figure A4-13).

Cat M31. Plasma samples from cat M31 were available from 3 time points and were tested against a pseudotype bearing an Env from time point A and 16 pseudotypes bearing Envs from time point C (Figure A4-14). Pseudotype M31A C253 was strongly neutralised by all plasma samples. Similarly, 6 pseudotypes bearing time point C Envs (M31C C4, C8, C12, C14, C15 and C16) were susceptible to neutralisation.
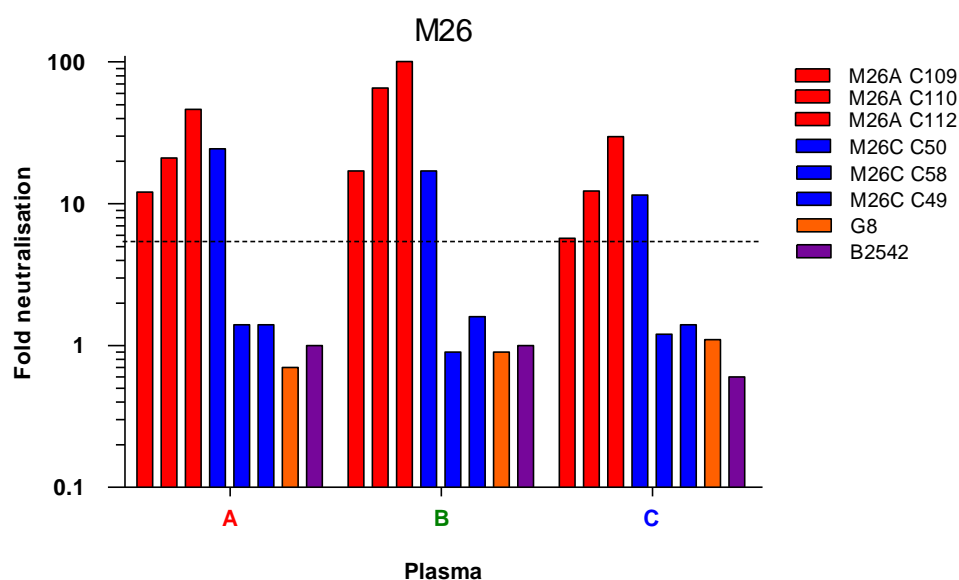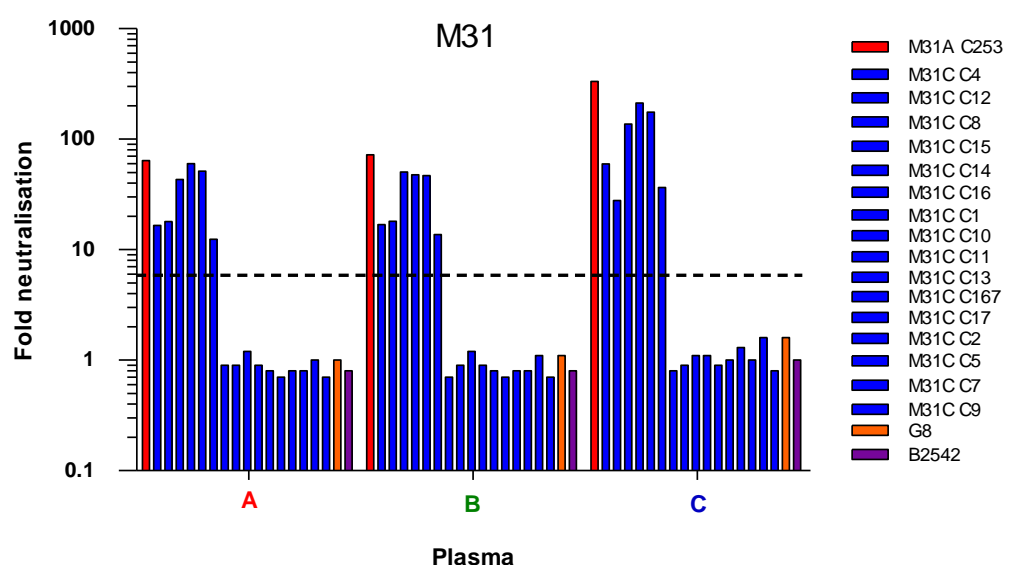

Figure A4-14 Autologous and Cr-NAb responses of plasma from cat M31. Plasma samples from each time point (A, B and C highlighted on the X axis in red, green and blue

respectively) were tested against pseudotypes bearing: 1) 1 Env variant isolated from time point A (highlighted in red) and 2) 16 Env variants from time point B (highlighted in blue). Cross neutralisation activity of each plasma sample was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (from left to right) in the same order as in the graph legend (top to bottom). A significant proportion of pseudotypes bearing time point C Env variants (10) resisted neutralisation shortly before the cat's death.

In contrast, the remaining 10 pseudotypes bearing time point C Envs remained neutralisation resistant until the cat's death (10.03.11). Although the Env variants had various N-linked glycosylation patterns (ranging from 21 to 23), neutralisation sensitivity appeared to be influenced by various amino acid substitutions within Env rather than by the presence or absence of PNGS. Cat M31 had been infected for approximately 6 years at the time of enrolment and CD4 lymphocyte counts decreased from 800 to 260 cells/μl recorder shortly before it died (ΔCD4 = -540 cells/μl) (Table 5-2). No cross neutralisation was observed against GL-8 or B2542 pseudotypes (Figure A4-14).

Cat P2. Plasma samples from cat P2 were available from two time points (A and B) and were tested against sixteen pseudotypes bearing Envs from time point B (Figure A4-15). Six pseudotypes (P2B C6, C18, C24, C52, C59 and C65) were susceptible to neutralisation, but the remaining 10 were resisted neutralisation by plasma collected until the cat's death (13.12.10). Resistance to neutralisation was most likely associated with the absence of a PNGS at position 554 compared to Env variants which were neutralisation sensitive.
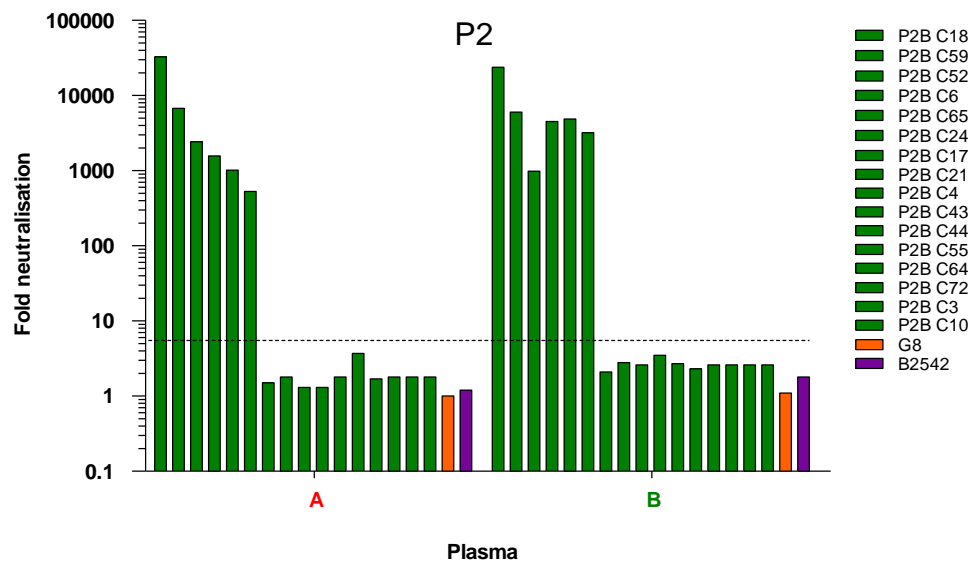
Figure A4-15 Autologous and Cr-NAb response of plasma from cat P2. Plasma samples from time points A and B (highlighted on the X axis in red and green respectively) were tested against 16 pseudotypes bearing Envs from time point B (highlighted in green). The cross reactivity of each plasma sample was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the graph legend (top to bottom). No Cr-NAb response was observed.

For the *env* genes isolated from cat P2, the genetic diversity, calculated for the entire *env* as the highest K2P pairwise distance, was 1.5%. Cat P2 had been infected for approximately 4 years at the time of enrolment and showed a slight decline in CD4 lymphocyte counts from 400 to 360 cells/μl over the 6 months observation period (ΔCD4 = -40 cells/μl) (Table 5-3). No cross neutralisation was observed against the GL-8 or B2542 pseudotypes (Figure A4-15).

