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The role of virus neutralisation in immunity to feline immunodeficiency virus infection

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Declaration

I hereby declare that all the work presented in this thesis is the result of my own investigation unless otherwise stated. This work has not already been accepted for any degree, and is not being currently submitted for any other degree.

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Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
aNAb	autologous Neutralising Antibody
ARC	AIDS-Related Complex
bNAb	broadly Neutralising Antibody
bp	base pair
BSA	Bovine Serum Albumin
C domain	Conserved domain
CA	Capsid
CD	Cluster Determinant
cDNA	Complementary DNA
CLL-CD134	Canine Lymphocytic Leukaemia cell line transduced with feline CD134
CrFK	Crandel Feline Kidney
C-terminal	Carboxy- terminal
CTL	Cytotoxic T Lymphocyte
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxynucleotide Triphosphate
DU	dUTPase
E.Coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
Env	Envelope glycoproteins
FBS	Foetal Bovine Serum
FeLV	Feline Leukaemia Virus
FIV	Feline Immunodeficiency Virus
gp120	Glycoprotein 120 with the molecular weight of 120 kilo daltons
gp41	Glycoprotein 41 with the molecular weight of 41 kilo daltons
HCl	Hydrochloric Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus

HRP	Horseradish Peroxidase
IFA	Immunofluorescence Assay
IFNγ	Interferonγ
IgG	Immunoglobulin G
IL	Interlukin
IN	Integrase
ISCOM	Immune Stimulating Complex
K	Lysin
kb	Kilobases
LB	Luria Bertani
LTNP	Long Term Non-Progressor
LTR	Long Terminal Repeat
M	Molar
MA	Matrix
mA	milliambeir
mAb	Monoclonal Antibody
mRNA	messenger RNA
N	Aspergine
NAb	Neutralising Antibody
NC	Nucleocapsid
nt	nucleotide
N-terminal	Amino- terminal
OD	Optical Density
p24	Capsid Protein with the molecular weight of 24 kilo daltons
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reacion
PE	Phycoerythrin
pH	Pleckstrin Homology
PHA	Phytohemagglutinin
PNGS	Potential N-Linked Glycosylation
PR	Protease
RBC	Red Blood Cell
RNA	Ribose Nucleic Acid
rpm	Revolutions per minute

RPMI	Rosewell Park Memorial Institute
RRE	Rev Response Element
RT	Reverse Transcriptase
S	Serine
SIV	Semian Immunodeficiency Virus
SOC	Super Optimal Broth
SU	Surface Unit
T	Threonine
TBE	Tris-Borate containing EDTA
TE	Tris-EDTA
TM	Transmembrane
Tris	hydroxymethyl aminomethane
Tween 20	polyoxyethylene-sobitanmonolaurate
U	Unit
URT	Upper Respiratory Tract
UV	Ultraviolet
V	Volt
V loop	Variable loop
VNA	Virus Neutralisation Assay
WT	Wild Type

Abstract

Feline immunodeficiency virus (FIV) is an important veterinary pathogen with comparative significance because of its similarities to its human counterpart HIV. Since FIV is the only non-primate lentivirus which induces AIDS-like symptoms in its natural host, it serves as a valuable animal model for both prophylactic and therapeutic studies of HIV.

It is accepted that the induction of neutralising antibodies (NAbs) is a key element in the control of lentiviral infection, since T-cell based vaccines alone failed to prevent infection in most experimental animal model systems. In this project a robust and reproducible *in vitro* neutralisation assay was developed and optimised, permitting the assessment of the NAb response in naturally infected cats and with the potential to evaluate candidate vaccines.

It was demonstrated that, in general, primary FIV strains in the UK belong to subtype A, and therefore the development of a regional, subtype A-specific, FIV vaccine could be considered for use in the UK. The identification of a neutralisation resistant isolate of FIV led to the finding that a linear neutralisation determinant was located within the V5 region of Env and mutations in this region may lead to immune evasion *in vivo*. In addition, a second neutralisation determinant was identified in the C3/V4 region of Env.

Finally, it was observed that a small proportion of naturally infected cats generated NAbs against FIV. Of these, only a very small proportion of the cats had antibodies with the potential to cross neutralise strains within the same subtype as the homologous isolate. Nonetheless, a plasma sample from a single cat was identified that neutralised all strains tested, including strains from different subtypes and geographical regions. It is likely that studies of the homologous isolate that induced the broad NAb response may be capable of inducing a similar broad response in vaccinated cats. Such a finding would have important implications for the design of potential novel lentiviral immunogens.

1 Introduction

1.1 An overview of FIV

Feline immunodeficiency virus (FIV) is a major veterinary pathogen that is an important cause of morbidity and mortality in domestic cats. It was first isolated in 1986 in Petaluma, California from a cat with an immunodeficiency-like syndrome (Pedersen *et al.*, 1987) and was soon recognised as the feline homologue of human immunodeficiency virus (HIV). Both FIV and HIV belong to the genus Lentivirus within the family Retroviridae, cause immunodeficiency in their natural hosts and share numerous biological, clinical and pathogenic features (Hartmann, 1998).

1.2 Epidemiological features of FIV

FIV is highly host specific to the Felidae family, which includes the lion, the tiger, the jaguar, the puma, the pallas cat, the leopard, the cougar, the cheetah, the serval, the lynx and the ocelot as well as the domestic cat. Seroconversion does not occur following exposure of humans to the virus, and therefore there is no risk of zoonotic transmission (Yamamoto *et al.*, 1989). The prevalence of infection is approximately 11% in cats worldwide (Courchamp & Pontier, 1994), varying from 1-14% in cats with no clinical signs to up to 47% in sick cats. In the UK the prevalence is approximately 5% in healthy and 47% in sick cats (Hosie & Beatty, 2007).

FIV strains are usually classified into five subtypes (designated A-E), based on nucleotide sequence. Inter-subtype recombinants A/B, A/C and B/C have been recognised (Bachmann *et al.*, 1997). Serological subtyping has not yet been conducted for FIV. As well as the prevalence differing greatly between geographical areas, the subtype distribution is variable between different regions (Hosie *et al.*, 2009) (Figure 1.1).

Transmission of virus occurs mainly by biting, via the saliva of infected cats, during fights or at mating (Pedersen *et al.*, 1989). Trans-mucosal (vaginal or rectal), *in utero* and trans-mammary infections have been recorded experimentally; however, it is unknown how often transmission occurs by these

routes in nature (Bishop *et al.*, 1996; O'Neil *et al.*, 1995). The prevalence of FIV infection is highest amongst male, free-roaming, older, and sick animals, reflecting the route of natural viral transmission and the immune status of the host (Courchamp & Pontier, 1994).

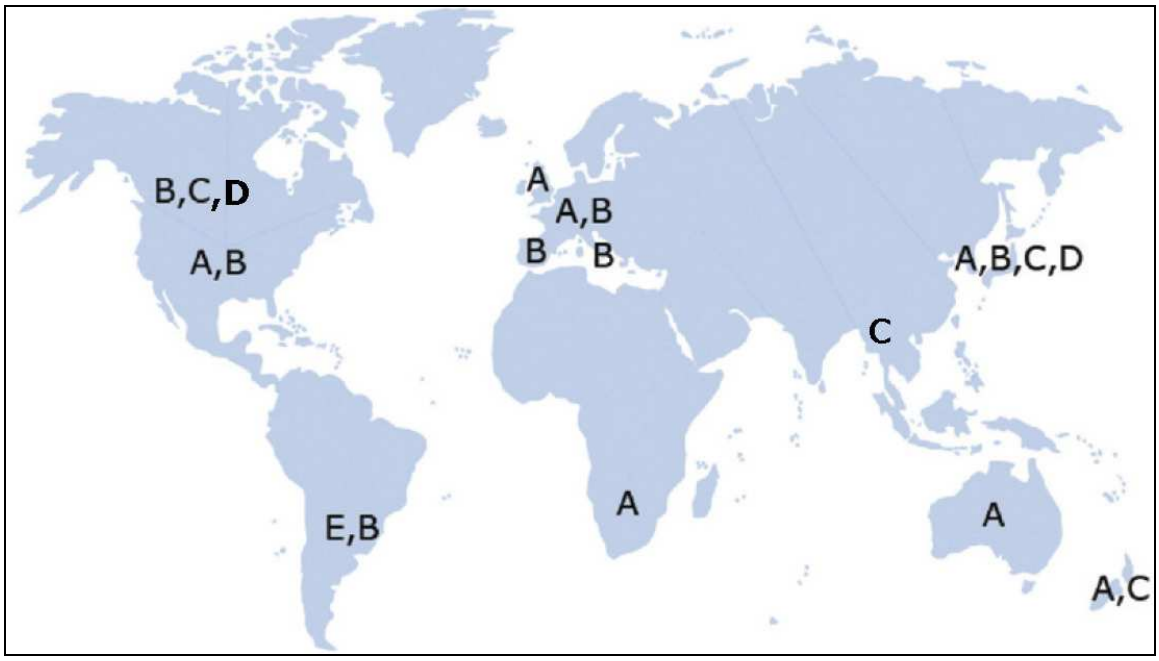


Figure 1.1. The global distribution of FIV subtypes. Adapted from (Hosie *et al.*, 2009).

1.3 Pathogenesis of FIV infection

Infection with FIV is associated typically with generalised lymphadenopathy, gingivitis/stomatitis, and cachexia (Callanan *et al.*, 1992). Infected cats undergo an acute viraemic phase, with varying degrees of fever, diarrhoea, conjunctivitis, and generalised lymphadenopathy developing a few weeks post infection, accompanied by leucopenia as $CD4^+$ T cells are rapidly depleted. This phase involves a burst of virus replication before seroconversion occurs (Callanan *et al.*, 1992). The acute phase is then followed by an asymptomatic phase, usually lasting for several years, during which $CD4^+$ T cell numbers decrease slowly, a balance having been reached between the virus and the host immune response. At the same time, $CD8^+$ T cell numbers increase; as a result the $CD4^+$:$CD8^+$ ratio becomes inverted (Diehl *et al.*, 1995a). FIV infection induces both antibody and cytotoxic T lymphocyte (CTL) mediated responses which are thought to maintain the viral load at a low and stable

level during the entire latent phase (Beatty *et al.*, 1996) (Figure 1.2). $CD4^+$ T cell depletion continues and infected cats may start to display non-specific clinical signs, including recurrent fever, generalised lymphadenopathy, apathy, leukopenia, anemia, wasting, stomatitis and behavioural abnormalities. This stage, AIDS-related complex (ARC), may last for several months to years (Matsumura *et al.*, 1993; English *et al.*, 1994). A proportion of FIV-infected cats develop AIDS-like symptoms with opportunistic infections and lymphomas (Shelton & Linenberger, 1995). $CD4^+$ T cell numbers may drop to 200 cells/μl or fewer in terminally ill cats (Bendinelli *et al.*, 1995).

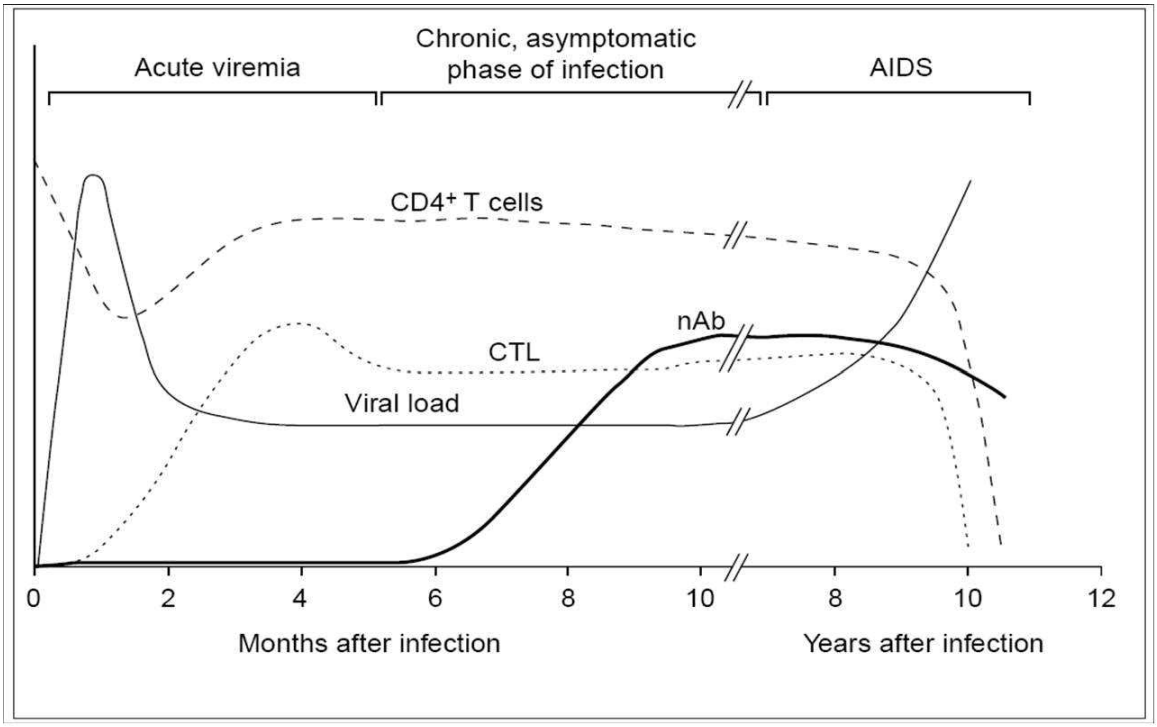


Figure 1.2. Clinical phases of FIV/HIV infection. During the acute phase, there is a peak of viraemia accompanied by a decrease in the number of CD4+ T cells, and a CTL-mediated response is initiated. A constant viral load and maintenance of CD4+ T cell numbers is associated with the asymptomatic phase following the peak CTL response. Maturation of NAbs occurs at 6-8 months of infection and levels are maintained throughout the course of infection (Richman *et al.*, 2003). AIDS-like clinical signs occur as the immune response collapses. Adapted from (Ferrantelli & Ruprecht, 2002).

1.4 FIV structure and genome organisation

The FIV virion is spherical to ellipsoid, 105 to 125 nm in diameter, with a typical lentivirus structure, containing two copies of single stranded, positive-sense RNA (Pedersen *et al.*, 1987; Miyazawa *et al.*, 1989a). The viral genome contains approximately 9400 nucleotides and is surrounded by a conical shell, the capsid (CA), and is attached to nucleocapsid (NC) proteins. Together, these form the viral core. The core is enclosed by the matrix (MA) and enclosed within the outer envelope which is composed of two layers of phospholipids (Olmsted *et al.*, 1989; Talbott *et al.*, 1989) containing the surface (SU) and transmembrane (TM) envelope glycoproteins (Env).

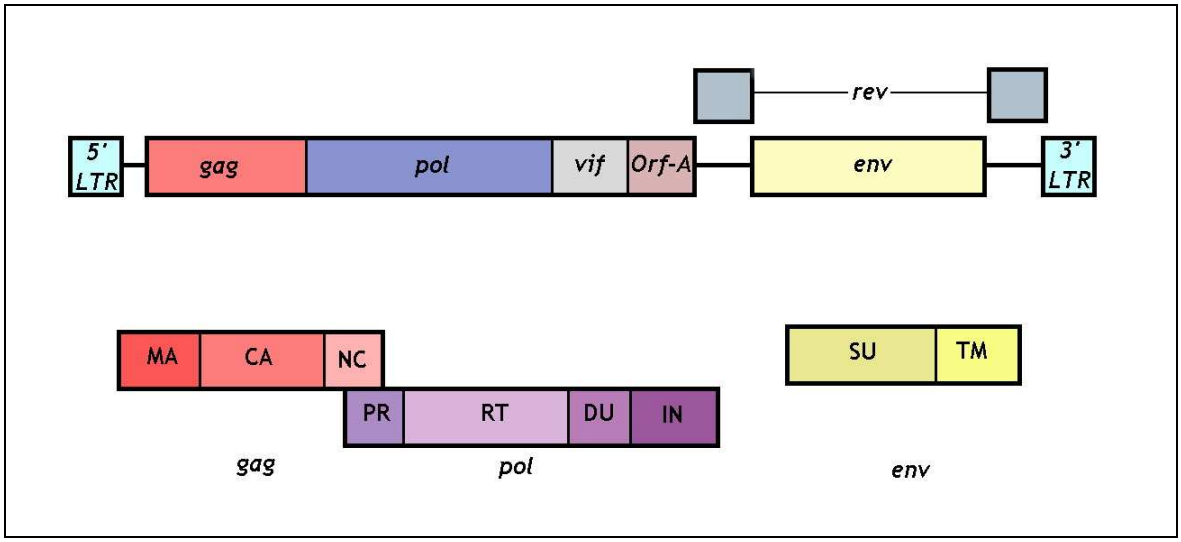


Figure 1.3. Structure of FIV genome. Diagram depicting the FIV genome (upper) and the gene products of the main three FIV genes (lower). MA: matrix, CA: capsid, NC: nucleocapsid, PR: protease, RT: reverse transcriptase, DU: dUTPase, IN: integrase, SU: surface unit, TM: transmembrane.

The FIV genome contains the three major lentiviral genes; *gag*, *pol*, and *env*, and a number of accessory genes, flanked on both sides by long terminal repeat (LTR) sequences (Figure 1.3). The *gag* gene encodes the internal structural proteins CA, NC, and MA, which are necessary for maintaining the integrity of the virion particle. The *pol* gene encodes enzymes that are necessary for virus replication and reproduction; the protease (PR), which cleaves newly synthesised polyproteins to create the mature protein components, the reverse transcriptase (RT), which transcribes single-stranded

viral RNA into double-stranded DNA, the integrase (IN), which integrates transcribed viral DNA into the DNA of the infected cell, and dUTPase (DU), which is absent in HIV and is believed to facilitate productive infection of non-dividing cells such as macrophages. The *env* gene encodes Env, comprising the SU and TM proteins. As well as protecting the viral particle, Env is responsible for viral entry into the host cells. The LTRs flanking the genome contain an untranslated 3' (U3) region, which involves promoter/enhancer elements, and an untranslated 5' (U5) region, which contains the polyadenylation repeat (Miyazawa *et al.*, 1994; Stephens *et al.*, 1991; Lerner *et al.*, 1995; Tomonaga & Mikami, 1996).

Other genes are *rev*, *vif*, and *orf-A* which encode the Rev, Vif, and Orf-A proteins respectively. Rev is involved in shuttling RNAs from the nucleus and the cytoplasm by its binding to the viral RNA Rev response element (RRE) (Phillips *et al.*, 1992), and Vif blocks the action of antiviral response by preventing cytidine deamination by APOBEC-3G (An *et al.*, 2004; Mangeat *et al.*, 2003), while Orf-A arrests host cell division (Gemeniano *et al.*, 2004). FIV particles lack the *vpu*, *vpr*, and *nef* genes that are present in HIV.

1.4.1 Structure of FIV Env

Env is responsible for viral entry to the host cells and has an important role in the pathogenesis of infection. Env is composed of variable regions, usually known as variable (V) loops, interspersed with conserved (C) domains. The conserved region forms inner and outer domains as well as a ß-bridging sheet, which includes the primary and chemokine receptors binding sites required for viral entry and considered to be major targets for neutralisation (Wyatt & Sodroski, 1998). Variable loops present potentially variable epitopes to the immune system. Nine variable loops have been identified in the FIV Env protein; V3-V6 in SU and V7-V9 in TM, while the V1-V2 region is located within the leader sequence and is not part of the mature Env of FIV (Pancino *et al.*, 1993; Verschoor *et al.*, 1993). As well as the sequence variation of the variable regions amongst FIV strains, which makes it difficult to generate broadly neutralising antibodies (bNAbs), the variable loops are heavily glycosylated, masking potential neutralising epitopes within and outside variable regions (Moulard *et al.*, 2002; Lusso *et al.*, 2005; Xiang *et al.*, 2005).

1.5 The replication of FIV

Env forms spike-like projections that appear on the surface of the envelope in trimers (consisting of three SU and three TM subunits) attached by non-covalent bonds. The SU protein, gp120, binds to the primary receptor CD134 (or OX40) on the surface of $CD4^+$ cells (Shimojima *et al.*, 2004). CD134 is a member of the tumour necrosis factor receptor family and binding of Env to CD134 leads to a conformational change in Env that enables a second binding step with the chemokine receptor CXCR4 to proceed, bringing the virus and cell membranes into close proximity. Further conformational changes allow the TM protein, gp41, to fuse with the cell membrane, resulting in complete fusion of the two membranes and allowing the virus to release the core into the cell cytoplasm (de Parseval *et al.*, 2005; de Parseval *et al.*, 2004; Garg *et al.*, 2004; Moore *et al.*, 2001). Once viral genome and enzymes are liberated into the host cell, the viral single stranded RNA genome is transcribed into double stranded DNA, which is then transported to the nucleus and integrated as a provirus into the host genome. Proviral DNA is transcribed into mRNA which is then translated into viral protein precursors. Viral proteins are processed and cleaved by proteolysis before assembly of the viral genome and proteins occurs, forming pre-mature virions. Virions are released from the host cell by budding; during this stage the viral cores are enveloped by parts of the cell membrane into which the viral glycoproteins are inserted (Haseltine, 1991; Frey *et al.*, 2001).

1.6 The virus-host interaction

Following an initial infection, vigorous immune responses are elicited in an attempt to defend the host against viral attack. Although unable to contain the massive burst of viral replication during the acute viraemic phase, it is not long before a state of equilibrium is reached, with the aid of humoral and cell mediated immune responses, and the host enters the asymptomatic phase of infection. Cytotoxic T cell-mediated (CTL) immunity is thought to play an important role in the initial viral response, since CTL activity may be detected indirectly as early as one week post infection, via the detection of chemokines and other soluble factors which efficiently block the interaction between viral particles and their receptors (Song *et al.*, 1992; Choi *et al.*, 2000; Flynn *et al.*,

2002). CTL activity is mediated mainly by $CD8^+$ T-cells and recognises FIV Gag and Env proteins (Flynn *et al.*, 1995). The $CD4^+$ T cell-mediated response starts strongly and effectively early in infection through the secretion of gamma interferon (IFN-γ) and interleukin 10 (IL-10), but gradually decreases with time as $CD4^+$ T cells are major targets for FIV (Liang *et al.*, 2000).

Neutralising antibodies (NAbs) are crucial in controlling the infection by FIV. Anti-FIV antibodies may be detected as early as six weeks post infection (Hosie *et al.*, 1998), but NAbs require longer to mature (Moog *et al.*, 1997). NAbs inhibit infection by blocking the entry of the virus into susceptible cells, presumably by binding gp120 trimers and so preventing attachment of the virus to the receptor on the target cell (Spenlehauer *et al.*, 2001; Binley *et al.*, 2004; Labrijn *et al.*, 2003). The generation of NAbs is a slow process, with NAbs effective against the homologous virus being detected first. It takes over 8 months following seroconversion before it is possible to detect NAbs for HIV-1 (Moog *et al.*, 1997; Richman *et al.*, 2003). It is considered that the long period required for NAbs to develop is due to an inability to induce an efficient, broadly neutralising response (Bower *et al.*, 2004).

Despite the strong immune responses generated by the host, lentiviruses are not cleared and persistently infected cats may proceed through ARC and enter the terminal AIDS phase. FIV has its own effective weapons to elude the host's defences; (i) Env is heavily glycosylated; sugar residues form a glycan shield which strongly protects the virus from antibody-mediated neutralisation (Burton *et al.*, 2004; Zolla-Pazner, 2004; Doria-Rose *et al.*, 2009), (ii) conserved regions, such as the primary and co-receptor binding sites, are flanked and masked by variable loops, (Burioni *et al.*, 2008; Huang *et al.*, 2005; Kwong *et al.*, 2002) (iii) steric masking of the epitopes renders them inaccessible to neutralising antibodies (NAbs) as they fail to achieve the necessary entropic changes that are required for high-affinity interaction (Decker *et al.*, 2005; Labrijn *et al.*, 2003; Richman *et al.*, 2003), (iv) the substitution of non-essential residues which do not form part of the functional epitope may block antibody binding (Burton *et al.*, 2004), (v) Env is internalised from the surface of infected cells via a process of receptor-mediated endocytosis and so is protected from the NAbs (Hosie *et al.*, 2005), (vi) the disintegration of Env glycoproteins elicits the production of non-

neutralising antibodies (Thali *et al.*, 1992; Chiarantini *et al.*, 1998), (vii) the emergence of new viral variants, a consequence of the high mutation rate in the viral genome, leads to escape from neutralisation (Douek *et al.*, 2006; Richman *et al.*, 2003).

It is commonly presumed that the asymptomatic state occurs when a balance is achieved between viral attack and host defence mechanisms, resulting in a relatively low and stable viral titre. However viral replication is not contained, spontaneous recovery following lentiviral infection has not been recorded and the host becomes persistently infected. Virus evolves in response to immune pressure and new variants emerge continuously. As the lentiviral reverse transcriptase (RT) lacks proof-reading functions, new mutations arise regularly; only those that do not affect the viability and infectivity of the virus will survive and contribute to genetic diversity. The population, comprising new variants of a viral strain within an individual, is known as a quasispecies (Shankarappa *et al.*, 1998; Lukashov *et al.*, 1995).

The increase in diversity often results in the emergence of more pathogenic strains (Balfe *et al.*, 2004), since neutralising responses are evaded. Factors that affect quasispecies diversity are both virus-related (such as the initial viral load, virulence and antigenic diversity) and host-related (availability of target cells and host immunocompetence). As diversification appears to occur at a constant rate, this may indicate that host parameters of variation are of less significance (Shankarappa *et al.*, 1998; Liu *et al.*, 1997). The diversity of viral *env* gene may be as high as 10% within an infected individual, but extraordinarily, may exceed 30% amongst the different subtypes, making it a real challenge for the immune system to deal with (Rong *et al.*, 2007b).

1.7 The diagnosis of FIV infection

Serological methods are used commonly in diagnostic laboratories for the detection of anti-FIV antibodies. Immunofluorescence assays (IFA) and ELISA-based kits are widespread with positive and negative predictive values approaching 100% (Levy *et al.*, 2003). Antibodies can be detected also by Western blotting using gradient purified tissue culture-grown virus as an antigen source (O'Connor, Jr. *et al.*, 1989). Antibodies may be detected as early as 2-4 weeks after experimental infection with FIV (Yamamoto *et al.*,

1988). Antibody testing is reliable whereas viral antigen levels are often below the threshold of detection in the blood of infected cats. However, the introduction of a commercial vaccine to some countries, which induces seroconversion against the structural proteins of FIV, invalidates the serological diagnosis of infection. Levy et al. (2008) described an ELISA method that can distinguish between vaccinated and infected cats; however, the method fails to detect reliably vaccinated cats that later became infected, which is important since vaccination is not 100% effective and infected vaccinated cats could transmit infection to other cats. In these circumstances virus isolation remains the method of choice to identify FIV-infected cats.

Virus isolation is considered the gold standard method for identifying FIV infected cats; however, it is expensive, time consuming and not suitable for routine diagnosis (Levy *et al.*, 2004). PCR-based tests can be used to diagnose FIV infection; viral RNA is reverse transcribed to cDNA which is then amplified using FIV- specific primers (Vahlenkamp *et al.*, 1995). This method has limitations; levels of free, circulating virus can vary considerably in an infected cat and viral RNA is highly labile. As an alternative, proviral DNA can be amplified directly from circulating peripheral blood mononuclear cells (PBMCs) (Leutenegger *et al.*, 1999). However, the sensitivity can be greatly affected by sample quality and quantity, sequence variation amongst field strains, and a lack of detectable provirus in PBMCs (Bienzle *et al.*, 2004). However, real time PCR methods have been shown to provide better sensitivity and allow the quantification of proviral DNA (Klein *et al.*, 1999).

1.8 FIV as a model for HIV infection

It is now widely accepted that FIV is a useful model for the study of HIV in terms of pathogenesis, prophylaxis, and therapeutics. FIV shares many similarities with its human counterpart; from its genome structure and organisation, to the mechanism of infection and the pathogenesis of infection (Pedersen *et al.*, 1987).

FIV and HIV have similar structural and functional proteins as both viruses share the three major lentiviral genes: *pol*, *gag* and *env*. FIV Env consists of variable and conserved domains similar to HIV, although FIV lacks the first two variable loops present in the mature HIV Env. Both FIV and HIV possess the

accessory genes *rev* and *vif* and, although FIV does not have the *vpr*, *vpu* or *nef* genes, it is believed that their function may be offset by ORF-A and DU (Gemeniano *et al.*, 2004; Chen *et al.*, 2004), and so the replication of FIV mirrors that of HIV.

Both viruses target $CD4^+$ T lymphocytes and induce a progressive immunosuppression in their hosts. Also, FIV and HIV share a similar cell tropism, infecting monocyte/macrophages, glial cells, B cells and $CD8^+$ T cells (Hein *et al.*, 2000; English *et al.*, 1993). Infection of $CD4^+$ T cells leads to inversion of the $CD4^+$:$CD8^+$ ratio, which is considered to be a hallmark of both HIV and FIV infections (Garg *et al.*, 2004; Diehl *et al.*, 1995a). While FIV does not utilise CD4 as its primary receptor, its entry mechanism resembles that of HIV. All isolates of FIV utilise the CXCR4 co-receptor that is used by isolates of HIV. As FIV and HIV have many shared features, such as Env structure, disease manifestation and pathogenesis of infection, FIV is an ideal model in terms of vaccine development for HIV.

As well as the many similarities between FIV and HIV, the cat model has the advantage over other animal models for HIV, such as chimpanzees and macaques, because of the small size of the cat and relative ease of handling. Cats are also less expensive, easier to breed and have a relatively short breeding cycle (Miller *et al.*, 2000). Moreover, the FIV-cat model provides an opportunity to test the *in vivo* effects of immunologic determinants in a relevant model system. The identification of such determinants has the potential to contribute towards the development of effective vaccines against both FIV and HIV infections.

1.9 FIV vaccine development

As a significant pathogen of the domestic cat and having a remarkable homology with HIV, FIV vaccine development has comparative as well as veterinary importance. It is likely that improving our understanding of immunity to FIV infection will contribute also to the development of an effective HIV vaccine, as FIV is widely considered to be a useful model for HIV (Willett *et al.*, 1997a; Uhl *et al.*, 2002).

The ideal vaccine against any pathogen should be highly efficacious, capable of inducing sterilising protection against the pathogen and safe, i.e. having no adverse side effects. Vaccine development against FIV, in common with HIV, faces a number of obstacles owing to the properties and characteristics of the virus so that an ideal vaccine has not yet been developed. Ideally, an effective vaccine against FIV should induce a broad and potent protective response against natural challenge.

1.9.1 Obstacles and challenges to vaccine development

Although the host mounts a vigorous immune response against FIV, the virus can still replicate, leading to progression of infection. Vaccine development faces a number of challenges:

First, due to the highly error-prone RT, diverse variants arise within infected individuals as infection proceeds. Continuous viral evolution results in the emergence of a massive number of epitopes which are presented to the immune system and neutralisation determinants on Env are often concealed or inaccessible (Zhang *et al.*, 2004). New variants may emerge also as a result of recombination due to co-infection (Kyaw-Tanner *et al.*, 1994; Hayward & Rodrigo, 2008). In addition, there is enormous genetic diversity between and within FIV subtypes. Diversity between subtypes has been estimated, based on the nucleotide sequence of the *env* gene, and may reach 30% (Burkhard & Dean, 2003; Rong *et al.*, 2007b). Though serotypes have not yet been defined, serological variation within subtypes appears to be very high and therefore it may not be feasible for a single subtype vaccine immunogen to protect against all FIV strains. An ideal vaccine would protect against infection with a wide range of isolates but the idea of developing multiple regional vaccines has been proposed as an alternative (Pistello *et al.*, 1997).

Second, FIV persists in host cells as a non-immunogenic provirus. When the virus replicates, proviral DNA becomes integrated within the cat's genome. Replication proceeds with the synthesis of the viral components, packaging and shedding of mature virions, or the virus becomes latent, with the virus harbouring within $CD4^+$ T-lymphocytes (Blankson *et al.*, 2002). This state of latency renders the virus unrecognisable by the immune system and serves as a reservoir; virus is shed throughout the course of infection, with no possibility of

removing the integrated provirus without damaging the host cell (Marcello, 2006). Viral latency thus prevents the occurrence of sterilising immunity. Ideally, effective vaccination would induce sterilising immunity (neither infectious virus could be isolated, nor viral nucleic acid detected by PCR following challenge) or at least reduce considerably the viral load, thereby reducing or preventing clinical signs and transmission of infection.

Third, spontaneous recovery from FIV infection is effectively unattainable (Yamamoto *et al.*, 1989; Ishida *et al.*, 1989) and therefore a protective vaccine cannot be designed that reproduces the immune responses in cats that have recovered from infection. However, the study of FIV pathogenesis may provide clues about how a successful vaccine could be developed. While the antibody response generally reduces the initial viral burst following infection, this does not appear to be the case for FIV, because NAbs are not yet mature in naïve cats and so the viral burst of replication at the acute phase is not controlled until the CTL response is mounted. As a result, virus disseminates quickly to host cells, and remains in a latent state until later in infection, when the immune response is impaired. Indeed, as the immune system is not capable of eradicating infection, an efficacious vaccine should evoke NAbs and boost immune responses prior to challenge and infection. But CTL responses appear to be important in reducing the viral set-point later in infection (Flynn *et al.*, 2002). Hence, it is generally believed that an ideal vaccine should influence both arms of the immune response and induce both humoral and cell-mediated immunity (Douek *et al.*, 2006; Walker & Burton, 2008; Pu *et al.*, 1997).

The fourth obstacle faced in vaccine development is the difficulty in mimicking natural infection for an effective vaccine challenge system. In order to evaluate accurately the efficacy of candidate vaccines, it is important to mimic the natural challenge in terms of route of infection, dose, and biological features. The dominant natural route of FIV transmission is via biting, which involves the inoculation of viral particles and perhaps also virus-infected cells into skin and/or subcutaneously or intramuscularly. Kusuhara et al. (2005) conducted a vaccine trial in which vaccinated and naïve cats were housed together with a group of infected cats. All of the cats were entire males, in order to promote fighting behaviour and hence transmission of FIV via biting. None of the cats vaccinated with the Fel-O-Vax FIV vaccine became infected,

whereas five of eight unvaccinated cats became infected, providing evidence that vaccination protected cats against a natural challenge. More usually, FIV vaccine trials use experimental routes of infection for challenge studies, infecting cats with either virus or virus-infected cells. The infectious dose in natural infection is likely to be variable and has not been defined. Typically, challenge doses of 10-50 median cat infectious doses of virus are used to ensure that all non-vaccinated control cats become infected (Pu *et al.*, 1997).

The biological features of natural challenge have been studied and it has been shown that viral strains vary significantly in their virulence, establishing different viral loads and inducing different effects on $CD4^+$ T-lymphocyte cell numbers after challenge (Diehl *et al.*, 1995a). It has been established that even a few *in vitro* passages of primary HIV-1 isolates in T-cell lines select for viral variants that are not only more adapted to growth in such cells but also are compromised in their innate resistance to antibody neutralisation (Sawyer *et al.*, 1994; Wrin *et al.*, 1995). Similarly, it has been shown that laboratory adapted FIV strains are less virulent than naturally occurring strains, and may not be representative of a field challenge with primary strains (Bendinelli *et al.*, 2001; Giannecchini *et al.*, 2001a). An effective vaccine should protect cats against challenge with a virulent strain representative of those circulating in the field; it is likely that challenge with less virulent isolates may lead to an overestimation of the protective response. GL8 is a virulent primary strain that was isolated from an asymptomatic cat (i.e. a cat likely to be in the early stages of infection) and is considered representative of field isolates (Hosie & Beatty, 2007). Consequently, GL8 has been used in many studies described in this thesis.

Finally, vaccination may induce adverse effects; vaccine-mediated enhancement of FIV infection has been encountered in several clinical trials (Karlas *et al.*, 1998; Elyar *et al.*, 1997; Siebelink *et al.*, 1995c), perhaps through the generation of antibodies that facilitate virus entry or expand viral tropism (Thieblemont *et al.*, 1993). Enhancement is usually observed as a higher viral load (and often more rapid disease progression) in vaccinates compared to non-vaccinated controls receiving the same challenge (Hosie *et al.*, 1992; Richardson *et al.*, 2002). Vaccine-mediated enhancement is a significant challenge to lentivirus vaccine development. For example, the

failure of Merck's clinical trial of an adenovirus-based HIV-1 vaccine caused major concerns for HIV vaccine development (Fauci, 2007). Enhancement of FIV infection is discussed in more detail in chapter three of this thesis.

1.9.2 Significance of NAbs in vaccine development

Virus neutralisation is defined as the reduction of infectivity by antibody or by soluble receptor in the absence of other inactivating factors. Two types of antibody neutralisation have been described; inhibition of virus binding to the target cell, and interference with post-binding events, including fusion of the virus and cell membranes (Dimmock, 1993). It is speculated that NAbs block the interaction between gp120 and the primary viral receptor, or inhibit fusion of the virus and host cell's membrane which, in either scenario, leads to virus inactivation (Poignard *et al.*, 1996; Labrijn *et al.*, 2003). The ability of an antibody to neutralise a virus depends on several factors, including the presence of the epitope on the virion, the accessibility and conformation of that epitope, the assay system used to measure the virus-antibody interaction and the presence or absence of accessory factors such as complement. Thus, there are variables at both the viral and the cellular levels which may alter neutralisation (Golding *et al.*, 1994).

There is evidence to support the hypothesis that the generation of NAbs will be essential for a successful FIV vaccine, since in most experimental animal model systems, T-cell based vaccines alone have been unable to prevent infection or provide sterilising immunity (Shiver *et al.*, 2002; Wilson *et al.*, 2006). Furthermore, sterilising immunity against FIV was achieved through passive immunisation, and through maternal transfer of antibody (Hohdatsu *et al.*, 1993; Pu *et al.*, 1995). Monoclonal antibodies (mAbs) have also been shown to potently neutralise a broad range of primary isolates (Parren *et al.*, 1995). However, many researchers debate the importance of NAbs in the control or prevention of infection *in vivo* (and thus in vaccine development also) as there is no direct, unequivocal evidence supporting their role. Most of the discussion relies on the fact that NAbs are not detected during virus clearance in the acute stage of infection (Moore *et al.*, 1994), whereas cytotoxic $CD8^+$ T cells are readily detectable (Borrow *et al.*, 1994) and effectively control the virus until the immune system collapses at the terminal AIDS stage. The role of CTL-

mediated interference is regarded as highly important in the control of infection. It has, however, been proven that the antibody response plays a key role in Inhibiting viral replication throughout the course of infection. It has been shown that the serum of HIV-infected long term non-progressors (LTNPs), defined as individuals with no AIDS symptoms over 10 years of infection, had higher titres of NAbs, contained NAbs that more frequently neutralised primary isolates and occasionally showed broad neutralising ability (Cao *et al.*, 1996; Binley *et al.*, 2008). It has also been shown that virus evolves in response to the immune pressure exerted by NAbs, in order to escape neutralisation (Sather *et al.*, 2009; Sagar *et al.*, 2006).

Although broadly neutralising antibodies are identified only rarely (Wyatt & Sodroski, 1998), a number of cross-neutralising mAbs that target HIV-1 Env have identified potential vaccine targets (Binley *et al.*, 2004). Antibody b12 targets the primary receptor binding site (CD4bs) and is considered to be the most potent broadly NAb; b12 effectively binds to oligomeric gp120 on the virion as well as monomeric gp120 (Parren *et al.*, 2001; Veazey *et al.*, 2003). Antibody 2G12 is another broadly NAb, unusually directed against the carbohydrate-covered outer domain, or the silent face of Env, indicating that humoral immunity can, to a certain extent, overcome the glycan mask (Trkola *et al.*, 1996; Sanders *et al.*, 2002). However, it has not yet been demonstrated that similar antibodies may be elicited via vaccination. Antibody 2G12 has lower neutralising breadth compared to antibody b12 and is unable to neutralise subtype C viruses, most likely due to the absence of one or more glycans that are required for efficient binding (Binley *et al.*, 2004). Antibody 447-52D targets the GPGR domain which is predominant in clade B isolates and is situated at the centre of the V3 region; this antibody neutralises a broader range of viruses than any known anti-V3 NAbs probably because of its unique mode of interaction with V3 (Stanfield *et al.*, 2004; Rosen *et al.*, 2005). However, its interaction with its target motif is difficult to reproduce following experimental immunisation and V3 may be inaccessible in some clade B viruses due to the presence of glycan moieties (Binley *et al.*, 2004).

Because conserved regions are relatively inaccessible, one approach to the design of a successful vaccine immunogen it to expose these highly immunogenic conserved epitopes to the immune system. To this end, several

studies have attempted either partial or complete truncation of one or more variable loops, or mutation of N-linked glycosylation sites to reduce glycan shielding (Lu *et al.*, 1998; Kim *et al.*, 2003). The outcomes have had varying degrees of success and the identification of key modifications that solve the puzzle is still awaited. In addition, it is likely that a successful Env construct should mimic the native protein which exists in trimers. Kim et al. (2005) and others have demonstrated that trimeric envelop glycoproteins were more efficient at inducing NAbs compared to simple monomeric constructs.