Cat M44. A single plasma sample was available from cat M44 and this was tested against two pseudotypes bearing contemporaneous time point A Envs (Figure A4-16). M44A C91 and C92 were strongly neutralised (12.9 and 17.6 fold respectively). The M44A C91 Env contained 24 PNGS, while the M44A C92 Env contained 25. Cat M44 had been infected for approximately 3.5 years and, shortly prior to death (14.04.10), displayed high CD4 lymphocyte counts (1240 cells/μl) (Table 5-2). Again, no cross neutralisation was observed against either the GL-8 or B2542 pseudotypes (Figure A4-16).
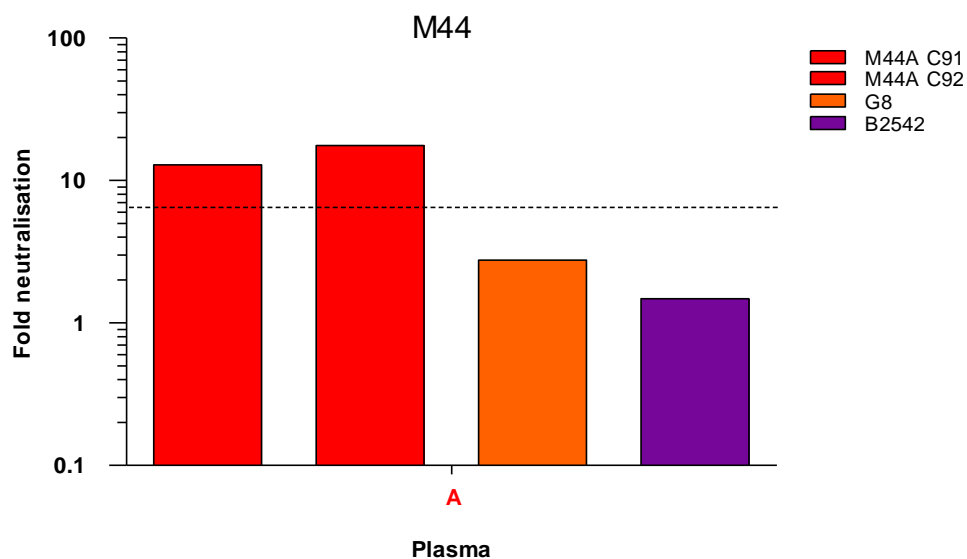
Figure A4-16 Autologous and Cr-NAb response of plasma M44. Plasma from time point A (highlighted on the X axis in red) was tested against pseudotypes bearing 2 Env variants isolated from time point A (highlighted in red). Cross neutralisation was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom). This cat died shortly after sampling A.

Moderate neutralisation

Cat M46. All plasma samples collected from cat M46 moderately to strongly neutralised all but one of the pseudotypes bearing autologous Envs (from 4.8 to 10.6 fold). The remaining pseudotype, bearing an Env from time point A (M46A C73), was weakly to moderately neutralised (from 2 to 4.2 fold) (Figure A4-17).

Figure A4-17. Autologous and Cr-NAb response of plasma from cat M46. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against: 1) 3 pseudotypes bearing Envs isolated from time point A (highlighted in red), 2) 2 time point B pseudotypes (highlighted in green) and 3) 3 time point C pseudotypes (highlighted in blue). Cross neutralisation was assessed against GL-8 (orange) and B2542 (purple) pseudotypes. Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. All autologous pseudotypes were moderately to strongly neutralised except M46A C73 (in red with dotted pattern).

The genetic diversity amongst the autologous *env* sequences was 0.2% and all Envs contained 23 PNGS. The decreased sensitivity to neutralisation of the pseudotype bearing the M46A C73 Env was associated with mutation W440L, unique to this Env and absent from all other Env variants tested. Cat M46 had been infected for approximately 3 years at the start of the study and demonstrated a slight increase in CD4 T lymphocyte numbers, from 150 to 180 cells/μl recorded shortly before death (23.07.11) (ΔCD4 = +30 cells/μl) (Table 5-2). No cross neutralisation against the GL-8 or B2542 pseudotypes was detected (Figure A4-17).

Cat P5. An increase in neutralisation potency of sequential plasma samples from cat P5 was observed. Whereas plasma A did not neutralise any pseudotypes tested, the neutralisation potency of subsequent plasma samples increased and ranged from moderate to strong neutralisation (up to 6.9 fold neutralisation) for pseudotypes bearing autologous Envs isolated from later time points (Figure A4-18).
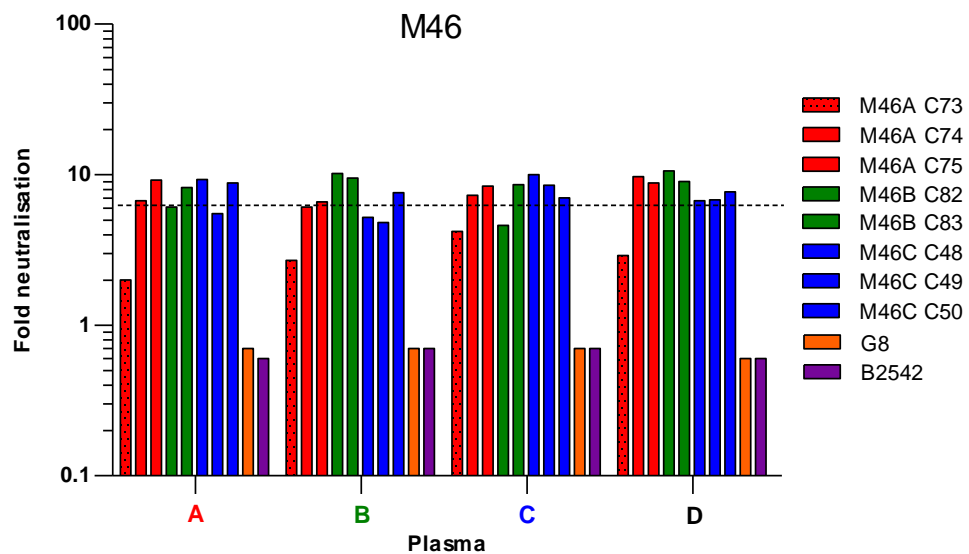

Figure A4-18 Autologous and Cr-NAb response of plasma samples from cat P5. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against: 1) 2 pseudotypes bearing Envs isolated from time point A (highlighted in red), 2) 3 pseudotypes from time point B (highlighted in green) and 3) 3 pseudotypes from time point C (highlighted in blue). Cross neutralisation was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line.

The genetic diversity amongst the *env* genes was 0.5% and the Env contained either 25 or 26 PNGS. (The Env variant P5 C118 of the least neutralised pseudotype; 0.4 to 3.5 fold neutralisation) contained 26 PNGS. Cat P5 had been infected for approximately 1.5 years at the start of the study and displayed a progressive decline in CD4 T lymphocyte numbers, from 790 to 300 cells/µl (ΔCD4 = -490 cells/µl) (Table 5-3). No cross neutralisation of the GL-8 or B2542 pseudotypes was detected (Figure A4-18).

Cat P9. Pseudotypes bearing autologous Envs from cat P9 were moderately to strongly neutralised by all plasmas (4 to 12.4 fold); however the neutralisation potency of plasma D against all pseudotypes was slightly less (Figure A4-19). The

genetic diversity amongst the autologous *envs*, expressed as highest K2P pairwise distance, was 0.4% and Envs had either 23 or 24 PNGS (Chapter 4). Cat P5 had been infected for approximately 3 years at the start of the study and developed a decline in CD4 T lymphocyte numbers, from 630 to 180 cells/µl over the 12 months of observation (ΔCD4 = -450 cells/µl) (Table 5-3). No cross neutralisation against the GL-8 or B2542 pseudotypes was detected (Figure A4-18).