A vaccine immunogen designed to elicit strong neutralising responses should thus be engineered to simulate the most relevant *in vivo* response, including the induction of NAbs. A rational approach would be to manipulate the immunogen in an attempt to shift immunodominance towards more conserved neutralising epitopes, rather than non-specific or highly variable epitopes; it is likely that vaccination with such an immunogen would result in the generation of a mature humoral response shortly after immunisation.

1.9.3 Significance of FIV subtypes to vaccine-induced protection

A number of vaccine trials have been conducted to date with variable outcomes, ranging from protection to enhancement (Willett *et al.*, 1997b). Although a vaccine has been developed that is available commercially in a number of countries outside Europe, a vaccine capable of eliciting a strong and potentially broad protective neutralising response against virulent primary isolates of FIV has not yet been developed. It is likely that much can be learnt for the development of a successful lentiviral vaccine by reviewing the outcomes of various approaches that have been tested in FIV vaccine trials.

1.9.3.1 Inactivated virus and infected cell-based vaccines

The most successful vaccine trials to date have used whole inactivated virus, inactivated virus-infected cells or a combination of these. Variable outcomes between different trials may be attributed to factors such as the vaccination schedule, the adjuvant, inactivation method, the cells used to propagate the vaccine virus and the challenge virus, the vaccine and challenge doses as well as the virulence of the challenge virus (Hosie *et al.*, 2000). The first successful vaccine trial using inactivated vaccines was conducted as early as 1991 when a

vaccine derived from cells infected with the inactivated Petaluma strain (FIV-PET) protected cats against homologous challenge (Yamamoto *et al.*, 1991). The main advantage of using infected cell vaccines is that cells do not require expensive purification or concentration techniques and so such vaccines are more easily produced commercially. Moreover, inactivated virus-infected cells may induce stronger humoral responses compared to inactivated virus vaccines, although the duration of protective immunity may be only short-lived (Matteucci *et al.*, 1997). The commercial vaccine, Fel-O-Vax®, consists of inactivated subtype A FIV-PET produced in the cell line FL4 (persistently infected with FIV-PET) and FeT-1 cells infected with the subtype D FIV-Shizuoka isolate; the *env* genes of the two isolates vary as much as 21%. This dual subtype vaccine contains abundant levels of highly stable Env protein, and protected cats against both subtype A and D challenges (Uhl *et al.*, 2008; Uhl *et al.*, 2002). The Fel-O-Vax® vaccine is approved in USA, Japan, UAE, Canada, Australia and New Zealand, but not in Europe.

1.9.3.2 Live attenuated vaccines

Attenuated virus was tested in a number of vaccine trials with some success. Vaccine attenuation leads to a reduction in the virulence of the virus, although the vaccine virus remains viable and replication leads to a more rapid development of an immune response. FIV can be attenuated in several ways, including propagation *in vitro*, selecting for less virulent strains (Pistello *et al.*, 1999), mutagenesis that renders the virus non-infective (for example by inducing mutation at the AP-1 binding site in the FIV transcriptional enhancer (Kohmoto *et al.*, 1998)), or targeted deletions in genes required for virulence, such as *orf-A* (Pistello *et al.*, 2005). It is notable that lion and puma FIV strains have been used as attenuated viruses to vaccinate cats, with relative success (Vandewoude *et al.*, 2002). On the other hand, attenuated vaccines are associated with safety concerns of potential reversion to virulence as well as recombination with live virus (Lockridge *et al.*, 2000) and therefore their use may be restricted to the identification of protective immune responses.

1.9.3.3 Subunit vaccines

Rather than presenting a whole inactivated or attenuated virus to the immune system, a subunit vaccine comprises an immunogenic protein fragment that, when combined with adjuvant, induces immune responses. The majority of FIV subunit vaccines have been based on structural proteins, such as Gag or Env. Vaccine trials have been conducted to test recombinant capsid protein p24 incorporated into immune stimulating complexes (ISCOMs) (Hosie *et al.*, 1992), as well as recombinant Env incorporated into ISCOMs (Huisman *et al.*, 1998). The advantage of this approach is that it permits the evaluation of the immunogenicity of particular protein subunits, or even a single or multiple polypeptides, such as the V3 domain (Lombardi *et al.*, 1994) or the membrane-proximal region of the extracellular domain of the transmembrane glycoprotein (Richardson *et al.*, 1998). Some success has been demonstrated using subunit vaccines, with immunisation leading to a reduction in the viral burden following challenge (Hosie *et al.*, 1996; Leutenegger *et al.*, 1998). However, there are examples also of vaccine trials testing viral subunits that have been led to vaccine-mediated enhancement (Flynn *et al.*, 1997; Osterhaus *et al.*, 1996; Pistello *et al.*, 2006).

1.9.3.4 Recombinant and DNA vaccines

Recombinant vaccines utilise bacterial or viral vectors to encode particular immunogen proteins by means of gene transfer. Feline herpesvirus type 1 (Verschoor *et al.*, 1996), canarypox (Tellier *et al.*, 1998), Salmonella (Tijhaar *et al.*, 1997) and Listeria (Stevens *et al.*, 2004) have been commonly used as vectors, with full or truncated viral genes as transgenes. Mutated proviral DNA bearing defective Vif, integrase, or reverse transcriptase coding genes, resulting in reduced viral replicative capacity, have conferred some protection (Flynn *et al.*, 2000; Lockridge *et al.*, 2000; Dunham *et al.*, 2006a; Dunham *et al.*, 2002). Notably, no potentiation of vaccine efficacy has been obtained when plasmids containing INFγ were included (Gupta *et al.*, 2007). Recombinant vaccines target cellular rather than humoral immunity, but may elicit humoral immunity depending on the biochemical features and the intensity of expression of the non-vectored targeted proteins (Uhl *et al.*, 2002). Suppression of viral burden, but not complete protection, following

challenge has been previously achieved (Hosie *et al.*, 1998). One approach to boost the effect of DNA vaccines is to administer DNA followed by either a dose of recombinant vaccine bearing genetically distant viral DNA or a viral subunit protein, aiming to broaden the immunogenicity and enhance the humoral response (Tellier *et al.*, 1998; Dunham *et al.*, 2006a). Nonetheless, compared to whole inactivated virus or cell vaccines, DNA vaccines have been less successful and additional studies are required.

1.9.4 Evaluation of vaccine trials

Many approaches to vaccine development have been tested for FIV, both as a veterinary vaccine and as a model for HIV vaccines. Sterilising immunity against homologous virus challenge has been induced by a number of vaccines. However, the unsuccessful or partially successful trials have also highlighted many important issues and have been valuable in understanding the mechanism of vaccine-induced protection. Success and failure are two sides of the same coin.

1.9.4.1 Protective vaccines

Most success has been achieved using inactivated vaccines, particularly against more severe challenge systems. In contrast, subunit vaccines performed feebly when used alone. The protection observed in studies using DNA vaccines is promising, although different studies have reported different outcomes which remain poorly understood; for example, Hosie et al. (1998) reported significant protection against challenge with the molecularly cloned homologous PET strain following the vaccination of cats with a DNA vaccine based on PET, while Richardson et al. (1997) reported enhancement of infection rather than protection using a similar DNA vaccine based on PET. Protection against homologous challenge was achieved using inactivated vaccines (Yamamoto *et al.*, 1993; Hosie *et al.*, 2000; Elyar *et al.*, 1997), and partially using DNA vaccines (Boretti *et al.*, 2000; Lockridge *et al.*, 2000). Furthermore, vaccines have successfully elicited neutralising responses in closely related heterologous challenge strains (Hesselink *et al.*, 1999; Hosie *et al.*, 1995). The major success in the field of FIV vaccination was afforded by the dual subtype vaccine

Fel-O-Vax®, which conferred protection against heterologous isolates within subtype B (Pu *et al.*, 2005; Kusuhara *et al.*, 2005).

1.9.4.2 Unsuccessful FIV vaccines

In several trials FIV vaccination did not confer protection against homologous challenge, even using vaccines based on laboratory-adapted strains (Matteucci *et al.*, 1996; Hesselink *et al.*, 1999). Also, vaccines which provided partial protection, although reducing the viral burden following homologous challenge, did not prevent infection and even vaccines that protected cats against homologous challenge did not protect against strains from other subtypes (Pu *et al.*, 2005; Pu *et al.*, 2001). Although there is evidence that protection extends to some heterologous virus strains, Fel-O-Vax® did not protect cats against challenge with GL8 (Dunham *et al.*, 2006b) and a similar, experimental, inactivated virus vaccine did not protect against either GL8 or FIV-AM6 (Hosie *et al.*, 2000; Hosie *et al.*, 1995). A major concern that has emerged from unsuccessful FIV vaccine trials has been one of safety, since the spectrum of outcomes has included enhancement of infection as well as protection. It is hoped that much may be learnt from unsuccessful trials that will assist in future vaccine development.

1.10 Project aims

The major aims of this study were as follows:

i. To investigate the role of NAb in immunity to infection with FIV in naturally and experimentally infected cats.

ii. To develop and optimise a robust, reproducible virus neutralisation assay to measure the NAb response generated *in vivo,* both in naturally infected cats and in vaccinates.

iii. To study *in vitro* enhancement of FIV infection and evaluate its potential impact on vaccine development.

iv. To examine naturally occurring UK isolates of FIV and assess susceptibility to neutralisation by autologous and heterologous plasmas.

v. To identify neutralisation determinants on Env, as potential FIV vaccine immunogens.

2 Materials and Methods

2.1 Materials and suppliers

AbD Serotec Ltd., Oxford, UK
Phycoerythrin-conjugated rabbit anti-mouse IgG, anti CD134, anti CXCR4.
Abgene, Epsom, Surry, UK
0.2 ml 96-well PCR plates
Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK
Protein A, protein G, Glutathione Sepharose 4B beads
Applied biosystem, Warrington, Cheshire, UK
BigDye terminator, v1.1 cycle sequencing kit, Hi-Di formamide, genetic analyser plate septa
BDH laboratory supplies, Poole, UK
Sodium acetate, Tween 20, sodium azide, glycerol
Becton Dickinson labware, Bedford, UK
10 cm^2 biocoat dishes, 6-well, biocoat plates, 5 ml polystyrene rounded bottom tubes, 14 ml polypropylene rounded bottom tubes
Bioline UK Ltd., London, UK
Agarose
Corning Inc., Corning, NY, USA
5 ml, 10 ml and 25 ml pipettes, 25 cm^2, 75 cm^2, 162 cm^2, 225 cm^2 vented tissue culture flasks, 100 mm and 150 mm dishes, 15 ml and 50 ml centrifuge tubes
Eurofins MWG GmbH, Ebersberg, Germany
All oligonucleotides used for PCR reactions
Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK
Tris base, boric acid, propan-2-ol, ethanol absolute, scalpel blades, NH_4Cl
IDEXX laboratories, Buckinghamshire, UK
FIV antigen ELISA kit
Invitrogen Life Technologies Ltd., Paisley, UK
DH5α supercompetent cells, DNA ligase, Kanamycin Sulphate, Geneticin 418, HEPES (1M), RPMI 1640, L-glutamine (200 mM), penicillin/streptomycin, 5% trypsin-EDTA, DMEM, PureLink HiPure Filter Plasmid Maxiprep kit, 1 kb ladder, dNTPs (25 mM each)

Merck Chemicals Ltd., Beeston, Nottingham, UK

KOD Hot Start DNA polymerase

Miltenyi Biotec, Bergisch Gladbach, Germany

MiniMACS separation columns, goat anti-mouse IgG MicroBeads, MACS magnetic Separator

New England Biolabs, Hitchin Herts, UK

Restriction enzymes, Phusion high fidelity Polymerase kit.

Oxoid Ltd., Basingstoke, Hampshire, UK

NaCl, KCl, KH_2PO_4, Na_2HPO_4, bactotryptone, yeast extract, glucose, $MgSO_4$, $MgCl_2$, phenol red, agar

Perbio Science Ltd., Cramlington, Northumberland, UK

Foetal Bovine Serum (FBS)

Perkin-Elmer Life & Analytical Sciences, Beaconsfield, Buckinghamshire, UK

Steadylite HTS luciferase substrate, 96-well CulturePlate, adhesive seal

Promega UK Ltd., Southhampton, UK

T4 DNA ligase, nuclease free water

Qiagen, Crawley, West Sussex, UK

SuperFect®Transfection kit, QIAquick gel extraction kit, QIAamp DNA Blood Mini Kit, QIAprep Spin Mini-Prep kit

Roche Diagnostics Ltd., Burgess Hill West Sussex, UK

High fidelity RT Polymerase kit, Complete Protease Inhibitors

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

BSA, EDTA, bromophenol blue, DMSO, spectrophtometer quartz cuvet, ethidium bromide

StarLab Ltd., Blakelands, Milton Keynes, UK

1.5 ml eppendorf tubes.

Sterilin Ltd., Aberbargoed, Coerphilly, UK

10 cm^2 agar dishes, 7 ml Bijou tubes, 30 ml Universal tubes

Stratagene, Amsterdam, Netherlands

QuikChange site directed mutagenesis kit, XL-1 Blue competent cells

Thermofisher scientific, Roskilde, Denmark

NUNC 1.8 ml cryo-tubes.

Vical Inc., San Diego, CA, USA

VR1012 retroviral plasmid vector

VWR International Lutterworth, Leicestershire, UK

22 mm x 22 mm coverslips

Weber Scientific International Ltd., Lancing, Sussex, UK

Haemocytometer

2.2 Buffers

- Gel loading buffer (10x): 60% glycerol, 0.25% bromophenol blue
- HEPES Buffered Saline (HBS) 2x: 50 mM HEPES, 250 mM NaCl, 1.5 mM $NaHPO_4$; pH 7.2
- LB agar: 1 % bactotryptone, 0.5% yeast extract, 1% NaCl, 7% agar
- Luria-Bertani (LB) medium: 1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.4
- L-broth: 1% bactotryptone, 0.5% yeast extract, 0.5% NaCl; pH 7.0
- MACS buffer: 2 mM EDTA, 0.5% BSA in PBS; pH 7.2
- PBAz: 1% bovine serum albumin (BSA) and 0.1% sodium azide in PBS
- PBSA: 1% bovine serum albumin (BSA) in PBS
- PBST: 0.1% Tween® 20 in PBS
- Phosphate buffered saline (PBS) 10x: 137mM NaCl, 27mM KCl, 1.5mM KH_2PO_4, 10.2mM Na_2HPO_4; pH 7.4
- SOC medium: LB broth, 0.04% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$
- TBE (10x): 1.25 M Tris, 27mM EDTA, 0.4 M boric acid
- TE buffer: 10 mM Tris.HCl pH 8.0, 1 mM EDTA
- Versene: 0.6 mM EDTA with 0.002% phenol red in PBS
- RBC lysis buffer: 0.88% NH_4Cl, 10 mM; pH 7.4

Other buffers provided within kits are indicated in methods.

2.3 Cell culture

All cell culture work was performed employing aseptic techniques with sterile equipment and reagents inside a laminar flow hood.

2.3.1 Plasma and PBMC collection

Blood samples were collected from FIV sero-positive samples submitted to the Companion Animals Diagnostic Services at the University of Glasgow. Blood samples were centrifuged at 2000 rpm for 10 minutes and the plasma fractions were dispensed in 200 µl aliquots and stored at -80ºC until required. To collect peripheral blood mononuclear cells (PBMCs), the remainders of blood samples were diluted in 1 ml PBS, transferred to a 30 ml universal container containing 20 ml pre-warmed RBC lysis buffer, incubated at room temperature until the solution became clear (approximately 10 minutes) and then centrifuged at 1000 rpm for 5 minutes. Supernatant was removed and cells were washed twice with PBSA buffer and pelleted in 1.5 ml eppendorf tubes. PBMCs were either used fresh for co-cultivation and virus isolation or stored frozen at -80ºC until required for molecular analyses.

2.3.2 Cell lines

All cells used in this study were grown at 37ºC under 5% CO_2 in a humidified atmosphere. To initiate growth of specific cell line, cells were recovered from frozen in liquid nitrogen, thawed rapidly at 37ºC and washed in 10 ml of warmed culture medium to ensure complete removal of the DMSO within the freezing mix. The appropriate medium was then used to resuspend the cells which were transferred into culture flasks and maintained for a period of time sufficient to allow full recovery prior to experimental use or manipulation.

2.3.2.1 MYA-1

The lymphoblastoid IL-2 dependent MYA-1 cell line (Miyazawa *et al.*, 1989b) was grown in RPMI 1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 100 IU/ml

human recombinant interlukin-2 (IL-2). Generally, cultures were subcultured 1 in 2 at confluence so that cell concentrations of $5x10^5$ to 10^6 per ml were maintained.

2.3.2.2 CLL-CD134

This cell line was derived from cells isolated from a canine chronic lymphocytic leukaemia that were transduced with a retroviral vector expressing feline CD134. Feline CD134 expressing cells were enriched twice using magnetic cell sorting (section 2.3.4). Cells grow in suspension and were routinely subcultured every 3-4 days by dilution (1:10) into fresh culture medium. Cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 25 mM HEPES, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 IU/ml penicillin, and 100 µg/ml streptomycin, and selected with 400 µg/ml Geneticin 418.

2.3.2.3 HEK293T

Human embryonic kidney cells stably expressing the SV40 large T-antigen, known as 293T cells (DuBridge *et al.*, 1987), were grown in DMEM with high glucose (4.5 g/l) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin, and selected with 400 µg/ml Geneticin 418. Cells were typically grown in 75 cm^2 flasks. For serial passage, the growth medium was removed and confluent cell monolayers were washed with 5 ml PBS to remove all traces of serum that contains trypsin inhibitor, then treated with 2 ml 10% trypsin in versene (2 ml 0.25% trypsin-EDTA in 20 ml versene) and incubated at 37ºC for 5-10 minutes. Once the cell layer had detached, cells were washed with 5 ml of fresh medium, centrifuged at 1000 rpm for 5 minutes, resuspended in fresh medium and subcultured at a ratio of 1 in 5.

2.3.3 Cell staining for flow cytometry

Cells were regularly examined for the expression of both CD134 and CXCR4 receptors. 10^6 cells were pelleted in a 5 ml polystyrene tube by centrifugation at 1000 rpm for 5 minutes and culture medium was removed. Cell pellets were re-suspended in 10 µl of 100 mg/ml anti-CD134 (7D6) or anti-CXCR4 antibody

(Ab) and stored in the dark at 4ºC for 30 minutes. The cells were then washed in 2 ml PBAz buffer to remove unbound Abs. Bound Ab was detected following incubation with Phycoerythrin (PE)-conjugated rabbit anti-mouse IgG for 15 min in the dark at room temperature. Finally, cells were washed again and re-suspended in 1 ml PBAz.

Samples were analysed using a Coulter Epics LX-MCL FACScan (Beckman Coulter Inc., Fullerton, CA, USA) with a laser at 488 nm and 'Expo32 v1.2 Cytometry List Mode Data Acquisition and Analysis Software'. Analysis was performed on 10,000 events.

2.3.4 Magnetic cell sorting

CLL-CD134 cells (approximately $2x10^7$) were pelleted in a 15 ml tube by centrifugation at 1000 rpm for 5 minutes and culture medium was removed. Cell pellets were re-suspended in 100 µl anti-CD134 (100 mg/ml) and incubated at 4ºC for 30 minutes. After being washed in 1 ml MACS buffer, cells were suspended in 20 µl goat anti-mouse IgG microbeads and incubated at 4ºC for 15 minutes. Meanwhile, a MACS column was placed in the MACS magnetic Separator and 500 µl MACS buffer was allowed to flow through by gravity. Cells were washed in 5 ml MACS buffer and then suspended in 500 µl before being applied to the column. The column was washed twice with 500 µl MACS buffer so that unlabelled cells were washed out and discarded. The column was then removed from the magnet prior to the addition of 1 ml MACS buffer and the depleted cells were collected by pressing a plunger into the column. Cells were suspended with RPMI 1640 and cultured at 37ºC in 5% CO_2. Receptor expression was estimated by flow cytometry (section 2.3.3).

2.3.5 Virus neutralisation assay

The assay was optimised as described in detail in chapter 3. Subsequently, assays were set up in triplicate wells of 96-well flat-bottom tissue culture plates, incubating 25 µl of heat-inactivated plasmas with 25 µl of luciferase HIV(FIV) pseudotype viruses (4 tenfold serial dilutions starting at 1 in 10 dilution) for 1 h at 37ºC and then adding 50 µl of $5x10^5$ cell/ml CLL-CD134 cells (or $1x10^6$ MYA-1). Neutralising activity was determined after 72 hours in culture, following the addition of 100 µl steadylite HTS substrate, by measuring

the luciferase activity in a 1450 MicroBeta Liquid Scintillation and Luminesence Counter (Perkin-Elmer). Data was analysed using SigmaPlot software, version 8.02. Percent neutralisation was calculated as follows:
(No plasma luc activity – plasma luc activity) / no plasma luc activity * 100.

Empirical cut-off values of greater than 80% or 60% were considered as strong and moderate neutralisation respectively.

2.3.6 Transient transfection and pseudotype production

To prepare pseudotypes, 293T cells were transfected with VR1012 expressing the FIV *env* clone and a second plasmid, pNL-Luc-E-R^+, that incorporates a luciferase reporter gene. For transient transfection, $2x10^6$ cells were seeded into a 10 cm^2 biocoat culture dish in 10 ml complete DMEM medium and grown overnight until 60-80% confluent. Transfection complex was then prepared by adding 7.5 µg of each of FIV *env*-expressing VR1012 and pNL-Luc-E-R^+ plasmids to 300 µl of serum free-DMEM and 60 µl of SuperFect® reagent, mixed well and left at room temperature for 15 minutes. The transfection complex was added dropwise to the cells and incubated for 3 hours at 37ºC in an atmosphere of 5% CO_2 before replacing the medium with fresh complete DMEM. Cells were washed with 4 ml PBS before and after applying the transfection complex. Cells were then grown for 48-72 hours at 37ºC in an atmosphere of 5% CO_2. Pseudotype-containing media were collected in 15 ml tubes and centrifuged at 2000 rpm for 10 minutes or passed through a 0.45 µm syringe-operated filter and each pseudotype titre was measured before stocks were aliquoted in cryo-tubes and stored at -80ºC until required.

To produce a large batch of GL8 pseudotype, 293T cells were transfected using the calcium phosphate co-precipitation method. Accordingly, $5x10^6$ cells were seeded into 15 cm^2 culture dishes in 20 ml complete DMEM and grown overnight to 80% confluency. On the day of transfection, 15 µg of each of VR1012 expressing GL8 *env* and pNL-Luc-E-R^+ plasmids were mixed in a 30 ml universal tube and the volume was brought to 4 ml by adding 0.01 M Tris-HCl buffer (pH 8). Then 560 µl of 2M $CaCl_2$ was added slowly and the mix was shaken vigorously for 15 seconds. Keeping continuous mixing by air-bubbling the mixture, 4.5 ml 2x buffer HBS was carefully added. The complex DNA/$CaPO_4$ was incubated 30 minutes at 37ºC and added dropwise to the cells. The cells

were incubated overnight at 37ºC in 5% CO_2 and then the medium was replaced with 20 ml fresh complete DMEM, before being incubated at 37ºC in 5% CO_2 for 48 hours. Pseudotype-containing medium was collected in a 50 ml tube and centrifuged at 2000 rpm for 10 minutes. The luciferase titre was measured before pseudotypes were aliquoted in cryo-tubes and stored at -80ºC until required.

2.3.7 Virus isolation

Fresh PBMC (section 2.3.1) were washed and co-cultivated with $2x10^6$ MYA-1 cells in 5 ml complete RPMI 1640 in a 25 cm^2 culture flask and observed for cytopathic effect. Once cytopathic effect was evident (cell swelling, syncytium formation and cell death) the culture was tested for p24 production by FIV antigen ELISA (section 2.3.8). Antigen (p24) positive cultures were centrifuged at 1000 rpm for 5 minutes and supernatants were aliquoted and stored at -80ºC until required. Cells were washed in PBS and either used fresh for DNA extraction or stored at -80ºC until required.

Virus could also be isolated by infecting CLL-CD134 cells with 1 ml of p24 positive tissue culture supernatant or 200 µl of plasma, as described above.

2.3.8 FIV p24 antigen detection

To confirm successful isolation of the virus, FIV antigen ELISA kits were used in accord with the manufacturer's instructions. Briefly, 200 µl cell-free culture fluid was incubated with 20 µl sample treatment solution in an anti-FIV antibody coated well for 30 minutes at room temperature. This allows viral lysis and FIV p24 capture. The solution was then aspirated off and the well washed thoroughly with PBST buffer before 200 µl of anti-FIV Horseradish Peroxidase (HRP) conjugate was added and incubated for 15 minutes before a second wash step. Then 200 µl of chromagen substrate was added and left for 30 minutes. Stop solution (100 µl) was added and the absorbance was measured at 650 nm using a spectrophotometer. All incubations were conducted at room temperature. For each test, positive and negative controls (provided with the kit) were tested in parallel, the difference in absorbance between the positive and negative being ≥ 0.500. The assay cut-off was estimated as the negative control absorbance plus 0.150

2.3.9 Cryogenic preservation and storage

To create frozen cell stocks, cells were grown in 225 cm^2 flasks and, when confluent, they were pelleted by centrifugation at 1000 rpm (800 rpm for MYA-1 cells) for 5 minutes and re-suspended in a solution containing 70% complete medium, 20% heat inactivated FBS and 10% dimethylsulphoxide (DMSO). Cells were aliquoted into cryo-tubes, each containing 10^7 logarithmically growing cells, properly labeled, placed in a cryo-freezing container (Nalgen, USA) jacketed with propan-2-ol and frozen at -80ºC overnight before being placed in the vapour phase of liquid nitrogen for long-term storage.

2.4 Molecular biology

2.4.1 DNA Extraction

QIAamp DNA Blood Mini Kits were used to extract DNA from PBMCs and cultured cells. All extractions were performed in a room designated for Category 2 work (pre-PCR room). Precautions were taken to avoid cross-contamination between samples, including cleaning the bench thoroughly, placing samples in multiple racks, keeping sufficient space between samples in the same rack, using aerosol-barrier pipette tips, changing pipette tips between all liquid transfers and regular glove changes during each procedure.

As per the manufacturer's instructions, up to 10^7 cells were suspended in 200 µl PBS and then added to 20 µl proteinase K (0.15 mg/ml) in a 1.5 ml eppendorf tube, before the addition of 200 µl lysis buffer (AL) (all buffers and components supplied with kit). The lysate was mixed using a vortex for 15 seconds and then incubated at 56ºC for 10 minutes; then 200 µl of 100% ethanol was added prior to mixing for 15 seconds using a vortex. The solution was transferred to a QIAamp Mini spin column and centrifuged at 8000 rpm for 1 minute. The column was washed twice with 500 µl AW1 and AW2 respectively and placed on a clean 1.5 ml eppendorf tube and centrifuged at 13000 for 1 minute. DNA was then eluted in 50 – 100 µl buffer EB and used immediately in PCR (section 2.4.2) after quantification (section 2.4.11), or aliquoted and stored at -20ºC until required.

2.4.2 Polymerase chain reaction

Polymerase chain reactions (PCR) were performed in a PCR workstation with laminar flow under conditions designed to prevent cross contamination. Reagents and DNA samples were stored at -20ºC and defrosted at room temperature. Equipment was sterilised under UV light for 10 minutes before the work commenced and was kept separate from that used for other techniques within the laboratory.

Conditions were altered according to the requirements of the PCR product, in order to optimise the purity and efficiency. The plasmid pBR-GL8-Mya, which

contains one full length GL8 genome, and distilled water were included in each batch of samples as positive and negative controls respectively. All PCR were performed using a GeneAMP PCR system 9700 Thermocycler (Applied Biosystems). FIV *env* genes were amplified from genomic DNA (gDNA) using the following PCR systems.

2.4.2.1 Phusion high fidelity polymerase kit

The following conditions were used in a 2 round nested protocol:
The first round mixture contained 4 µl high fidelity (HF) Buffer (supplied in the kit), 0.16 µl 25 mM dNTPs, 1 µl (10 mM stock) primers, 0.8µl DMSO, 0.2 µl (1U) Phusion Polymerase, 0.2-1.2 µg purified gDNA and nuclease-free water to a total volume of 20 µl. First round thermocycling conditions were as shown in Table 2.1a.

The second round mixture contained 4 µl HF Buffer, 0.16 µl 25 mM dNTPs, 1 µl (10 mM stock) primers, 0.6 µl DMSO, 0.2 µl (1U) Phusion polymerase, 2 µl first round PCR product and nuclease free water to a total volume of 20 µl. Second round thermocycling conditions were as shown in Table 2.1b.

Table 2.1a.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	98ºC	2 minutes	1
Denaturation	98ºC	10 seconds	35
Annealing	58-64ºC	30 seconds	
Extension	72ºC	90 seconds	
Final extension	72ºC	10 minutes	1
Hold	4ºC	∞	1

Table 2.1b.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	98ºC	1 minute	1
Denaturation	98ºC	10 seconds	30
Annealing	64-68ºC	30 seconds	
Extension	72ºC	75 seconds	
Final extension	72ºC	10 minutes	1
Hold	4ºC	∞	1

Table 2.1. Thermocycling conditions for polymerase chain reaction using phusion high fidelity polymerase kit, (a) shows the first round and (b) shows the second round.

2.4.2.2 Hot start KOD high fidelity polymerase kit

PCR reactions contained 2 µl 10x buffer, 2 µl 2 mM dNTPs, 1 µl (10 mM stock) primers, 1.2 µl $MgSO_4$, 1 µl DMSO, 0.4 µl (1U) KOD polymerase, 0.2-1.2µg purified gDNA and nuclease free water to a total volume of 20 µl. PCR was performed under the conditions described in Table 2.2. Primers used to clone and sequence the FIV *env* genes are shown in Table 2.3.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	95ºC	2 minutes	1
Denaturation	95ºC	20 seconds	35
Annealing	69ºC	10 seconds	
Extension	70ºC	13 minutes	
Final extension	70ºC	10 minutes	1
Hold	4ºC	∞	1

Table 2.2. Thermocycling conditions for polymerase chain reaction using Hot start KOD high fidelity polymerase kit.

5'→ 3' sequence	Name	Description of use	Binding site (GL8–antisense)
GTCGACTGGCAGGTAAGTAGAGA GCTCTTTGC	5340Sal1	*env* cloning	5244 - 5275
GCGGCCGCCATCATTCCTCCTCTT TTTCAG	XG8Not1	*env* cloning	8812 - 8841
GGATGAGTGACGAAGATTGGCAG	AF2	PCR amplification	5233 - 5255
CCTTGTAACCAAGTATCTACTC	AnaSeqR	*env* sequencing	7126 - 7147
TATTATTGGCA(AG)TTGCAATCTA C(AC)TTATC	2F2	PCR amplification	6183 - 6211
TGTAATCAACG(CT)TTTGT(AG)TC TCCTTACAG	1F4	PCR amplification	5892 - 5920
CCAATA(AC)TCCCAGTCCACCCTT	1R4	PCR amplification	9103 - 9124
GGGTCGACACCATGGCAGAAGGG TTTGCAGCA	G8Sal1F	*env* cloning	6256 - 6285
GGGCGGCCGCCATCATTCCTCCTC TTTTTCAGAC	G8Not1R	*env* cloning	8810 - 8843
AGACTTTACAAAGACTCGCTATGT TGGC	1F5	PCR amplification	5830 - 5857
CTATGAAAATAGAGGACC	JAPSeqF	Confirm mutagenesis	7789 – 7806*
CCTCAAAGGGAAGAAATCAGCTCA	XR2	PCR amplification	8869 - 8892
CTGGGTTATACCAATTTCTTAAG	V6RSeq	*env* sequencing	7967 - 7999
GGTGTGGAGATTGTTGGAATTTAA TG	AF1	PCR amplification	5782 - 5807
GCTAACATAACATGAATAGC	1790SeqR	*env* sequencing	8097 - 8116
CATCCTAATTCTTGCATAGC(AG)A AAGCG	AySeqR	*env* sequencing	8330 - 8357
AACCTAACCTTTGCAATGAGAAGT	V3R	*env* sequencing	7527 - 7550
GTATCTGTGGGAGCCTCAAGG	AR1	PCR amplification	9352 - 9380
TTTTTTGGGATTGTTGGGC	550SeqF	*env* sequencing	6841 - 6859

Table 2.3. Oligoneucleotides used in env gene cloning and sequencing, a brief description of use and their binding sites based on the 5' sequence of GL8 virus.

* sequence based on TOT1 sequence.

2.4.3 DNA gel electrophoresis

PCR products or digested DNA samples (section 2.4.5) were examined by gel electrophoresis to ensure correct ligation of constructs, to isolate a plasmid vector or to separate and identify PCR products. A 0.8% agarose gel was prepared by mixing agarose with 1x TBE buffer and heating until the agarose had melted, then 1 µl of ethidium bromide (10 mg/ml) was added and the gel was poured into trays with the appropriately-sized comb. The gel was left to set before immersion in 1x TBE buffer in a horizontal gel tank. Samples were mixed with 10x DNA loading buffer and loaded alongside a 1 Kb ladder to assess the fragment sizes and gel were electrophoresed at 100 V / 75 mA for 40-60 minutes. DNA fragments were visualised under UV transillumination (Syngene Bio Imaging, Cambridge, UK) and photographed; otherwise DNA bands were excised and gel purified (section 2.4.4).

2.4.4 DNA purification from agarose gels

DNA was visualised under UV transillumination (UltraViolet Products Inc., San Gabriel, CA, USA). Appropriate DNA fragments were excised from the gel using a clean scalpel blade and purified using the QIAquick gel extraction kit as per the manufacturer's instructions. Briefly, DNA-gel fragments were dissolved in buffer QG for 10 minutes at 50ºC (all buffers and components supplied with kit). One gel volume of propan-2-ol was added and the solution transferred to a QIAquick column and spun at 13000 rpm for 1 minute. The column was washed using buffer PE and DNA was eluted in 50 µl buffer EB.

2.4.5 Restriction endonuclease digestion

Restriction digests of DNA were performed before PCR fragments and purified plasmid DNA were sub-cloned, in order to ensure successful ligation. Digests were usually prepared in a final volume of 20 µl, containing 2 µl buffer and restriction enzymes as directed by the manufacturer. Restriction enzymes and DNA concentration were adjusted, assuming that 1 U of the enzyme is capable of completely digesting 1 µg of DNA in 1 hour at the appropriate temperature. Enzyme was occasionally added in excess, based on the purity of the DNA. Purified PCR products or plasmid DNA were double digested with *Not*I and *Sal*I.

Reactions were incubated at 37ºC for a minimum of 1 hour before being examined by DNA gel electrophoresis (section 2.4.3).

2.4.6 DNA ligations

Ligated constructs were generated by ligating *Not*I *Sal*I double digested PCR fragments into plasmid vector using T4 DNA ligase. For each construct, a typical ratio of 1:3 of vector to PCR product was used in a volume of 20 µl containing 1 µl of ligase and 2 µl of the supplied buffer. The reaction was incubated at 15ºC approximately for 16 hours. The ligation reactions were transformed as detailed in section 2.4.8. The remainders were stored at -20ºC.

2.4.7 Preparation of antibiotic agar plates

LB agar was autoclaved and allowed to cool prior to the addition of kanamycin as a selection antibiotic at the final concentration of 30 µg/ml. The medium was mixed gently and approximately 25 ml poured into 10 cm^2 petri dishes. The dishes were left to allow the agar to set at room temperature before being either used or stored at 4ºC. Unused plates were disposed of two weeks following preparation.

2.4.8 Transformation

E.Coli DH5α supercompetent cells or XL-1 Blue supercompetent cells stored at -80ºC were thawed on ice and aliquoted in 40 µl amounts into pre-chilled plastic 14 ml Falcon round bottomed tubes. DNA ligations were diluted in TE buffer (pH 7.5) at 1:5 and then 4 µl (0.5-1 µg) was added to the cells and incubated on ice for 30 minutes. The cells were then subjected to a heat shock at 42ºC for 45 seconds, incubated on ice for 2 minutes and then 400 µl of SOC medium was added and cells were incubated for one hour at 37ºC in a shaking incubator at 225 rpm. The mixture was spread onto two agar plates (section 2.4.7). Plates were then incubated for 24-48 hours at 30ºC, to allow slow bacterial growth because of the instability of the primary FIV *env* genes. Colonies were picked from the plates and cultured as described in section 2.4.9. For long-term storage, 812 µl of bacterial culture were mixed with 188 µl of 80% sterile glycerol in a cryo-tube and stored at -80ºC.

2.4.9 Small scale DNA preparation

Starter cultures were generated by inoculating 4 ml L-broth containing kanamycin (30 µg/ml) with a single bacterial colony. This was placed in a shaking incubator (200 rpm) at 30ºC for 24 hours before 1.5 ml of the bacterial culture were transferred into a clean 1.5 ml eppendorf tube. The bacteria were pelleted by centrifugation at 3000 rpm for 5 minutes and plasmid DNA was extracted using the QIAprep Mini-Prep Kit according to manufacture's guidelines, as follows:

The bacterial pellet was re-suspended in 250 µl buffer P1 and the cells lysed by the addition of 250 µl buffer P2 (all buffers and components supplied with kit). The resulting lysate was neutralised with 350 µl of buffer N3 to precipitate any unwanted chromosomal DNA. Precipitated chromosomal DNA was removed by centrifugation at 13,000 rpm for 10 minutes and the supernatant was applied to a QIAprep spin column. The column was washed with 500 µl buffer PB, and then 750 µl buffer PE. DNA was eluted in 50 µl buffer EB and was analysed by digestion with the appropriate restriction endonucleases (section 2.4.5).

2.4.10 Large scale DNA preparation

To achieve greater amounts of purified DNA, PureLink HiPure Filter Plasmid Maxiprep kit was used according to the manufacturer's instructions. Briefly, 2 ml of the starter culture was used to inoculate a 200 ml LB culture containing 30 µg/ml kanamycin in a conical flask and grown for 24-48 hours at 30ºC in a shaking incubator at 200 rpm. Bacterial cultures were spun at 5000 rpm using a Beckman Coulter JA10.500 rotor (Galway, Ireland) for 10 minutes at 4ºC, and the resulting supernatant was discarded. The bacterial pellet was re-suspended in 10 ml of chilled buffer R3 and lysed with 10 ml buffer L7 (all buffers and components supplied with kit). The lysate was incubated at room temperature for 5 minutes and then neutralised by 10 ml buffer N3 in order to precipitate any unwanted chromosomal DNA. Meanwhile, a HiPure Filter column was equilibrated by adding 30 ml buffer EQ1 into the filter cartridge, placed inside the column. The lysate was then passed through the filter cartridge into the column. The filter was removed and the column was washed with 50 ml of buffer W8. DNA was eluted in 15 ml buffer E4 in a clean 50 ml centrifuge tube, precipitated by adding 10.5 ml isopropan-2-ol and spun at

3500 rpm for 30 min at 4ºC. The DNA pellet was washed with 1.5 ml 70% ethanol, transferred to a 1.5 ml eppendorf tube and centrifuged at 13000 rpm for 5 min. The supernatant was carefully removed and the DNA pellet was air dried prior being resuspended in 500 µl buffer TE and transferred to a fresh eppendorf tube. DNA was quantified, aliquoted, and stored at -80ºC.

2.4.11 DNA quantification

Quantification of DNA samples was performed by measuring the absorbance of a 1:200 dilution of the sample at a wavelength of 260 nm to give a spectrophotomeric measurement of the amount of ultraviolet radiation absorbed by DNA bases. An absorbance at 260nm (OD_{260} value) of 1 unit was assumed to correspond to 50 µg/ml of double stranded DNA.
DNA concentrations were calculated using the following formula:
Sample concentration = OD_{260} X dilution factor (200) X 50 (µg/ml)
The OD_{280} value of each sample was also measured to assess the purity of the DNA solution. A DNA solution with OD_{260}/OD_{280} ratio of 1.8 represents a pure preparation. Values between 1.7 and 2.0 were considered sufficiently pure.

2.4.12 DNA sequencing of PCR products

Purified PCR products were sequenced using the Big Dye Terminator V1.1 kit. The reaction mixture consisted 100 µg/µl purified DNA, 0.32 µl sequencing primers (10 mM stock), 4 µl sequencing buffer, 2 µl sequencing enzyme and nuclease-free water to a total volume of 20 µl. Cycling conditions were as shown in Table 2.4.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	96ºC	1 minute	1
Denaturation	96ºC	10 seconds	25
Annealing	50ºC	5 seconds	
Extension	60ºC	4 minutes	
Hold	4ºC	∞	1

Table 2.4. Thermocycling conditions for sequencing.

Sequencing reactions were then purified using the following procedure. The volume of each reaction was adjusted to 100 µl by adding 80 µl sterile distilled water and the mixes were transferred to 1.5 ml eppendorf tubes. To precipitate DNA, 200 µl 100% ethanol and 1 µl 3M sodium acetate were added, the solutions were mixed well and then incubated at -70ºC for 30 minutes. Tubes were centrifuged at maximum speed for 10 minutes and supernatants were discarded. DNA pellets were washed with 200 µl 70% ethanol and centrifuged again at maximum speed for 10 minutes. The supernatants were carefully removed and DNA pellets were air dried, re-suspended in 20 µl buffer Hi-Di, transferred to a 0.2 ml 96-well PCR plate and covered with a stratum. Sequencing was performed using the ABI3700 automated capillary array sequencer. Raw chromatograph data were analyzed using 'Contig Express' sequence analysis software within the Vector NTI suite of programs.