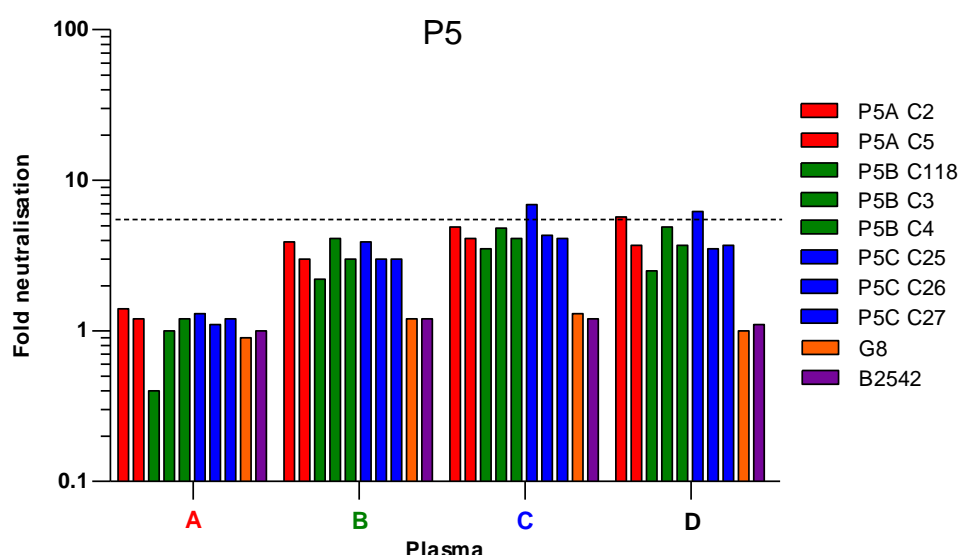**Figure A4-19 Autologous and Cr-NAb responses of plasma samples from cat P9. Plasma samples from each time point (A,B,C and D highlighted on the X axis in red, green, blue and black respectively) were tested against: 1) 3 pseudotypes isolated from time point A (highlighted in red), 2) 2 pseudotypes from time point C (highlighted in blue). Pseudotypes from time point B were not available. Cross neutralisation assessed against two pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line.**

Cat P11. Pseudotypes bearing autologous Envs from cat P11 were weakly neutralised, with the exception of the P11C C88 pseudotype which was moderately to strongly neutralised by all plasmas tested (5.4 to 6.5 fold) (Figure A4-20). The genetic diversity amongst *envs*, expressed as highest K2P pairwise distance, was 0.1% and all isolates had 25 PNGS (Chapter 4). Pseudotype P11C C88 was most susceptible to neutralisation; this Env contained an E511G mutation which was not present in other autologous Envs. Cat P11 had been infected for approximately 1.5 years at the start of the study and maintained a stable CD4 T lymphocytes count at 450 cells/µl over the 12 month study period (Table 5-3). Interestingly, plasma from this cat moderately to strongly

neutralised the heterologous GL-8 pseudotype (from 4.9 to 9.2 fold) as well as B2542 pseudotype (4.2 to 8.3 fold; Figure A4-20).
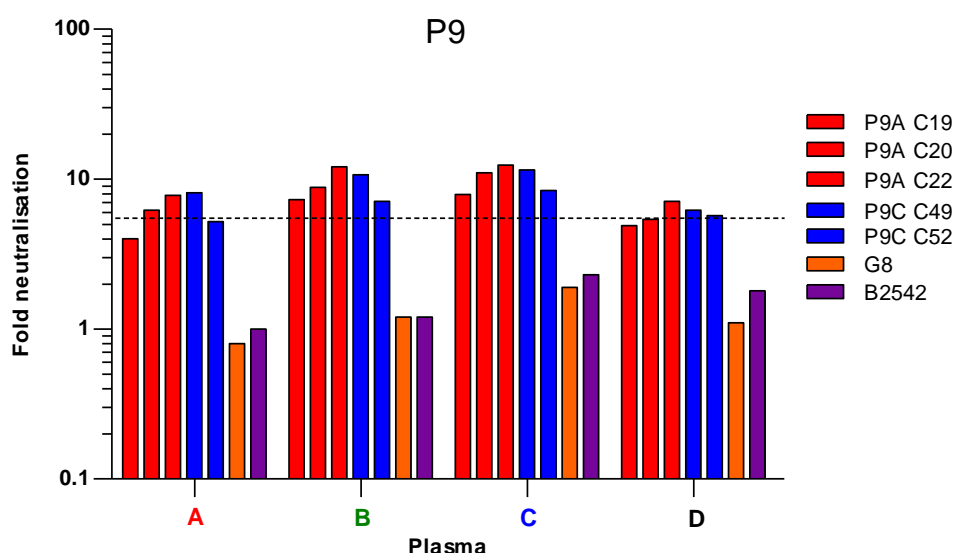

Figure A4-20 Autologous and Cr-NAb responses of plasma samples from cat P11. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against: 1) 3 pseudotypes bearing Envs isolated from time point B (highlighted in green) and 2) 4 pseudotypes bearing Envs from time point C (highlighted in blue). Pseudotypes bearing Envs from time point A were not available. Cross neutralisation was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Note moderate to strong cross-reactive neutralising activity was observed against the GL-8 and B2542 pseudotypes.

Cat P7. Two pseudotypes bearing Envs from cat P7 were available: one each from time point B and C. Both pseudotypes were moderately (4.9 fold for P7B C50) or strongly neutralised (7.5 fold for P7C C61) by plasma D but not plasma A, B or C (Figure A4-21). The *envs* on the sequence level differed from each other by 0.5%. Cat P7 had been infected for approximately 5.5 years at the start of the study and stably maintained its CD4 T lymphocytes count at 400 cells/µl over the 12 month observation period (Table 5-3). No cross neutralisation was observed against pseudotypes bearing the heterologous GL-8 or B2542 Envs (Figure A4-21).

Cat P8. A single pseudotype bearing an autologous Env from cat P8 was tested and found to be weakly to moderately neutralised (3.1 to 4.4 fold; Figure A4-22). Notably, plasma from cat P8 cross neutralised pseudotypes bearing heterologous Envs; the GL-8 pseudotype was moderately neutralised (3.1 to 4.5 fold), while the B2542 pseudotype was strongly neutralised (8.1 to 14.6 fold).

Cat P8 had been infected for approximately 3.5 years at the start of the study (suspected super-infection) and over the 12 month follow up, an increase in CD4 T lymphocytes count, from 500 to 600 cells/µl (Table 5-3), was observed.
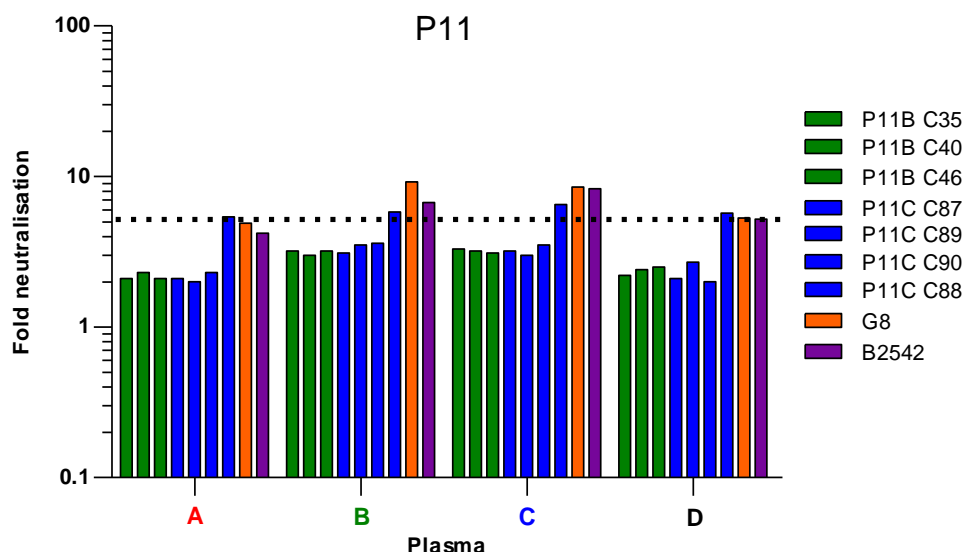

Figure A4-21 Autologous and Cr-NAb response of plasma from cat P7. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against: 1) 1 pseudotype bearing Env isolated from time point B (highlighted in green), 2) 1 pseudotype bearing Env from time point C (highlighted in blue). Pseudotypes bearing Envs from time point A were not available. Cross neutralisation was assessed against GL-8 (orange) and B2542 (purple) pseudotypes. Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line.

Figure A4-22 Autologous and Cr-NAb responses of plasma from cat P8. Plasma samples from time points A, B and C (highlighted on the X axis in red, green and blue respectively) were tested against 1 pseudotype bearing an Env variant from time point A (highlighted in red). Cross neutralisation activity was assessed against two reference pseudotypes bearing the Envs of GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom). Moderate to strong Cr-NAb responses were observed against the GL-8 and B2542 pseudotypes respectively.

Absent neutralisation

No NAbs in cats that remained alive during the study.