2.4.13 Multiple alignments and phylogenetic analysis

Nucleotide sequence analysis was performed on a 651 nucleotide fragment spanning the V3-V5 region of the FIV *env* gene. The generated consensus sequence comprised sequences of isolates included in the study, as well as reference sequences. Multiple alignments were performed using the ClustalX (version 2.0.1)(Larkin *et al.*, 2007) and BioEdit (version 7.0.9.0) applications, followed by manual adjustment to maximise similarities. Alignments were translated and the resulting amino acid-based alignments were used as an exact guide for re-positioning of improper gapping, particularly where sequences differed in length. DNA distance matrices were calculated with DNADIST from the PHYLIP software package (version 3.68, 2008)(Felsenstein, 1989) using the F84 model and an empirical transition/transversion ratio of 2. Phylogenetic trees were constructed by the neighbour-joining method. Robustness of the tree was evaluated by bootstrap analysis on 1000 replicates to assess the support at each of the internal nodes of the neighbour-joining and minimum evolutionary trees. To compare different measures, additional trees were created by the maximum likelihood using DNAML with the substitution model of HKY85, assuming two rates of variability along the alignment. Amino-acid alignments were analysed with PROTDIST to create distance matrices based on the Dayhoff PAM model prior to constructing the neighbour-joining

tree. The phylogenetic trees were committed for final editing and graphical representation using MEGA 4 (Tamura *et al.*, 2007).

2.4.14 Site-directed Mutagenesis

The QuickChange II site-directed mutagenesis kit was used to induce mutations in the FIV *env* gene. According to the manufacturer's protocol, a 50 µl reaction contained 50 ng of DNA (VR1012 plasmid carrying FIV *env* gene), 125 ng of each of the primers, 10 × *Pfu* reaction buffer, 1 µl of dNTP mix (0.2 µM), and 2.5 U *PfuUltra*® high-fidelity DNA polymerase.
Reactions were cycled under the cycling conditions described in Table 2.5.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	95ºC	30 seconds	1
Denaturation	95ºC	10 seconds	12-18
Annealing	50ºC	1 minute	
Extension	68ºC	13 minutes	
Hold	4ºC	∞	1

Table 2.5. Thermocycling conditions for site-directed mutagenesis PCR.

The product was treated with 1µl (10U) *Dpn*I endonuclease at 37ºC for 1 hour, in order to digest the methylated parental DNA template. *Dpn*I treated DNA was then transformed into XL1-Blue Supercompetent® cells, and the *env* gene was sequenced to confirm the mutation. Primers sequences are detailed in Table 2.6.

Primer sequence (5' → 3')	Description of use
CAAATGGAACTGATAATAGTAAGACAAAAATG GCATGCCCTGAG	F primer, to induce 6 bp insertion AAGACA (Arg & Thr) in V5
CTCAGGGCATGCCATTTTTGTCTTACTATTAC AGTTCCATTTG	R primer, to induce 6 bp insertion in V5
GTGACATTTCAGTGTCACAGAACACAAAGTCA ATC	F primer, to induce substitution Gln→His in V3
GATTGACTTTGTGTTCTGTGACACTGAAATGT CAC	R primer, to induce substitution (Gln→His) in V3
GATATTTGAATTGTAATTGTACAAATGGAACT GAT	F primer, to induce substitution Ser →Asn in V5
ATCAGTTCCATTTGTACAATTACAATTCATATA TC	R primer, to induce substitution Ser →Asn in V5
CAAATGGAACTGATAATAGTAAGAAAATGGCA TGCCCTGAG	F primer, to induce 3 bp insertion AAG (Arg) in V5
CTCAGGGCATGCCATTTTCTTACTATTACAGT TCCATTTG	R primer, to induce 3 bp insertion in V5
GTACAAATAGCACAGTATACAGTGATACTAAA ATGAC	F primer, to induce substitution NGNDN → DNSDT in V5
GTCATTTTAGTATCACTGTTATCTGTGCTATTT GTAC	R primer, to induce substitution NGNDN → DNSDT in V5
CAAGGAAAAGTAAGTATATCATTATGTC	F primer, to reinstate PNGS in V1/V2 analogue
GACATAATGATATACTTACTTTTCCTTG	R primer, to reinstate PNGS in V1/V2 analogue
GGATGTCATAGAAACAAAAGCCAATTTCATGA TG	F primer, to induce substitution Asn→Lys in C3
CATCATGAAATTGGCTTTTGTTTCTATGACAT CC	R primer, to induce substitution Asn→Lys in C3

Table 2.6. Oligonucleotides used in mutagenesis in this research, with a brief description of use.

3 The development and optimisation of an *in vitro* assay to detect virus neutralising antibodies

3.1 Introduction

It is generally accepted that it will be important for an effective lentiviral vaccine to elicit neutralising antibodies (NAbs). Therefore, it is important to develop an assay that is robust, sensitive, quantitative and reproducible, in order to monitor adequately neutralisation breadth and potency and to compare the efficacy of candidate FIV immunogens.

The first neutralisation assays to be developed for FIV used a Crandell feline kidney (CrFK) cell-based assay system (Fevereiro *et al.*, 1991; Siebelink *et al.*, 1995b; Tozzini *et al.*, 1992) because infection with isolates adapted for growth in CrFK cells produced syncytia that could be enumerated. Using CrFK-based assays, it was demonstrated that sera collected from naturally and experimentally infected cats in the United States and Europe contained NAbs. Subsequently it was shown that assays employing CrFK-adapted isolates detected antibodies that bound a linear neutralisation site within the third hypervariable (V3) region of the FIV envelope (Env) (Lombardi *et al.*, 1993). During adaptation for growth in CrFK cells, an E to K mutation within V3 was observed (Siebelink *et al.*, 1995a; Verschoor *et al.*, 1995) and, consequently, CrFK-based neutralisation assays emphasised the role of antibodies directed at the V3 region of Env.

CrFK-adapted primary isolates such as GL8 were poorly cross-neutralised by antibodies raised against the laboratory adapted isolate FIV-PET (Osborne *et al.*, 1994; Talbott *et al.*, 1989). A survey of plasma samples from naturally infected cats demonstrated that primary viruses with the reactivity of GL8 were common (Osborne *et al.*, 1994) and it was necessary to develop other neutralisation assay formats that did not require prior adaptation of primary isolates to CrFK cells. When assays were developed using IL2-dependent T-cell lines, viruses resisted neutralisation by sera with high titres of NAbs on CrFK-based neutralisation assays (Baldinotti *et al.*, 1994), suggesting that regions of Env other than V3 could be targets also for NAbs. Subsequently other lymphoid cell-based assays have been developed using the IL-2 dependant cell lines MBM

(Baldinotti *et al.*, 1994; Del Mauro *et al.*, 1998) and MYA-1 (Inoshima *et al.*, 1998).

For HIV, a number of neutralisation assays has been developed to detect antibodies elicited by either potential vaccine immunogens or following natural infection and two assay formats have emerged. The "gold standard" utilises primary peripheral blood mononuclear cells (PBMCs) as the target cell and uncloned primary clinical (field) isolates. In this assay system, freshly isolated PBMCs are stimulated with phytohemagglutinin (PHA) and cultured with IL-2 before infection in the presence or absence of test antibodies. The endpoint measure is the inhibition of viral infection based on the detection of HIV p24 antigen in the culture fluids. The main limitation of this system is the differential susceptibility of PBMCs to infection, which affects the reproducibility of the assay system (Brown *et al.*, 2005).

An alternative assay format utilises pseudotyped viruses, generated by co-transfection of an *env*- and *rev*-deleted HIV backbone, together with the *env* clone of interest; the resulting pseudotypes are then used to infect a transformed cell line expressing the appropriate viral receptors, such as TZM-bl, a genetically engineered HeLa cell clone that expresses CD4, CXCR4 and CCR5 and contains a Tat-responsive reporter gene encoding the firefly luciferase enzyme (Platt *et al.*, 1998; Wei *et al.*, 2002). This pseudotype-based assay has proved to be robust and highly reproducible (Mascola *et al.*, 2005; Li *et al.*, 2005). Other advantages of this system include: shorter assay length (2-3 days compared to 4-6 days for the PBMC-based system) and no cell-cell transmission (Brown *et al.*, 2005). However, it has also been demonstrated more recently that primary cell-based assays and pseudotype virus assays may each reveal different, distinct patterns of cross-neutralisation (Brown *et al.*, 2008).

Developing a reliable virus neutralisation assay (VNA) to detect neutralising antibodies is complex; there are many variables requiring standardisation and optimisation, including the cell substrate, cell count, prior cell adsorption, virus titre, antibody dilution, virus dose and antibody incubation times, length of culture incubation, the inclusion of complement, volumes of components added and the endpoint measured.

It was proposed that, using this assay, it would be possible to measure the NAb response generated *in vivo,* both in naturally infected cats and in vaccinates, in order to help evaluate the efficacy of potential vaccines. In addition, antibodies that enhance viral infectivity may be detected. This chapter describes studies undertaken to develop and optimise a novel, luciferase-based neutralisation assay employing viral pseudotypes bearing FIV Envs in order to detect FIV NAbs.

3.2 Methods

3.2.1 Plasma adsorption to remove anti-cellular antibodies

Plasma samples were adsorbed with CLL-CD134 cells before being used in the neutralisation assay, using the method described in Tozzini *et al*. (1992). Briefly, plasma samples were heat inactivated at 56ºC for 30 minutes and ten-fold serial dilutions starting at 1 in 5 were prepared in complete RPMI. An equivalent volume containing $5x10^5$ CLL-CD134 cells was added to each dilution, so the final dilution of 1 in 10 is maintained, and incubated at 4ºC for one hour with continuous shaking. Cells were removed and adsorbed plasmas were added to 1.5 ml eppendorf tubes containing a pellet of $5x10^5$ of CLL-CD134 cells. Tubes were incubated at 37ºC for one hour on a shaker. Adsorbed plasmas were removed to fresh 1.5 ml eppendorf tubes and the assay was conducted according to the standard protocol.

3.2.2 Effect of hypotonicity on neutralisation sensitivity

Non-ionic distilled water was used to prepare hypotonic solutions of growth media, according to the method used by Russell (1978), resulting in a series of solutions containing 10%, 25%, 50%, 75%, and 100% RPMI-1640. Plasma samples were diluted in each of the hypotonic solutions, and 25 µl of each plasma dilution were tested in the neutralisation assay.

3.2.3 CD134 receptor blocking assay

Dilutions of the anti-CD134 monoclonal antibody 7D6 were prepared, starting from 1.2 µg/ml in complete RPMI. Equivalent volumes containing CLL-CD134 cells (10^6 /ml) were added to each dilution of monoclonal antibody and incubated at 4ºC for one hour with occasional shaking. Fifty µl of cells were added to the equivalent volume of plasma/pseudotype mixture into a 96-well culture plate and subsequent steps of the assay were followed according to the normal protocol. Cells treated in parallel with anti-CD8 antibody served as negative controls.

3.2.4 Immunoglobulin separation

Immunoglobulins from enhancing plasma samples were purified using Protein-A Sepharose beads. The beads were washed twice with 10 volumes of PBS containing Complete Protease Inhibitors, before being suspended in the enhancing plasma and incubated at 4ºC overnight with rotation. The beads were carefully pelleted following centrifugation at 2000 rpm for 5 minutes, and the supernatant carefully removed and transferred to a fresh 1.5 ml eppendorf tube. The beads were then washed twice with 10 volumes of PBS containing protease inhibitors and immunoglobulins were eluted using 0.1M citrate pH 3. The eluate was dialysed overnight against an isotonic solution (PBS), and the eluted immunoglobulins were tested for neutralising activity.

3.3 Results

3.3.1 Optimisation of virus neutralisation assay (VNA)

A luciferase-based neutralisation assay was designed to detect NAbs with activity against FIV. Figure 3.1 shows a cartoon depicting the major steps involved in the standard assay. HIV(FIV) luciferase pseudotypes based on an *env*- and *rev*-deleted HIV pNL-Luc-E$^-$-R$^-$ plasmid (Conner *et al.*, 1995), and carrying a luciferase reporter gene were prepared as described in section 2.3.6, bearing a series of FIV Envs. Serial dilutions of each test plasma were incubated with HIV(FIV) pseudotype for an hour in 96-well culture plates in order to permit neutralisation of the pseudotype to proceed. Following the addition of substrate cells and incubation for two to three days, non-neutralised pseudotype was quantified by measuring the luciferase activity of each well. The percent neutralisation was calculated with reference to control wells which contained no plasma, and plasmas were classified as for HIV, as strongly neutralising (≥80%), moderately neutralising (60-79%), and weakly neutralising 40-59%). Various parameters were examined as described in the following sections. Reproducibility was ascertained with the low error bars of the raw data of luciferase activity counts (see Appendix 3).

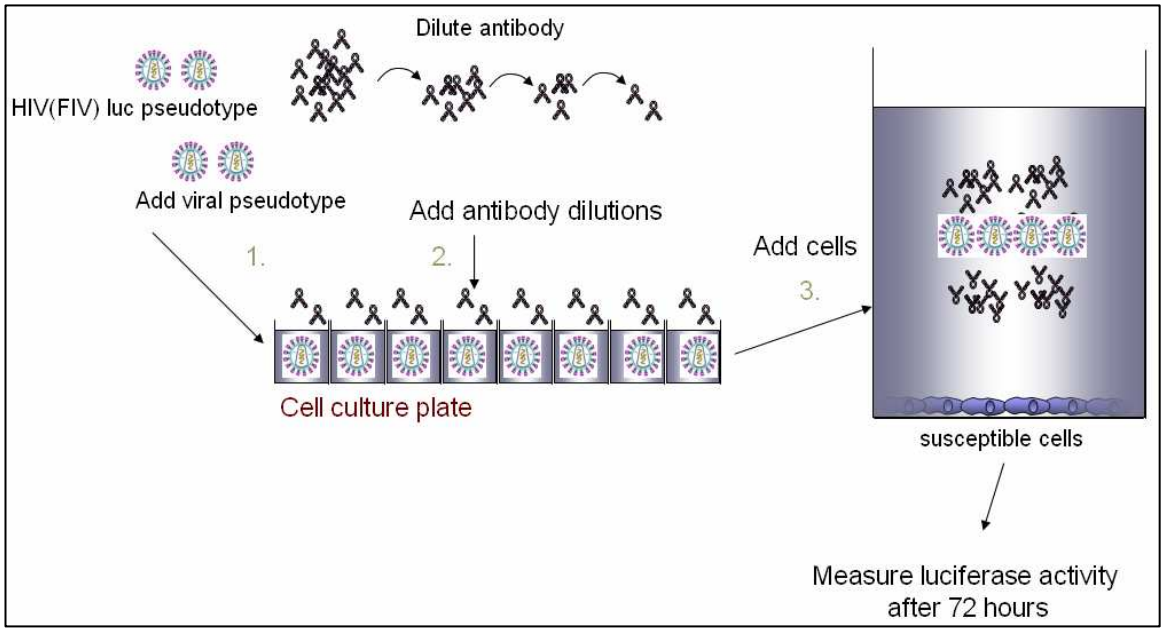


Figure 3.1. Cartoon depicts the steps of the neutralisation assay.

3.3.1.1 Effect of cell substrate on neutralisation

For productive infection to occur, FIV utilises the chemokine CXCR4 as a co-receptor in addition to the primary receptor CD134. The MYA-1 cell line, a feline lymphoblastoid, IL-2 dependent cell line, has been utilised previously in FIV neutralisation assays. However, its low expression of CXCR4 (~10%), slow growth, sensitivity to manipulation, and IL-2 dependency are all considered limitations for the use of this cell line for routine neutralisation assays. As an alternative and to overcome these limitations, the cell line CLL-CD134, derived from a canine chronic lymphocytic leukaemia and transduced with feline CD134 was tested (Willett *et al.*, 2008). Prior to use, the cells were enriched for CD134 expression on two occasions (as described in section 2.3.4) and selected using Geneticin 418, and high expression of CD134 (over 99%) was maintained over 50 passages (Table 3.1).

Passages #	Percent expression	
	CXCR4	CD134
4	99.50	99.75
8	99.07	99.59
14	98.96	98.85
22	99.42	99.53
32	99.87	99.82
38	99.81	99.79
52	99.37	99.79
60	74.58	99.11

Table 3.1. Expression of CD134 and CXCR4 receptors on CLL-CD134 cell line with passage number over a 30 week period. Both CD134 and CXCR4 receptor expression was maintained over 99% over 50 passages.

CLL-CD134 is an IL-2 independent, easily manipulated and robust cell line, with a high growth rate compared to MYA-1; as a result higher luciferase activity was observed in CLL-CD134 compared to MYA-1 cells (Figure 3.2).

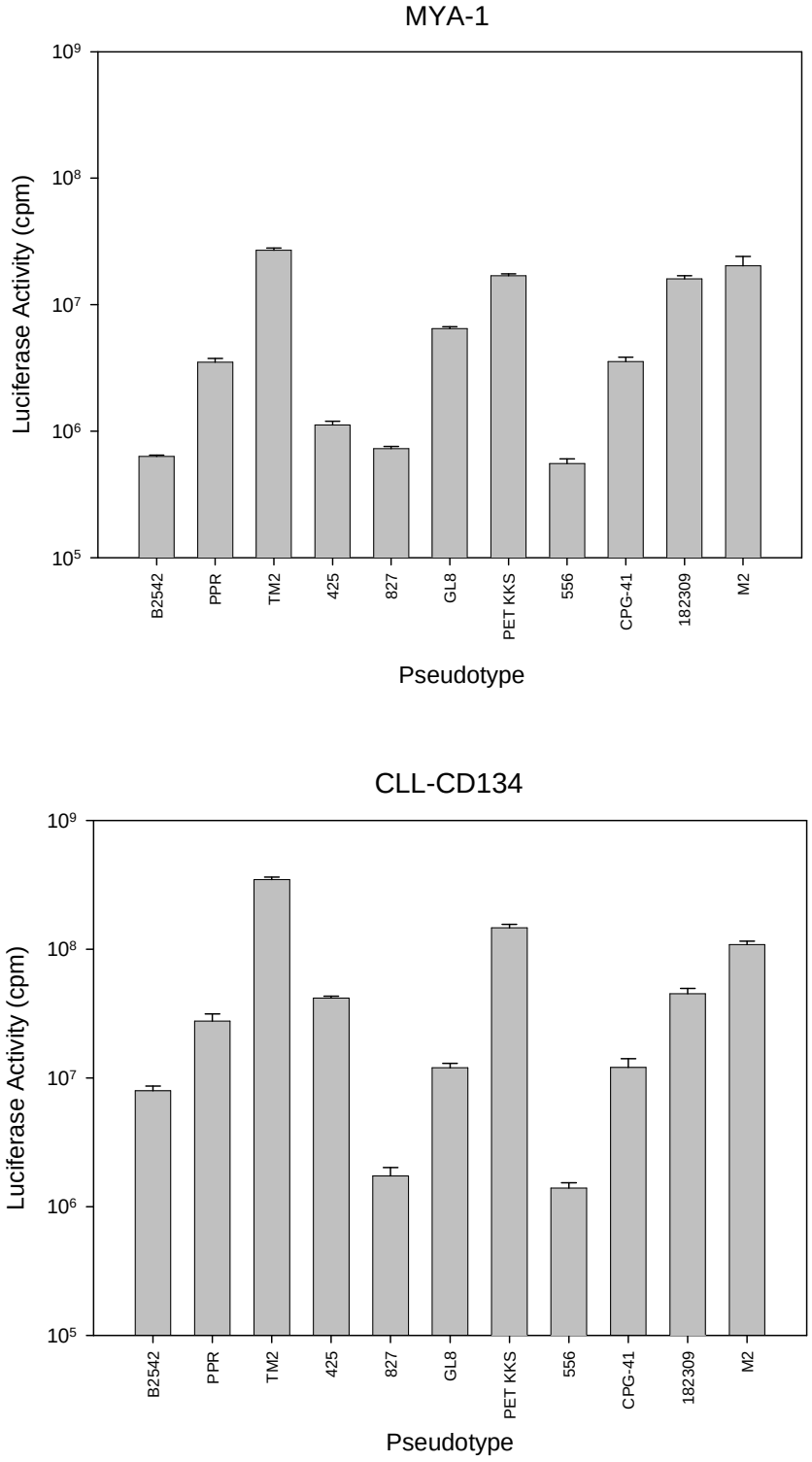


Figure 3.2. Infection of MYA-1 and CLL-CD134 cell lines with a panel of HIV(FIV) pseudotypes. B2542, M2, and TM2 are subtype B strains; PET KKS, GL8, and PPR are subtype A strains; CPG-41 is subtype C strain; and 425, 827, 556, and 182309 are primary subtype A strains. A similar pattern of infectivity was seen but, in general, higher luciferase activity was observed following infection of CLL-CD134 compared to MYA-1 cells. Each column represents the mean (n=3) +/- SEM.

Next, the CLL-CD134 concentration was optimised using two HIV(FIV) luciferase pseudotypes, bearing Envs of GL8 and CPG-41. The optimum concentration was found to be within the range of $2x10^5$ – $5x10^5$ per ml, equivalent to 10^4 – $2.5x10^4$ cells per well (Figure 3.3).

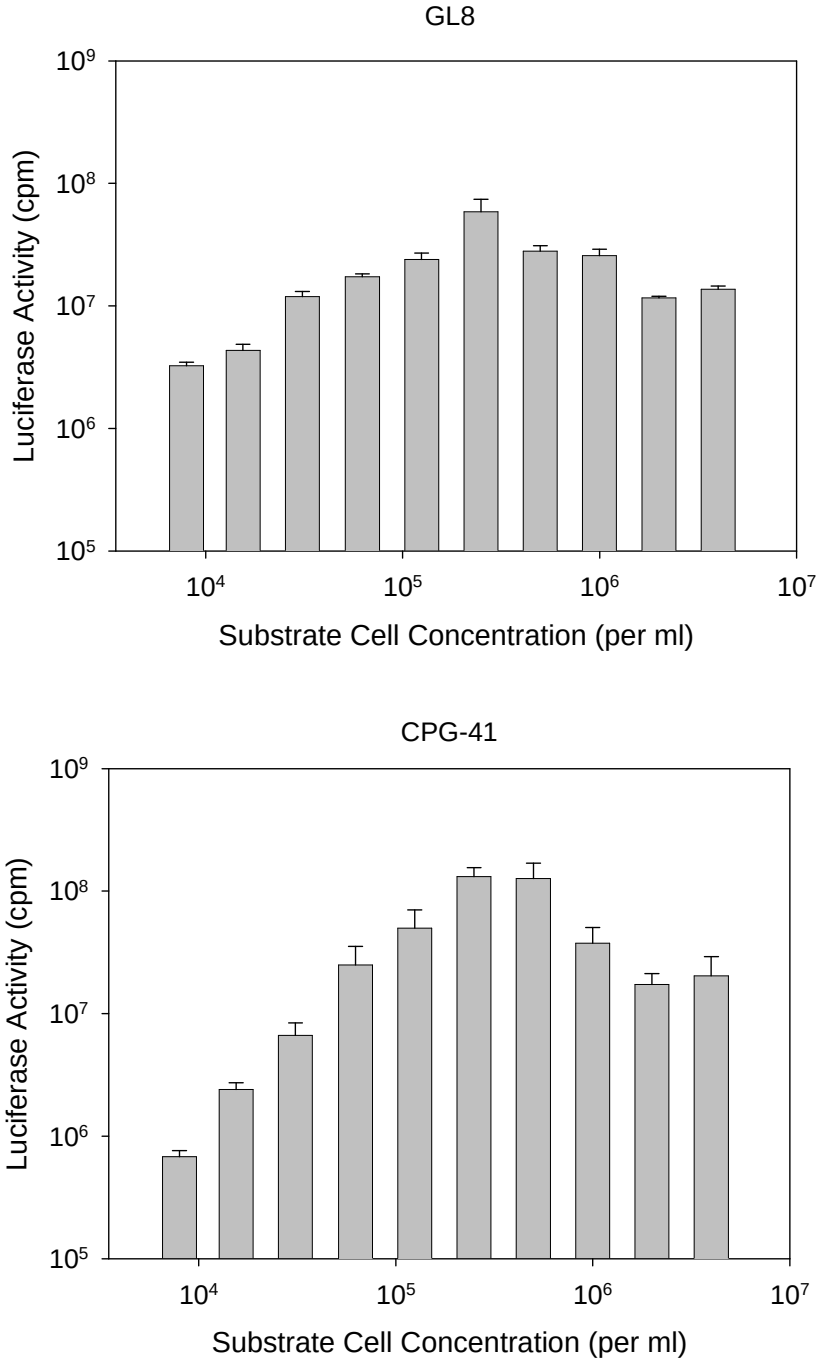


Figure 3.3. Comparison of different CLL-CD134 cell concentrations. The luciferase activity was compared when different concentrations of CLL-CD134 cells were infected with a constant dose of HIV(FIV) luciferase pseudotype, bearing the Env of either GL8 or CPG-41. Each column represents the mean (n=3) +/- SEM.

3.3.1.2 Titration of pseudotype luciferase activity

As described in chapter two, pseudotyped viruses were produced comprising HIV structural proteins, bearing FIV Envs and carrying the luciferase reporter gene (HIV(FIV)luc). The minimum pseudotype titre result in a reproducible, productive infection was determined by titrating the GL8 pseudotype, using a constant concentration of strongly neutralising plasma. The % neutralisation remained stable, or was only slightly reduced, with a pseudotype titre of $2x10^5$, but dramatically changed at pseudotype titres lower than $4.5x10^4$, indicating that below this titre, pseudotypes were not sufficiently abundant to produce a reliable infection. The assay reproducibility was greatly improved at pseudotype titres of 10^6 or greater (Figure 3.4).

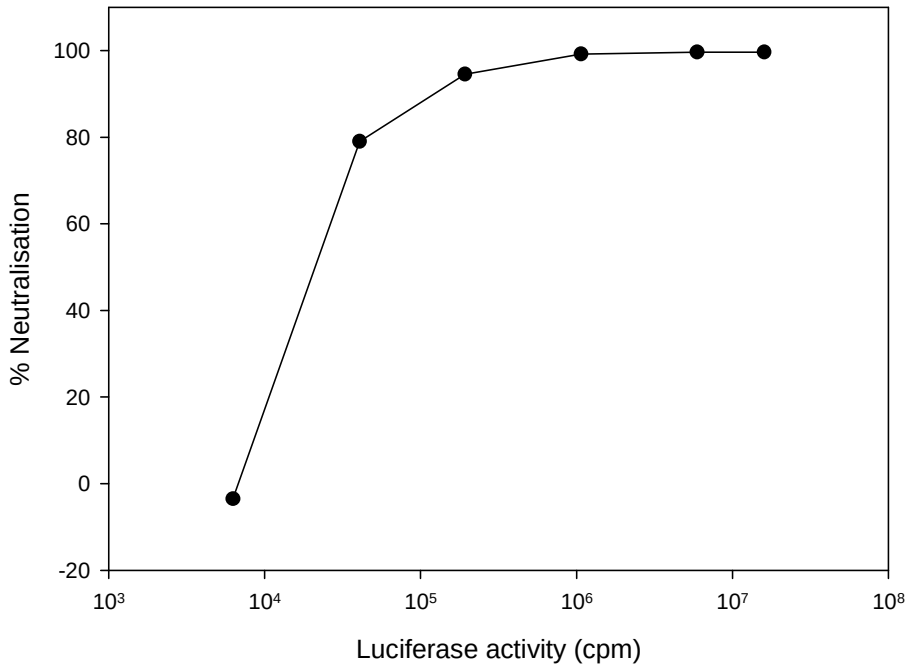


Figure 3.4. Pseudotype titration. The GL8 pseudotype was five-fold serially diluted starting from $1.6x10^7$ and then tested against homologous neutralising plasma, Q253, diluted at 1 in 10. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

3.3.1.3 Other variables

Optimisation of the virus neutralisation assay involved testing additional variables, including the incubation periods. It was shown that an incubation period of one hour prior to addition of the substrate cells was sufficient for neutralisation of the HIV(FIV) pseudotypes. Shorter incubation periods may not allow sufficient time for neutralisation, and longer incubation periods at 37ºC led to reduced luciferase activity (data not shown). The second incubation time was dependent on the growth rate of the substrate cells, since cells would overgrow the assay plate wells if the incubation was too long. As shown in Table 3.2, CLL-CD134 cells grow well for the first three days after being set up in fresh medium, whereas the growth rate declined dramatically after the fourth day. It was concluded that a three day incubation period, using a final cell concentration of $1.5x10^5$ cells per well, was optimal.

Days	Percent growth rate
First	179
Second	190
Third	188
Fourth	138
Fifth	39

Table 3.2. Growth curve for CLL-CD134 cells. It was observed that cells grow actively during the first three days following subculture; however, the growth rate declined dramatically after day 4 when cells became over-confluent.

Next, the inclusion of a plasma adsorption step was examined. Thirty nine plasma samples from vaccinated cats, and twenty nine plasma samples from naturally or experimentally infected cats were adsorbed against uninfected CLL-CD134 cells, prior to being tested for neutralisation activity in order to remove antibodies recognising cellular antigens that could have the potential to affect the detection of NAbs *in vitro*, as reported by Tozzini *et al*. (1992). No significant differences were detected in the neutralisation activity of plasmas before and after adsorption, as shown in Figure 3.5. It was concluded that there was insufficient evidence to include plasma adsorption routinely prior to conducting virus neutralisation assays on plasmas from naturally infected cats.

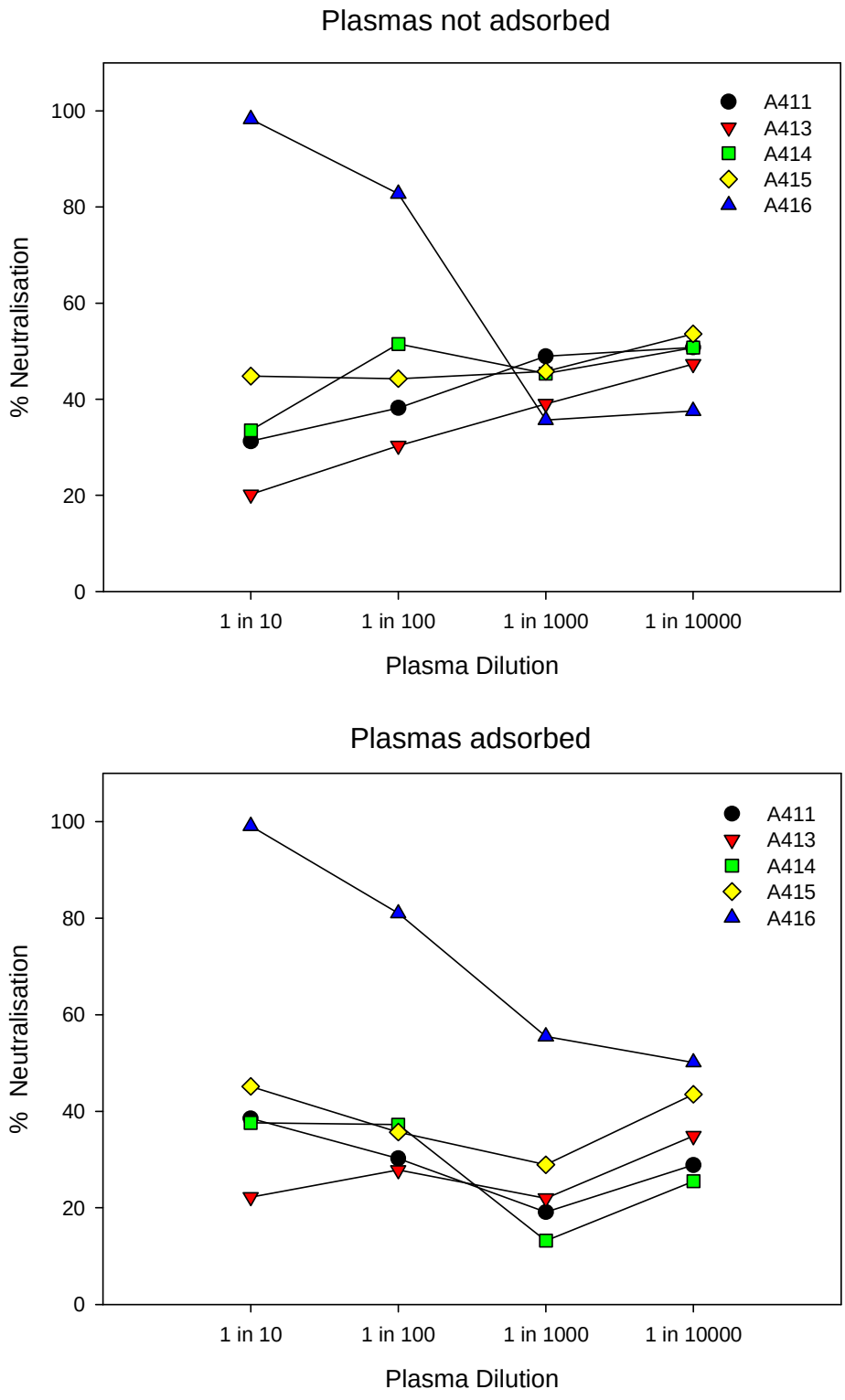


Figure 3.5. The effect of plasma pre-adsorption on sensitivity to neutralisation. Plasma samples from experimentally infected cats (A411, A413, A414, A415, A416 - see appendix 4) were adsorbed prior to being tested for neutralising activity in order to remove antibodies recognising cellular antigens which could potentially affect the detection of NAbs *in vitro*. Samples were taken at 32 weeks post infection. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

Consistent with other protocols for the detection of HIV NAbs, plasma samples were heat inactivated at 56ºC for 30 minutes in order to inactivate complement before dilutions were incubated with the HIV(FIV) pseudotypes (Derby *et al.*, 2006; Kraft *et al.*, 2007). However, there was no evidence that complement played a significant role as non-heat inactivated plasma samples displayed a similar neutralisation pattern to untreated samples (Figure 3.6). Nevertheless, a heat inactivation step was included for consistency, in order to cancel any potential effects of complement and other labile proteins in viral lysis, allowing a direct comparison with previously published studies (Fevereiro *et al.*, 1993).

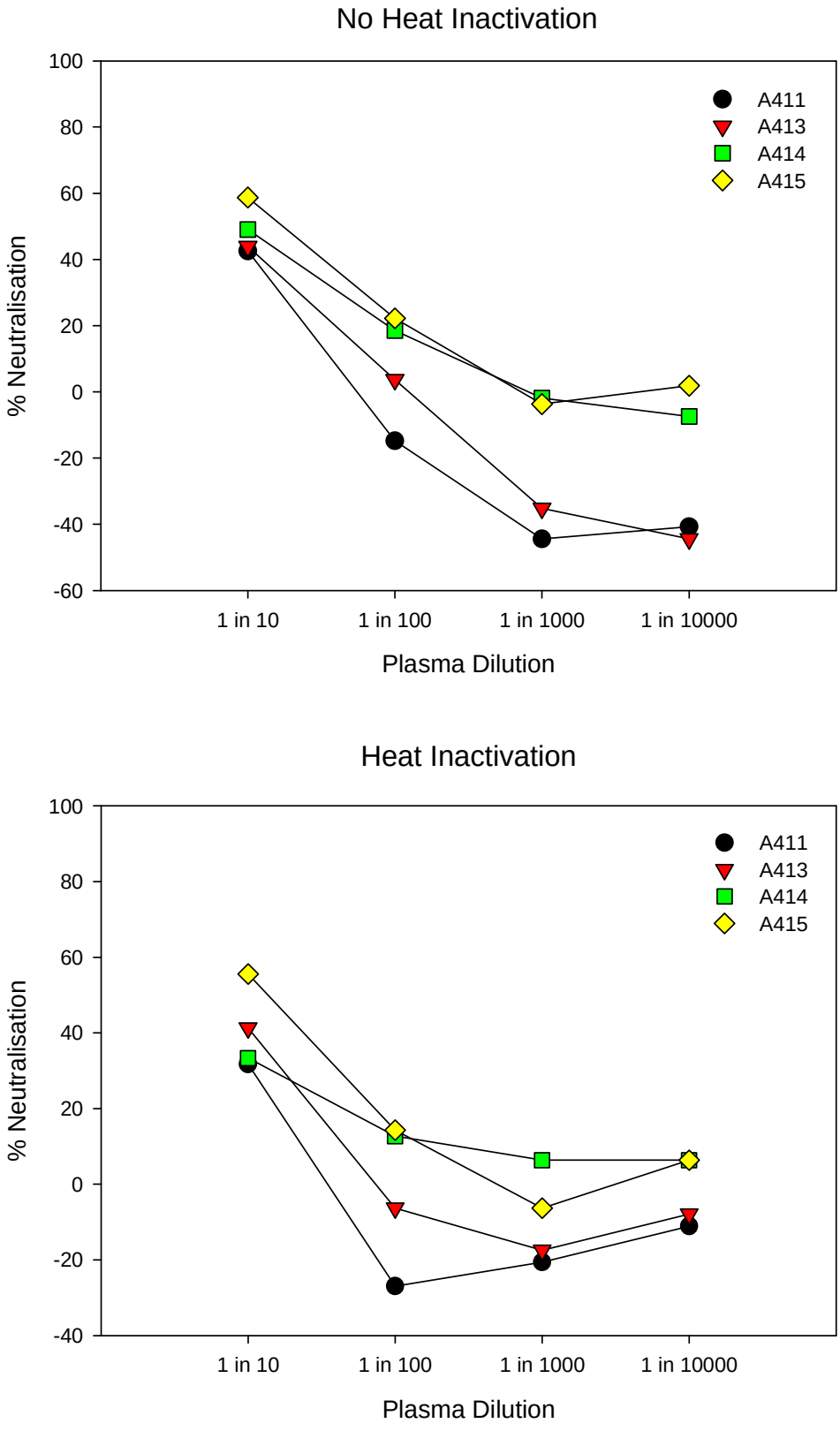


Figure 3.6. Comparison of neutralisation activity with and without heat inactivation of plasma samples from four experimentally infected cats, A411 & A413, which were infected with PET, and A414 & A415 which were infected with GL8. Samples were taken at post mortem at 48 weeks post infection. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

Russell (1978) reported a method in which the neutralisation of feline leukaemia virus (FeLV) was potentiated when plasma samples were diluted in a mixture containing 50% hypotonic solution and 50% culture medium. A similar methodology was applied using weakly neutralising plasmas, and a slight potentiation of neutralisation at 50% RPMI (and 50% non-ionised distilled water) was observed in some of the plasmas tested (Figure 3.7). Given the lack of compelling evidence for a significant improvement in the neutralisation sensitivity afforded by this approach, the use of hypotonic diluent was not pursued further in FIV neutralisation assay development.

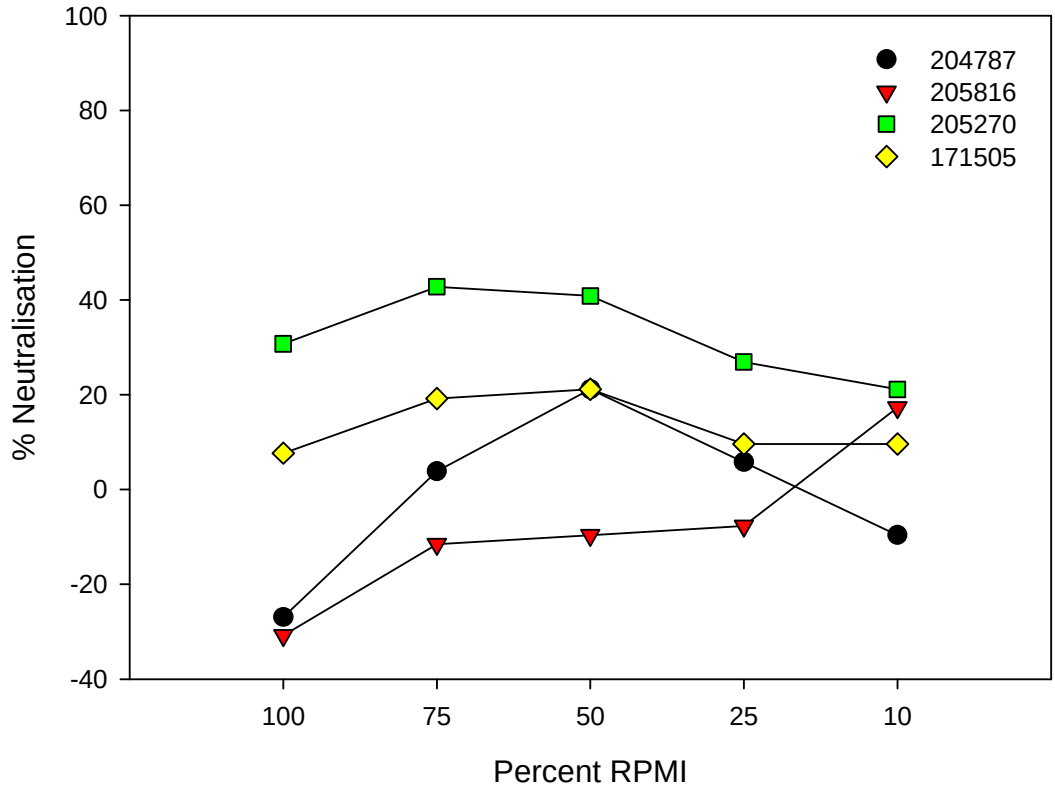


Figure 3.7. The effect of hypotonic solution on FIV neutralisation. Four plasma samples from naturally infected cats, 294787, 205270, and 171505 were diluted 1 in 10 in RPMI adjusted to be hypotonic by dilution in water as shown and the effect on neutralising activity was measured. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

3.3.2 Time course of NAb production

Anti-FIV antibodies can be detected as early as 6 weeks post infection in experimentally infected cats (Hosie *et al.*, 1998). Similarly, seroconversion can typically be detected between 2 and 4 weeks of HIV infection (Sierra *et al.*, 2005; Pantaleo *et al.*, 1993). However, it takes longer for a NAb response to mature and in order to estimate the time required, sequential samples collected from an experimentally infected cat at 23, 26, 32 & 37 weeks post challenge were examined. The results demonstrated that NAbs were generated between 26 and 32 weeks post infection (Figure 3.8). Subsequent assays used the strongly neutralising plasma sample A416.

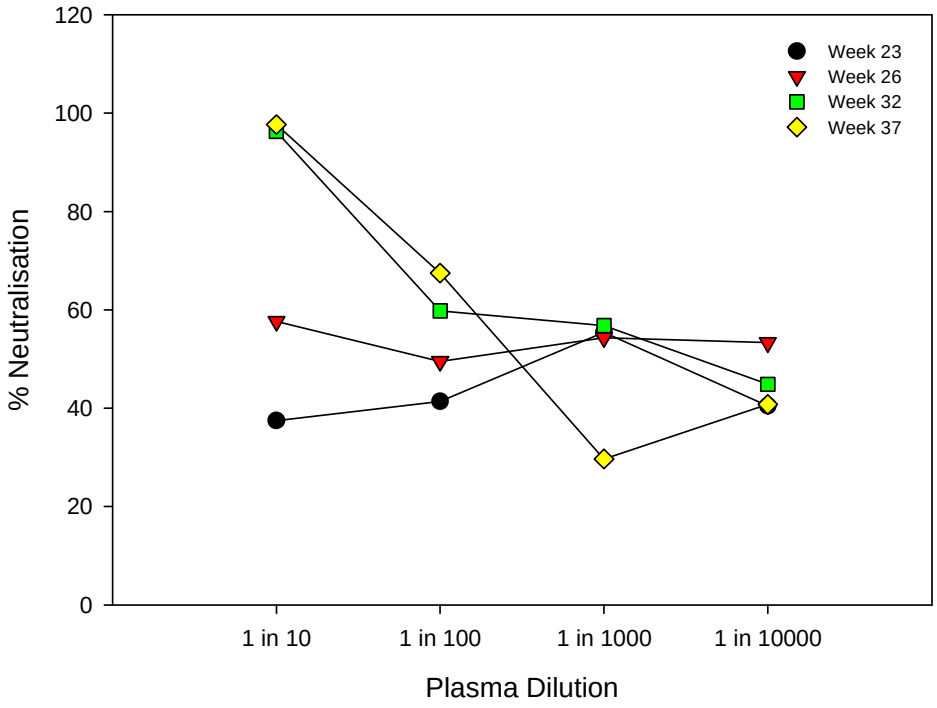


Figure 3.8. The development of NAb response. Sequential samples collected from cat (A416) experimentally infected with a molecular clone of homologous GL8 were tested and NAbs were first detected 32 weeks post infection. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

3.3.3 Variation in NAb response amongst viral isolates

HIV(FIV) luciferase pseudotypes bearing the Envs of the GL8, PPR (Phillips *et al.*, 1990), B2542 (Diehl *et al.*, 1995a) and CPG-41 (Diehl *et al.*, 1995b) isolates of FIV were tested for neutralisation by plasma from a cat experimentally infected with the GL8 isolate of FIV. GL8 is a representative UK isolate (Hosie *et al.*, 2000; Dunham *et al.*, 2006a), belonging to subtype A (see chapter 4), while PPR, B2542 and CPG-41 were isolated in the US and belong to subtypes A, B, and C, respectively. The pattern of neutralisation observed with a strongly neutralising plasma sample collected from a cat experimentally infected with GL8 varied dramatically amongst the pseudotypes, as shown in Figure 3.9. While the GL8 pseudotype was strongly neutralised at 1 in 10 plasma dilution by its homologous plasma, B2542 was only weakly neutralised. A similar profile was achieved by plasma samples from naturally infected cats (data not shown). Furthermore, enhancement rather than neutralisation was observed for both B2542 and CPG-41. The enhancement was dose-dependent, being most marked at lower dilutions of plasma. Such a spectrum of activities displayed by a plasma sample, from neutralisation to enhancement of infection, indicates the major challenge faced in FIV vaccine development, since ideally an effective and safe vaccine should protect against a wide range of primary isolates with no adverse effects such as enhancement of infection.

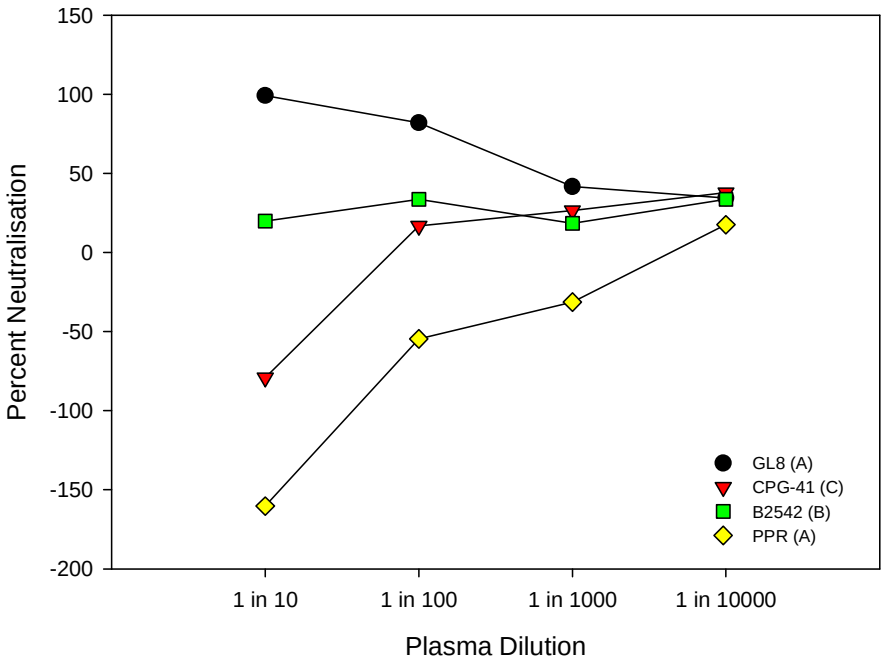


Figure 3.9. Plasma A416 neutralised a pseudotype bearing the homologous GL8 Env but enhanced infection with pseudotypes bearing the heterologous PPR and CPG-41 Envs (appendix 4). Subtype classification between parentheses.

3.3.4 *In vitro* enhancement of FIV infection

Steps were taken to investigate further the enhancement phenomenon whereby, on occasions, increased luciferase activity was observed in wells in which pseudotypes were incubated with heterologous plasma samples compared to "no plasma" control wells. One possible mechanism for antibody-mediated enhancement was via T-cell activation mediating upregulation of CD134, the primary receptor for FIV (Shimojima *et al.*, 2004), and the co-receptor CXCR4. Accordingly, we attempted to block the enhancement effect using antibody 7D6 recognising CD134. As shown in Figure 3.10, the enhancement of CPG-41 infection displayed by plasma samples A411 and 170338 was blocked by the addition of increasing doses of antibody 7D6 (starting concentration 600ng/ml). In contrast, enhancement was not blocked by an isotype-matched control antibody recognising CD8. It was concluded, however, that this reduction occurred by blocking viral entry, since plasma 170305 was not enhancing but showed a similar pattern of reduction in luciferase activity. Concentrations of 7D6 greater than 600 ng/ml did not result in further reduction of the enhancement.

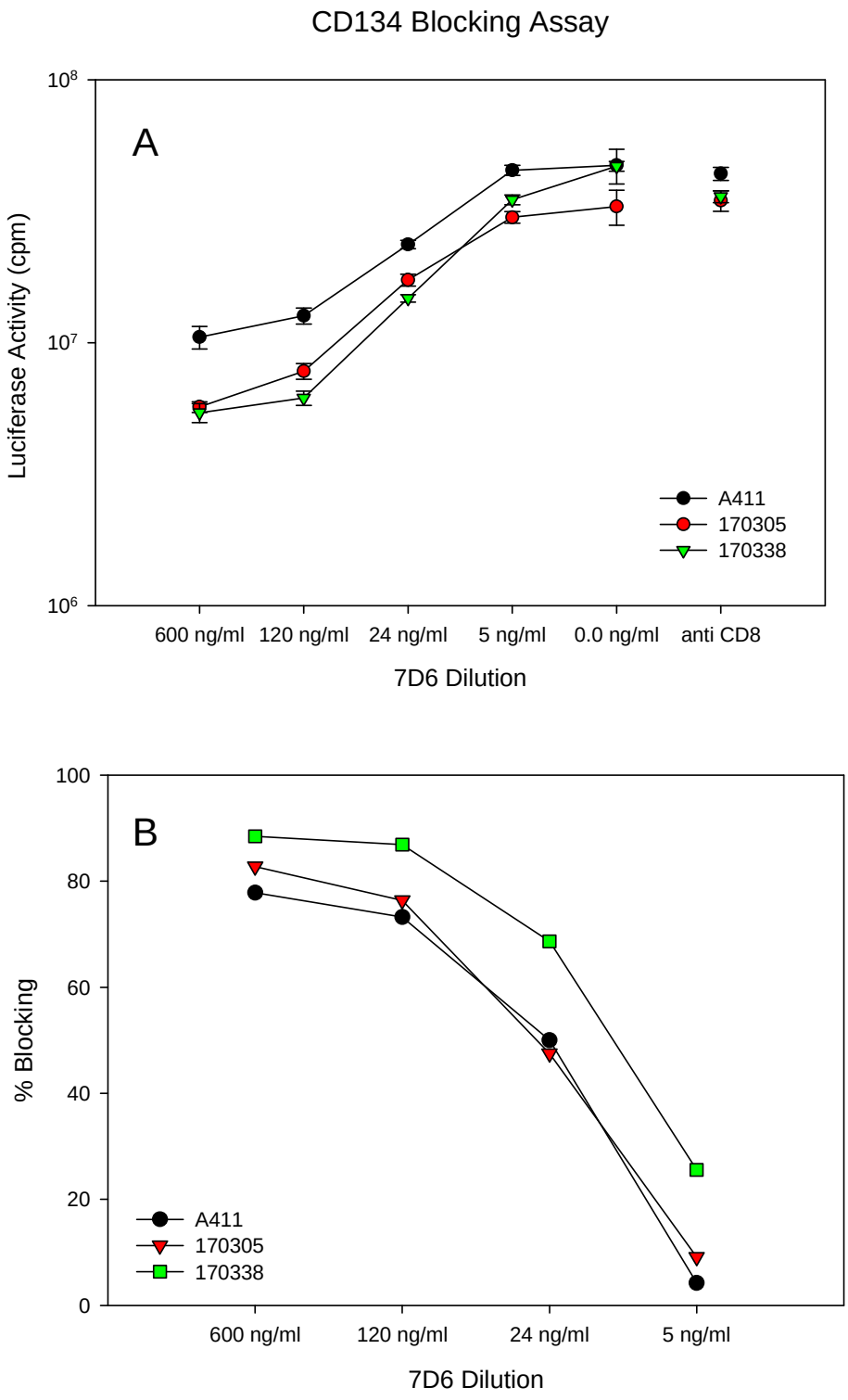


Figure 3.10. CD134 blocking. This assay aimed to block enhancement by preventing the activation of the primary (CD134) at a 1:10 plasma dilution. A.) Luciferase activity (counts per minute) with 1:5 dilutions of 7D6. Anti CD8 was used as a negative control. B.) Percent blocking values. Each point represents the mean (n=3) +/- SEM.

To test whether the enhancement was antibody-mediated, antibodies were separated from other soluble factors within a representative enhancing plasma (A411) using either protein A-Sepharose or Sepharose-G25 as a control. The eluted fractions were tested for the ability to neutralise the CPG-41 pseudotype. It was demonstrated that only the fraction eluted from protein A Sepharose and whole plasma enhanced infection (Figure 3.11), confirming that the enhancement was antibody-mediated. Furthermore, when a panel of anti-FIV Env monoclonal antibodies was tested, it was observed that incubation with vpg67, a monoclonal antibody targeting the V3 loop of FIV, also led to enhancement of infection with the GL8 and TM pseudotypes (data not shown).

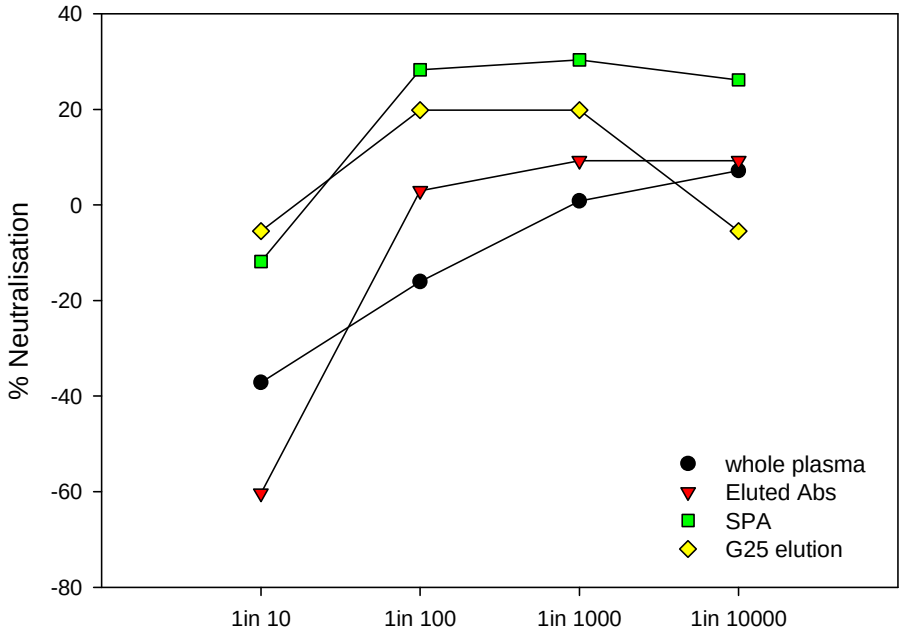


Figure 3.11. Enhancement is antibody-mediated. Immunoglobulin was separated from enhancing plasma using protein A-Sepharose (Eluted Abs). Eluted antibodies enhanced infection whereas the fraction that could not be eluted from protein A-Sepharose (SPA) and the eluate from Sepharose G25 with no Protein A attached (G25 elution) did not enhance infection, suggesting that enhancement was antibody-dependent. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

Plasma Q253 was identified as having strongly neutralising activity against pseudotypes bearing the GL8 Env. To investigate the mechanism of enhancement observed with the anti-FIV Env antibody vpg67, a series of dilutions of plasma Q253 in RPMI were set up, each containing 2 µg/ml of the anti-FIV Env monoclonal antibody vpg67. Enhancement of infection in the presence of monoclonal antibody vpg67 was observed only in the absence of NAb, i.e. at the highest dilutions of the neutralising plasma Q253 (Figure 3.12). Therefore it is likely that an immunogen which induces high titres of broadly cross-neutralising antibodies may overcome the effect of enhancing antibodies.

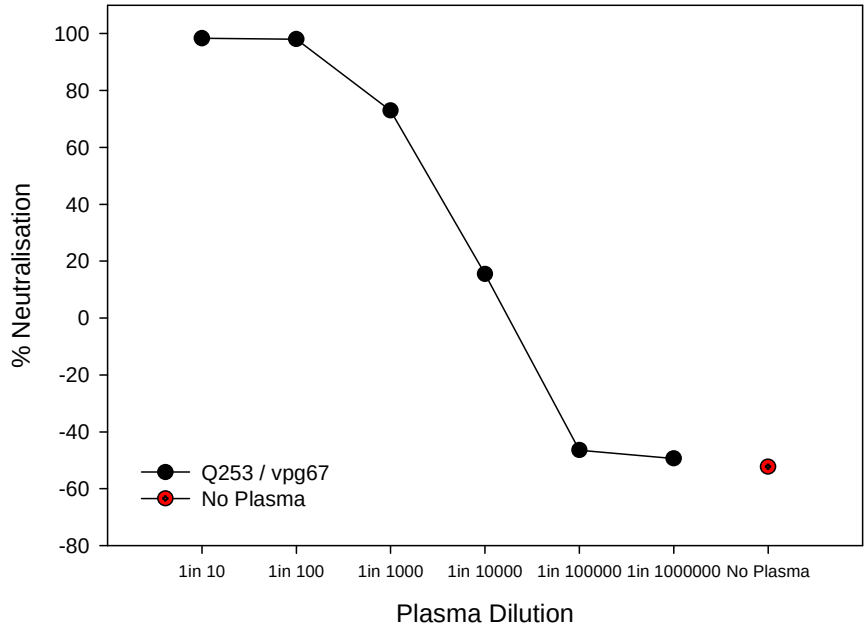


Figure 3.12. Strong neutralisation overcomes the enhancement. Strongly neutralising plasma is titrated against the GL8 pseudotype in the presence of a constant concentration of an enhancing antibody (vpg67), showing that enhancement occurs at plasma dilutions greater than 1 in 10000. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

3.4 Discussion

A robust assay for the detection of FIV NAbs is highly desirable, to understand more fully the humoral immune response in naturally infected cats and to determine the potential value of candidate vaccine immunogens, either experimentally or in clinical trials. Here we developed and optimised a highly reproducible FIV neutralisation assay using pseudotypes bearing FIV Envs and carrying a luciferase gene. The pseudotyped viruses were efficiently produced by HEK293T cells transfected with the HIV pNL-Luc-E^--R^- expression plasmid. Assay variable parameters were optimised and adjusted for robustness and reproducibility.

The luciferase based assay is likely to replace PBMC assays which utilise uncloned viruses produced in PBMCs. Both assays are based on similar principles, measuring reductions in virus infectivity, but the former has the advantage of being more reliable and robust, using shorter assay periods and excluding cell-cell transmission.

One application for the luciferase based assay for FIV NAbs is likely to be in testing vaccine efficacy. While the aim of vaccination against FIV infection is to protect cats from virus challenge, it has been observed that some immune responses elicited by vaccination may result in deleterious responses that actually enhance viral pathogenicity in immunised animals (Hosie *et al.*, 1992; Karlas *et al.*, 1999; Osterhaus *et al.*, 1996; Giannecchini *et al.*, 2002; Wang & Teng, 1994; Richardson *et al.*, 2002). The induction of such immune responses must be avoided in the production of efficacious and safe vaccines.

Generally speaking, lentiviral vaccine-based enhancement has been well-known; several lentiviral vaccines enhanced rather than decreased susceptibility to challenge (Willett *et al.*, 1997b), with vaccinated subjects showing enhanced levels of viraemia, more rapid disease progression and often increased disease severity compared to control subjects (Montefiori *et al.*, 1996). Such outcomes are of major concern for vaccine development, illustrated by the failure in September 2007 of the clinical trial of an adenovirus-based HIV-1 vaccine (Fauci, 2007).

Enhancement of viral infection is speculated to be immune-mediated, mainly through the Fc receptor or the complement receptor (Fust, 1997; Robinson, Jr. *et al.*, 1988; Perno *et al.*, 1990; Wu *et al.*, 1995). It has been demonstrated that antibody-dependent enhancement of HIV-l infection occurs via IgG Fc receptors on monocytes, and that enhancement was blocked by the presence of a potent ligand for Fc receptors (Takeda *et al.*, 1988). Other immune-mediated enhancement mechanisms can be attributed to dendritic cells (Izquierdo-Useros *et al.*, 2007), soluble CD4 (Sullivan *et al.*, 1995), or perhaps other, as yet undefined, soluble factors.

Indeed, the enhancement phenomenon does not appear to be solely vaccine-mediated; in this study enhancing plasmas were identified from naturally and experimentally infected cats. The finding that vaccinated cats showed enhanced viraemia compared to control cats could be attributed to the fact that the immune system of vaccinated cats has encountered the immunogen earlier than that for control cats, resulting in an earlier and enhancing response. Antibodies generated early in infection are not neutralising. We observed that in an experimentally infected cat, NAbs required several months to mature and, in the absence of NAbs, antibody-mediated enhancement may occur.

Antibody-mediated enhancement has been described *in vitro* (Fust, 1997; Sullivan, 2001). Enhanced infection after viral challenge was established in naïve cats following the passive transfer of plasma from vaccinated cats; such findings indicated that enhancement was antibody-mediated (Siebelink *et al.*, 1995c). In this chapter it was observed that the enhancement was attributable to the immunoglobulin fraction of an enhancing plasma sample, confirming that enhancement is likely to be antibody-mediated. The mechanism of enhancement is not known, but it has commonly been assumed that enhancing antibodies promote viral attachment and subsequent internalisation by acting as adaptors between viral particles and Fc or complement receptors on target cell membranes, or that enhancing antibody binds to Env, mimics the interaction with the primary receptor and allows the direct interaction with the co-receptor (Trischmann *et al.*, 1995; Thomas *et al.*, 2006; Takada & Kawaoka, 2003). However, further investigations are required to understand more fully the exact mechanism of enhancement.

The data described in this chapter were consistent with the epitope, recognised by the enhancing monoclonal antibody vpg67, being sited within the V3 loop. Indeed, the V3-V5 region was predicted previously to include epitopes for enhancing antibodies, and thus was deleted from a recombinant protein tested in a vaccine trial (Huisman *et al.*, 2004). Nonetheless, enhancement occurred, indicating that epitopes for enhancing antibodies are not confined to this region. Indeed, epitopes for enhancing antibodies were identified previously in other domains within HIV-1 Env (Robinson, Jr. *et al.*, 1990), including an epitope in the transmembrane glycoprotein gp41 (Takeda *et al.*, 1992). Moreover, monoclonal antibodies directed against the principal immunodominant domain may enhance infection (Eaton *et al.*, 1994). Hence, enhancing epitopes may be distributed throughout Env, and it may not be feasible to locate and exclude all enhancing epitopes for the purpose of designing the ideal safe and effective vaccine. However, when the significance of enhancing antibodies was assessed in the presence of NAbs in this study, it was observed that NAbs can efficiently block viral infection, even in the presence of enhancing antibodies. Therefore it appears that enhancement cannot occur when a strong neutralising response has been induced.

The complex neutralisation profile displayed by the plasma sample that strongly neutralised the GL8 pseudotype but enhanced infection with the PPR and CPG-41 pseudotypes may be attributed to the fact that this plasma contained both neutralising and enhancing antibodies. While it strongly neutralised its homologous pseudotype, it appeared to lack sufficient potency to neutralise a broader range of genetically distinct pseudotypes; these were unlikely to share common neutralisation epitopes and therefore NAbs effective against GL8 Env were not effective against PPR or CPG-41 Env and, accordingly, enhancement occurred.

In light of these findings, and in terms of vaccine development, it appears more feasible to identify common neutralising epitopes, and to solve the spatial structure that renders such epitopes exposed to the host's immune system, rather than to identify and delete epitopes for enhancing antibodies. Accordingly, more extensive investigations were directed towards identifying key components capable of inducing broad NAb responses against FIV and the results of these investigations are presented in the following chapters.

4 Phylogenetic subtyping in the United Kingdom

4.1 Introduction

Like other retroviruses, FIV has high mutation rate, mainly due to the error-prone reverse transcriptase (Shankarappa *et al.*, 1998); accordingly, diverse viral variants emerge continually. Since the *env* gene is the key determinant of viral diversity amongst FIV strains (Olmsted *et al.*, 1989), FIV phylogenetic studies have focussed mainly on *env* sequences. According to the nucleotide sequence diversity of the V3-V5 region of *env*, FIV is classified currently into five distinct phylogenetic subtypes, designated A to E (Pecoraro *et al.*, 1996; Sodora *et al.*, 1994); subtypes A and B are the most commonly occurring worldwide (Martins *et al.*, 2008). Furthermore, similar to HIV-1, several inter-subtype FIV recombinants have been recognised in natural populations following co-infection; inter-subtype recombinants A/B, A/C and B/D have been identified (Hayward & Rodrigo, 2008; Bachmann *et al.*, 1997).

Phylogenetic studies on FIV sequences have revealed significant heterogeneity (up to 30%) in the sequence of the *env* gene of FIV isolates worldwide (Burkhard & Dean, 2003), similar to that estimated for HIV-1 (Rong *et al.*, 2007b). Subtype A isolates are common in Australia, New Zealand, western United States, South Africa and northwestern Europe (Steinrigl *et al.*, 2009; Kann *et al.*, 2006a; Kann *et al.*, 2007; Kann *et al.*, 2006b; Bachmann *et al.*, 1997). Subtype B isolates have been identified in the central and eastern United States, central Europe, Brazil and eastern Japan (Pistello *et al.*, 1997; Nishimura *et al.*, 1998; Sodora *et al.*, 1994; Kakinuma *et al.*, 1995; Steinrigl & Klein, 2003; Martins *et al.*, 2008). Subtype C has been recognised in Canada, New Zealand and southeast Asia (Nakamura *et al.*, 2003; Reggeti & Bienzle, 2004; Uema *et al.*, 1999; Hayward *et al.*, 2007). Finally, subtypes D and E are infrequent but were identified originally in southwestern Canada and Japan, and Argentina, respectively (Pecoraro *et al.*, 1996; Nishimura *et al.*, 1998) (Figure 1.1).

Such heterogeneity in *env* gene sequence poses problems for the design of a broadly protective vaccine. It has been reported previously that effective

protection was obtained following a homologous FIV challenge using an inactivated whole virus vaccine, although, despite the relative success of Fel-O-Vax against heterologous subtype B isolates (Pu *et al.*, 2005), protection did not extend to a heterologous challenge (Dunham *et al.*, 2006a). Such different outcomes highlight the impact of genetic diversity on vaccine strategies against FIV and the importance of assessing the genetic diversity of local subtypes for vaccine development, or before introducing a commercial vaccine to a particular geographical area. Furthermore, identification of the predominant strains in a particular region is necessary in order to develop appropriate reagents for the molecular diagnosis of FIV infection (Hosie *et al.*, 2002; Leutenegger *et al.*, 1999).

Based on the strains previously isolated in the UK, there is a general agreement that all UK strains of FIV belong to subtype A. Nonetheless, there have not been any phylogenetic studies conducted describing accurately the strains of FIV circulating within the UK. This phylogenetic study was conducted to investigate the distribution of UK FIV subtypes, based on the sequence of the V3-V5 region of *env*, examining 45 novel sequences from the UK and including one sequence from of the Republic of Ireland and another from Jersey.

4.2 Methods

4.2.1 Blood samples, PBMC collection and DNA extraction

Blood samples were examined from 47 FIV sero-positive, naturally infected domestic cats (*Felis catus*). The blood samples were collected over the period from April 2007 to August 2008 and had been submitted to the Companion Animals Diagnostic Services at the University of Glasgow for the diagnosis of FIV, either because the cat was sick or for screening purposes. The remainders of blood samples were centrifuged at 2000 rpm for 10 minutes and the plasma was removed and stored at -80^{o}C. Peripheral blood mononuclear cells (PBMCs) were collected as described in section 2.3.1. Approximately 10^{7} PBMC were used directly for DNA extraction using QIAamp DNA Blood Mini Kit (Qiagen, West Sussex, UK), as described in section 2.4.1.

4.3 Results

4.3.1 The sample population

In total, 47 left-over clinical blood samples were examined from cats that had been infected naturally with FIV; the cats were either pets or stray cats waiting to be re-homed from animal protection shelters. Samples were submitted from across the UK; the majority originated from southern Scotland, England or Wales but one sample came from Jersey (171069) and another from the Republic of Ireland (179200) (Figure 4.1). The majority of the cats (35 of 47) were male (74%), a finding that is consistent with other studies (Hayward *et al.*, 2007; Reggeti & Bienzle, 2004), and likely to be associated with aggressive social behaviour in males increasing the risk of transmission via biting (Natoli *et al.*, 2005). The ages were known for only 34 of the 47 cats and these ranged from 6 months to 16 years, with only 3 cats less than 1 year of age. The infected cats were likely at different stages of infection, with clinical signs ranging from asymptomatic to symptomatic; clinical signs recorded on the submission forms included lethargy, pyrexia, anaemia, stomatitis, gingivitis, upper respiratory tract infections, urinary tract infections and central nervous system disorders (Table 4.1).

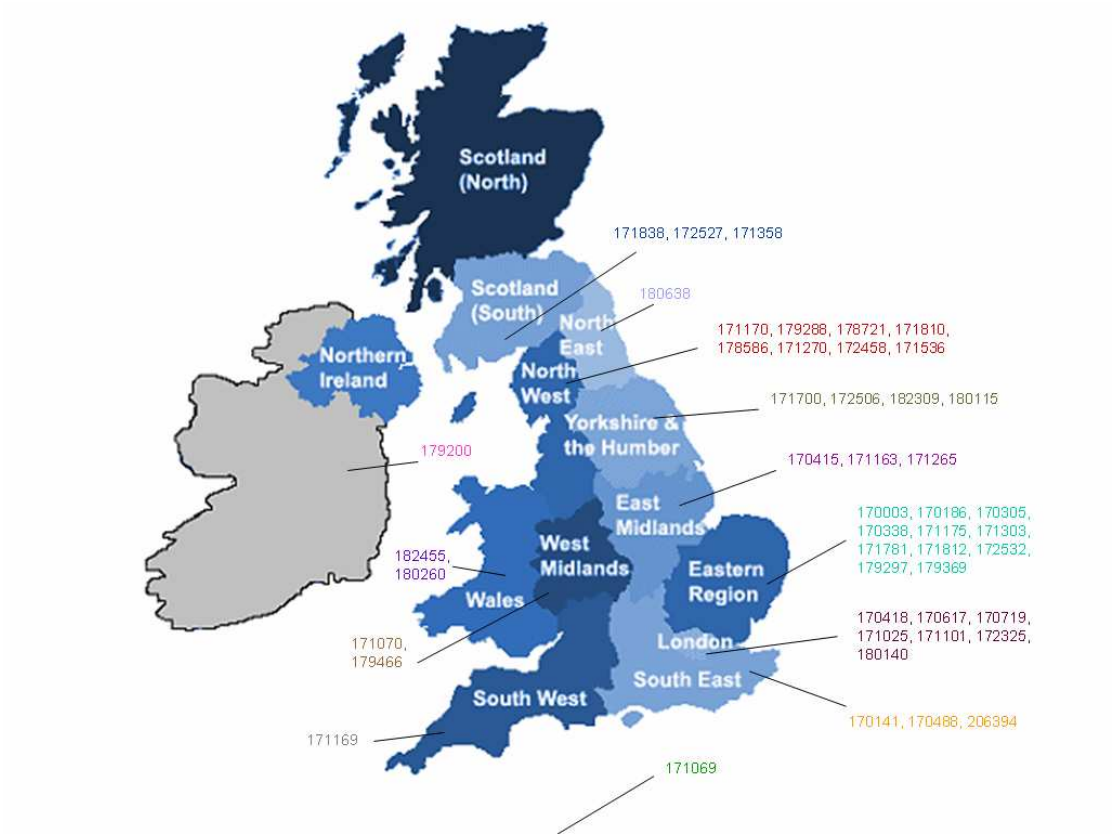


Figure 4.1. Distribution of FIV isolates included in the study. This map shows the locations of the veterinary practices where blood samples were collected from FIV infected cats and sent to the University of Glasgow for the diagnosis of FIV infection.

Strain	Location	Age	Gender	Health Status	Clinical Signs	Subtype
170003	Essex	2-3 years	Male	NK	NK	A
170141	Kent	12 years	Male	NK	NK	A
170186	Norfolk	15 years	Male	Sick	anaemia	A
170305	Suffolk	NK	Female	NK	NK	A
170338	Essex	11 years	Male	Sick	NK	A
170415	Lincolnshire	Adult	Male	NK	covered in scabs and scars	A
170418	London	1 year	Male	NK	NK	A
170488	Kent	4 years	Male	NK	pale - poor condition	A
170617	London	Adult	Female	Sick	NA	A
170719	London	9 years	Male	Sick	anaemia - dehydration	A
171025	London	2.5 years	Male	NK	NK	A
171069	Jersey	NK	Male	Healthy	No aparant lesion	A
171070	Worcestershire	8 years	Male	Sick	ulcerative pharyngitis	A/C
171101	London	6 months	Male	Sick	alopecia	A
171163	Leicester	9 months	Female	Sick	cyclical pyrexia	A
171169	Cornwall	1.5 years	Female	Healthy	slight conjunctivitis	A
171170	Lancashire	Adult	Male	Healthy	No aparant lesion	A
171175	Norfolk	16 years	Female	Sick	upper respiratory tract signs	A
171265	Nottinghamshire	NK	Male	Healthy	No aparant lesion	A
171270	Cheshire	3 years	Male	Healthy	No aparant lesion	A
171303	Norfolk	Adult	Male	Sick	NK	A
171358	Lanark	16 years	Male	Sick	persistant non-regenerative anaemia - leucopenia	A
171532	Cumbria	5 years	Male	Sick	recurrent gingivitis/ stomatitis	A
171700	Hull	NK	Male	NK	NK	A
171781	Norfolk	10 years	Male	Sick	anorexia - recurrent gingivitis	A
171810	Lancashire	NK	Female	Sick	change of coat colour	A

171812	Norfolk	5 years	Male	Healthy	No aparant lesion	A
171838	Glasgow	1.5 years	Female	Sick	dullness – stopped eating	A
172325	London	12 years	Female	NK	NK	A
172458	Wirral	4 years	Male	Sick	gingivitis - stomatitis	A
172506	Hull	10 years	Male	Sick	NK	A
172527	Glasgow	9 years	Female	sick	ataxia	A
172532	Essex	NK	Male	NK	NK	A
178586	Manchester	5 years	Female	Sick	severe gingivitis	A
178721	Lancashire	4-5 years	Male	Sick	large abscess on side of face	A
179200	Dublin / Ireland	10 years	Male	Sick	weight loss - lethargy - fever	A
179288	Lancashire	3 years	Male	Sick	mild gingivitis - neck lesions	A
179297	Norfolk	8 years	Male	Healthy	No aparant lesion	A
179369	Hertfordshire	Adult	Male	NK	NK	A
179466	Worcestershire	5 years	Male	NK	NK	A
180115	Dewsbury	12 years	Female	sick	mucopurulent ocular + nasal discharges - stomatitis - bilateral conjunctivitis	A
180140	London	Adult	Male	Healthy	No aparant lesion	A
180260	Denbigh	10 years	Male	NK	NK	A
180638	Durham	> 2 years	Male	sick	severe periodontitis - swollen and lysed jaw bones	A
182309	Hull	9 years	Male	sick	loss of appetite - halitosis	A
182455	Bridgend	NK	Female	NK	NK	A
206394	Kent	11 years	Male	sick	Urinary tract infection	A

Table 4.1. Details of the naturally FIV-infected cats included in the study. NK: Not known

4.3.2 Phylogenetic Analysis of naturally occurring UK isolates of FIV

An unrooted neighbour-joining phylogenetic tree was generated based on a 651 nucleotide alignment of the V3-V5 region of the *env* gene, comprising 47 novel sequences from naturally occurring isolates of FIV together with reference sequences from subtypes A, B, C, D and E (Figure 4.2). The tree demonstrated that all the novel sequences, with the exception of 171070, cluster within subtype A with a bootstrap value of 99%, while 171070 was classified as belonging to subtype C, albeit with low bootstrap support (36%). Sequences within subtype A were grouped on a branch from which two sequences (179200 and 179297) emerged as a separate group. This demonstrated that both the Irish and British sequences (179200 and 179297), in spite of originating from different geographical areas (Table 4.1), were closely related. Consistent with the phylogenetic distance between FIV isolate 179200 and the other subtype A isolates, a pseudotype bearing the 179200 FIV Env was not highly susceptible to neutralisation by a subtype A cross-neutralising plasma. Subsequently the neutralisation resistance of 179200 was found to be associated with a mutation in C3-V4 domain (see section 6.2.5). These data highlight the role of the V3-V5 region of Env as a target for neutralisation.

The genetic distance between any two sequences within subtype A ranges between 0.0 and 0.272 (between 171810 and 179297), while the maximum difference in base composition bias per site (Kann *et al.*, 2006b) is 1.86 (between 171810 and 179200). The pairs 180140, 180115; 182455 and 179288; 171536 and 171069 have similar V3-V5 sequences, despite having being isolated from geographically distinct locations (Figure 4.1), in England and the island of Jersey. Each sample of these three pairs had been collected and manipulated on different days; hence any possibility of sample mix-up or cross-contamination was highly unlikely. The average evolutionary divergence within subtype A sequences, which refers to the number of base substitutions per site from averaging over all sequence pairs based on the pairwise analysis, was calculated as 0.091 using the MEGA-4 software (Tamura *et al.*, 2007); this value was lower than the divergence for other subtypes described in previous studies (Duarte & Tavares, 2006; Sodora *et al.*, 1994), indicating that isolates within subtype A may be less genetically heterogeneic compared to other FIV subtypes.

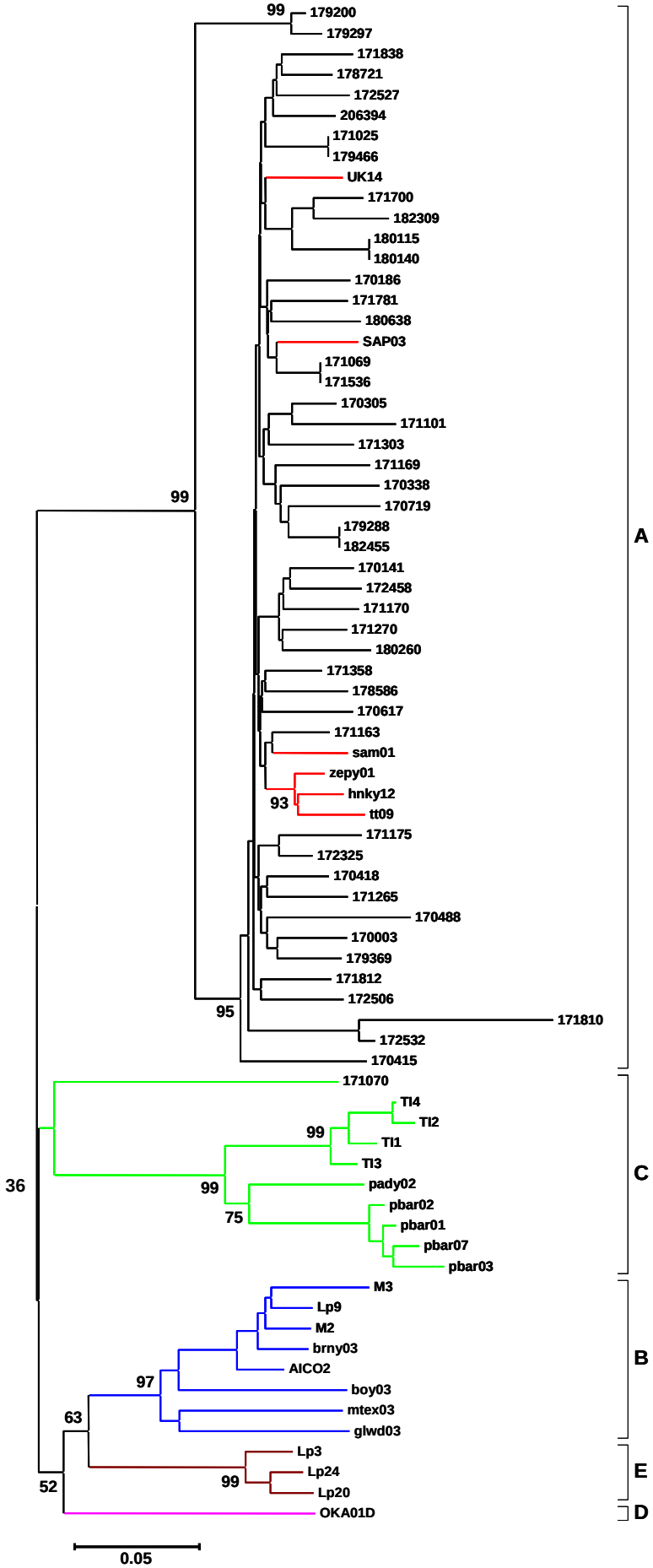


Figure 4.2. An unrooted neighbour-joining phylogenetic tree from 651 nucleotides of the V3-V5 region of *env*. Bootstrap values based on 1,000 replications are shown at the nodes. The tree was constructed using Kimura's two-parameter model and includes the 47 novel sequences as well as representative sequences from subtypes A, B, C, D, and E. Branches are shown in red for subtype A reference sequences, blue for subtype B reference sequences, green for subtype C reference sequences, pink for subtype D reference sequence, and brown for subtype E reference sequences.

A Maximum likelihood tree (Figure 4.3) was also constructed in order to confirm the consistency of subtyping. The tree was similar to the neighbour-joining tree, with the exception that 171070 did not cluster with subtype C isolates. To investigate further the inconsistent subgroup assignment of 171070, a minimum evolutionary tree was constructed and this showed a similar pattern as the maximum likelihood tree (data not shown). Because it could not be assigned to a particular subtype, the 171070 sequence was submitted for recombination analysis.