Cat M28. None of the pseudotypes bearing Env variants isolated from cat M28 were neutralised by autologous plasma (Figure A4-23). The diversity amongst the *envs* was 0.6% and each Env had either 24 or 25 PNGS. Cat M28 had been infected for approx. 4 years and, despite the lack of NAb response, remained healthy during the 18 month observation period. This cat did, however, display a progressive decline in CD lymphocyte numbers, from 1230 to 900 cells/µl, over the 18 month observation period (ΔCD4 = -330 cells/µl) (Table 5-2). No cross neutralisation of the GL-8 or B2542 pseudotypes was detected (Figure A4-23).
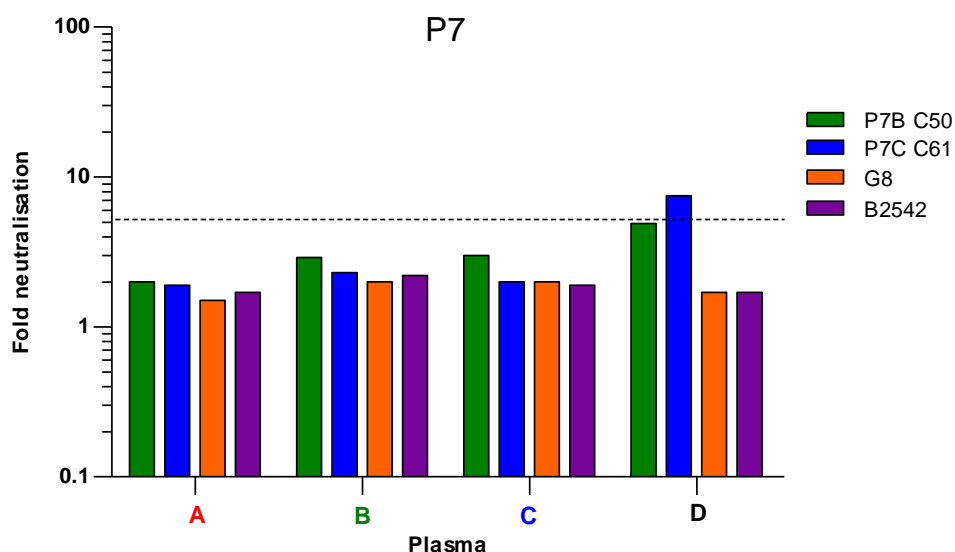
Figure A4-23 Autologous and Cr-NAb response of plasma collected from cat M28. Plasma samples from time points A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 3 Envs from time point A (in red), 2) 5 Envs from time point B (in green), and 3) 3 Envs from time point C (in blue). Cross neutralisation was assessed against two reference pseudotypes, bearing the clade A GL-8 (orange) and clade B B2542 (purple) Envs. Values indicative of strong neut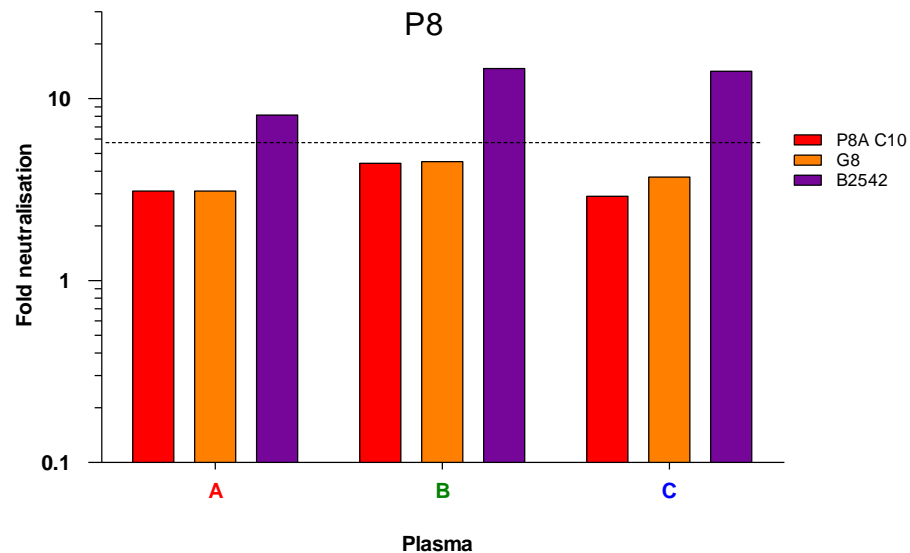ralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom). No Cr-NAb responses were observed.

Cat P17. None of the pseudotypes bearing Env variants from cat P17 were neutralised by autologous plasma samples (Figure A4-24). The *env* diversity was 0.5% and Env variants had 23 or 24 PNGS. Cat P17 had been infected for approx. 5 years and demonstrated a progressive decline in CD4 lymphocyte numbers, from 490 to 280 cells/μl, over the 18 month observation period (ΔCD4 = -210 cells/μl) (Table 5-3). No cross neutralisation of the GL-8 or B2542 pseudotypes was detected (Figure A4-24).

Figure A4-24 Autologous and Cr-NAb response of plasma collected from cat P17. Plasma samples from time points A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 2 Env variants from time point A (highlighted in red), 2) 5 Env variants from time point B (highlighted in green), and 3) 2 Env variants from time point C (in blue). Cross neutralisation was assessed against two reference pseudotypes bearing GL-8 (orange) or B2542 (purple) Envs. Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom). No Cr-NAb responses were observed.

Cat P6. Similarly, no autologous or cross-reactive NAb responses were observed in plasma collected from cat P6 (Figure A4-25). Intra-host *env* diversity was 0.4% and all Env variants contained 24 PNGS. Cat P6 had been infected for 3.5 years and, despite the lack of NAbs, displayed increased numbers of CD4 lymphocytes from 480 to 570 cells/µl over 12 months of study (ΔCD4 = +90 cells/µl) (Table 5-3). No cross neutralisation of the GL-8 or B2542 pseudotypes was detected (Figure A4-25).
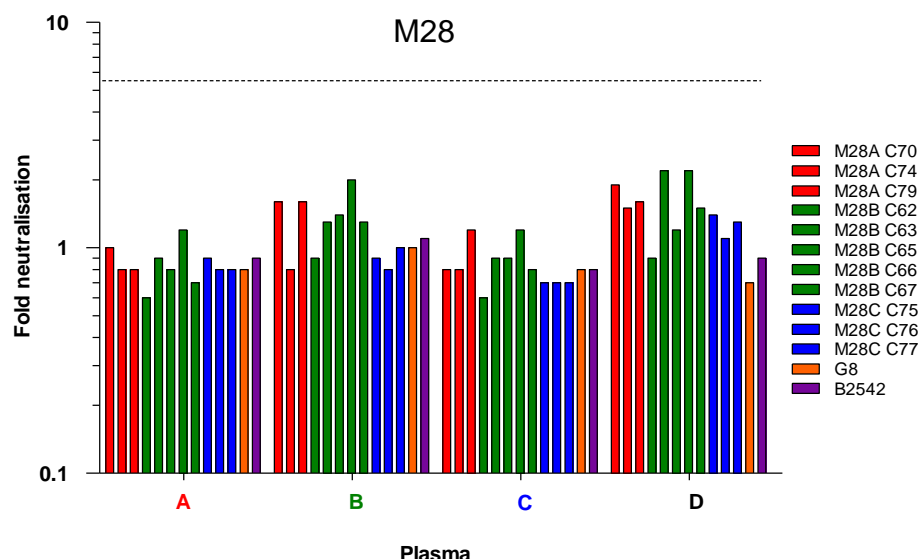
Figure A4-25 Autologous and Cr-NAb response of plasma from cat P6. Plasma samples from time points A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 3 Env variants from time point A (in red), 2) 3 Envs from time point B (in green), and 3) 2 Env variants from time point C. Cross neutralisation was assessed against GL-8 (orange) and B2542 (purple) pseudotypes. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom).

Cat M1. Pseudotypes bearing one and three Envs from time points A and C respectively were tested for neutralisation but none was neutralised by homologous plasma from cat M1 (Figure A4-26). The genetic diversity amongst the *envs* was 0.4%. Cat M1 had most likely acquired its infection following vertical transmission from its mother and at the start of the study was 1.5 years old. M1 displayed a progressive decline in the number of CD4 lymphocytes, from 870 to 260 cells/µl (ΔCD4 = -610 cells/µl) (Table 5-2), but remained healthy during the 18 month study period. No cross neutralisation of the GL-8 or B2542 pseudotypes was detected (Figure A4-26).
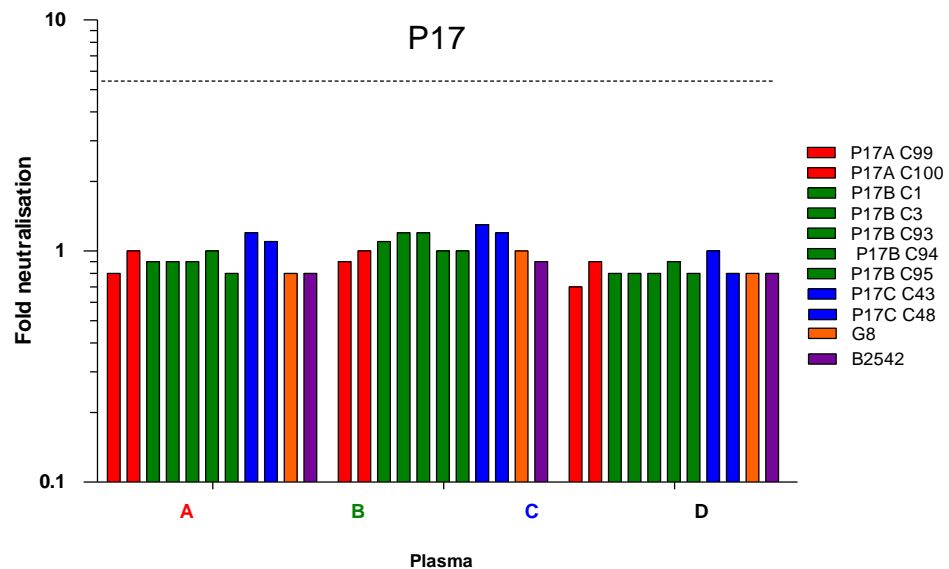
Figure A4-26 Autologous and Cr-NAb response of plasma from cat M1. Plasma samples from each time point (A, B, C and D highlighted on the X axis in red, green, blue and black respectively) were tested against pseudotypes bearing: 1) 1 Env variant isolated from time point A (red) and 2) 3 Env variants from time point C (blue). Cross neutralisation was assessed against the GL-8 (orange) and B2542 reference pseudotypes (purple).