Given its importance as a target for neutralisation, the V3-V5 *env* sequences were translated in order to calculate a neighbour-joining tree based on the amino acid sequence. The tree was not significantly different from the nucleotide-based trees, but demonstrated also that 171070 did not cluster within any of the subtypes (Figure 4.4).

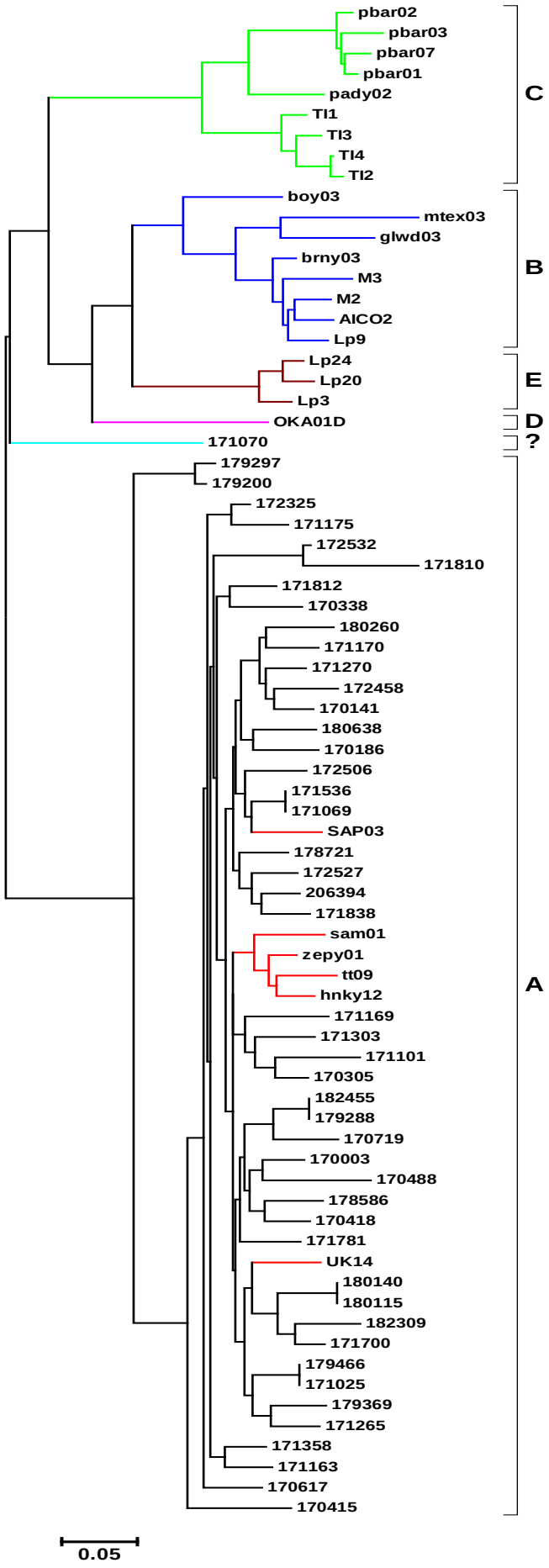


Figure 4.3. Maximum likelihood tree based on a 651 nucleotide alignment of V3-V5 *env* gene. The tree was generated using DNAML with the substitution model of HKY85.

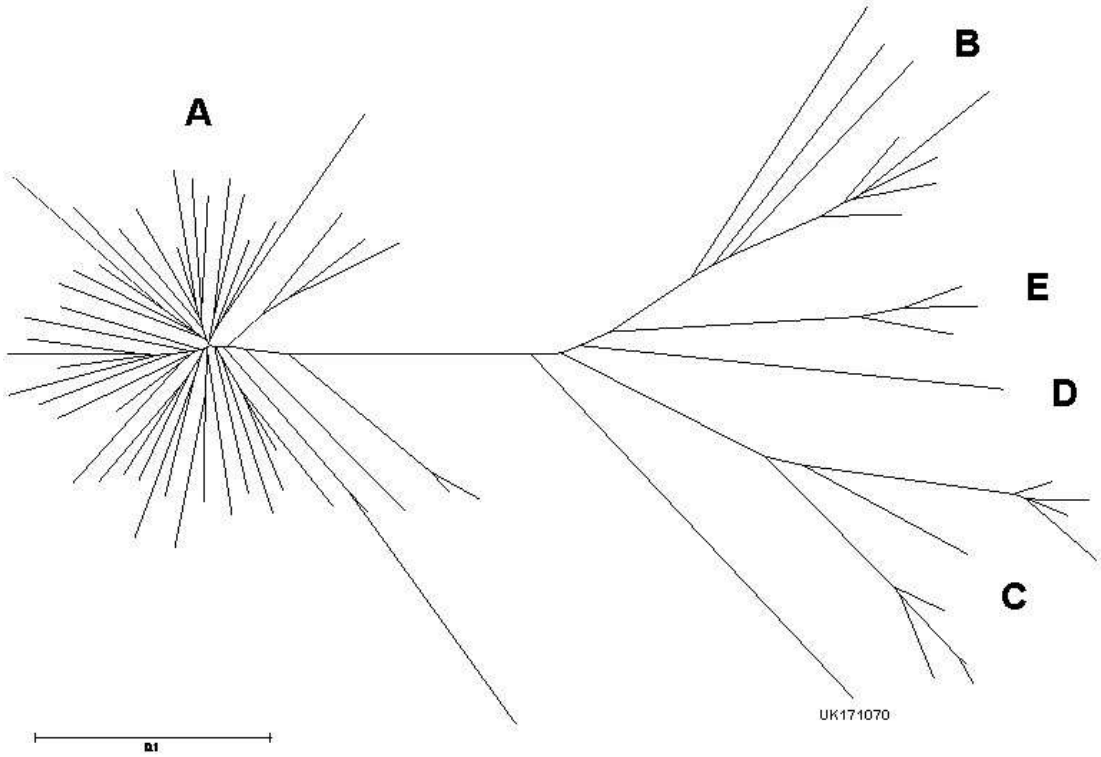


Figure 4.4. Phylogenetic tree inferred by neighbour-joining from amino acid sequence. Tree was generated from an alignment of 217 amino acids including the UK sequences and reference sequences from all subtypes A-E.

4.3.3 Recombination analysis

The sequence of 171070 *env* was examined for possible recombination using SimPlot (Lole *et al.*, 1999). Software analysis confirmed that 171070 is a recombinant of subtypes A and C, with a putative breakpoint approximately 260 nt from the start of the *env* sequence (Figure 4.5). Phylogenetic trees were constructed based on the criteria of minimum evolution for the sequences before and after the putative breakpoint and indicated that the 5' moiety of the sequence clustered within subtype A, whereas the subtype assignment of the second moiety was not clear (Figure 4.6). Interestingly, plasma from cat 171070 significantly neutralised a pseudotype bearing the subtype C Env CPG-41, whereas a pseudotype bearing the subtype A GL8 Env was not neutralised, suggesting that the major neutralisation determinant is more likely to be located within the subtype C sequence of the strain, i.e. within the V4-V5 region (Figure 4.7).

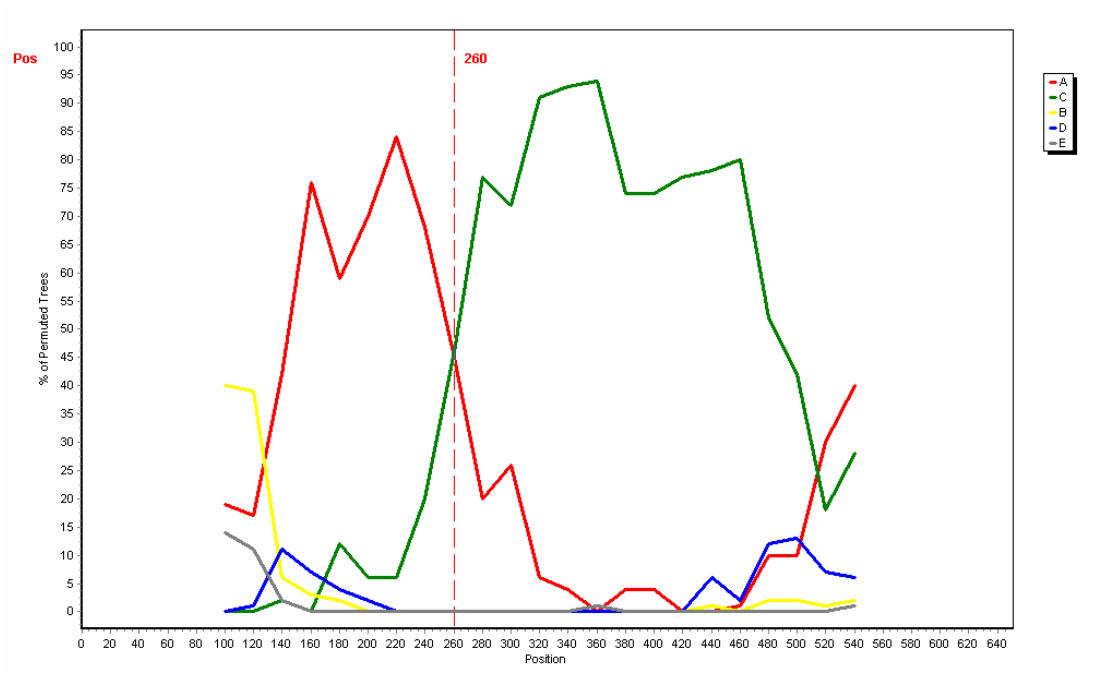


Figure 4.5. Bootscan analysis of *env* V3-V5 from 171070. Bootscanning analysis was performed using a window of 200 nucleotides and a step of 20 nucleotides, by the neighbour-joining algorithm based on the Kimura two-parameter model and 1000 bootstrap replicates. Strain 171070 was used as a query in the analysis and the subtype A-E reference sequences used in the phylogenetic analysis were used as reference groups for the bootscan.

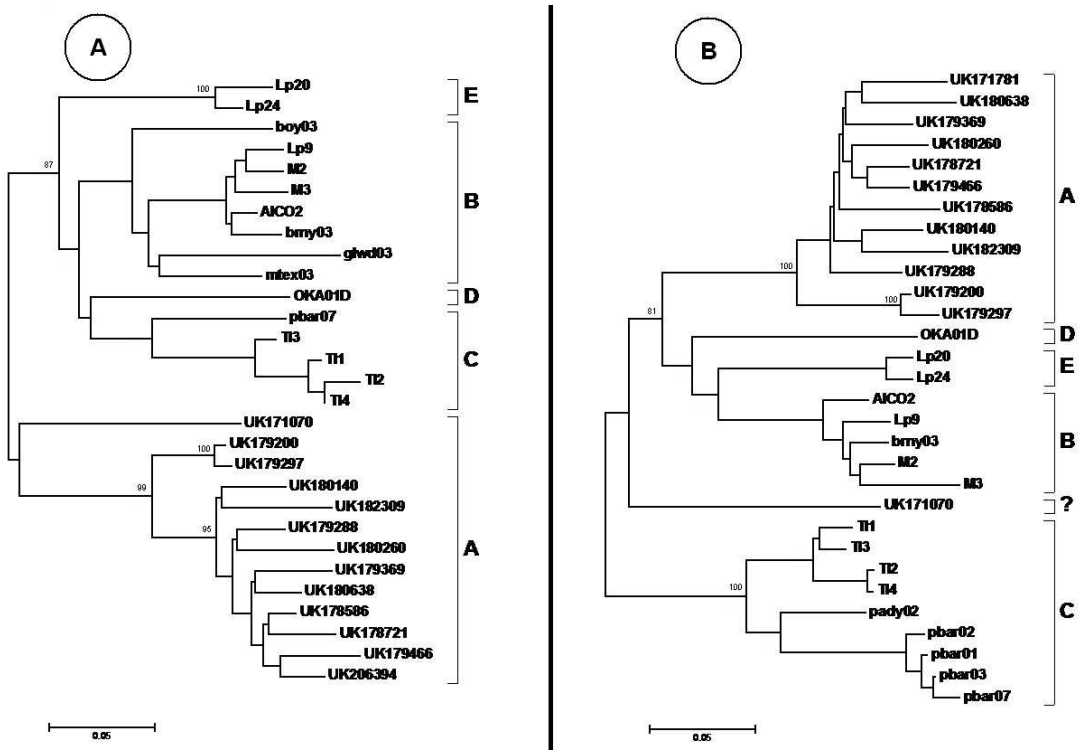


Figure 4.6. Recombination analysis of the A/C recombinant 171070. Minimum evolutionary trees based on the nucleotide sequence of (A) 5' terminus to putative breakpoint sequence (B) breakpoint to the end of the sequence. Bootstrap frequencies are shown at the main nodes.

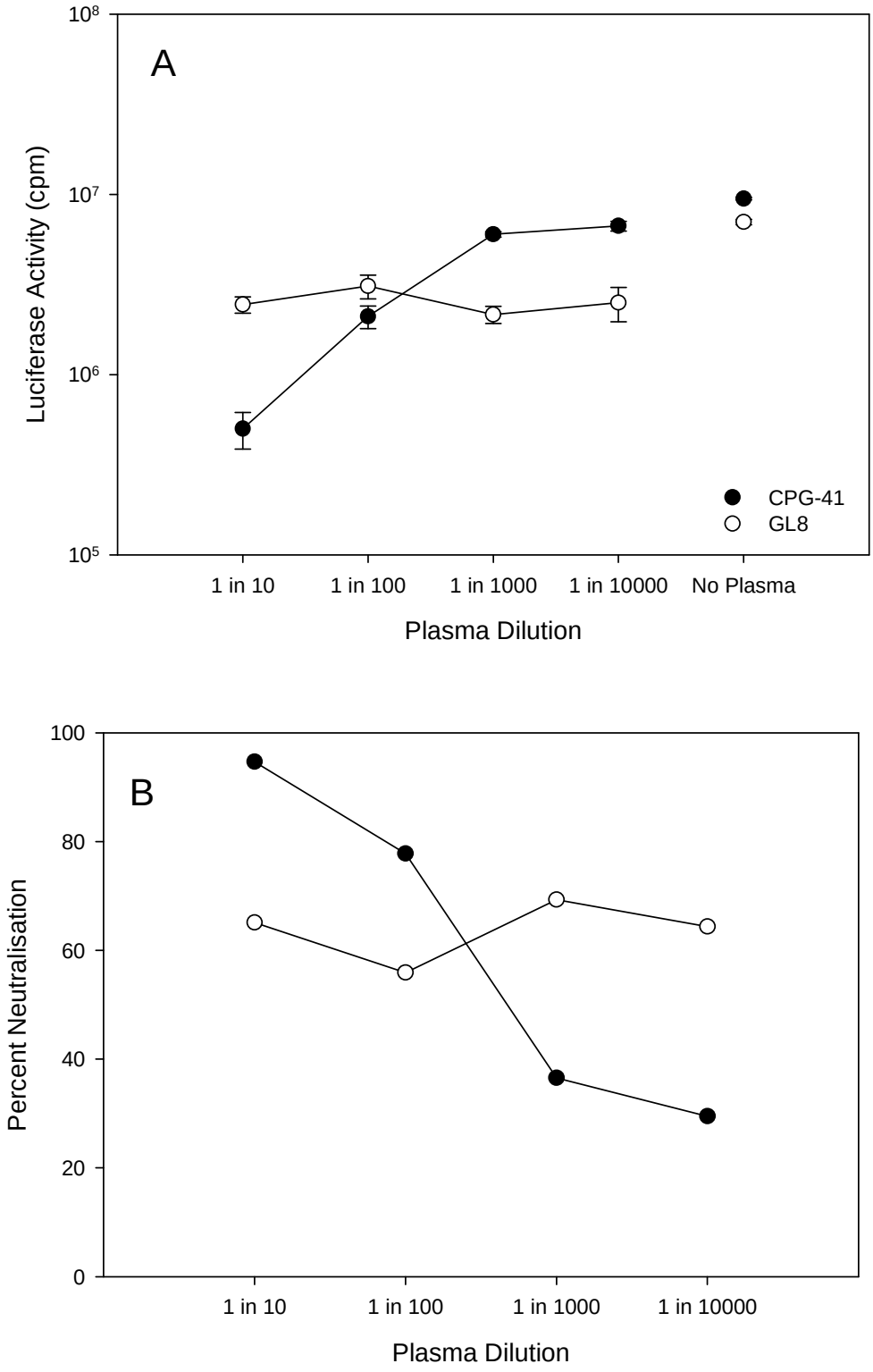


Figure 4.7. Neutralisation profile of plasma 171070 against GL8 and CPG-41 pseudotypes. A.) Luciferase activity (counts per minute) with successive dilutions of TOT1 serum (dilution factor 1/10, 1/100, 1/1000 and 1/10,000, no serum=∞). B.) Percent neutralisation relative to no serum control. Each point represents the mean (n=3) +/- SEM.

4.4 Discussion

Nucleotide sequences of the V3-V5 region of the *env* gene have been used extensively in the genetic subtyping of FIV strains as well as in molecular epidemiology studies. Although there is general agreement that the FIV isolates circulating within the UK are assigned exclusively to subtype A (Hosie *et al.*, 2009), this is the first phylogenetic study to have been performed in the UK. The results of the current study, based on the sequence of 45 naturally occurring isolates of FIV from across the UK, confirm that subtype A predominates. However, isolate 171070, was identified as an A/C recombinant. The cat from which 171070 was isolated was a feral cat that had been adopted in a suburb near a sea port, raising the possibility that the cat had originated from, and perhaps become infected, outside the UK.

It is possible that recombination arose via PCR-mediated recombination. However, this highly unlikely in this case, since PCR-mediated recombination occurs in heterogeneous genetic populations and in this study all the UK sequences appear to belong to subtype A. Furthermore, the high fidelity DNA polymerase used to amplify the *env* gene has a low error rate and overcomes many of the limitations of Taq polymerase (Gilje *et al.*, 2008).

Hence it appears likely that this is the first isolation of a subtype C isolate of FIV from the UK or Western Europe. Reviewing the database containing FIV sequences from Eastern Europe confirmed that no sequences had been recorded from this region. The finding that plasma 171070 cross-neutralised CPG-41, a subtype C isolate, but none of the subtype A isolates tested may be highly significant, suggesting that 171070 Env contains a cross-neutralisation determinant in a location beyond the putative breakpoint, i.e. within the V4 or V5 regions.

The V3-V5 region of Env has been shown to play a significant role in viral neutralisation for both FIV and HIV-1 (Lombardi *et al.*, 1993; Draenert *et al.*, 2006; Bendinelli *et al.*, 2001). The neutralisation resistance by 179200 to the cross-neutralising plasma due to a mutation at the start of V4 loop confirms that the V3-V5 region (see chapter six), which has been examined in FIV phylogenetic studies, is a vital target for neutralising antibodies. Furthermore, the ability of 171070 plasma to cross-neutralise a subtype C FIV is consistent with the

existence of a neutralisation determinant in the V4 loop or further downstream, since its apparently recombinant homologous strain shares sequence homology with subtype C isolates between the V4 region and the 3' end of the *env* gene. Therefore phylogenetic subtyping based on the V3-V5 region of *env* are likely to be important for vaccine development.

In conclusion, this study has demonstrated that FIV subtype A, if not exclusively the circulating subtype, predominates in the UK. Further characterisation of the genetic diversity of FIV investigating a greater number of isolates may provide more information about whether subtype A is the only subtype circulating within the UK, since an A/C recombinant isolate has been recognised, albeit from a cat of unknown origin.

5 Neutralisation of FIV by antibodies targeting the V5 Loop of Env

5.1 Introduction

The induction of an effective humoral response against lentiviral infection is a key element in the immunological control of the disease and the primary target for neutralising antibodies is the viral envelope glycoprotein (Env) (Binley *et al.*, 2004; Wei *et al.*, 2003; Frost *et al.*, 2005). The FIV Env varies by up to 30% amongst the FIV subtypes (Hosie & Beatty, 2007) and thus the preparation of an immunogen capable of inducing broadly neutralising antibody responses against such diverse isolates of FIV would be of great value to the development of an FIV vaccine. Further, the development of a vaccine against FIV would have implications extending beyond veterinary medicine; FIV is the only non-primate lentivirus which induces AIDS-like symptoms in its natural host and as such is a valuable animal model for both prophylactic and therapeutic studies for HIV (Okada *et al.*, 1994; Elder *et al.*, 1998; Bendinelli *et al.*, 1995). Moreover, cats have the advantage of being easier to breed and have shorter life cycles than other animal models currently used for HIV research (Miller *et al.*, 2000; Desmaris *et al.*, 2005).

Previous studies showed that vaccination with whole-inactivated vaccines or DNA vaccines can protect cats against challenge with either low virulence/ homologous strains of FIV, but not against virulent/heterologous primary strains (Hosie *et al.*, 1995). A vaccine capable of protecting cats against infection with a wide range of virulent primary isolates remains a challenge, primarily because of the limited number of conserved neutralisation epitopes that have been identified and the failure to present such epitopes appropriately in vaccines. Steric factors may render neutralisation epitopes inaccessible to neutralising antibodies (NAbs) (Chiarantini *et al.*, 1998; Labrijn *et al.*, 2003) while most of the antibodies generated by vaccination may be directed against non-neutralising epitopes (Dhillon *et al.*, 2007; Richman *et al.*, 2003). Accordingly, the identification of conserved determinants for neutralisation will be an important advance in the design of the next generation of FIV vaccines.

Understanding the biological basis for the induction of antibodies with broad neutralising activity is pivotal to the development of efficacious lentiviral vaccines and to the generation of vaccines that will protect against a broad range of primary isolates. Encouraging results have indicated that it is possible to protect cats against challenge with primary FIV isolates depending on their regional distribution. By vaccinating cats with a whole inactivated vaccine based on the subtype B Pisa M2 strain, cats were protected from natural challenge with subtype B isolates of FIV (Pistello *et al.*, 1997). It has been suggested that subtype B isolates may be more ancient and accordingly more host-adapted (Bachmann *et al.*, 1997), hence they may be more readily neutralisable by the host humoral response. In contrast, while subtype A viruses display similar levels of non-synonymous mutations, such viruses display half as many synonymous site mutations, suggesting a more recent spread and lower level of host-virus adaptation (Bachmann *et al.*, 1997). As the major FIV subtypes are restricted in their global distribution, it has been proposed that it may be necessary to design several regional vaccines rather than a single worldwide protective vaccine (Steinrigl & Klein, 2003).

In this study, we investigated the observation that a subtype B strain resisted neutralisation by its homologous serum. By applying targeted mutagenesis across the *env* gene, we identified the V5 loop as the primary determinant of escape from neutralisation and localised the primary determinant to a lysine-threonine (K-T) motif. Additional subtype B strains bearing the same motif were also sensitive to neutralisation. Furthermore, incorporation of these residues into a neutralisation-resistant subtype B strain rendered the virus more sensitive to neutralisation, confirming the mono-specificity of the neutralising serum. These data indicate that the functional neutralising response in natural infection with FIV may be highly specific and that immune evasion *in vivo* may stem from the mutation of as few as two amino acids in the V5 loop.

5.2 Methods

5.2.1 Blood samples and collection of peripheral blood mononuclear cells (PBMC) and sera

All blood samples were collected from five naturally-infected cats from three different regions of Japan (isolate NG4 originated in Isikawa, KNG1 and KNG2 from Kanagawa and TOI1 and TOT1 from Tokyo). Four of the cats, KNG1, NG4, TOT1, and TOI1 were apparently healthy and displaying no clinical signs (analogous to the asymptomatic stage of HIV infection) whereas one cat, KNG2, presented with severe gingivostomatitis (analogous to the symptomatic stage of HIV infection). PBMC were fractionated from 5 ml of heparinised whole blood by centrifugation over Ficoll-Paque density separation medium (GE Healthcare, Little Chalfont, U.K.). Sera were aliquoted and stored at -80ºC prior to use in neutralisation assays.

5.3 Results

5.3.1 Phylogenetic analysis

An unrooted neighbour-joining phylogenetic tree was generated based on 651 nucleotides spanning the *env* V3-V5 sequence from the five isolates included in the study, as well as reference sequences from subtypes A, B, C, D and E. The phylogenetic tree (Figure 5.1) demonstrated that the five Japanese isolates were genetically related and clustered within subtype B with a bootstrapping support of 100%. KNG1 and TOT1 were highly similar at the nucleotide level and indeed had identical V3-V5 amino acid sequences (100% identity) and clustered together on a single tree branch (Figure 5.1).

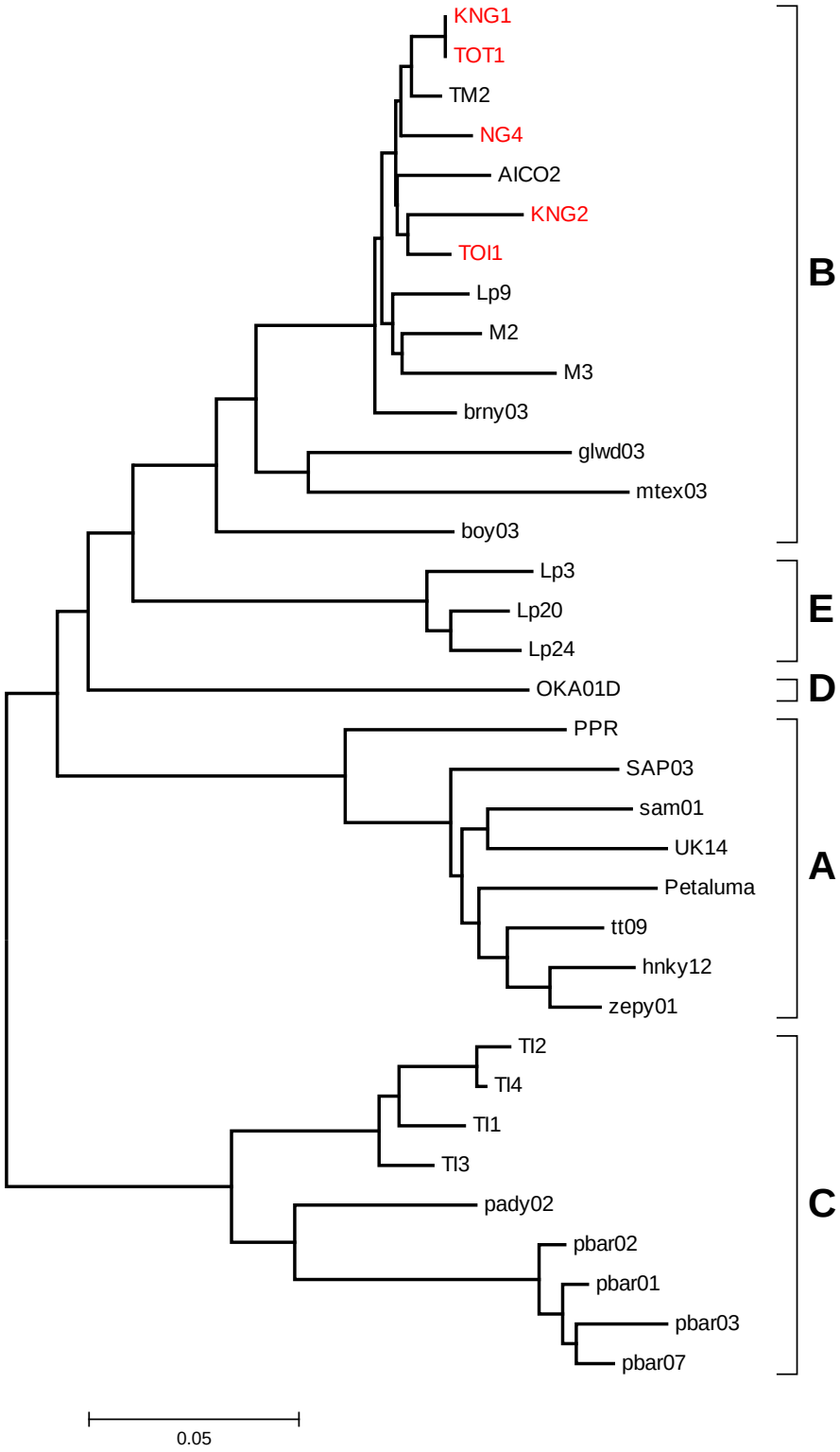


Figure 5.1. Midpoint-rooted neighbour-joining tree of the FIV V3-V5 *env* gene sequence. The tree shows the five Japanese sequences included in the study (in red typeface) as well as reference sequences from subtypes A, B, C, D, and E.

5.3.2 Cats show different NAb responses to homologous and heterologous strains

Sera from cats KNG1 and TOI1 showed no evidence of neutralising activity against any of the pseudotypes tested (except moderate neutralisation of KNG2) including those bearing their homologous Envs and serum from cat KNG2 showed no neutralising activity against the KNG1, NG4 or TOT1 pseudotypes, moderate neutralising activity against the KNG2 pseudotype and weak neutralising activity against the TOI1 pseudotype (Table 5.1). In contrast, sera from cats NG4 and TOT1 showed strong neutralising activity against pseudotypes expressing Envs of the heterologous KNG1, TOT1 and TOI1 strains, and moderate responses against pseudotypes expressing the Env of strain KNG2. The pseudotype expressing the NG4 Env resisted neutralisation by four heterologous sera and showed only weak neutralising activity against its homologous serum (Table 5.1). Although KNG1 and TOT1 shared similar *env* sequences (97% similarity), they did not induce similar humoral immune responses in their respective hosts.

Pseudotype	Serum				
	KNG1	**KNG2**	**NG4**	**TOT1**	**TOI1**
KNG1	-7	-12	86	99	-78
KNG2	67	71	73	64	61
NG4	4	25	46	-31	-15
TOT1	-31	-48	80	99	-57
TOI1	15	41	98	96	-8

Table 5.1. Percent neutralisation values (at 1:10 serum dilution) for five HIV(FIV) pseudotyped viruses tested against homologous and heterologous sera for neutralisation, neutralising activity is categorised as weak (40-59%, highlighted in green), moderate (60-79%, highlighted in yellow) or strong (80-100%, highlighted in orange). Negative values indicate *in vitro* enhancement of infection and are not significant.

5.3.3 Escape from homologous neutralisation by NG4

NG4 was only weakly or resisted neutralisation by the two cross-neutralising sera NG4 and TOT1 respectively. The finding that NG-4 was only weakly neutralised by its homologous serum, a serum that cross-neutralised the other four viruses, may have indicated the evolution *in vivo* of a neutralisation escape mutant that, at the time of sampling, was the dominant species in the periphery since five independent Env clones for each virus were sequenced, all of which gave rise to identical V5 amino acid sequences (data not shown). However, as blood samples from cat NG4 at earlier time points in infection were not available, it was not possible to identify earlier strains which would most likely have contributed to the generation of the NAbs. However, given the similarity between the five subtype B isolates, sites of amino acid divergence from the consensus sequence that were identified in NG4 would be likely to contribute to the relative resistance of this strain to neutralisation by its homologous serum (Figure 5.2). Accordingly, these non-synonymous residues were targeted by site-directed mutagenesis in an attempt to render NG4 more sensitive to neutralisation by its homologous serum. Initially, Env was mutated at the V3 loop, a known target for neutralising antibody (Osborne *et al.*, 1994; Lombardi *et al.*, 1993). A glutamine residue (377) was substituted with a histidine (Q377H), resulting in a net increase in the charge of the V3 loop. However, the Q377H mutation did not render NG4 more sensitive to neutralisation (Figure 5.3). Next, the NG4 Env was mutated at the crown of the predicted V5 loop (Figure 5.2), targeting serine residue 550 (S550N), creating a potential site for N-linked glycosylation. When tested against its homologous serum, the S550N mutant did not show enhanced sensitivity to neutralisation (Figure 5.3, WT = 46% neutralisation at 1 in 10 dilution; S550N = 38% at 1 in 10 dilution), indicating that the absence of asparagine 550 alone, and ablation of the predicted site for N-linked glycosylation did not contribute to the escape from neutralisation by homologous serum. The NG4 Env was then mutated within the V5 loop with the insertion of two amino-acids, lysine and threonine, at positions 556 and 557 (S556(KT)557K). Interestingly, this mutation rendered NG4 sensitive to neutralisation, with strong neutralising activity (98%) observed at 1:10 dilution (Figure 5.3A,B). Moreover, the S556(KT)557K insertion also rendered NG-4 sensitive to the broadly neutralising TOT1 serum (Figure 5.3C,D) indicating that despite the independent origins of the two viruses, both cats responded to infection by targeting V5.

Next, we asked whether the increase in polarity afforded by the inserted lysine residue contributed to the formation of the neutralising determinant. An S556(K)557K mutant was prepared in which only a lysine residue was inserted. The NG4 S556(K)557K mutant was tested against NG4 serum and showed an intermediate pattern of neutralisation (Figure 5.3) indicating that while the lysine residue was clearly important, the additional threonine residue enhanced the formation of the neutralising determinant. These data emphasise the dominance of V5 in the neutralisation of FIV and are consistent with a hypothesis whereby the broadly neutralising NG4 serum selected for mutant viruses in the host animal bearing a deletion in V5. Fold neutralisation was calculated based on the mean (n=3) of the luciferase activity of no plasma control relative to that for each plasma dilution. Fold neutralisation was used to clarify the image when relatively strong neutralisation is obtained.

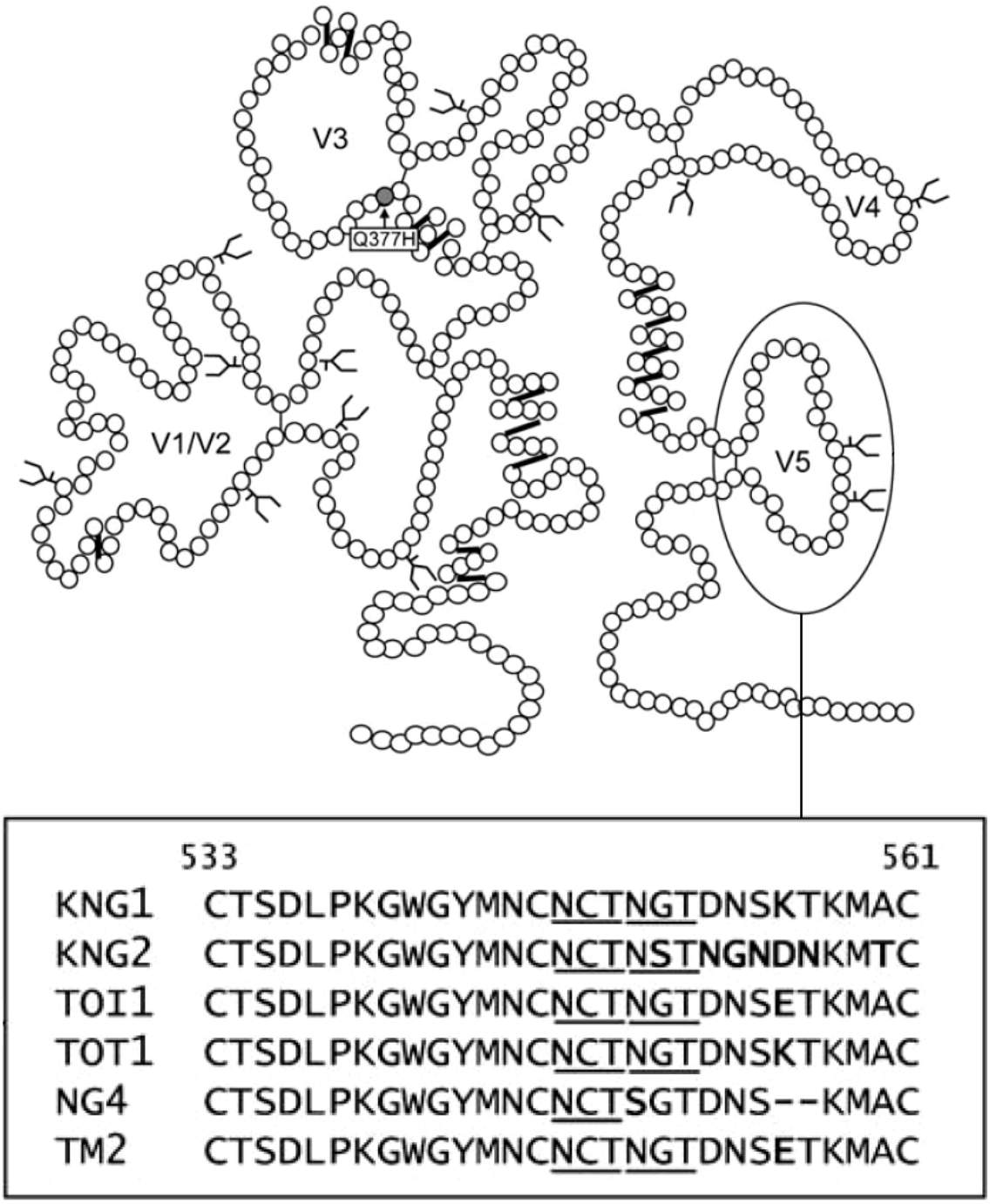
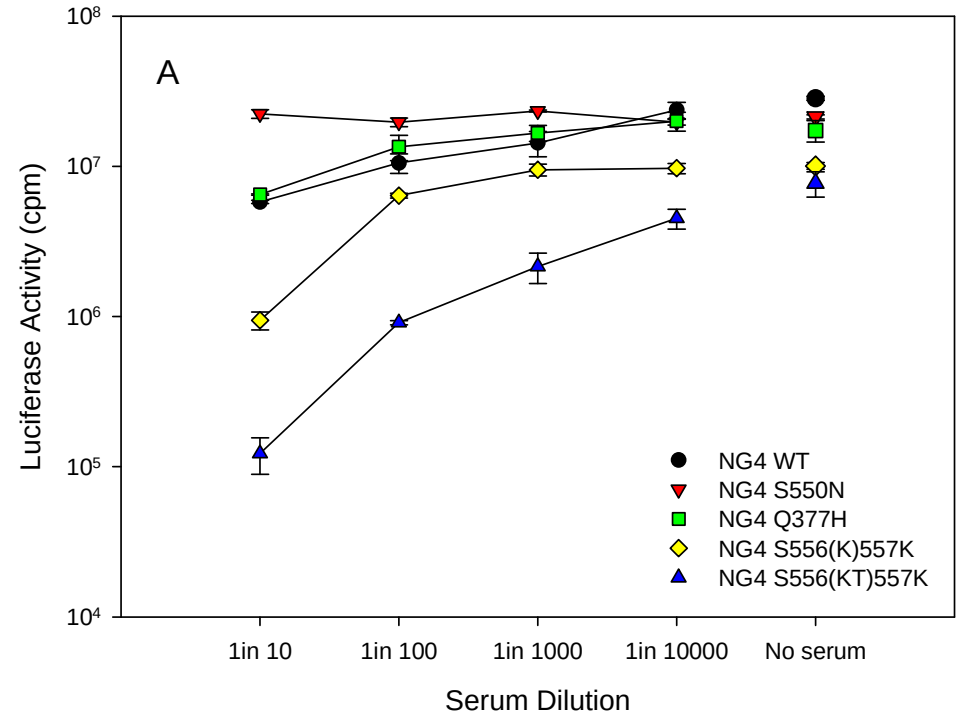
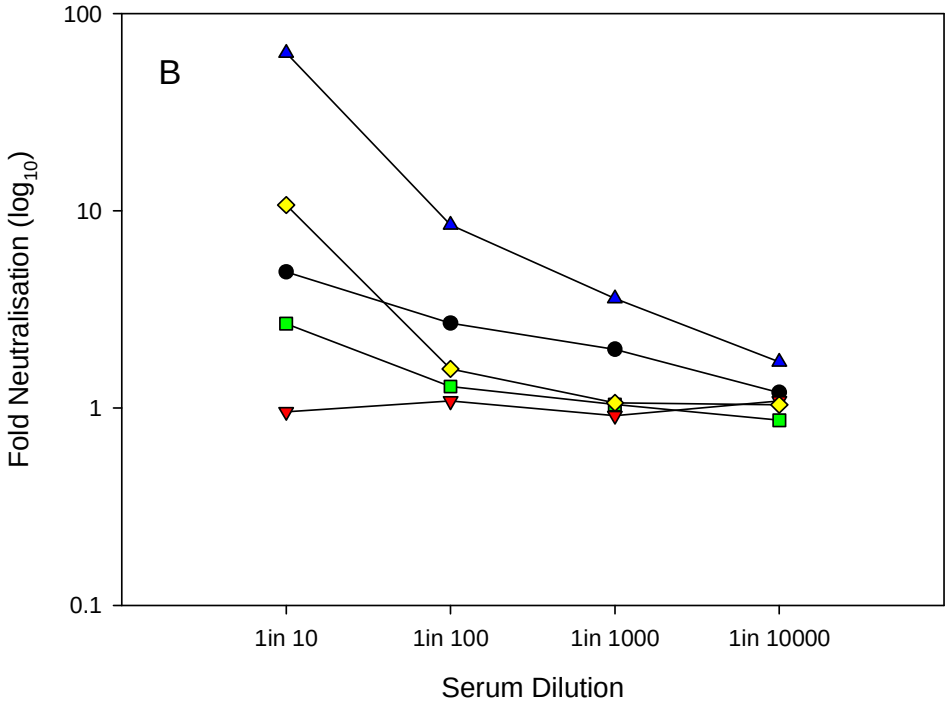


Figure 5.2. Schematic structural model of the FIV SU protein illustrating the location of the Q377H mutation in V3, and the V5 loop. Inset displays a comparison of the sequence from the five study strains KNG1, KNG2, TOI1, TOT1 and NG4, and the reference strain TM2 (Genbank: M59418).