Cat P21. Plasma from cat P21 did not neutralise any of the 6 pseudotypes bearing either autologous Envs or 2 heterologous Envs (Figure A4-33). The autologous *env* diversity was 11.9% and this cat was thought to have been super-infected. Despite the possibly high antigenic stimulation by the clade B (P21B) and recombinant A/B (P21C) strains that were isolated from this animal, no NAb responses were detected. Cat P21 had been infected for 1 year at recruitment and, during 6 months of observation, showed a decline of the CD4 lymphocyte count from 930 to 570 cells/µl (ΔCD4 = -360 cells/µl) (Table 5-3).

Figure A4-27 Autologous and Cr-NAb response of plasma P21. Plasma samples from time points B, C and D (highlighted on the X axis in green, blue and black respectively) were tested against pseudotypes bearing: 1) 4 Env variants from time point B (highlighted in green), and 2) 2 Env variants from time point C. Cross neutralisation was assessed against the GL-8 (orange) and B2542 (purple) reference pseudotypes. Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom). There was no Cr-NAb response observed.

Cat M32. No neutralising activity by plasma collected from cat M32 was observed (Figure A4-28). The *env* diversity was 0.1% and all Envs contained 25 PNGS. Cat M32 had been infected for 8 years and, despite this long duration of infection, no NAbs were detected. During the 18 month follow up period, the CD4 lymphocyte count declined from 380 to 210 cells/µl (ΔCD4 = -170 cells/µl) (Table 5-2).
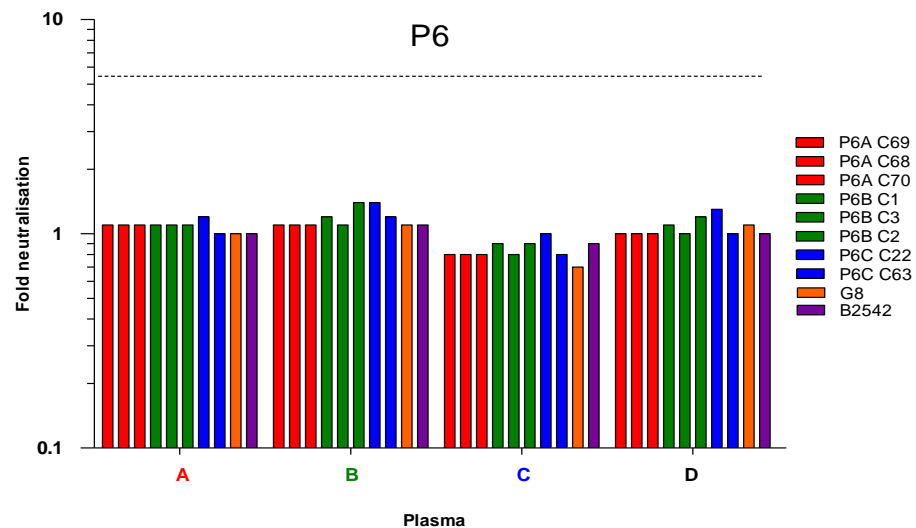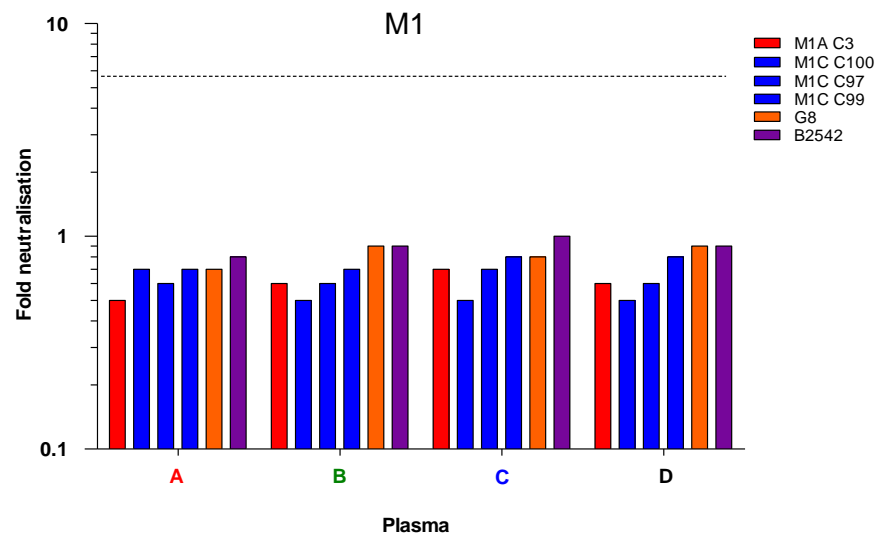
Figure A4-28 Autologous and Cr-NAb response of plasma from cat M32. Plasma samples from time points A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against 3 pseudotypes bearing Env variants from time point A (red). Cross neutralisation was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom).

Cat M29. No NAbs were observed in plasma collected from this animal (Figure A4-29). The *env* diversity was 0.1% and all Envs contained 25 PNGS. The cat had been infected for 3.5 years and its CD4 lymphocyte count decreased from 1480 to 620 cell/μl over the 18 month observation period (ΔCD4 = -560 cells/μl) (Table 5-2).

Cat M8. One pseudotype (M8C C144) bearing an autologous Env from this animal was tested and plasma M8 did not neutralise either this autologous pseudotype or the two heterologous reference pseudotypes (Figure A4-30). Cat M8 had been infected for 5.5 years at the time of recruitment and demonstrated a progressive decline in CD4 lymphocyte counts, from 550 to 150 cells/μl over the 18 month follow up period (ΔCD4 = -400 cells/μl) (Table 5-2).

Figure A4-29 Autologous and Cr-NAb response of plasma M29. Plasma samples from time points A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against 3 pseudotypes bearing Env variants isolated from time point C (in blue). Cross neutralisation was assessed against two reference pseudotypes, bearing clade A GL-8 (orange) or clade B B2542 (purple) Envs. Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom). No Cr-NAb response was observed.
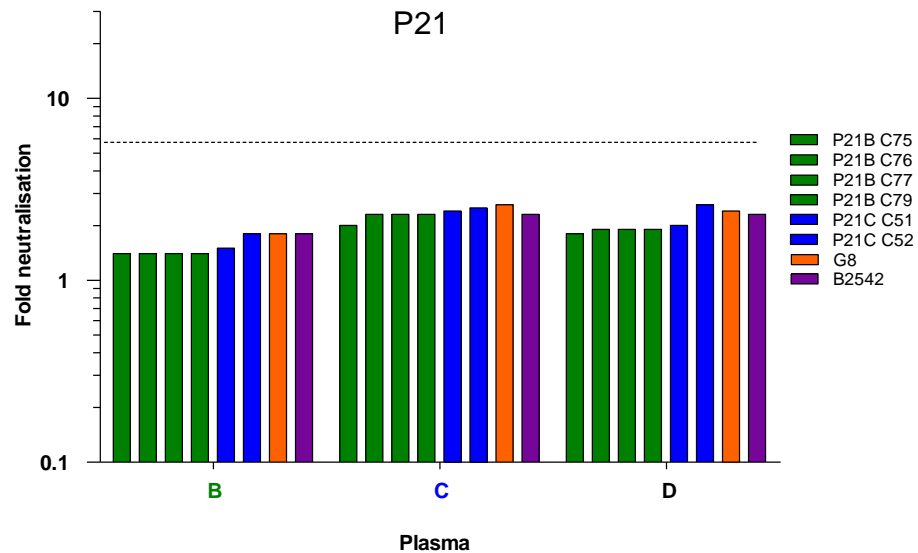

Figure A4-30 Autologous and Cr-NAb response of plasma M8. Plasma samples from time points A, B, C and D (highlighted on the X axis in red, green, blue and black respectively) were tested against a pseudotype bearing an Env variant from time point C (in blue). Cross neutralisation was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom). No Cr-NAb response was observed.