A
Luciferase Activity (cpm)
10^8
10^7
10^6
10^5
10^4
1in 10
1in 100
1in 1000
1in 10000
No serum
Serum Dilution
NG4 WT
NG4 S550N
NG4 Q377H
NG4 S556(K)557K
NG4 S556(KT)557K


B
Fold Neutralisation (log_{10})
100
10
1
0.1
1in 10
1in 100
1in 1000
1in 10000
Serum Dilution

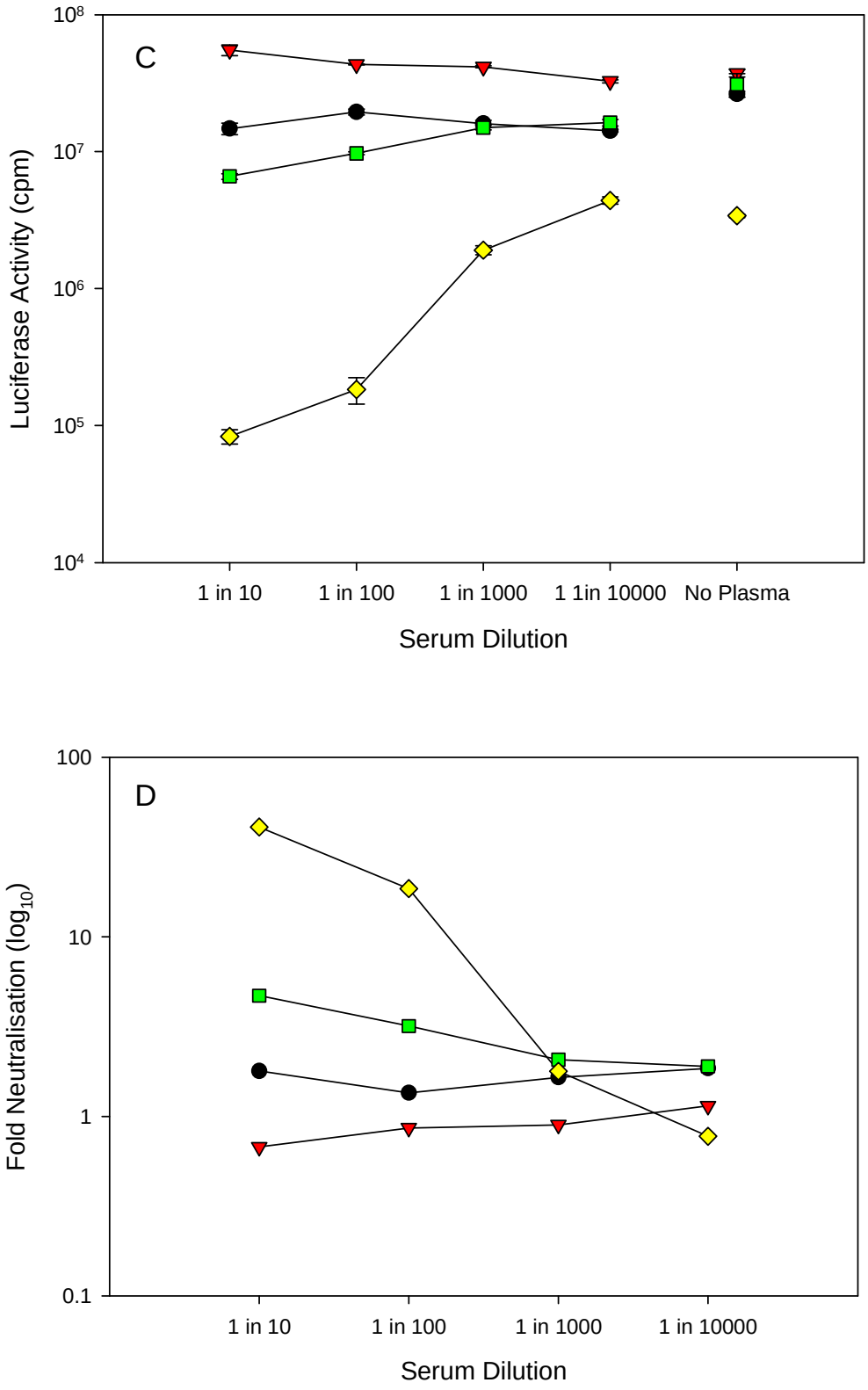


Figure 5.3. Neutralisation of wild type (WT) NG4 and NG4 mutants by homologous serum (A,B) and by TOT1 serum (C,D). A,C. Luciferase activity (counts per minute) with successive dilutions of NG4 serum (dilution factor 1/10,1/100, 1/1000 and 1/10,000, no serum=∞). B,D. Fold neutralisation relative to no serum control. Each point represents the mean (n=3) +/- SEM.

5.3.4 Neutralisation sensitivity of KNG2

Of the five isolates studied, KNG2 was the most divergent at V5. In comparison with the other strains, KNG2 was moderately sensitive to neutralisation by all the sera tested, including the NG4 and TOT1 sera that showed strong neutralising activity against KNG1, TOT1 and TOI1. Based on our finding that NG4 could be rendered sensitive to neutralisation by a single change in the amino acid sequence of the V5 loop, we asked whether the partial resistance of KNG2 was conferred by its divergent V5 loop. Firstly, a KNG2 mutant was prepared bearing a DN556KT mutation targeting the same residues shown earlier to confer neutralisation sensitivity upon NG4. The DN556KT mutation did not display improved sensitivity to neutralisation relative to the wild type. Since the sequence of KNG2 diverged from the consensus at 7 amino acids (Figure 5.5), we considered that the context of the mutation may have been important for formation of the epitope. Accordingly, the V5 loop was mutated in stages towards identity with the (highly sensitive) KNG1 and TOT1 sequences; initially a 5 amino acid change was created (553NGNDN558→553DNSDT558) followed by the full mutation (551STNGNDNKMT558→551GTDNSKTKMA558). Both KNG2 mutants were then tested for sensitivity to neutralisation by the TOT1 serum. The 551STNGNDNKMT558→551GTDNSKTKMA558 variant was neutralised efficiently by the TOT1 serum while the 553NGNDN558→553DNSDT558 mutant showed an intermediate sensitivity (Figure 5.4). The conversion of KNG2 from neutralisation-resistant to neutralisation-sensitive by changing the sequence of V5 to a sequence similar to the neutralisation-sensitive KNG1 and TOT1 strains is consistent with V5 being a primary determinant of neutralisation sensitivity and the specificity of the neutralising sera.

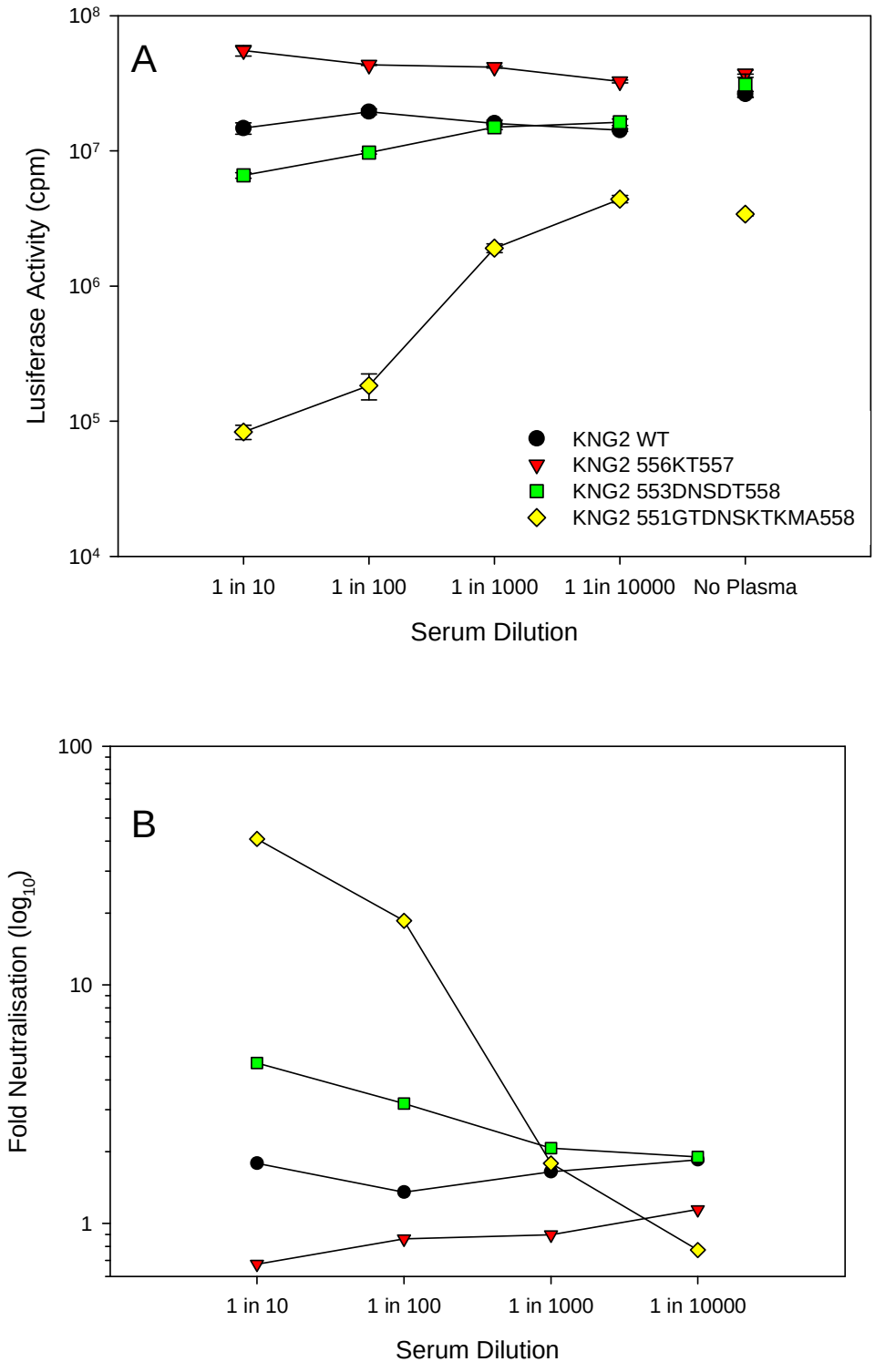


Figure 5. 4. Conferral of neutralisation sensitivity to KNG2 by V5-loop transfer. A.) Luciferase activity (counts per minute) with successive dilutions of TOT1 serum (dilution factor 1/10, 1/100, 1/1000 and 1/10,000). Each point represents the mean (n=3) +/- SEM. B.) Fold neutralisation relative to no serum control.

The results of this study indicate the importance of V5 to antibody-mediated neutralisation of FIV. While anti-V5 antibodies can neutralise primary isolates of FIV very efficiently, the virus appears able to escape neutralisation readily by mutating V4. What then are the prospects for designing an FIV vaccine that induces broadly neutralising antibodies targeting V5? A comparison of published V5 sequences (Figure 5.5) would suggest that the V5 region has highly conserved structural features such as the cysteine residues at 533, 546, 548 and 561 (numbering as in KNG1&2, TOI1 and TOT1, Figure 5.5). However, the region between cysteine residues 548 and 561 appears to be remarkably flexible in its ability to tolerate amino acid substitutions, glycosylation (potentially O and N-linked) and length polymorphisms, suggesting that it is under pressure to vary from the host immune response. The relative conservation of the region between cysteines 533 and 548 may indicate that this region is either inaccessible or structurally constrained, perhaps through interactions with adjacent regions of Env. If possible, targeting the humoral response to this region may induce more broadly neutralising antibodies.

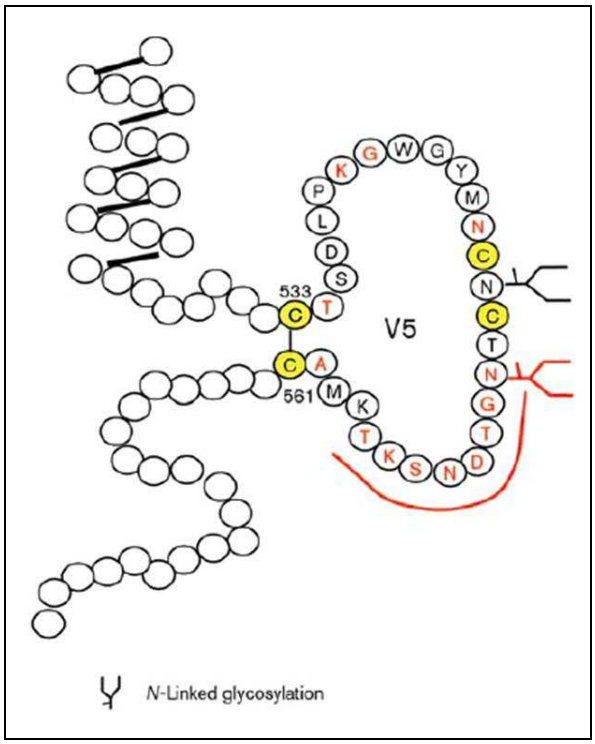


Figure 5.5. Amino acid alignment of the V5 region from diverse FIVs. Within the framework of the predicted loop bounded by cysteines 533 and 561 (numbering as in KNG1&2,TOI1 and TOT1), extensive variation is evident, the majority of which resides between cysteine residues 548 and 561.

5.4 Discussion

In this chapter, it was demonstrated that the V5 loop of FIV Env is a primary target for virus neutralising antibodies. Although previous studies *in vitro* indicated that the V3 loop was a major determinant for virus neutralisation, subsequent studies revealed that the significance of antibodies targeting this region to the neutralising response had been over-emphasised by the cellular substrate used to quantify neutralisation. Formerly, assays were based on inhibiting infection of CrFK cells with "CrFK-adapted" strains of virus. Prior to measuring neutralisation in this system, the challenge virus had to be selected for growth in CrFK cells, thereby selecting for viruses that were capable of CD134-independent infection through a direct interaction with CXCR4 (analogous to CD4-independent infection with HIV). The V3 loop plays a critical role in the FIV Env-CXCR4 interaction; accordingly, CrFK-based assays exaggerated the importance of neutralising antibodies binding this region. Subsequent studies revealed that when primary (non-CrFK-adapted) isolates were assayed on IL2-dependent T cells, the correlation between the efficiency of virus neutralisation and the response to V3 was diminished. This study extended previous studies that indicated that in biologically relevant *in vitro* systems, the variable loops V4 and V5 appear to be the major targets for effective neutralising antibodies. Moreover, it was found that in polyclonal sera from infected cats, deletion of as few as two amino acids from V5 converted a virus from a strongly neutralised to a weakly neutralised or neutralisation resistant phenotype. Each of the isolates described in the current study were confirmed as CD134-dependent, by plating the viral pseudotypes on target cells expressing CD134. No evidence of CD134-independent infection via CXCR4 alone was detected (data not shown), confirming that the isolates were primary and not cell-culture adapted.

Amongst the variable regions in FIV Env, V5 appears to be highly polymorphic between strains, with variations in amino acid sequence, glycosylation, and extended stretches of serine and threonine residues being common. These data suggest that there is a selective pressure within the host for variation in V5, consistent with escape from an effective immune response. That minimal variation in V5 is sufficient to either reduce substantially or ablate completely the neutralising capacity of the host humoral response is of concern for the design of effective FIV vaccines. How then are we to elicit broadly neutralising

antibodies *in vivo*? Clearly, the V4 and V5 loops are exposed and immunogenic and antibodies targeting these regions neutralise virus. However, it would appear that these regions may represent little more than a decoy for the virus, presenting the immune system with an easy target for neutralising antibodies. By the time functional neutralising antibodies develop, viral variants that can escape the mono-specific serum may have evolved already *in vivo*. The solution to this dilemma would appear to lie in the identification of framework determinants in Env that induce antibodies that are broadly active, refocusing the immune response by either silencing the immunodominant (but ultimately ineffectual) determinants in V4 and V5, or by presenting them in a such a way as to induce antibodies that recognise structures or motifs that are difficult or impossible for the virus to vary.

Previous studies have demonstrated the importance of V5 in virus neutralisation (Pistello *et al.*, 2003b; Siebelink *et al.*, 1995a). V5 was mapped using synthetic peptides for a possible linear epitope based on the sequence of the 19k1-560 strain (Siebelink *et al.*, 1995a), and demonstrated that a single mutation at position 560 may be involved concurrently with another mutation at 483 (V4 loop) in one or more conformational epitopes; but no linear epitope was mapped in V5. Further, two independent mutations have been reported that resulted in the creation of potential N-linked glycosylation sites in V4 (K481N) and V5 (S557N) and which contributed to the conversion from a neutralisation sensitive to neutralisation resistant phenotype (Giannecchini *et al.*, 2001b; Pistello *et al.*, 2003b). In comparison, the conversion from a weakly neutralised or neutralisation resistant to a strongly neutralised phenotype described herein was mediated by mutation of V5 alone. The V5 loop of FIV Env is highly variable among isolates. Variation occurs not only within the amino acid sequence but also in the length of the central region (up to 14 residues) towards the end of the loop formed by disulfide bond linkage, indicating that this particular region may play a significant role in viral evolution and escape from neutralisation. It is notable that the length polymorphisms in this region are attributable to a reiteration of codons encoding serine and threonine residues. Threonine and serine have hydroxyl side chains and may, potentially, undergo O-linked glycosylation. It is generally believed that due to their small molecular size, O-linked oligosaccharides have only minor effects on the formation of the glycan shield on HIV Env compared to N-linked oligosaccharides. However, these

oligosaccharides usually have diverse structures with no common carbohydrate core and it is difficult to predict how efficient they may be in shielding neutralisation epitopes, especially when there are several adjacent molecules (Bernstein *et al.*, 1994). Moreover, X-ray crystallography studies on HIV-1 gp120 showed that glycosylation may have significant effects on the conformational stability of Env as well as an indirect effect on more distant sequences along the secondary structure. This in turn would affect the interaction between the epitope and neutralising antibodies, rendering epitopes less accessible for antibody binding (Huang *et al.*, 1997).

In conclusion, we identified a neutralisation determinant in the FIV V5 loop which is associated with the conversion of strongly neutralised to weakly neutralised or neutralisation-resistant strains. The determinant was apparently linear and involved at least 10 amino acids (GTDNSKTKMA) within the V5 region, a highly variable region amongst FIV strains. The identification of this neutralisation determinant will inform vaccine design in the future in order to induce more potent neutralising antibody responses.

6 Identification of neutralising antibodies in clinical samples

6.1 Introduction

There are many similarities between the clinical manifestations and progression of disease with FIV and HIV infections; following the acute phase of infection, which lasts a few weeks, infected subjects enter the asymptomatic phase. During this phase virus replication is minimal in PBMCs and the immune response develops and matures (Stebbing *et al.*, 2004; English *et al.*, 1994). Both humoral and cell mediated immune responses have roles in restraining the virus at this stage of infection.

It has been documented that, several months after sero-conversion, HIV infected individuals develop NAbs effective against the homologous challenge strains, commonly named autologous NAbs (aNAbs) (Sather *et al.*, 2009; Frost *et al.*, 2005). An inverse correlation between the presence of NAbs and disease manifestation has been well documented in HIV-1 infection, where progression of infection to clinical AIDS is dependent on viral evasion of antibody-mediated neutralisation (Kwong *et al.*, 2002). Moreover, sera from long term non-progressors (LTNPs) contain high titres of NAbs and more frequently develop stronger neutralising responses compared to short term progressors (Cao *et al.*, 1996), highlighting the protective role of humoral immunity.

The continuous emergence of new viral variants in response to immune pressure reduces the efficacy of NAbs (Mahalanabis *et al.*, 2009; Bunnik *et al.*, 2008). Although it is an additive challenge for the immune system to respond to a highly heterogeneic population or viral quasispecies, such viral heterogeneity broadens the scope of NAb recognition such that even heterologous strains may be neutralised in some LTNPs (Simek *et al.*, 2009). Factors involved in the generation of broad NAbs have not yet been well defined but may include the virulence of the infecting strain and the initial viral load that is established, quasispecies complexity, the status of the host immune system, as well as other factors (Balfe *et al.*, 2004; Krachmarov *et al.*, 2006).

A small proportion of sera from HIV-infected individuals may contain cross-neutralising antibodies, usually after two years of sero-conversion (Kraft *et al.*,

2007; Moore & Trkola, 1997). Broad NAb responses are well-developed in LTNPs, whereas NAbs titres are usually low in rapid disease progressors (Pilgrim *et al.*, 1997; Carotenuto *et al.*, 1998). It is therefore reasoned that NAb impede disease progression, and that a decline in HIV-specific antibody responses often predicts a poor prognosis (Cecilia *et al.*, 1999). For FIV, little is known about the ability of infected cats to either mount a NAb response or develop NAbs that may cross-neutralise either related or unrelated primary strains of FIV.

The variable (V) loops of Env play a key role in viral evasion from NAbs, shielding conserved epitopes that would be targets for neutralisation (Etemad-Moghadam *et al.*, 1999). For example, the V1/V2 region in HIV-1 occludes important conserved epitopes such as the co-receptor binding site (Krachmarov *et al.*, 2005; Pinter *et al.*, 2004). The V3 loop of HIV-1 is considered to be a principal neutralising determinant (Moore & Trkola, 1997) and is involved in co-receptor binding and determines cell tropism (Sundstrom *et al.*, 2008; Cormier *et al.*, 2001; Hoffman & Doms, 1999). The role of the V4 and V5 loops in neutralisation resistance is unclear but it is thought that these regions impact upon Env conformation and glycan packing (Wei *et al.*, 2003). For both FIV and HIV-1, mutations in the V4 loop alter the viral neutralisation profiles (Bendinelli *et al.*, 2001; Kinsey *et al.*, 1996).

This chapter describes studies undertaken using the remainders of blood samples collected from cats naturally infected with FIV. Plasma samples were screened for NAbs against a representative primary UK isolate of FIV, GL8. Subsequently, samples which strongly neutralised GL8 were tested against other isolates to assess the breadth of cross-reactivity. Furthermore, viral pseudotypes were constructed bearing the Envs of novel primary isolates and these pseudotypes were tested for susceptibility to neutralisation by homologous plasma as well as the broadly neutralising plasma samples that were identified. It was possible to estimate the proportion of naturally infected cats that had developed NAbs and to assess the cross-reactivity of these NAbs, thereby providing an insight into the ability of the feline immune system to generate effective responses against FIV. As a result of these studies, a neutralisation epitope was identified in the C3-V4 domain

6.2 Methods and Results

6.2.1 Description of the sample population

Plasma samples were collected from 345 FIV sero-positive, naturally infected cats that have been submitted to the the Companion Animals Diagnostic Service at the University of Glasgow. Samples were tested for the presence of antibodies against FIV using an immunofluorescence assay (IFA). Information included on the sample submission forms such as the age, sex, health status, and clinical signs of the cats is presented in Appendix 2.

The cats' ages ranged from 5 months to 18 years but many ages were either unknown or estimated by the cats' veterinarians. Many of the FIV-infected cats were held in cat protection shelters, strays awaiting adoption, or were free roaming. Cats within these groups are at greater risk of FIV infection, according to epidemiological studies (Courchamp & Pontier, 1994). The FIV-infected cats in this study were five times more likely to be male than female, consistent with previous studies which demonstrated that males were at a higher risk of FIV infection due to their more aggressive nature (Natoli *et al.*, 2005). Approximately one third of the cats displayed no clinical signs and were apparently healthy. However, not all cats had been examined thoroughly by veterinarians and so this proportion may have been over estimated. Clinical signs recorded for the sick cats included weight loss, dullness, anorexia, vomiting, inappetance, malaise, lethargy, pyrexia, anaemia, jaundice, keratitis/uveitis, upper respiratory tract (URT) signs, stomatitis/gingivitis, neurological signs and enlarged lymph nodes.

6.2.2 Identification of NAbs against HIV(GL8)

The remainders of 345 samples that tested positive for FIV were screened for NAbs against the HIV(GL8) pseudotype, since GL8 has been shown to be representative of primary FIV strains in the UK (Dunham *et al.*, 2006a). As the aim of the study was to estimate the proportion of cats naturally infected with FIV that develop NAbs, samples were tested subsequently for the ability to cross-neutralise other pseudotypes. Since the plasma samples were collected from clinical cases, the time of FIV infection was unknown and clinical staging based

on the signs recorded on the submission forms was unlikely to be accurate. Since older cats may have been infected for longer, we examined the data collected from 214 cats of known age to determine whether older cats had higher levels of NAbs (Figure 6.1). The neutralisation profiles from these cats ranged from slight enhancement to strong neutralisation, with no relationship evident between age and cross-neutralisation of the GL8 pseudotype. For example, plasma sample 179028 (collected from an 11 year old male cat with clinical signs including chronic gingivitis) only poorly neutralised the GL8 pseudotype (28% neutralisation at a plasma dilution of 1 in 10), whereas plasma sample 171838 (belonging to an 18 month old male cat) was strongly neutralising (90% neutralisation at a plasma dilution of 1 in 10). Furthermore, the latter plasma from the younger cat displayed strong cross-neutralising activity against pseudotypes expressing Envs from a panel of primary isolates (see section 6.2.6).

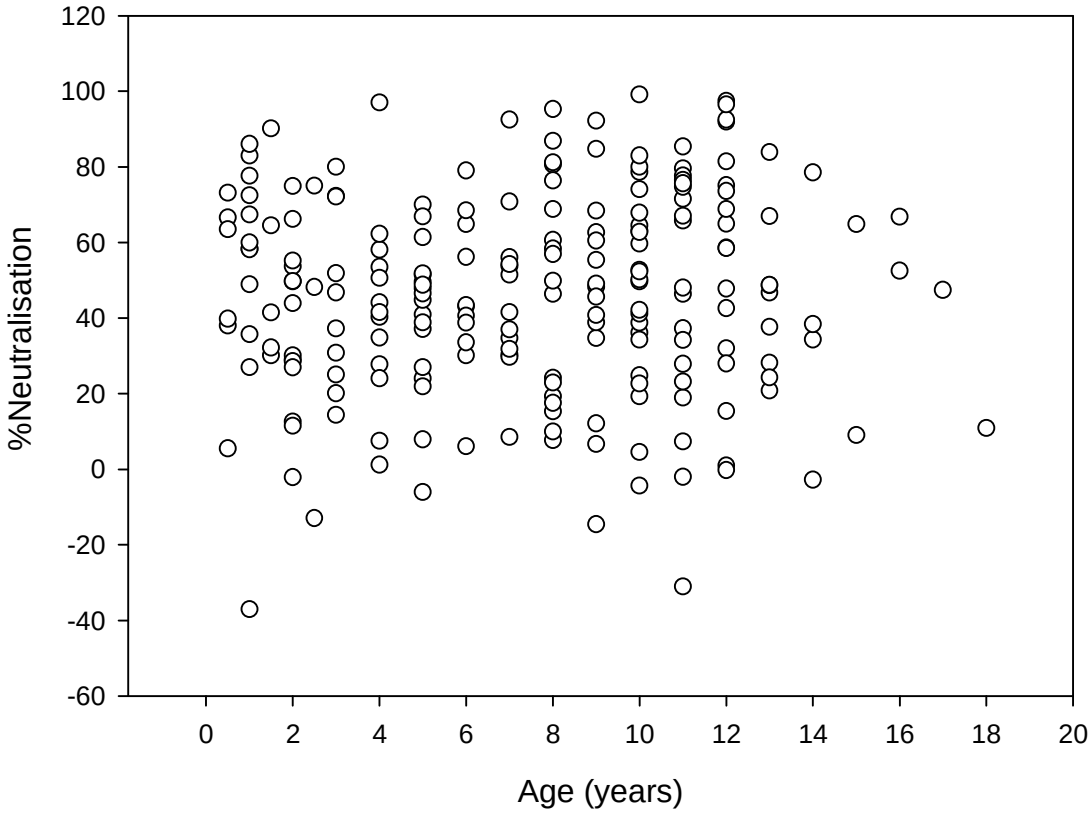


Figure 6.1. The relationship between age and levels of FIV NAb. Plasma samples from cats of known ages were screened against HIV(GL8). Values for % neutralisation (at a 1:10 dilution of plasma) are shown in relation to the cats' ages.

It was observed that 30 of the 345 plasma samples examined (8.7%) strongly (i.e. ≥ 80% at a 1 in 10 dilution) neutralised HIV(GL8). The samples containing NAb were collected from cats aged between 18 months and 14 years (Table 6.1). Hence it was concluded that 8.7% of plasma samples collected from cats naturally infected with FIV contained NAbs against the heterologous primary isolate GL8. It is likely that the proportion of cats with NAbs against their homologous isolates may have been greater but, nevertheless, it appears that, as for HIV-1 infected individuals (Beirnaert *et al.*, 2001), NAbs generated during natural infection are not commonly detected in FIV infected cats.

Number	Age (years)	% Neutralisation	Number	Age (years)	% Neutralisation
169248	7	92	179035	NK	92
169422	9	92	180644	10	85
169630	12	92	180779	NK	84
170141	12	93	180871	1	86
170305	NK	83	181380	8	95
170338	11	85	181516	NK	85
170764	12	81	181650	8	81
170777	13	84	181793	12	97
170962	8	81	203126	NK	99
170973	NK	89	203135	4	97
171806	8	87	203179	NK	98
171838	2	90	205687	10	80
172279	12	97	206394	14	95
172532	NK	100	209971	3	80
178639	10	99	210101	10	83

Table 6.1. Identification of plasma samples that strongly neutralised (≥ 80%) HIV(GL8). Values shown represent % neutralisation at a 1:10 dilution of plasma. Cats' ages are also shown where known; NK: not known.

6.2.3 Identification of samples containing broadly neutralising antibodies

The 30 plasma samples containing NAbs that strongly neutralised GL8 (Table 6.1) were tested next for the ability to neutralise HIV(FIV) pseudotypes bearing Envs from primary isolates such as PPR and CPG-41. Selected samples are shown in Table 6.2. Next, samples displaying broad cross-reactivity were tested also against pseudotypes bearing Envs from a panel of primary isolates. Three samples exhibited broad neutralising antibody activity against most or all of the pseudotypes tested (Table 6.3), representing 10% of the plasma samples containing NAbs. Unfortunately there was insufficient sample available from cat 171838 to test more pseudotypes; this sample contained high levels of broadly NAbs against the majority of pseudotypes tested and is discussed later in this chapter.

Plasma	Pseudotype		
	GL8	PPR	CPG-41
169630 (17)	87	99	86
203135	97	99	82
203179	98	60	ND
203292	95	99	99
209971	80	74	ND
210101	83	100	21

Table 6.2. Screening plasma samples for broad neutralisation. The values shown represent the % neutralisation at a 1 in 10 dilution of plasma. Samples which strongly neutralised GL8 were subsequently tested against the pseudotype bearing the Env of PPR, a subtype A strain isolated in the USA. Samples which strongly or moderately neutralised PPR were further tested against the CPG-41 pseudotype, a subtype C strain isolated in the USA. Values for plasma 169630 (17) are recorded for the sample collected 17 months after the first sample.

ND: not done

Pseudotype	Subtype	Plasma		
		178639	206394	171838
180638	A	99	90	83
171838	A	ND	99	99
180260	A	96	96	97
182309	A	100	100	98
180140	A	90	97	93
178721	A	99	97	99
179200	A	83	97	16
206394	A	83	93	89
KNG2	B	96	99	ND
TM2	B	99	99	ND
GL8	A	99	95	90
B2542	B	99	99	ND
CPG-41	C	96	99	ND
PPR	A	98	99	ND
0425	A	99	96	ND
M2	B	93	100	ND
0827	A	99	95	ND
1419	A	95	96	ND
Leviano	B	ND	100	ND

Table 6.3. The identification of broadly neutralising plasmas. Values of % neutralisation at plasma dilutions of 1:10 are shown. B2542, M2, KNG2, Leviano and TM2 are subtype B strains; PET KKS, GL8, and PPR are subtype A strains; CPG-41 is subtype C strain; and 425, 1419, 827, 556, 180638, 171838, 180260, 180140, 178721, 179200, 206394 and 182309 are primary subtype A strains (also see appendix 4). ND: not done.

6.2.4 The development of NAb with time following natural infection

In order to monitor the development of immunity as infection proceeds, we examined sequential plasma samples collected from naturally infected cats. However, few sequential samples were obtained as a large number of the FIV infected cats had been euthanised following diagnosis. Follow-up samples were obtained from nine FIV infected cats as a part of an undergraduate student project. These samples were collected at intervals of between 10 months and 18 months after the first sample collection and the sample pairs were tested for the presence of NAb against GL8. As shown in Table 6.4, in general the follow-up samples showed either little change in neutralisation (or even decreased

neutralisation) in the later samples. It had been speculated that the NAb response would mature as infection proceeds; however the results did not confirm this hypothesis. For example, although cat 171505 showed an increase in neutralising activity after 15 months (neutralisation increased from 40% to 80% at a 1:10 dilution of plasma), the follow-up sample from cat 172279 showed only moderate neutralisation although the earlier sample had been strongly neutralising (neutralisation decreased from 97% to 61%). Cat 181809 also showed reduced neutralising efficacy in the follow-up sample (neutralisation decreased from 68% to 21%).

The most likely explanation for the reduction in neutralisation observed in follow-up samples from cats 172279 and 181809 is that both cats had progressed to the late stage of infection; the former had chronic stomatitis and laryngeal swelling, and the latter was lethargic, pyrexic, inappetant, and slightly ataxic. Perhaps these two cats were terminally ill and their immune systems were failing at the time of the follow-up sampling. Other samples did not show significant changes in neutralising ability, perhaps because insufficient time had elapsed between samplings to permit the maturation of cross-reactive NAbs. Plasma from cat 169630 displayed a strong NAb response against GL8 at both the first sampling as well as at the follow-up, 17 months later. Furthermore, the follow-up sample showed broader NAb activity, with stronger neutralising activity against CPG-41 (neutralisation increased from -7% to 86%) (data not shown). Therefore, it appeared that, in this cat, the NAb response had matured in both efficacy and breadth during the 17 month period between samplings.

Plasma number	First sample	Follow up	Time interval (months)
169630	92	87	17
172279	97	61	14
169421	43	14	18
170003	48	33	16
172471	30	29	13
169244	15	35	18
181809	68	21	10
170901	79	62	15
171505	40	80	15

Table 6.4. The effect of time on NAb maturation. Values show % neutralisation at a 1:10 dilution for plasma samples tested against HIV(GL8).

6.2.5 Neutralisation of homologous pseudotypes by clinical samples

In order to assess the neutralisation of field isolates by their homologous plasmas, pseudotypes were prepared bearing the Envs of primary field isolates from naturally infected cats. The *env* genes of these primary field isolates were cloned and HIV(FIV) pseudotypes were constructed, using the methods described in Section 2.3.6. Eight viable pseudotypes were prepared, bearing the Envs of 171838, 179200, 178721, 182309, 180638, 180140, 180260, and 206394. As shown in Table 6.5, plasma samples from cats 171838, 182309, and 206394 strongly neutralised (>80% neutralisation at 1:10 dilution of plasma) pseudotypes bearing their homologous Envs, while the remaining plasmas only weakly neutralised or did not neutralise their homologous pseudotypes. While plasma samples from cats 171838 and 206394 contained NAbs that cross neutralised strongly (>90%) the GL8 pseudotype, plasma from cat 182309 only weakly neutralised GL8 (34%).

Plasma	Homologous pseudotype [a]	GL8 pseudotype [b]
206394	93	95
171838	99	90
178721	29	45
179200	38	41
180140	53	63
180260	-30	23
180638	-4	-13
182309	83	34

Table 6.5. The ability of plasma samples from 8 naturally infected cats to neutralise pseudotypes bearing either a.) homologous or b.) GL8 Env. Values shown are % neutralisation at 1:10 plasma dilution.

6.2.6 Characterisation of a broadly neutralising plasma

Plasma collected from the 18 month old cat 171838 contained NAb that showed strong cross-reactivity (>80% neutralisation) against the GL8 pseudotype and 7/8 pseudotypes bearing the Envs of novel primary isolates (Table 6.3 and Figure 6.3). In order to investigate the factors that may have contributed to the induction of this broad NAb response, the sequence of the *env* gene cloned from MYA-1 cells co-cultivated with PBMC from cat 171838 was compared to the proviral *env* sequence amplified directly from PBMCs. It was striking that these two *env* sequences displayed significant differences in amino acid sequence (Figure 6.2), suggesting that perhaps cat 171838 was infected with a diverse FIV quasispecies.

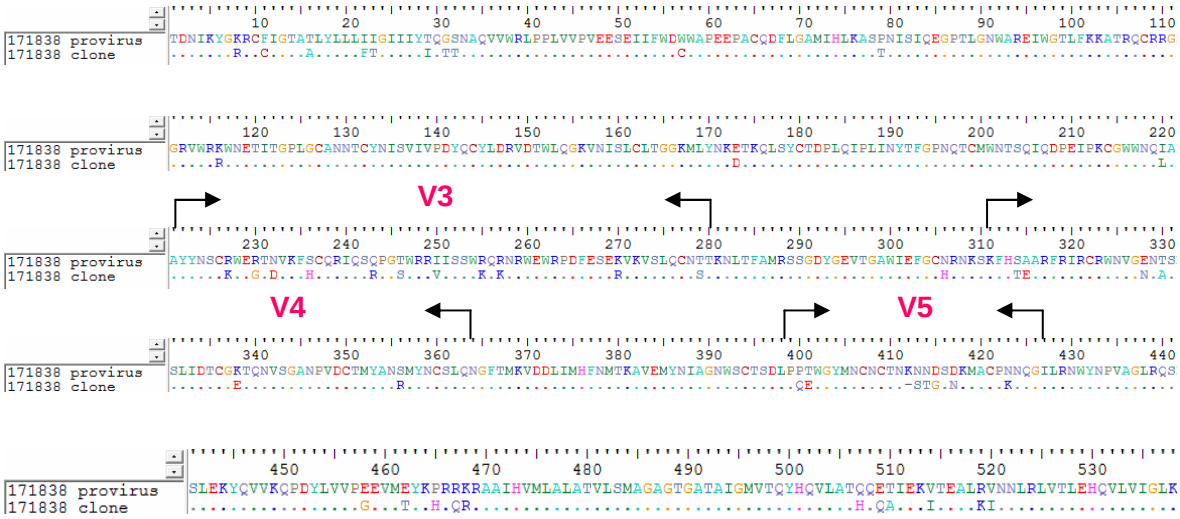


Figure 6.2. Sequence diversity of 171838 Env. Amino acid sequences of Env 171838 cloned from virus recovered from MYA-1 co-cultivated with the cat's own PBMC (171838 clone), compared to the sequence of the provirus cloned directly from PBMC (171838 provirus). The V3, V4 and V5 loop sequences are located between the arrows as shown.

6.2.7 The identification of a neutralising epitope in the C3-V4 region of Env

Plasma from cat 171838 neutralised all pseudotypes bearing the Envs of primary strains that were prepared in this project, with the exception of the pseudotype bearing the Env of 179200 (Figure 6.3). Unfortunately, no follow up samples were available from cat 171838 as the cat was euthanised following the diagnosis of FIV infection and therefore further tests could not be conducted.