Cat M20. Similarly no neutralisation was observed with plasma collected from cat M20 (Figure A4-31), although plasma collected from time point C was not available. Cat M20 had been infected for 2.5 years and during the 18 month observation period its CD4 lymphocyte count decreased from 1500 to 1250 cells/µl (ΔCD4 = -250 cells/µl) (Table 5-2).
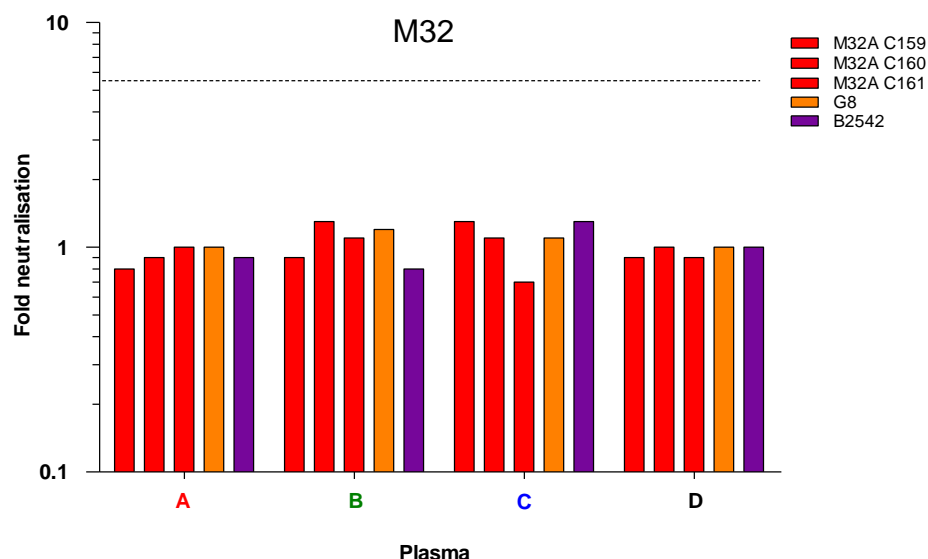

Figure A4-31 Autologous and Cr-NAb response of plasma from cat M20. Plasma samples from time points A, B and D (highlighted on the X axis in red, green and black respectively) were tested against a pseudotype bearing an Env variant from time point A (highlighted in green). Plasma C was not available. Cross reactivity was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (from left to right) in the same order as in the legend (top to bottom). No Cr-NAb response was observed.

No neutralisation in cats that died during the study.

Cat M11. None of pseudotypes bearing autologous Envs (n=15) or heterologous Envs (n=2) was neutralised by plasma from cat M11 (Figure A4-32). The intra-host *env* diversity was 0.7% and Env variants contained either 22 or 23 PNGS. Cat M11 had been infected for 2 years at the time of recruitment and, during the 12 months of observation, displayed a progressive decline in CD4 lymphocyte counts from 980 to 480 cell/µl recorded shortly before death (04.02.11) (ΔCD4 = -500 cells/µl) (Table 5-2).
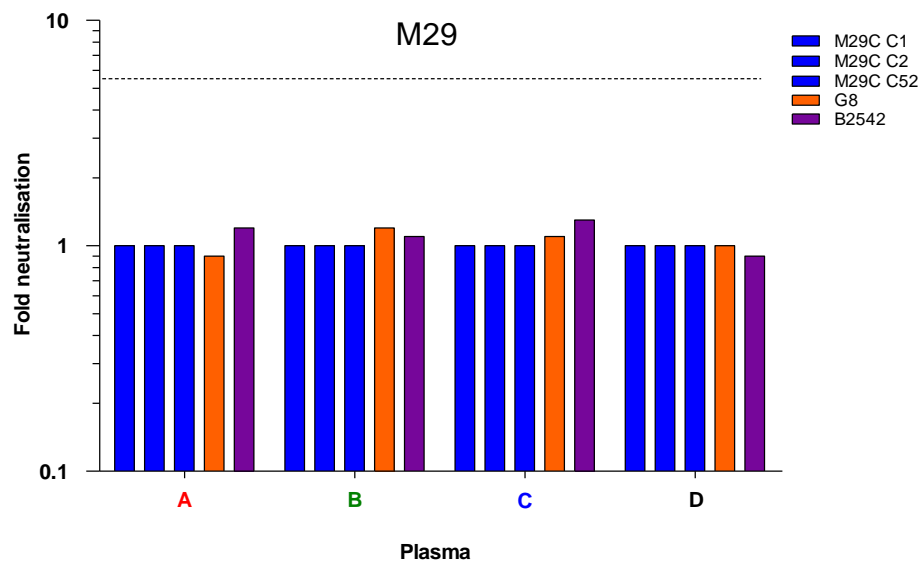
Figure A4-32 Autologous and Cr-NAb response of plasma M11. Plasma samples from each time point (A, B and C highlighted on the X axis in red, green and blue respectively) were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (red), 2) 3 Env variants from time point B (green) and 3) 9 Env variants from time point C (highlighted in blue).

Cat M5. No neutralisation of pseudotypes bearing autologous Envs was observed in cat M5 (Figure A4-33). Cat M5 had been infected for approx. 2.5 years prior to enrolment in the study and likely became superinfected between samplings B and C. The *env* diversity was as high as 4.2% and the number of PNGS ranged from 21 to 23 amongst Env variants. CD4 lymphocyte counts decreased from 560 to 160 cell/µl recorded at sampling C, 4 months before death (18.05.11) (ΔCD4 = -400 cells/µl) (Table 5-2).

Cat M41. None of the 11 pseudotypes bearing autologous Env variants that were tested was neutralised by plasma collected from cat M41 (Figure A4-34). The *env* diversity was 4.1% but all Env variants contained 22 PNGS. This cat had been infected for approx. 4.5 years at the time of enrolment and, during the 12 months of observation, displayed a decline in CD4 lymphocytes, from 340 to 120 cells/µl recorded shortly before death (03.02.11) (ΔCD4 = -220 cells/µl) (Table 5-2).
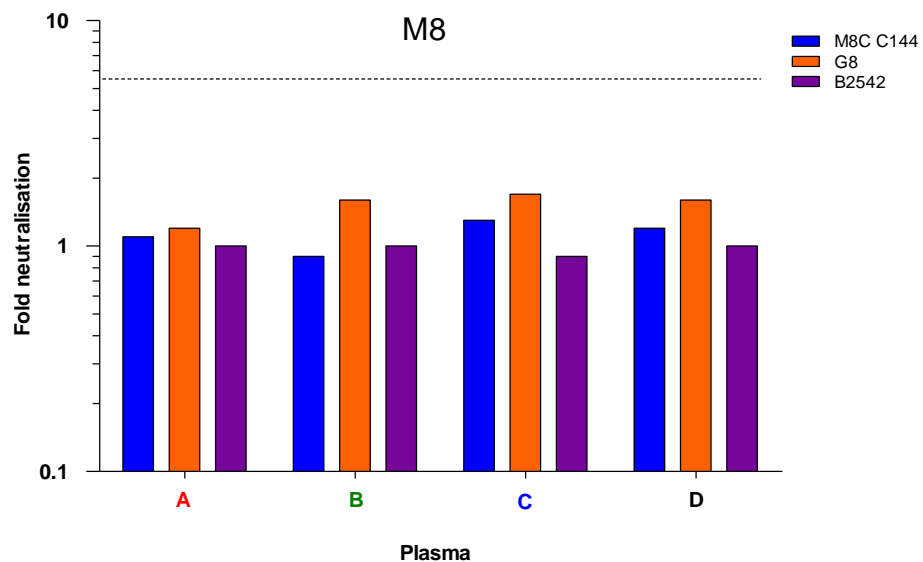
Figure A4-33 Autologous and Cr-NAb response of plasma from cat M5. Plasma samples from each time point (A, B and C highlighted on the X axis in red, green and blue respectively) were tested against pseudotypes bearing: 1) 3 Env variants isolated from time point A (highlighted in red), 2) 2 Env variants from time point B (highlighted in green) and 3) 3 Env variants from time point C (highlighted in blue). Cross neutralisation was assessed against GL-8 (orange) and B2542 (purple) reference pseudotypes.

Figure A4-34 Autologous and Cr-NAb response of plasma from cat M41. Plasma samples from time points A, B and C (highlighted on the X axis in red, green and blue) were tested against pseudotypes bearing: 1) 2 Env variants isolated from time point A (highlighted in red), 2) 9 Env variants from time point C (highlighted in blue). Env variants isolated from time point B were not available. Cross neutralisation was assessed against the GL-8 (orange) and B2542 (purple) reference pseudotypes.