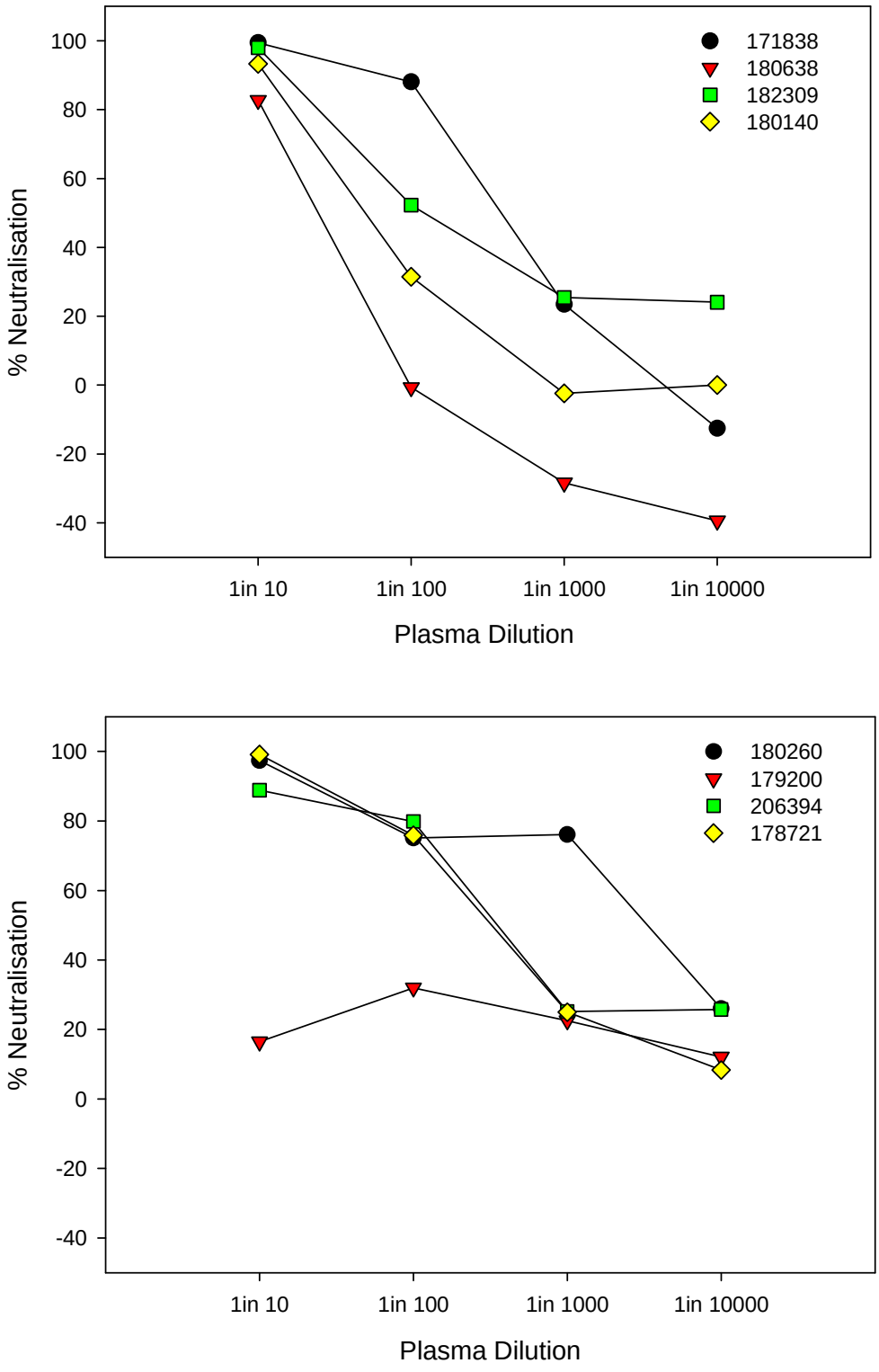


Figure 6.3. Plasma from cat 171838 was broadly neutralising. Plasma from cat 171838 was tested for the ability to neutralise a panel of HIV(FIV) pseudotypes bearing the Envs of eight naturally occurring UK isolates (see key), showing % neutralisation. All pseudotypes except 179200 were strongly neutralised (>80% at 1:10 plasma dilution). Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

Since 179200 resisted neutralisation by the broadly neutralising plasma 171838, the Env amino acid sequence of 179200 was compared with sequences derived from other *env* clones encoding Envs that were susceptible to neutralisation by plasma 171838 (Figure 6.4). We looked for evidence of sequence variation within the variable loops, especially for amino acid substitutions which changed the polarity and/or net charge of the domains, as well as substitutions resulting in the creation or deletion of potential N-linked glycosylation sites (PNGS). PNGS are covalently attached to aspargine residues identified within the following sequence: N-X-S/T, where N is aspargine, X is any amino acid except proline, S is Serine, and T is Threonine. Using these criteria, a number of unique substitutions were identified in Env 179200. Firstly, a PNGS close to the start of the V4 region of Env, at aspargine-447, was ablated (Figure 6.4). This PNGS was conserved in most of 47 naturally occurring strains that were sequenced as part of this project (see Appendix 1), as well as in strains from subtypes A,B,C, D and E.*

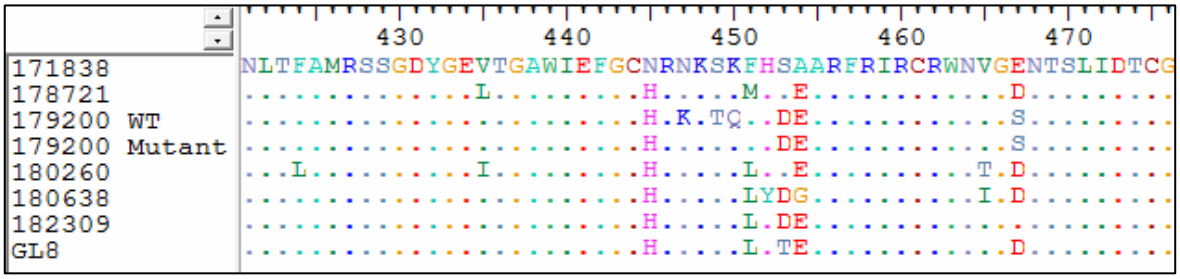


Figure 6.4. Amino acid sequence of wild-type and mutant variants of 179200. This region of C3-V4 sequence shows that the 179200 Env contains a unique KKTQ motif located between residues 447 and 450. The 179200 mutant variant contains the sequence NKSK, similar to the other Envs.

Accordingly, a mutant variant Env of 179200 was prepared, repairing the deleted PNGS (179200-K447N), and an HIV(FIV) pseudotype bearing the 179200-K447N mutant Env was tested for sensitivity to neutralisation by plasma 171838. It was

* GenBank accession numbers for strains that retain the PNGS at N-447 are as follows. Subtype A: U02403 (hnky12); U02410 (sam01); U02413 (tt09); U02417 (zepy01); NC001 (Petaluma); AB010404 (SAP03); X57002 (Swiss Z1). Subtype B: U02418 (brny03); U02419 (boy03); U02420 (glwd03) U02422 (mtex03); D84497 (Lp9); AB010397 (AICO2). Subtype C: AB016025 (TI1); AB016026 (TI2); AB016027 (TI3); AB016028 (TI4); U02392 (pady02). Subtype D: AB010400 (OKA01D). Subtype E: D84496 (Lp3); D84497 (Lp9); D84498 (Lp20); D84500 (Lp24).

observed that the K447N mutation alone did not render 179200 sensitive to neutralisation (neutralisation of 16% was observed for the wild-type 179200 pseudotype compared to 23% for the 179200-K447N mutant at a 1:10 dilution of plasma 171838). The mutation was then extended to include the threonine-449 and glutamine-450 residues, constructing a mutant with a sequence similar to the remaining sequences (the WT sequence 447KKTQ450 was mutated to 447NKSK450), and an HIV(FIV) pseudotype bearing the 447NKSK450 mutant Env was tested for susceptibility to neutralisation by plasma 171838. The 447NKSK450 mutant pseudotype was neutralised strongly by plasma 171838 compared to the wild-type pseudotype (Figure 6.5a). It was concluded, therefore, that the four amino acid sequence 447KKTQ450 rather than the absent PNGS had an important role in rendering the 179200 Env neutralisation-resistant. Subsequently, the likelihood of N-linked glycosylation occurring at position 447 was calculated using NetOGlyc 3.1 software (Julenius *et al.*, 2005); the results obtained by applying the full sequence of *env* were found to be minimal (<50%), consistent with our observation. The 447NKSK450 mutant was tested also for susceptibility to neutralisation by the homologous plasma from cat 179200. The results showed no improvement in neutralisation sensitivity (Figure 6.5b), suggesting that plasma 179200 did not contain mature NAbs. Furthermore, no significant differences in neutralisation sensitivity were observed when the neutralisation of the wild-type 179200 Env was compared with the neutralisation of the 447 NKSK 450 mutant of 179200 Env, using the broadly neutralising plasma 206394 (Table 6.6). Pseudotypes bearing both Envs were strongly neutralised.

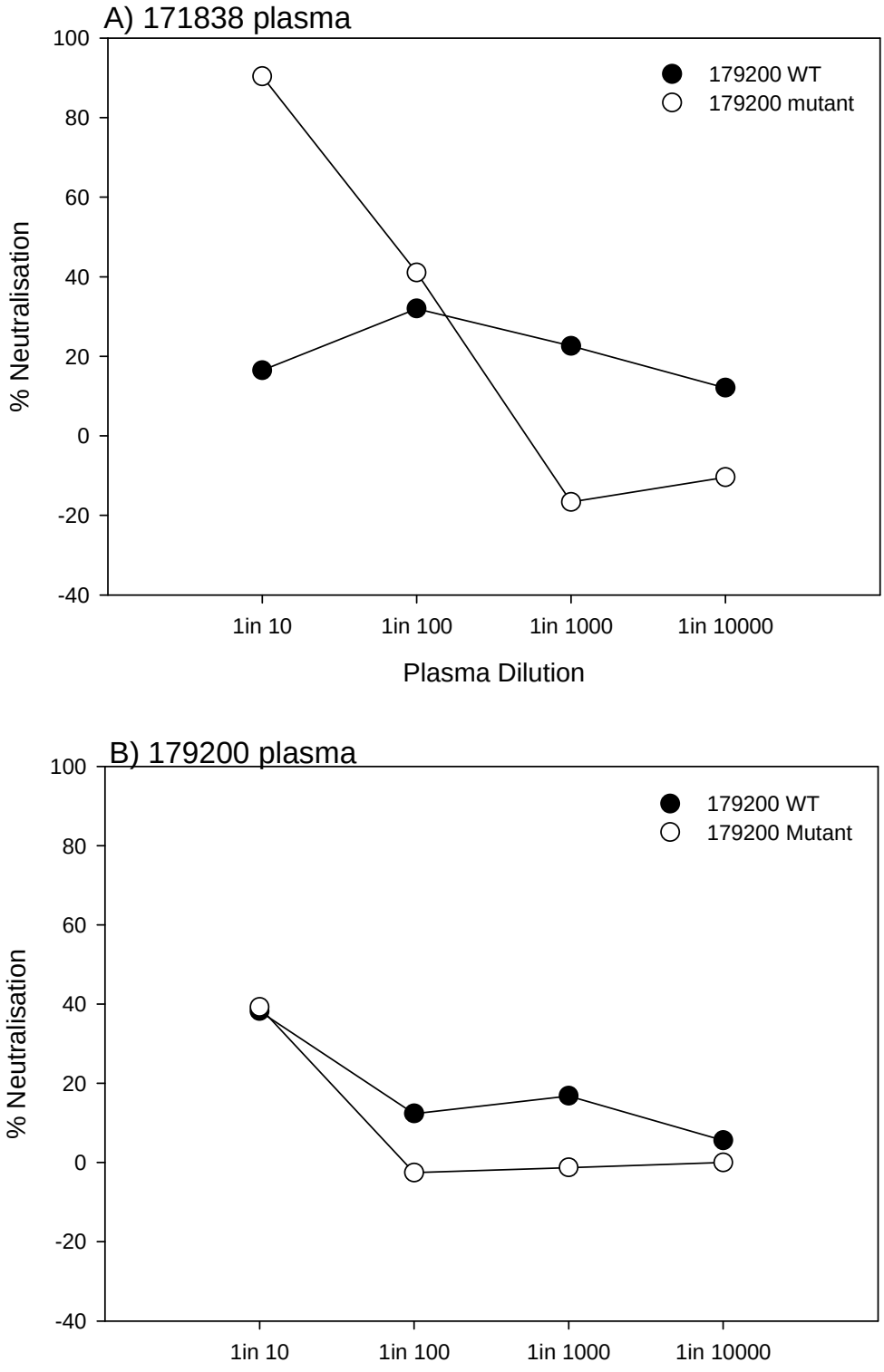


Figure 6.5. Neutralisation of pseudotypes bearing the Env of wild type (WT) 179200 or its mutant (179200 mutant) by A.) the heterologous broadly neutralising plasma 171838 and B.) the homologous plasma 179200. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

Plasma	179200 WT	179200 mutant
179200	38	39
171838	16	90
206394	99	97

Table 6.6. Summary of % neutralisation of wild type and mutant 179200 pseudotypes by homologous plasma and two broadly neutralising plasma samples tested at 1:10 dilution of plasma.

6.3 Discussion

In this chapter the generation of NAbs in response to naturally occurring FIV infection was investigated. It was demonstrated that out of eight plasma samples, two (171838 and 206394) have shown strong neutralising activity against pseudotypes bearing homologous or heterologous Envs. Furthermore, one plasma sample (182309) strongly neutralised a pseudotype bearing the homologous 182309 Env but not the pseudotype bearing the GL8 Env (Table 6.5). In HIV-1 infected subjects, in whom the time of infection was more accurately determined, it has been shown that aNAbs were generated in almost all infected subjects a few months after sero-conversion (Gray *et al.*, 2007; Li *et al.*, 2006). It is possible, therefore, that the proportion of plasma samples from FIV infected cats containing NAbs may have been greater if follow up samples had been available from cats at later stages of infection. However, it was not possible to determine the time of infection since all the cats studied were naturally infected. It is possible that NAbs may not be detectable early after sero-conversion, as has been demonstrated for HIV-1 infection (Moog *et al.*, 1997).

It is likely that the generation of aNAbs is less important than the induction of broadly NAbs; certainly, in terms of vaccine development, an effective immunogen must be capable of inducing a broad neutralising response. The data presented in this chapter demonstrated that cats with broadly cross-reactive NAbs are not common (less than 3% of samples tested). Similarly, it has been shown that sera capable of neutralising a diverse panel of HIV-1 viruses are not common (Kraft *et al.*, 2007; Li *et al.*, 2007). An investigation of the specificities of these broadly NAbs may reveal neutralisation determinants that are conserved amongst viral isolates and between subtypes.

As the NAb response matures with time, in terms of potency and breadth, follow-up plasma samples were collected from FIV sero-positive cats at intervals of between 10 months and 18 months after the first sample collection. While no significant improvement was observed in neutralisation potency in most samples tested, it was shown that one follow up plasma sample (plasma 171505 collected 15 months after the first sample) was able to strongly neutralise GL8. This finding may suggest that a smaller proportion of plasma samples contain NAbs against FIV compared to HIV-1; alternatively it may require longer for heterologous NAbs against FIV to mature. Moreover, one of two plasmas which

strongly neutralised GL8 (169630) showed a broader response after follow-up, strongly neutralising CPG-41 (86%) and PPR (99%) in addition to GL8 (Table 6.2), whereas the other plasma (172279) showed no increase in the breadth of cross-reactivity (data not shown). However, plasma 169630 did not neutralise pseudotypes from primary UK isolates, suggesting that the cross-reactivity of the neutralising response was still limited.

Plasma sample 171838 displayed strong cross-neutralising activity against pseudotypes expressing the Env of GL8 as well as a panel of Envs from novel primary isolates. As cat 171838 was young, it was possible that this cat had become infected from its dam *in utero*; however the case history of the cat was not available and therefore the route and timing of infection could not be confirmed. Other factors that may have affected the development of broadly NAbs in this cat included the virulence of the transmitted virus, the viral load and divergence of the evolved quasispecies.

In order to analyse further the cross-reactivity of plasma 171838, a potential sequence motif was identified as conferring neutralisation resistance to the Env of one of the novel field isolates. Previous studies have indicated that the variable loops V4 and V5 appear to be major targets for effective neutralising antibodies and that substitution of a single amino acid in either or both loops may alter the phenotypic characteristics of neutralisation for primary FIV isolates (Bendinelli *et al.*, 2001; Siebelink *et al.*, 1995a). Here, we have demonstrated that a motif containing at least four amino acids (KKTQ) over the C3-V4 region contributed to the resistance of the 179200 Env to neutralisation by plasma 171838, rather than the loss of a PNGS within the same region.

The V4 loop of the HIV-1 Env has been shown to be highly variable, with the variability involving the numbers and locations of PNGSs (Belair *et al.*, 2009). Furthermore, enormous sequence variability within the V4 loop of simian immunodeficiency virus (SIV) has been associated with rapid disease progression in macaques (Rivera-Amill *et al.*, 2007). Moreover, the C3-V4 region in HIV-1 is considered to be a major target for NAbs (Moore *et al.*, 2008). Indeed, it has been shown that mutations within the α2-helix of C3, which is located in the outer domain of gp120, were associated with resistance to neutralisation (Rong *et al.*, 2007b). It has been speculated that the α2-helix plays a role in viral

evasion from NAbs and may participate in the maintenance of the tertiary structure of the gp120 outer domain or the quaternary structure of the trimer (Rong *et al.*, 2007a; Gnanakaran *et al.*, 2007).

7 Analysis of broad neutralisation

7.1 Introduction

Although an effective vaccine was developed shortly after the initial isolation of swine influenza, vaccination against persistent lentiviral infections represents a more significant challenge. Although the human immunodeficiency viruses (HIV) have been recognised for decades, such infections cannot yet be controlled by vaccination. A major obstacle to the development of an effective HIV vaccine is the resistance of primary viral isolates to antibody-mediated neutralisation (Wyatt *et al.*, 1998). Similarly, for FIV, it was reported in chapter six that only a small proportion of plasma samples from naturally infected cats contained virus neutralising antibodies (NAb). The studies described in the present chapter were designed to investigate mutations arising within the envelope glycoprotein of FIV that affected the induction of NAb.

It is debatable whether NAb play a major role in the clearance of the immunodeficiency virus infections; it requires several months for the humoral immune response to mature, whereas cell-mediated immunity requires only a few weeks (Koup *et al.*, 1994). It has, however, been proven that sterilising immunity against FIV, HIV-1 or SHIV can be achieved through passive immunisation with sera containing NAb (Shibata *et al.*, 1999; Hohdatsu *et al.*, 1993; Mascola *et al.*, 2000). Furthermore, in most experimental animal model systems of viral infection, T-cell based vaccines alone have been unable to prevent infection by providing sterilising immunity (Shiver *et al.*, 2002; Wilson *et al.*, 2006). It is thus believed that a successful vaccine must elicit high titres of broadly NAbs in addition to cell-mediated immune responses (Walker & Burton, 2008; Chakrabarti *et al.*, 2002; Schulke *et al.*, 2002).

Shortly after infection, antibodies recognising the viral envelope glycoprotein (Env) can be detected, although none have neutralising activity (Stamatatos *et al.*, 2009; Richman *et al.*, 2003); it takes the NAb response several months to mature. At this stage of infection, the generated NAbs are extremely limited in both potency and breadth, and are highly isolate-specific, that is neutralisation is restricted to the homologous isolate (Li *et al.*, 2006; Richman *et al.*, 2003). Later in infection, a small proportion of plasmas cross-neutralises various

isolates to varying degrees (Kraft *et al.*, 2007; Li *et al.*, 2007; Stamatatos *et al.*, 2009). Both neutralising and non-neutralising antibodies contribute to a high proportion (up to 5%) of total immunoglobulin in some HIV-infected individuals later in infection (Binley *et al.*, 1997).

Env is the target of neutralising antibodies; however, it is poorly immunogenic (Kolchinsky *et al.*, 2001) and evolves rapidly in response to immune pressure and selection, such that neutralisation determinants continuously diminish with time post infection. Furthermore, the masking of variable loops on conserved epitopes, such as the primary and co-receptor binding sites, the occlusion of protein surfaces through trimer formation, the glycan shield formed by extensive N-linked glycosylation, and the use of a two-receptor entry mechanism all contribute to the poor immunogenicity of Env (Koch *et al.*, 2003; Chackerian *et al.*, 1997). In particular, the co-receptor binding site is exposed only transiently following the conformational change that is triggered by the engagement of the primary receptor (Willett *et al.*, 2009), minimising the time during which the binding site may be recognised by the immune system.

Env is heavily glycosylated, such that carbohydrate comprises a great proportion of its molecular weight, estimated as more than 50% in HIV-1 (Leonard *et al.*, 1990; Geyer *et al.*, 1988). N-linked glycosylation of Env plays a shielding role and is essential for the correct folding of Env (Land & Braakman, 2001). It has been proposed that carbohydrate forms a glycan shield which creates a fluid-like shelter overlying the neutralising epitopes, thereby protecting the virus from the host immune system (Wei *et al.*, 2003). It has been speculated that potential N-linked glycosylation sites (PNGSs) maintain the integrity of the glycan shield. Since Env evolves in response to pressure exerted by NAbs, new PNGSs may be initiated or deleted throughout the sequence of Env. It has, however, been demonstrated that numerous PNGSs are highly conserved amongst viral isolates, indicating that specific glycans may be vital for the integrity and conformation of Env, while simultaneously masking key targets for NAb (Cheng-Mayer *et al.*, 1999). A proposed mechanism of HIV escape from NAbs is that the repositioning of glycosylation sites across the Env sequence limits recognition by NAbs but does not affect the ability of Env to interact with the primary and co-receptors (Wei *et al.*, 2003).

This chapter describes the examination of plasma samples from field cats containing broadly NAbs, identified and described in chapter six. An isolate, prepared from the PBMC of a cat which had generated a broadly neutralising plasma, lacked a conserved PNGS in the V1/V2 loop homologue. Therefore the effect of repairing this PNGS was investigated, utilising HIV(FIV) pseudotypes bearing modified Envs to assess susceptibility to neutralisation. Understanding the mechanism of cross-neutralisation and the generation of broadly neutralising antibodies could provide valuable insights for Env-based lentivirus immunogen design and facilitate the development of novel immunisation protocols.

7.2 Results

7.2.1 Broad neutralising activity is rare in FIV infected cats

As reported in chapter six, a panel of over 300 plasma samples collected from naturally infected cats was screened to detect NAb against the HIV(FIV) pseudotype bearing the GL8 Env pseudotype. The majority of the samples either did not neutralise or only weakly neutralised (<60%) the GL8 pseudotype that was used for screening (see Appendix 2). However, two plasma samples were identified (namely 178639 and 206394) that neutralised strongly (>80%) not only GL8 but all pseudotypes tested (Table 7.1), representing 0.67% of the plasma samples examined.

Env	Subtype	Origin	% Neutralisation		Pseudotype	Subtype	Origin	% Neutralisation	
			178639	206394				178639	206394
180638	A	UK	99	90	GL-8	A	UK	99	95
171838	A	UK	99	99	B-2542	B	USA	99	99
180260	A	UK	96	96	CPG-41	C	USA	96	99
182309	A	UK	100	100	PPR	A	USA	98	99
180140	A	UK	90	97	425	A	UK	99	96
178721	A	UK	99	97	M2	B	Italy	93	100
179200	A	UK	83	97	827	A	UK	99	95
206394	A	UK	83	93	1419	A	UK	95	96
KNG2	B	Japan	96	99	Leviano	B	Brazil	90	100
TM-2	B	Japan	99	99	LLV-B	B	Africa	51	16

Table 7.1. Percent neutralisation values for 178639 and 206394 plasmas at 1 in 10 dilution using a panel of 19 HIV(Env) pseudotyped viruses. The Env subtypes and country of origin are shown.

Plasma 178639 was collected from a 10 year old cat that was terminally ill (and was euthanised shortly after FIV infection was diagnosed), whereas plasma 206394 was collected from a 14 year old cat with chronic age-related renal problems but was otherwise healthy. The duration of infection for each cat was unknown as both had been adopted from the feral population. Both plasma samples were tested for neutralisation against pseudotypes bearing the Env of lion lentivirus B (LLV-B) as a control, in order to exclude the possibility that the plasmas may have contained substances that were toxic to the substrate cells used in the neutralisation assay. A panel of HIV(FIV) pseudotypes bearing the

Envs of 20 FIV isolates, including primary isolates derived from naturally infected cats, was used to test the plasma samples for broadly NAb (Figure 7.1). Since the volume of each sample was limited and follow-up samples were not available from cat 178639, it was possible to isolate virus only from sample 206394. Subsequently, the predominant *env* gene was cloned and HIV(206394) pseudotypes were prepared.

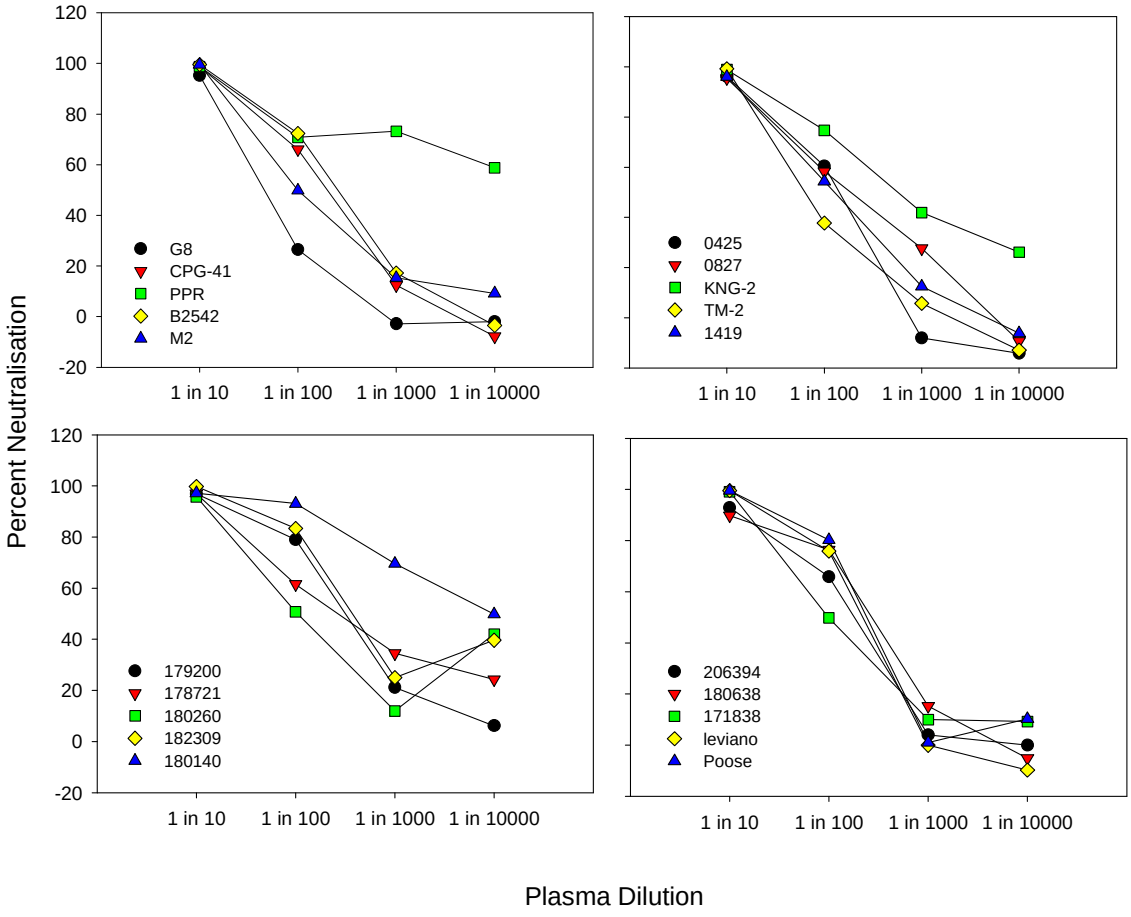


Figure 7.1. Neutralisation by HIV(FIV) pseudotypes (denoted by FIV Env genes) by successive dilutions of plasma 206394. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

7.2.2 Pseudotype bearing 206394 Env moderately neutralised by non homologous plasma samples

Susceptibility to NAbs is different amongst FIV isolates; for example, primary isolates are more refractory to neutralisation compared to laboratory adapted isolates. Consequently, it has been suggested that vaccines based on laboratory adapted isolates of FIV may fail to protect cats against challenge with more virulent primary isolates (Hosie *et al.*, 1995). Since plasma 206394 displayed broadly neutralising activity, it is conceivable that its homologous isolate was of low virulence and, as a consequence, readily neutralised by antibodies in plasmas from genetically related and unrelated isolates. Hence, neutralisation of the HIV(FIV) pseudotype bearing the Env of 206394 was examined, using a panel of plasmas collected from cats infected with isolates of FIV from different countries. The results of the luciferase-based neutralisation assay showed that the pseudotype bearing the Env of 206394 was neutralised strongly by both its homologous plasma and plasma 178639, one of the broadly neutralising plasma samples identified previously. In contrast, 4 plasma samples (from cats infected with FIV isolates of other subtypes or from different regions) did not neutralise 206394, as shown in Table 7.2. These results suggest that NAbs in weakly neutralising plasmas, while neutralising isolates within the same subtype, may not target common epitopes, as a result of genetic divergence amongst isolates. It was likely that plasma 206394 was broadly neutralising because it contained NAbs that targeted key epitope(s) that were conserved amongst isolates across the subtypes.

Plasma	Subtype	Country	% Neutralisation
Petaluma KKS	A	USA	12
LP20	E	Japan	-9
178639	A	UK	83
206394	A	UK	93
171070	AC	UK	41
TOT-1	B	Japan	51

Table 7.2. Neutralisation of pseudotype bearing 206394 Env by neutralising plasmas from cats infected with different subtypes of FIV. Values show % neutralisation when plasma samples were tested at 1 in 10 dilutions.

7.2.3 Loss of conserved PNGS in V1/V2 homologue of 206394 Env

Since plasma 206394 contained broadly NAbs that apparently targeted conserved epitope(s), the amino acid sequence of 206394 Env was examined in order to identify a potential sequence motif in Env that may have led to this significant observation. It was noted that the 206394 Env contained relatively few glycan residues and lacked two PNGS in the region analogous to the V1/V2 domain of HIV and SIV (Figure 7.2). One of the PNGS was ablated as a result of the T271A mutation, similar to the mutation in the FL4 strain of FIV (from which the Fel-O-Vax FIV vaccine was derived); the other PNGS was absent as a result of the N298S mutation. This is a unique mutation that has not been encountered in any other FIV Env sequence published to date. Figure 7.3 illustrates the sequence of 206394 Env compared to the Env sequences of the novel FIV isolates described in chapter six, all of which contained the PNGS at residue 298.

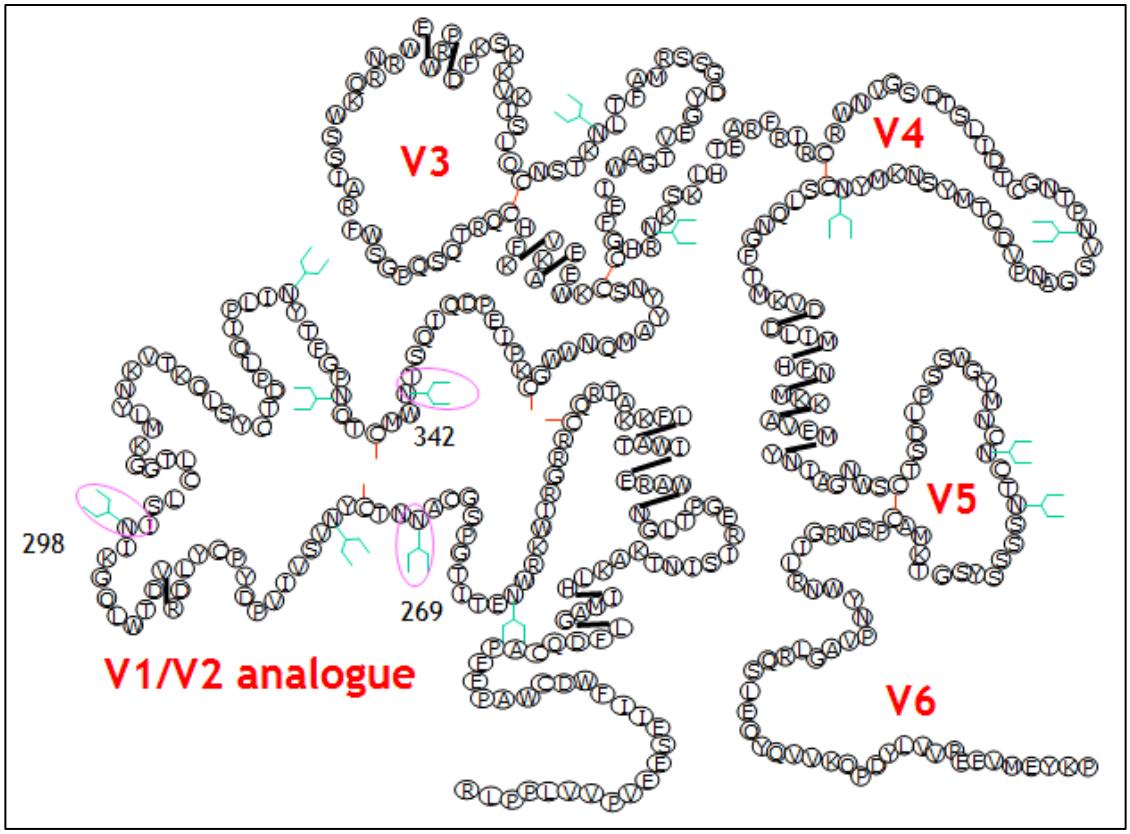


Figure 7.2. A schematic drawing of FIV Env showing the locations of PNGSs within variable loops in GL8. PNGSs 269 and 342 which were ablated in FL4 (GenBank: NC001), the vaccine isolate, and the PNGS at asparagine-298 that was ablated in 206394 are highlighted in circles.

```
                          260       270       280       290       300       260       270       280
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
UK206394         RIWRRW NETITGPLGC ANKACYNISV IVPDYQCYLD RVDTWLQGKV SISLCLTGGK MLYNKETKQL SYCTDPLQIP LINY
GL8              ...K.. .......... ..NT..... .......... .......... NV........ .....Y.... .......... ....
UK171838         .V..K. .......... ..NT..... .......... .......... N......... .......... .......... ....
UK178721         ..... .......... ..NT..... .......... .......... N......... .....Y.... .......... ....
UK179200         .T.... .......... ..NT..... .......... .......... N......... .....H.... .......... ....
UK180140         ..... .......... ..NT..... .......... .......... N......... .....Y.... .......... ....
UK180260         .T.... .......... ..NT..... .......... .......... N......... .....Y.... .......... ....
UK180638         ..... .......... ..NT..... .......... .......... N......... .....D.... .......... ....
UK182309         ..... .......... ..NT..... .......... .......... N......... .......... .......... ....
```

Figure 7.3. Amino acid sequence of V1/V2 homologous Env regions for the pseudotypes tested in this thesis. The 206394 sequence shows the loss of two PNGSs compared to the other sequences (shown within boxes).

7.2.4 PNGS at residue 298 affects susceptibility to neutralisation

Pseudotype 206394 displayed markedly lower luciferase activity on CLL-CD134 cells compared to other pseudotypes tested (Figure 7.4). In order to investigate this phenomenon, the ablated PNGS at residue 298 in Env 206394 was repaired using site-directed mutagenesis, resulting in the 206394-S298N mutant. Next, the PNGS at this site was eliminated from GL8 Env, resulting in the GL8-N298S mutant. Pseudotypes were prepared bearing the two mutated Envs and it was shown that the presence of a PNGS at residue 298 was associated with enhanced infectivity, since the luciferase activity was markedly lower for pseudotypes bearing Envs in which the PNGS was ablated compared to those containing the PNGS (Figure 7.5). Consistent results were obtained for both pairs of HIV(FIV) pseudotypes based on either the GL8 or the 206394 Envs.

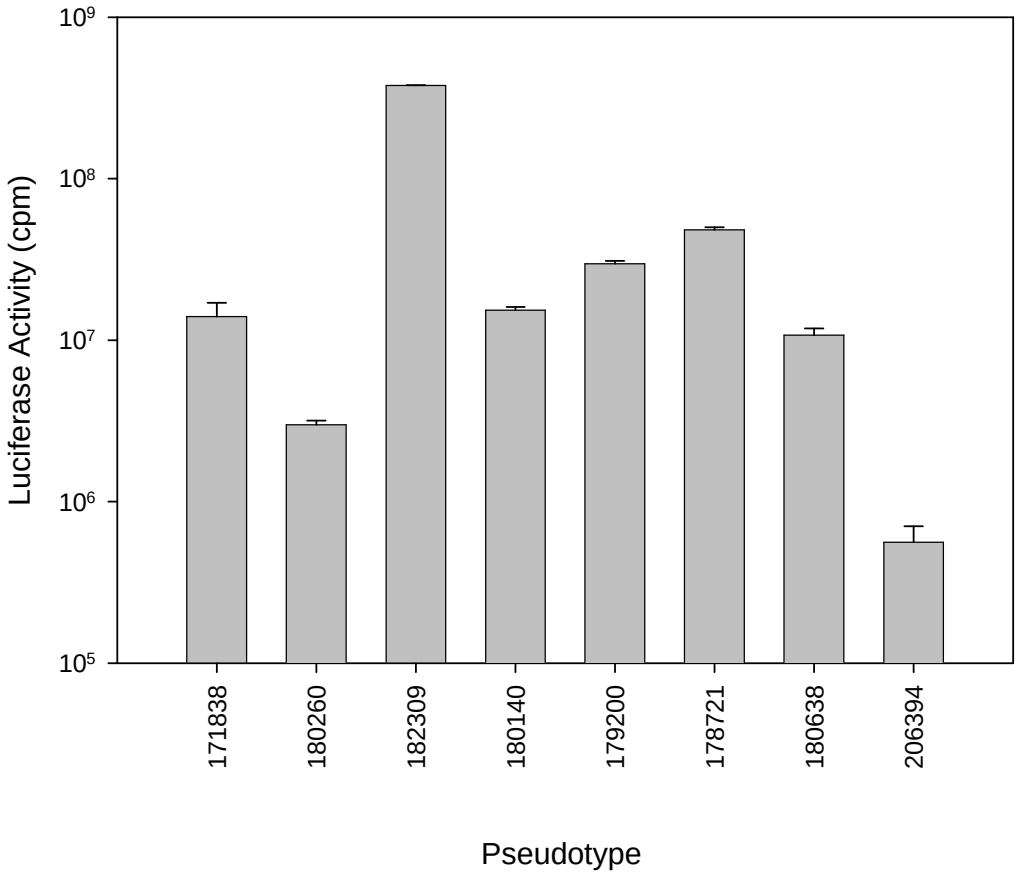


Figure 7.4. Comparison of the luciferase activity of the pseudotypes tested in this thesis. Pseudotypes were incubated with CLL-CD134 cells in triplicate. Each column represents the mean (n=3) +/- SEM.

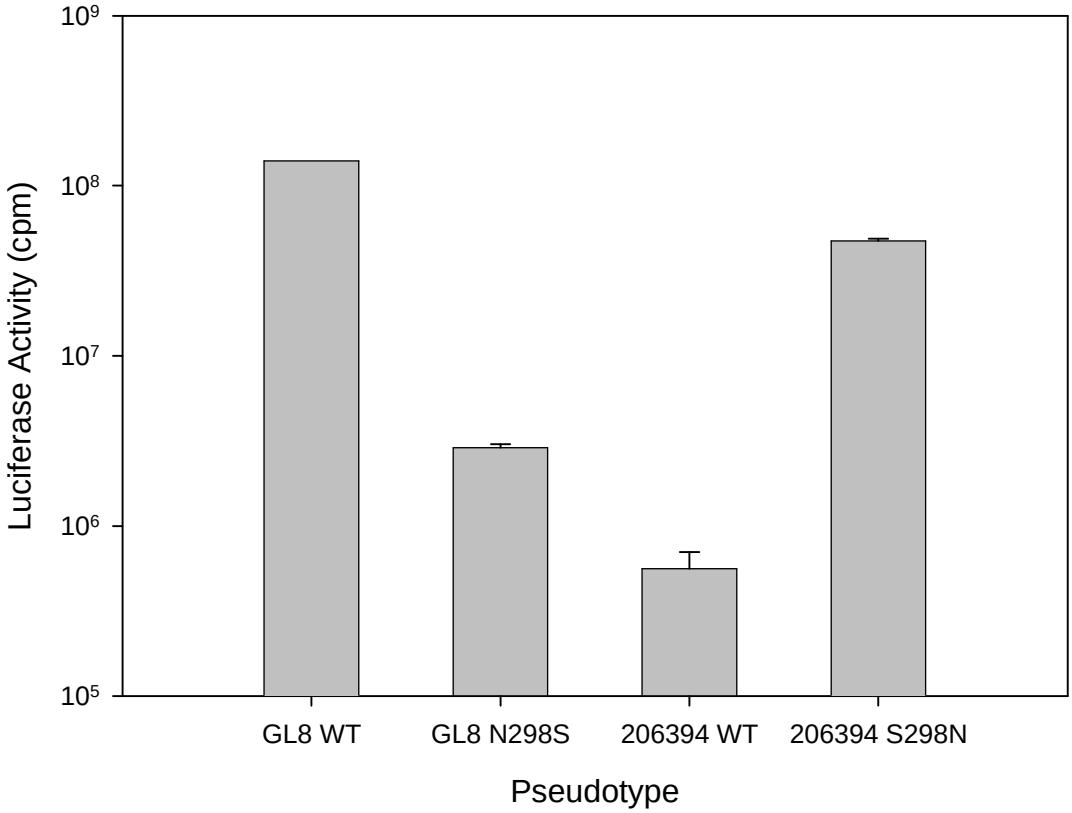


Figure 7.5. Comparison of luciferase activity of pseudotypes GL8 and 206394 with and without the PNGS at residue 298. The differences in infectivity, as indicated by luciferase activity, of each pair of pseudotypes bearing WT and variant Envs, indicate that the elimination of the glycan at residue 298 reduces viral infectivity. Each column represents the mean (n=3) +/- SEM.

Furthermore, it was observed that ablation of the PNGS at residue 298 was associated with marked enhancement of susceptibility to neutralisation. When the neutralising activity of two broadly neutralising plasmas (178639 and 206394) was compared against pseudotypes bearing the Env of either the GL8 wild-type or its mutant containing an ablated PNGS (GL8-N298S), the pseudotype bearing the GL8-N298S Env was approximately five fold more susceptible to neutralisation compared to the pseudotype bearing the wild-type GL8 Env (Figure 7.6). This result could not be reproduced with pseudotypes bearing the Env of 206394 as the titre of the pseudotype bearing the 206394 wild-type Env was too low to give valid results in the neutralisation assay when comparing the neutralisation sensitivity of the WT and mutant 206394 Envs (data not shown).