Cat M16. Plasma from cat M16 did not neutralise any of the pseudotypes bearing autologous or heterologous Env variants (Figure A4-35). The *env* diversity was 0.4% and all Env variants contained 24 PNGS. Cat M16 had been infected for 5.5 years prior to enrolment in the study and, during the 12 months of observation, maintained a stable CD4 lymphocyte count at around 350 cells/µl until the time of death (19.06.10) (Table 5-2).

Cat M33. Plasma from cat M33 did not neutralise any of the 19 pseudotypes bearing autologous Envs (Figure A4-36). The *env* diversity was 1.1 % and Env variants contained either 23 or 24 PNGS. The cat had been infected for 5.5 years prior to enrolment in the study. The last CD4 lymphocyte count, recorded 4 months before death, was 200 cells/µl (Table 5-2).
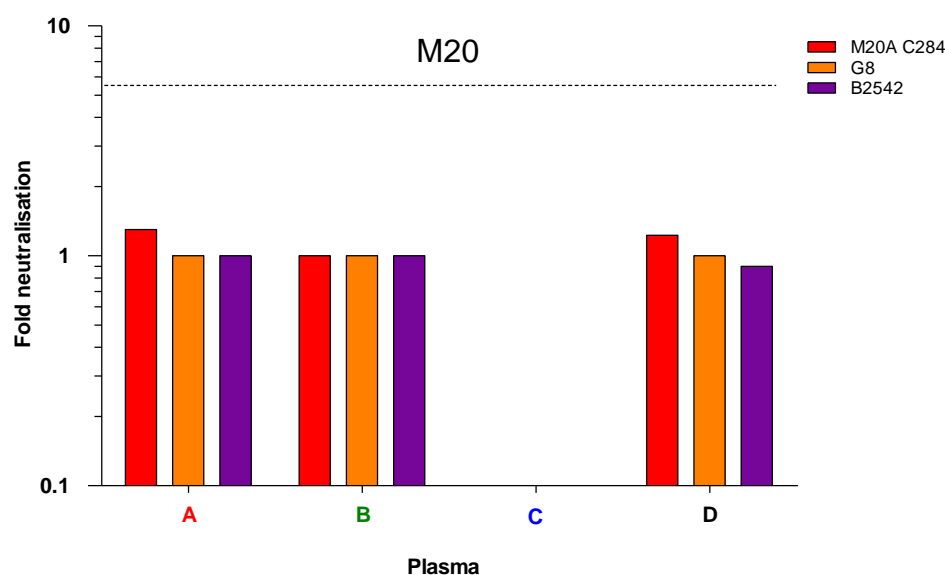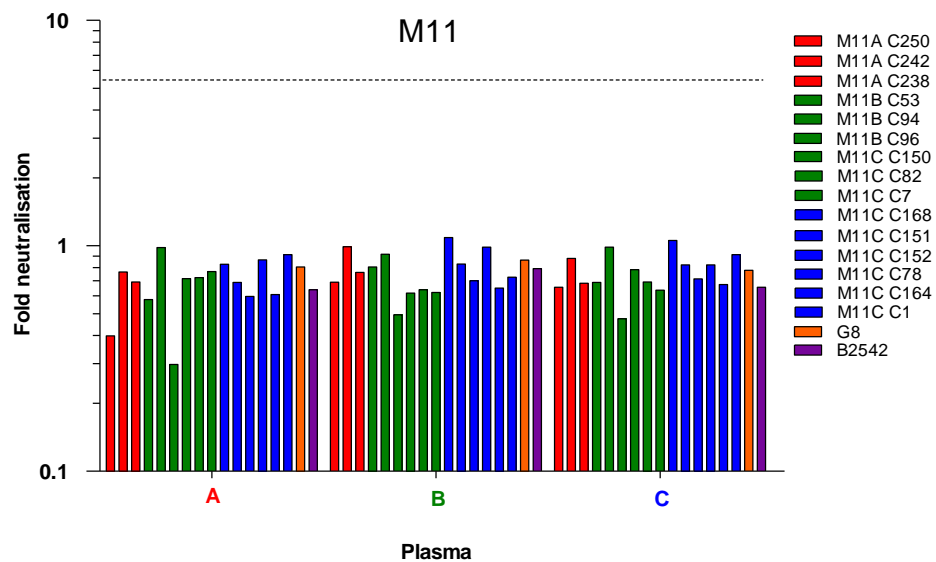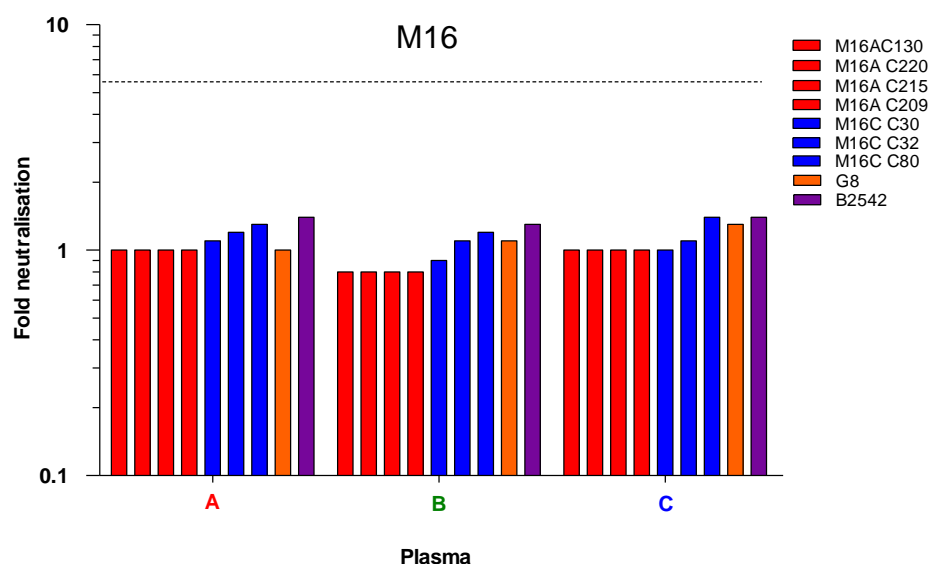

Figure A4-35 Autologous and Cr-NAb response of plasma from cat M16. Plasma samples from each time point (A, B and C highlighted on the X axis in red, green and blue respectively) were tested against pseudotypes bearing: 1) 4 Envs isolated from time point A (highlighted in red) and 2) 3 Envs from time point C (highlighted in blue). Cross neutralisation was assessed against two reference pseudotypes, GL-8 (orange) and B2542 (purple). Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line.

Cat M50. Cat M50 had been infected for approx. 2 years and its plasma did not neutralise pseudotypes bearing 2 autologous Envs. M50 died on 27.10.10 (10 months into the study) and its CD4 lymphocyte count 3 months earlier had been 1320 cells/µl (Table 5-2).
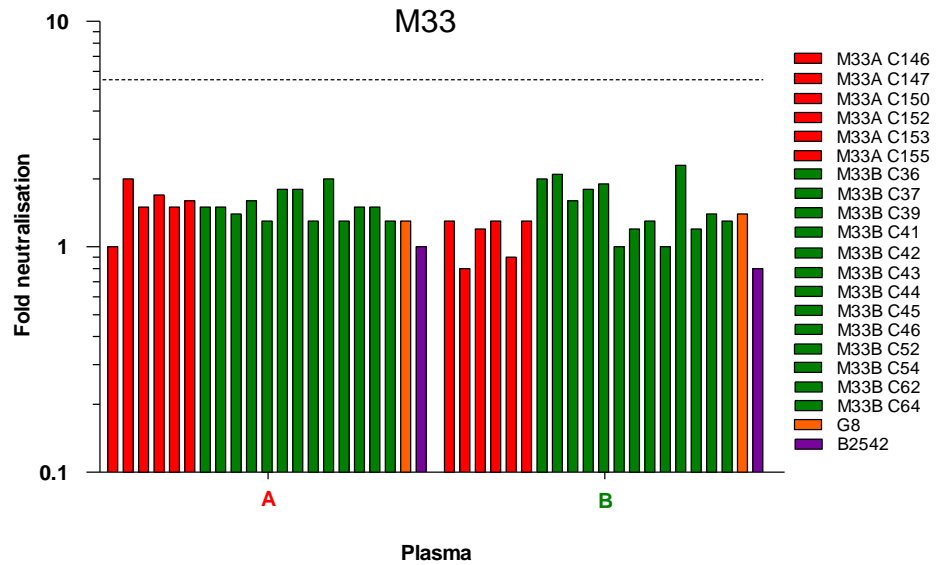
Figure A4-36 Autologous and Cr-NAb response of plasma from cat M33. Plasma samples from time points A and B (highlighted on the X axis in red and green respectively) were tested against pseudotypes bearing: 1) 6 Envs isolated from time point A (highlighted in red) and 2) 13 Envs from time point B (highlighted in green). Cross neutralisation was assessed against two reference pseudotypes bearing the clade A GL-8 (orange) and the clade B B2542 (purple) Envs. Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Samples from further time points were not available as the cat died after sample B was collected.
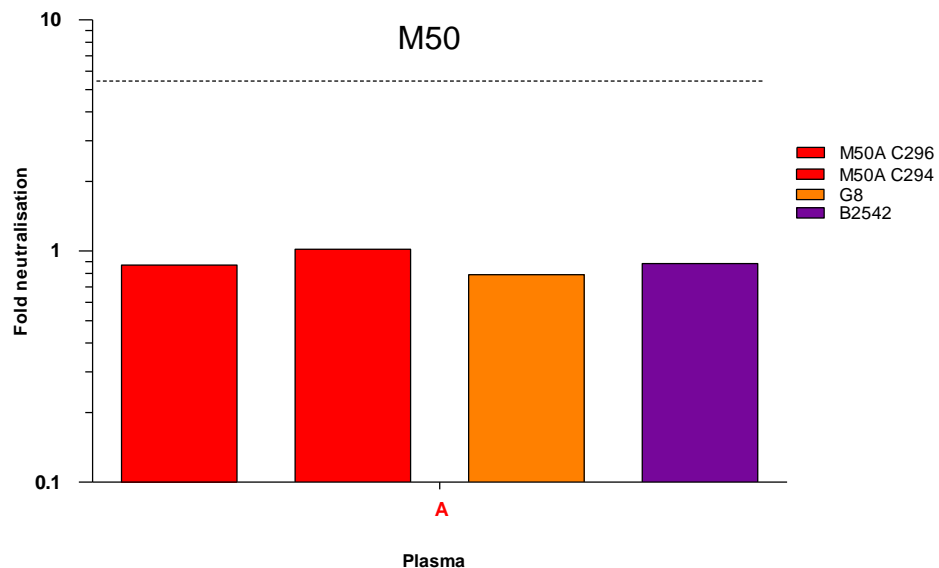
Figure A4-37 Autologous and Cr-NAb response of plasma from cat M50. Plasma from time point A (highlighted on the X axis in red) was tested against pseudotypes bearing 2 Envs from time point A (highlighted in red). Cross neutralisation activity was assessed against two reference pseudotypes bearing the clade A GL-8 (orange) and the clade B B2542 (purple) Envs. Values indicative of strong neutralisation (>5.6 fold) lie above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the graph legend (top to bottom).

Cat M3. Plasma from cat M3 failed to neutralise a pseudotype bearing a homologous Env but moderately neutralised a pseudotype bearing the heterologous GL-8 Env (3.8 fold), despite M3 having been infected with a non-recombinant clade B virus. Cat M3 had been infected for almost 9 years prior to enrolment in the study and its CD4 lymphocyte count, recorded 2 months before death, was 330 cells/µl (Table 5-2).
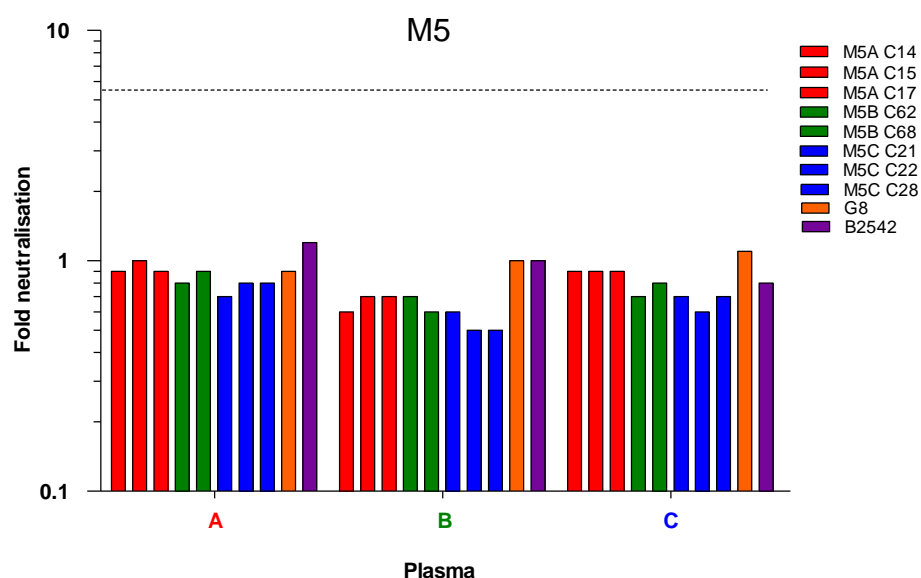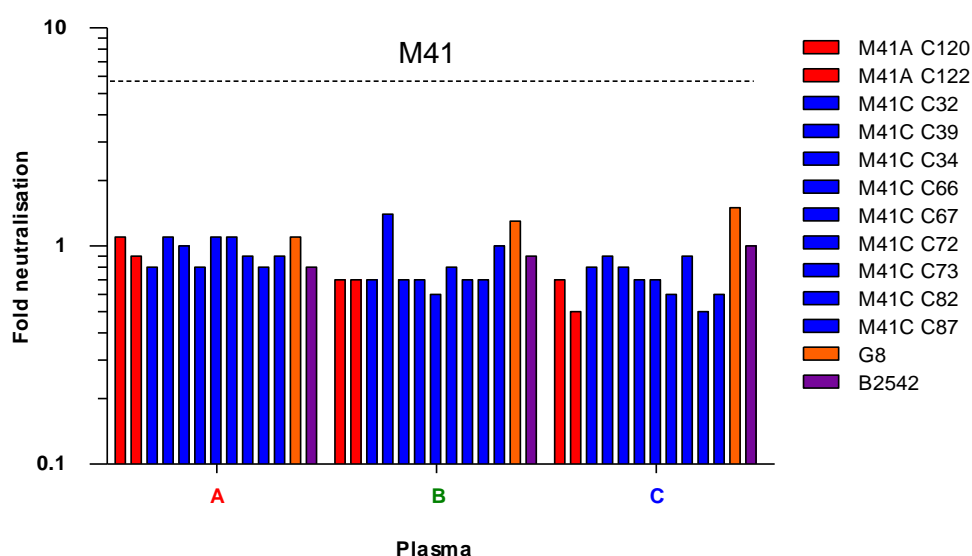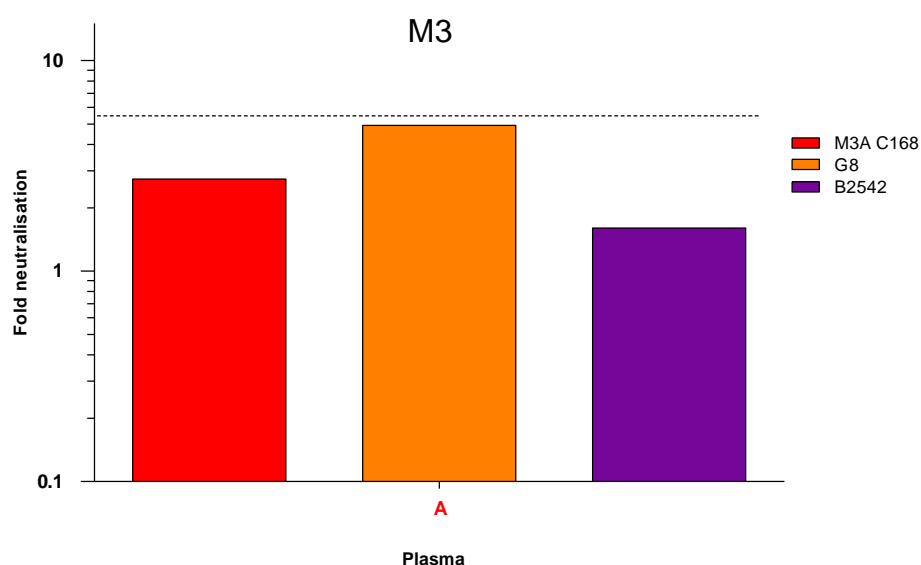

Figure A4-38 Autologous and Cr-NAb response of plasma from cat M3. Plasma collected at time point A (highlighted on the X axis in red) was tested against 1 pseudotype bearing homologous Env from time point A (highlighted in red). Cross reactivity was assessed against two reference pseudotypes bearing GL-8 (orange) or B2542 (purple) Envs. There were no values indicative of strong neutralisation (>5.6 fold) above the dashed horizontal line. Pseudotypes are shown (left to right) in the same order as in the legend (top to bottom).

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