In contrast, the elimination of two further PNGSs, at positions 269 and 342 (located at the predicted stem of the V1/V2 loop homologue) did not improve the neutralisation sensitivity of Env; only the elimination of the PNGS at residue 298 (located at the tip of the V1/V2 loop homologue) led to enhanced susceptibility of GL8 N298S to neutralisation compared to the wild-type (Figure 7.7). The PNGSs at residues 269 and 342 are missing from the FIV-FL4 Env, the vaccine isolate, where they are located towards the N- and C-terminals of the V1/V2 homologue respectively. The PNGS at residue 298, on the other hand, is located at the crown of the loop; hence, a glycan residue at this location may serve to mask a conserved epitope on Env.

A follow up sample was obtained from cat 206394 five months after the first sample was collected. Three Env variants were isolated (by Pawel Beczkowski), all of which had reinstated the PNGS at residue 298 (Figure 7.8). These findings are consistent with a role for the PNGS at residue 298 in escape from neutralisation.

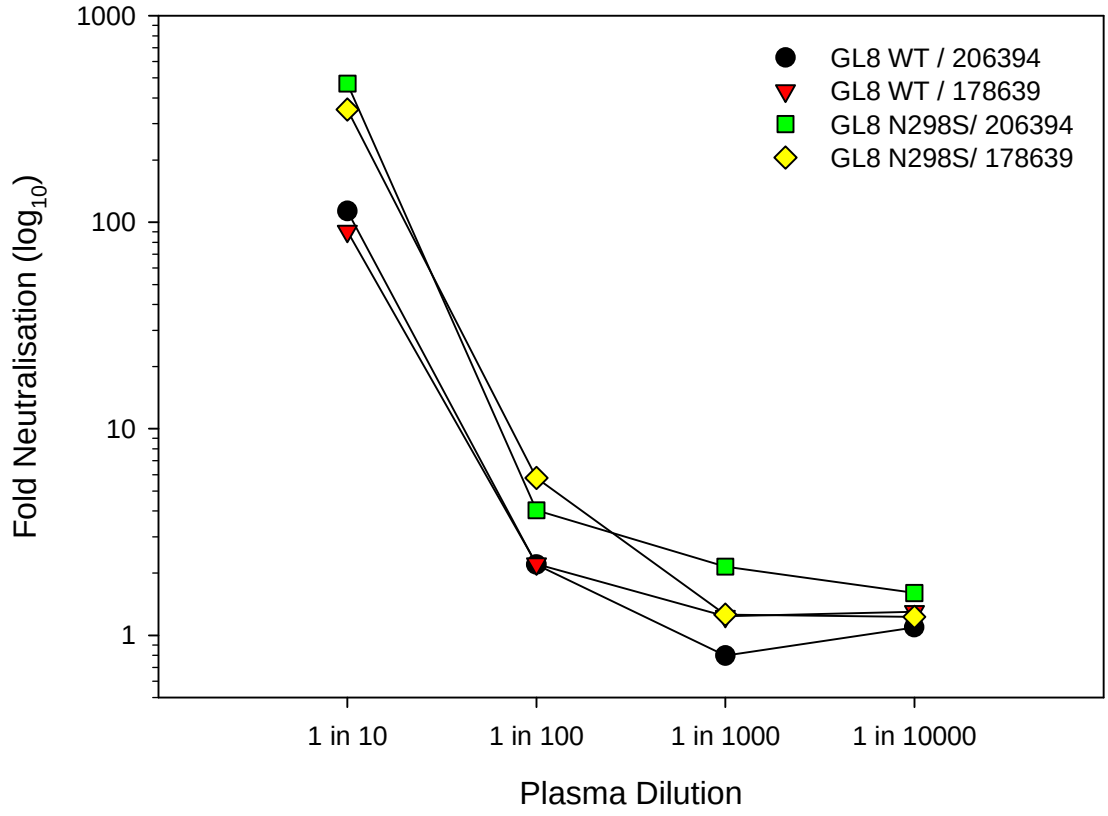


Figure 7.6. N298S mutation is associated with increased sensitivity of GL8 to neutralisation. GL8 N298S mutant was approximately 5-fold more sensitive to neutralisation by broadly neutralising plasmas 206394 and 178639 compared to the GL8-WT. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

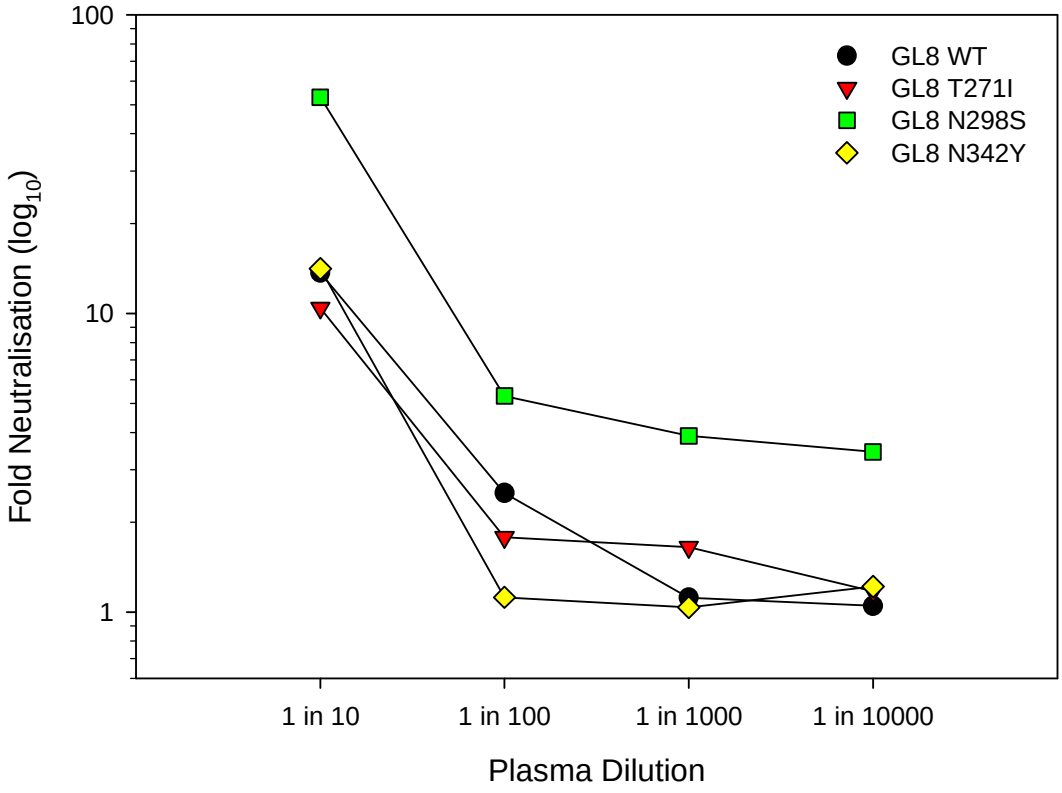


Figure 7.7. Neutralisation of GL8 wild-type and mutants by 206394 plasma. The effect of glycan removal within the V1/V2 loop homologue on the neutralisation sensitivity of Env by introducing three separate mutations that eliminated glycan residues at positions 269, 298, and 342. Each point is derived from the mean (n=3) +/- SEM of luciferase activity counts.

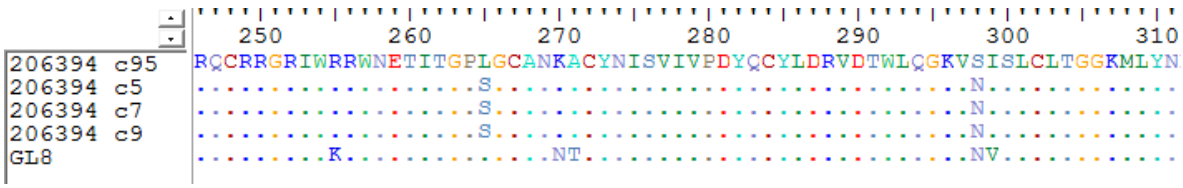


Figure 7.8. Amino acid sequence of the 206394 clones. 206394 clones c5, c7 and c9 were isolated 5 months after the isolation of the first variant (206394 c95). The PNGS at residue 269 was still absent from all 206394 variants whereas PNGS at residue 298 was reinstated. The sequence of GL8 is shown for comparison.

7.3 Discussion

This chapter describes studies focussing on the elimination of an asparagine-linked glycosylation site at residue 298 which is sited at the crown of the V1/V2 loop homologue of FIV Env and is highly conserved amongst published FIV sequences. A mutation at this site (N298S) was observed in the Env isolated from cat 206394, a cat naturally infected with FIV that had developed broadly neutralising antibodies (bNAbs). The N298S mutation ablated a PNGS and led to a reduction in infectivity of the HIV(FIV) luciferase pseudotype bearing the 206394 Env, while the same pseudotype was neutralised more readily by both the homologous plasma and heterologous plasma from other FIV-infected cats.

As has been well documented for HIV-1 infection, few subjects develop bNAbs during the course of infection (Beirnaert *et al.*, 2001; Binley *et al.*, 2008); similarly we observed that sample 206394 was amongst only a small proportion of plasma samples (0.67%) from cats naturally infected with FIV that contained bNAbs. Identifying the target for such bNAbs is important, having implications for the design of effective lentiviral vaccines.

It has been demonstrated for HIV infection that viral load and duration of infection correlate with the generation of a broadly cross-reactive humoral response (Sather *et al.*, 2009). However, other factors may contribute to the development of a bNAb response, including the virulence of the initial challenge virus, the strength of the host immune response, the occurrence of super-infection or recombination, and quasispecies diversity. Although the mechanisms leading to the development of bNAbs are not fully understood, high titres of NAbs have been observed in long-term HIV-1 non-progressors (Cao *et al.*, 1995; Cao *et al.*, 1996; Montefiori *et al.*, 1996) and bNAbs have been identified only in non-progressors (Carotenuto *et al.*, 1998; Dreyer *et al.*, 1999). Since non-progressors develop more potent and broader NAb responses, it is likely that the continuous evolution of Env leads to the recognition of more epitopes by the immune system, thereby increasing the breadth of the NAbs generated. Furthermore, the NAb response matures with time, becoming more focused on conserved epitopes and thereby increasing the titre of high quality NAbs. Simultaneously, higher titres of NAbs are associated with increased neutralisation potency. On the other hand, plasma samples from patients in whom infection progresses contain a less heterogeneous population of

antibodies, perhaps because the immune system has been exposed to a more homogeneous viral population, and such plasma samples have a narrower spectrum of neutralising activity.

It has been shown that viral evolution in response to immune pressure involves the addition and repositioning of PNGS within regions sometimes termed "hot spots" (Zhang *et al.*, 2004). The V1/V2 domain of HIV Env has been the subject of extensive investigations, since it varies greatly in both amino acid sequence and length amongst viral strains (Wang *et al.*, 1995; Fox *et al.*, 1997) and has a role as an immunological shield, covering the bridging sheet between the inner and outer domains of Env (Kwong *et al.*, 1998) and thereby protecting conserved regions of Env such as the primary and co-receptor binding sites. A review of the literature reveals that several studies have investigated the removal, repositioning, and addition of potential glycosylation sites, or even partial or complete deletions of variable loops, in HIV and SIV. Many of these studies have shown that glycosylation often results in the masking of certain neutralisation epitopes (Cheng-Mayer *et al.*, 1999; Reitter *et al.*, 1998; Stamatatos & Cheng-Mayer, 1998; Chackerian *et al.*, 1997; Lue *et al.*, 2002). Nevertheless, not all modifications result in an increase in viral susceptibility to neutralisation.

For example, Saunders et al. (2005) demonstrated that the deletion of the V1 loop of HIV-1 renders the virus more resistant to neutralisation, and it was concluded that perhaps deletion of the V1 loop focused the immune response on the non-functional epitopes of the V2 domain. Moreover, Gzyl et al. (2004) has reported that partial deletion of the V1/V2 loop of HIV-1 Env renders the virus less virulent, most likely by redirecting the immune response towards unshielded immunodominant epitopes and so broadening NAb responses to Env that cannot be afforded by the full removal of the variable domains. Single and multiple alterations in the numbers and locations of PNGSs resulted in variable outcomes. While no significant improvements in the immunogenicity of Env were obtained with mutants compared to the wild-type Envs in some studies (Quinones-Kochs *et al.*, 2002; Li *et al.*, 2008; Lue *et al.*, 2002), other studies demonstrated significant roles for particular PNGSs in viral susceptibility to neutralisation (McCaffrey *et al.*, 2004; Reitter *et al.*, 1998; Li *et al.*, 2008). Similarly, in this study, the PNGS at residue 298 had a major effect on the immunogenicity of FIV

Env as well as viral infectivity. However, no significant effects were observed when the PNGS at residues 269 and 342 were reinstated.

Cheng-Mayer et al. (1999) indicated that there are highly conserved PNGSs within the Env of HIV-1 and SIV, and thus a systematic study of these sites may be valuable in identifying immunogenic epitopes on Env. Pinter et al. (2004) demonstrated that exchanging the V1/V2 loops of two Envs switched viral sensitivity to neutralisation by several polyclonal and monoclonal NAbs directed against the V2 region, the V3 loop and the CD4 binding domain and concluded that the structure of the V1/V2 domain rather than sequence changes altered the neutralisation phenotype. On the other hand, although shorter V1/V2 HIV Env domains and fewer glycosylation sites were associated with higher susceptibility to neutralisation, Kraft et al. (2008) demonstrated that neither the length, nor the extent of glycosylation, of the variable regions affected the breadth of the NAbs generated against an immunogen based on a subtype A Env. These findings indicate that neither the size of the variable domain nor the quantity of PNGS but rather it is their positions within Env that are important in shielding immunodominant determinants and controlling viral susceptibility to neutralisation. Hence the removal (or shifting the locations) of key PNGSs, as well as exposing localised epitopes, also confers structural changes on the entire Env that may increase recognition by NAbs. Consistent with this hypothesis, the elimination of the PNGS at residue 298 in this study may have induced structural modifications on Env which led to either the exposure of key determinants in other domains to NAbs, or reduced masking of a neutralisation determinant on the V1/V2 domain, as was suggested following a similar observation by Krachmarov et al. (2006).

It has been reported by several research groups that, as infection proceeds, the NAb response matures in both potency and breadth in response to the continuously evolving virus (Cecilia *et al.*, 1999; Binley *et al.*, 2008). Viral evolution may involve the addition of PNGSs, particularly where immunodominant determinants can be guarded (Zhang *et al.*, 2004). It appears that FIV isolate 206394 evolved in this way, since all 3 of the newly-emerged variants had evolved by reinstating the PNGS at residue 298 during the 5 month period between samplings.

The mechanism of induction of bNAbs has always been poorly understood; bNAbs occur only rarely in natural infections and their target epitopes on Env have not been defined (Sather *et al.*, 2009). The matter is even more complicated when it comes to inducing NAbs following vaccination as vaccine-induced NAbs are far less potent than NAbs generated in natural infection (Bontjer *et al.*, 2009; Li *et al.*, 2008). It has been suggested that vaccine-induced NAbs are more likely to target epitopes that are either occluded or do not exist, in native virus (Derby *et al.*, 2007).

Promising results were achieved recently following immunisation with a mutant HIV Env lacking a single PNGS; the mutant induced stronger NAbs against both the mutant and wild-type variants (Li *et al.*, 2008). In order to evaluate the effect of the elimination of the PNGS at residue 298 in the FIV Env, it would be interesting to determine the efficacy of an FIV immunogen containing the N298S mutation in inducing NAbs in cats. Moreover, following the identification of PNGSs within Env that are conserved amongst published FIV sequences, particularly those located in the V1/V2 homologue, it should be possible to evaluate the contribution of each PNGS to FIV neutralisation by a process of systematic mutation. In this way it may be possible to understand more fully the effect of each glycan on Env immunogenicity and viral infectivity. Subsequently it may be possible to engineer an immunogen in which one or more PNGSs are eliminated, in order to generate an immunogen capable of inducing bNAbs.

8 Final Discussion

The principal aim of this project was to investigate the role of NAb in immunity to infection with FIV. Having developed a robust assay to detect NAb, bNAbs were identified in clinical samples collected from naturally infected cats and studies were conducted to address the mechanisms which may have led to the generation of such NAbs.

Other issues tackled during this project included an investigation of immune-mediated enhancement of FIV infection, a description of the FIV strains circulating in UK cats by generating phylogenetic trees based on the genetic and amino acid variation of Env and an analysis of Env sequences derived from several primary strains of FIV and identifying critical mutations in variable loops which altered viral sensitivity to NAbs. As a result of this project, a major question arose: what impact would these observations have on vaccine design for FIV and HIV?

8.1 Impact of FIV studies on HIV vaccine design

The value of FIV as a model for HIV has been discussed in detail in Section 1.8. In addition, a commercial vaccine for FIV has been developed and has shown promise, protecting cats against challenge with heterologous as well as homologous strains (Pu *et al.*, 2005). The comparative success in developing a protective vaccine against FIV has not, however, been coupled with a similar success for HIV vaccine development, raising the concern: are FIV vaccine studies relevant to HIV vaccine design?

In considering this question it is important to recognise that the Fel-O-Vax FIV vaccine does not induce protective immunity against challenge with virulent primary strains such as GL8 (Dunham *et al.*, 2006a), an isolate which is likely to be representative of naturally occurring isolates transmitted in the field. Given this limitation to the success of the Fel-O-Vax FIV vaccine, the relatively sparse information available related to its capacity to induce protection, and the as yet unidentified mechanism of protection, current investigations in terms of designing the ideal vaccine for either FIV or HIV are still at a relatively early stage. Another significant factor is that the Fel-O-Vax FIV vaccine is an

inactivated vaccine and therefore a similar approach cannot be reproduced for HIV vaccine trials for safety reasons, since HIV is the causative agent of an incurable human disease. Only limited success has been observed using other approaches to FIV vaccination, such as subunit or DNA vaccines (Hosie *et al.*, 1996; Leutenegger *et al.*, 1998; Dunham *et al.*, 2002); such approaches are more likely to be implemented for HIV vaccine development but success against FIV challenge has been very limited and has not, to-date, exceeded that for HIV trials.

In spite of there being differences between HIV and FIV, with the latter being a feline-specific virus, having a different route of transmission and host-cell receptor usage system, the two viruses share many similarities in terms of biological properties, genome organisation, the general structure of Env, the pathogenesis of infection and mechanisms of evasion from host immunity. As a result, FIV is a good candidate for the purpose of designing a highly effective lentiviral vaccine and provides a challenge system in which it is possible to dissect the mechanisms by which broadly neutralising responses may be induced in a natural host model system.

8.2 The development of regional vaccines

Since Env, the target for neutralisation, displays immense diversity (which can reach 30% and 35% between subtypes for FIV and HIV-1, respectively (Burkhard & Dean, 2003; Binley *et al.*, 2004), it has been proposed that it may be necessary to develop regional vaccines that are each based on the sequence of the predominant circulating viruses in particular regions or countries, at least as a preliminary controlling measure (Pistello *et al.*, 1997; Steinrigl & Klein, 2003).

In order to achieve this goal, the subtype of the strains circulating in particular regions or countries must first be identified. For example, it has been demonstrated that circulating FIV strains belong to subtype B in Italy (Pistello *et al.*, 1997), subtypes A and B in Germany and Austria (Steinrigl & Klein, 2003), subtype B in Portugal (Duarte *et al.*, 2002), subtype A predominates in Australia (Kann *et al.*, 2006b), subtypes A and C in New Zealand (Hayward & Rodrigo, 2008), subtype C in both Taiwan and Vietnam (Uema *et al.*, 1999; Nakamura *et al.*, 2003), subtype A in South Africa (Kann *et al.*, 2006a) subtypes A and C in Canada (Reggeti & Bienzle, 2004), subtype B in Brazil (Martins *et al.*, 2008), and

subtype E in Argentina (Pecoraro *et al.*, 1996). In chapter four of this thesis it was shown, based on the sequence of the V3-V5 region of Env for over 45 viruses collected from cats across the UK, that subtype A is the predominant FIV subtype in the UK.

There is some evidence that protection can be achieved against an intra-subtype B heterologous FIV challenge using an attenuated FIV vaccine (Pistello *et al.*, 2003a). However this trial was conducted solely with FIV isolates from subtype B and this finding has not been reproduced in trials involving other subtypes or in a wider geographical area. It has been suggested that subtype B isolates may be more ancient and host adapted and as a consequence may be less virulent (Bachmann *et al.*, 1997). In countries where a more heterogeneous FIV population exists, an effective vaccine would be required to protect against diverse strains which may belong to more than one subtype. Moreover, in such circumstances, recombination between FIV isolates of different subtypes may occur as a result of co-infection and result in chimaeric strains which may be resistant to vaccine-induced protection.

Another consideration is whether genetic subtyping is actually relevant for the development of regional vaccines. Several studies, that were conducted to investigate cross-neutralisation in HIV-1, have demonstrated that a more accurate means of linking the capability of NAbs to cross-neutralise heterologous isolates is to classify viral strains into 'serotypes' (Kostrikis *et al.*, 1996b; Nyambi *et al.*, 1996). Although there is little known about the relationship between genetic subtypes and neutralisation serotypes, it has been speculated that there may be no or only little significant correlation (Weber *et al.*, 1996; Kostrikis *et al.*, 1996a). The evidence in support of there being a correlation between genetic subtypes and neutralisation serotypes is that it has been shown that protective NAbs are directed towards determinants throughout Env that are highly conserved amongst isolates from different subtypes (Kessler *et al.*, 1997). Indeed, serotype classification requires neutralisation determinants to be mapped; as a result, viral strains can subsequently be classified according to their antigenic features. However, it has been proposed that conformational epitopes are common throughout Env and so antigenic mapping may not be straightforward. Little work has been conducted to date with regards to FIV

serotyping but, once highly conserved epitopes have been mapped, epitope-focused antigen design could be considered.

8.3 Significance of Env variable loops in viral resistance to NAbs

In order to escape the host's immune response, both FIV and HIV have developed effective mechanisms in order to maintain viral survival and replication. An important protective mechanism of immune escape occurs via evolution within the hyper-variable domains throughout Env that serve as a defence line in several ways. For example, the V1/V2 region in HIV-1 occludes important conserved epitopes such as the co-receptor binding site (Pinter *et al.*, 2004). It has been speculated also that the V1/V2 analogue in FIV may play a similar role in shielding the FIV co-receptor binding site, which has been mapped recently within the V3 hypervariable region (Sundstrom *et al.*, 2008). Another means by which variable domains (or loops) could serve as an efficient defence is by tolerating extreme sequence variation, especially at positions that are presumed to directly encounter the host's NAbs. As the virus evolves in response to immune pressure exerted by NAbs, new viral variants appear continuously, forcing the immune system to deal with newly emerged epitopes and rendering the humoral responses generated previously to be potentially ineffective. A striking example is displayed in the sequence at the crown region of the V5 loop of FIV Env; this region is highly variable even amongst strains from the same subtype and the same geographical region (see Appendix 1).

The variation in the V4 loop region of HIV-1 Env is similar to that observed for the V5 region in FIV Env. Variation in the amino acid sequence, the length of the domain, and the numbers and locations of PNGSs (Belair *et al.*, 2009), all appear to contribute to viral resistance (Moore *et al.*, 2008) making the V4 loop an interesting target for epitope mapping. The V3 loop of HIV-1, on the other hand, appears to be more conserved (Gorny *et al.*, 2004), and has been shown to be a target for neutralisation in both HIV-1 (Javaherian *et al.*, 1989; Li *et al.*, 2005; Binley *et al.*, 2004) and FIV (Lombardi *et al.*, 1993). Moreover, the HIV-1 V3 loop contributes to viral tropism (Hwang *et al.*, 1991; Cheng-Mayer *et al.*, 1988), cell fusion (Rusche *et al.*, 1988), virus infectivity (Freed *et al.*, 1991), and co-receptor interaction (Suphaphiphat *et al.*, 2003), perhaps accounting for V3 tolerating less variation compared to other loops on Env. The importance of the

V3 region to viral productivity suggests that the virus would be susceptible to NAb directed against this target. However, it is evident that the V3 region is less exposed to the immune system compared to the other variable regions (Bou-Habib *et al.*, 1994), being occluded within the trimeric Env structure (Hartley *et al.*, 2005).

In this thesis, neutralisation determinants were identified in the C3-V4 (see chapter six), and V5 (see chapter five) domains of FIV. In addition, other studies have demonstrated that single or double mutations in either or both the V4 and V5 loops of the FIV Env are responsible for neutralisation resistance in subtype A and B viruses (Verschoor *et al.*, 1995; Pistello *et al.*, 2003b). Indeed, the contribution of the variable loops to neutralisation resistance of viruses highlights that as well as playing a role in masking conserved epitopes that are important for viral infectivity, the variable regions also contain neutralisation epitopes. Whether such epitopes are conserved among viral strains should be established, as this would impact upon vaccine design. It is important also to define the role of key amino acids that have been shown to be responsible for the conversion of the viral phenotype and sensitivity to neutralisation. For example, residues 483 and 560 described by Bendinelli et al. (2001) and Siebelink et al. (1995a), could form part of a conformational epitope or these residues may be vital to maintain the correct folding of Env such that conserved neutralisation epitopes are inaccessible to NAbs.

It must be highlighted that it was possible to identify two neutralisation epitopes in the C3-V4 and V5 domains of FIV Env as a result of the neutralisation resistance of novel, naturally occuring isolates (observed in tests using a broadly neutralising plasma sample). Cats containing such broadly reactive NAbs do not appear to be encountered commonly, as described in chapter six. The role of residues 483 and 560 in viral resistance to NAbs was also recognised following the natural selection of an isolate that bore one or both mutations at these residues and was resistant to neutralisation. Consequently it is likely that a systematic approach may be useful to identify key residues that are responsible for viral resistance to NAbs (and probably maintaining the structure of Env) without affecting viral infectivity, using site-directed mutagenesis. Such an approach would require a critical analysis of Env sequences, focusing particularly on the variable regions, and then targeting key residues that change polarity,

net charge, or PNGSs. Mutant variants could then be tested alongside their wild-type (WT) counterparts for susceptibility to neutralisation by broadly NAbs. Once a variant is isolated that demonstrates neutralisation resistance compared to its susceptible WT variant, then that particular mutation can be investigated further. In this way, a library comprising key amino acid residues throughout Env would be generated, and subsequently key amino acids (and probably sequence motifs) may be identified using peptide mapping.

8.4 Significance of conserved PNGSs in resistance to NAbs

It is well-established that the immunologically silent carbohydrate residues scattered throughout Env serve to protect neutralisation epitopes against NAbs (Burton *et al.*, 2004). Longitudinal studies have shown that, with viral evolution, there is an accumulation of PNGSs on Env (Zhang *et al.*, 2004), suggesting that natural selection in response to immune pressure may favour the existence of strains with carbohydrate-shielded neutralisation epitopes.

However, the importance of N-linked glycosylation is not restricted to shielding important epitopes of Env; the carbohydrate moieties contribute also to viral structure by maintening the correctly folded conformational structure of Env (Li *et al.*, 1993). The number of PNGSs amongst viral isolates appears to be maintained within certain limits (proposed to range between 18 and 33 in HIV-1 (Zhang *et al.*, 2004), but they may occur in any number of positions throughout Env. Depending on their frequency and site of occurrence, PNGSs may be either conserved or variable. While certain PNGSs are essential for viral viability, it was observed in this thesis that the deletion/creation of PNGSs was associated with changes in luciferase activities of HIV luciferase pseudotypes bearing FIV Envs, as well as their susceptibility to neutralisation. These PNGSs may be involved in complex molecular interactions that may alter the accessibility of epitopes on Env to NAbs, shielding conserved epitopes, or affecting the orientation of the variable loops. Such different outcomes appear to be dependent on the positioning of more than one PNGS, or even by the interplay between PNGSs and amino acid residues (or motifs) dispersed throughout Env.

For a more detailed understanding of their importance in maintaining the structure of Env, PNGSs may be targeted by site-directed mutagenesis so that the structure of mutant variants with altered phenotypes (e.g. luciferase

activity, receptor usage, cell tropism or susceptibility to neutralisation) may be observed by nuclear magnetic resonance (NMR) or X-ray crystallography. An investigation of concomitant changes in structural and phenotypic features may shed more light on the mechanisms by which broad neutralisation can be induced, or provide information that is crucial for the development of more effective immunogens.

8.5 What are the prospects for a vaccine based on isolate 206394?

In this thesis plasma 206394 was shown to contain NAbs with broad cross-reactivity against HIV(FIV) luciferase pseudotypes bearing FIV Envs from different subtypes and geographical locations. Although the mechanism by which cross-reactivity can develop with time is still uncertain, it can be speculated that the ablation of a PNGS at residue 298 in 206394 Env played a role in the achievement of such broad reactivity, perhaps due to the exposure of a conserved epitope, early in infection, to the host's immune system. Since this key PNGS was repaired in all viral variants isolated from the same cat a few months after the initial isolation, it was likely that this PNGS had a significant role in protecting the virus from NAb.

Interestingly, cat 206394 did not display the typical clinical signs of FIV infection during the course of infection, but it was not possible to conduct a long term follow up of this case since the cat succumbed to age-related urinary tract problems. It is, however, thought that spontaneous recovery from FIV is unattainable (Ishida *et al.*, 1989), and persistent infections are established following both FIV and HIV infection.

It has been shown that broad NAb responses are well-developed in HIV-infected long term non-progressors (LTNPs), in contrast to rapid disease progressors (Pilgrim *et al.*, 1997; Carotenuto *et al.*, 1998). The serum of LTNPs contained not only higher titres of NAbs, but also NAbs that more frequently neutralised primary virulent isolates (Cao *et al.*, 1996; Binley *et al.*, 2008). Such a natural resistance that keeps patients clinically well may provide clues to protective immune mechanisms. Thus, an understanding of the mechanisms controlling this response would be valuable for lentiviral vaccine design.

Following the observations described in this thesis, it is tempting to speculate that an FIV vaccine trial testing an immunogen based on 206394 may induce bNAbs in vaccinated cats that may protect cats from challenge. A comparison with an immunogen based on a mutant variant of 206394, containing the PNGS that was reinstated at residue 298, would confirm whether the PNGS has a significant role in the generation of a bNAb response. It is conceivable, however, that individuals vaccinated with an HIV vaccine may not develop NAbs of similar activity or potency to those in naturally infected individuals (Doria-Rose *et al.*, 2009), and it may not be possible to predict the outcome of vaccination using such a vaccine in the human population.

The question that remains is: will such a vaccine impact upon future vaccine design for an improved FIV vaccine or for a successful vaccine against HIV? It is evident that the similarities in the structure of the Envs of FIV and HIV are significant, which perhaps means that the PNGS at residue 298 of FIV Env, if it were proven to be responsible for the resistance to cross-neutralising NAbs, may have a counterpart in HIV Env playing a similar role in protecting the virus from NAbs. And finally, the cat model enables researchers to test directly *in vivo*, in a model system with a natural host, novel and potentially improved immunogens. Studies such as these may, one day, lead to the development of a highly effective HIV vaccine.

Appendices

Appendix 1. Amino acid sequence of V3-V5 region of Env for all FIV strains sequenced for this project.

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            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                5          15         25         35         45         55         65         75
170003      QMAYYNRCRW ESTDVKFHCQ RTQSQPGTWL RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
170141      QIAYYNSCRW ESTDVKFHCQ RTQSQPGSWN RAISSWKQRN RWVWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVMG
170186      QIAYYNSCRW ESTDVKFHCQ RTQSQPGSWL RAISSWRQRN RWEWKPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
170305      QKAYYNSCRW ESTDVKFHCQ RTQSQPGSWL RTISSWRQRN RWEWRPDFES DKVKVSLQCN STRNLTFAMR SSGDYGEVTG
170338      QIAYYNSCKW EQTNVTFQCQ RTQSQPGSWI RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTSAMR SSGDYGEVTG
170415      QVAYYNSCRW EETDVKFNCQ RTQSRPGTWV RAISSWRQRN RWEWRPDFES EKVKISLQCN STQNLTFAMR SAGDYGEVTG
170418      QIAYYNSCRW ESTDVKFHCQ RTESQPGTWL RAISSWKQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEITG
170488      QRAYYNSCRW EQTDVKFHCQ RTQSQPGSWL RTISSWRQRN RWEWRPDFES EKVKISLQCN STKNLTSAIR SSGDYEEVVA
170617      QIAYYNSCRW EQTDVKFHCQ RNQSQPGSWV RTISSWRQGN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
170719      QIAYYNSCRW ERTNVKFRCQ RTQSQPGSWI RAISSWRQRN RWEWRPDFEN DKVKVSLQCN STKNLTFAMR SSGDYGEVTG
171025      QMAYYNSCRW ESTNIKFHCQ RTQSQPGSWR RAISSWRQRN RWEWRPDFES ERVKVSLQCN STENLTFAMR SSGDYGEVTG
171069      QIAYYNSCRW ENTDVKFHCQ RTQSQPGSWL RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR GSGDYGEVTG
171070      QIAYYNSCKW EQANVTFQCQ RKQSQPGTWS RAISSWKQRN RWEWRPDFES ERVKVSLQCN ATKNLTFAMR SSGDYGEVTG
171101      QWAYYNSCRW ESTDVKFHCQ RKQSQRGSWI RAISSWKQRN RWEWRPDFES EKVKVSVQCN STRNLTFAMR SSGDYGEVTG
171163      QIAYYNSCRW EQTDVKFHCQ RTQSQPGSWI RAVSSWRQRN RWEWRPDFES EKVKISLQCN STKNLTFAMR SSGDYGEVVG
171169      QMAYYNSCRW ESTNVTFHCQ RIQSQPGSWI RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYEEITG
171170      QIAYYNRCRW ESTDVKFHCQ RTQSQFGSWV RAISSWKQRH RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVMG
171175      QMAYYNSCRW EQTEVQFQCQ RTQSLPGSWH RAISSWRQKN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVMG
171265      QLAYYNSCKW EGTDVKFHCQ RIQSRPGSWR RVISSWKQKN RWEWRPDFES ERVKVSLQCN STKNLTFAMR SSGDYGEVTG
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171270      QMAYYNSCKW ESTDVKFHCQ RTQSQPGSWI RAISSWRQRN RWEWKPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEITG
171303      QIAYYNSCNW EKTDVKFHCQ RTQSQSGSWL RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
171358      QGAYYNSCRW ERTDVKFHCQ RIQSQPGSWI RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEITG
171536      QIAYYNSCRW ENTDVKFHCQ RTQSQPGSWL RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR GSGDYGEVTG
171700      QNAYYNSCRW ESTDVKFHCQ RKQSQPGSWR RAISSWKQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSSDYGEVTG
171781      QIAYYNSCRW ESTDVKFHCQ RTQSQPGSWL RTISSWRQRN RWEWRPDFES DKVKVSLQCN STKNLTFAMR SSGDYGEVTG
171810      QGAFFTVFGW DQAVLSFFFQ GPRVRLGPWV GLFCSWNQRI GWDWGPDFES EKVKISLQCN STNNLTFAMR SSGDYGEVTG
171812      QLAYYNSCKW EQTNVTFHCQ RKQSQPGAWR RAIASWKQEN RWVWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
171838      QIAYYNSCRW ERTNVKFSCQ RIQSQPGTWR RIISSWRQRN RWEWRPDFES EKVKVSLQCN TTKNLTFAMR SSGDYGEVTG
172325      QMAYYNSCRW ETTDVKFHCQ RTQSQPGSWR RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
172458      QIAYYNSCRW ESTDVKFHCQ RIQSRPGSWI RAIASWRQRN RWEWRPDFES EKVKISLQCN STKNLTFAMR SSGDYGEVTG
172506      QVAYYNSCKW EKTDVKFHCQ RTQSQPGSWR RAISSWKQKN RWEWRPDFES EKVKVSLQCN STQNLTFAMR SSGDYGEVTG
172527      QIAYYNSCRW ESTDVKFYCQ RTQSQPGSWH RTISSWKQKN RWVWRPDFES EKVKVSLQCN TTKNLTFAMR SSGDYGEVTG
172532      QMAYYTSCSW EQADVKFFCQ RTQSQPGSWL RVISSWKQKN RWEWRPDFES EKVKISLQCN STNNLTFAMR SSGDYGEVTG
178586      QIAYYNSCRW ESTDVKFHCQ RTQSQPGTWL RTISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEITG
178721      QIAYYNSCRW ESTDVKFHCQ RTQSQLGSWM RVISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGELTG
179200      QIAYYNSCKW EKTDVKFQCQ RTQSQPGTWN RIISSWKQRN RWEWRPDFES DKVKISLQCN STKNLTFAMR SSGDYGEVTG
179288      QRAYYNSCRW ESTDVKFHCQ RTQSQPGSWI RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
179297      QIAYYNSCKW EKTDVKFQCQ RTQSQPGTWS RIISSWKQRN RWEWRPDFES DKVKISLQCN STKNLTSAMR SSGDYGEVTG
179369      QIAYYNSCRW ESTDVKFHCQ RTQSQPGSWL RTISSWRQRN RWEWRPDFES DKVKVSLQCN STKNLTFAMR SSGDYGEVTG
179466      QMAYYNSCRW ESTNIKFHCQ RTQSQPGSWR RAISSWRQRN RWEWRPDFES ERVKVSLQCN STENLTFAMR SSGDYGEVTG
180115      QNAYYNSCKW EKTDVKFQCQ RTQSQPGSWR RAISSWKQGN RWVWRPDFES EKVKISLQCN STKNLTFAMR SSSDYGDVTG
180140      QNAYYNSCKW EKTDVKFQCQ RTQSQPGSWR RAISSWKQGN RWVWRPDFES EKVKISLQCN STKNLTFAMR SSSDYGDVTG
180260      QNAYYNSCRW EHTDVKFHCQ RKQSQAGSWN RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTLAMR SSGDYGEITG
180638      QIAYYNSCRW ESTDVKFHCQ RTQSQPGSWV RTISSWKQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
182309      QNAYYNNCKW ESTDVKFHCQ RKQSQPGSWL RAISSWRQRN RWEWRPDFES EKVKVSLQCN STRNLTFAMR SSGDYGEVTG
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182455      QRAYYNSCRW ESTDVKFHCQ RTQSQPGSWI RAISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSGDYGEVTG
206394      QMAYYNSCRW ESTNVKFHCQ RTQSQPGSWR RIISSWRQRN RWEWRPDFES EKVKVSLQCN STKNLTFAMR SSADYGEVTG

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                85         95        105        115        125        135        145        155
170003      AWIEFGCHRN KSKRHTEARF RIRCRRNVGN NTSLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQDGFTM KVDDLIMHFN
170141      AWIEFGCHRN KSIHHSAARF RIRCRWNTGK NSSLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
170186      AWIEFGCHRK KSSLHTDTRF RIRCRWNIGD NTSLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQDGFTM KVDDLIMHFN
170305      AWIEFGCHRN KSKLHSEARF RIRCRWNEGD NTSLIDTCGE TQNVTGANPV DCTMYTNKMY NCSLQDGFTM KVDDLIMHFN
170338      AWIEFGCHRK KSNLHSEARF RIRCRWNVGD NTSLIDTCGE NKNVSGANPV DCTMYANKMY NCSLQDGFTL KVEDLIMHFN
170415      AWIEFGCHRK KSKLHSEARF RIRCRWNAGD NTSLIDTCGN SQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
170418      AWIEFGCHRN KSKLHTEARF RIRCRWNEGN NASLIDTCGK TQNVSGANPV DCTMYANTMY NCSLQNGFTM KVDDLIVHFN
170488      AWIEFGRHSN KSKLHTEARF RIRCRWNTGD NTSLIDTCGD TKNVSGANPV DCTTYANKMY NCSSQDGFTM KIDDLIMHFN
170617      AWIEFGCLRN KSKLHSEARF RIRCRWNIGD NTSLIDTCGK TQNVSGANPV DCTMYTNTMY NCSLQNGFTM KVDDLIMHFN
170719      AWIEFGCRRN KSRLHTEARF RIRCRWNVGD NTSLTDTCG- TQNVTGANPV DCTMYANKMY NCSLQDGFTL KVEDLIMHFN
171025      AWIEFGCHRN KSKLHSEARF RIRCRWNVGD NTSLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
171069      AWIEFGCHRN KSKFHSEARF RIRCRWNMGD NASLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
171070      AWIEFGCMRN KSRRHSTARF RIRCRWNMGN NISLIDTCGK DQNVSGANPV DCTMKANTLY NCSLQDGFTM KIEDLIVHFN
171101      AWIEFGCHRN KSKFHSDARF RIRCRWNIGE NTSLIDTCGN TPNVTGANPV NCTMYANKMY NCSLQDGFTM KVDDLIVHFN
171163      AWIEFGCHRN KSKLHTETRF RIRCRWNEGD NTSLIDTCGK NQNVTGANPV DCTMYANKMY NCSLQDGFTM KVDDLIMHFN
171169      AWIEFGCHRQ KSRFHTEARF RIRCRWNVGD NTSLIDTCGK TPNVTGANPV DCTMYANKMY NCSLQNGFTM KVEDLIMHFN
171170      AWIEFGCHRN KSKLHSEARF RIRCRWNIGD NTSLIDTCGK TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
171175      AWIEFGCHRN KSKLHSEARF RIRCRWNVGN NTSLIDTCGK TQNVSGANPV DCTMYAHKMY NCSLQSGFTM KVDDLIMHFN
171265      AWIEFGCHRN KSKFHTEARF RIRCRWNVGN NASLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
171270      AWIEFGCHRN KSKLHSEARF RIRCRWNTGN NASLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQNGFTM KVDDLIMHFN
171303      AWIEFGCHRN KSKFHTEARF RIRCRWNVGD NTSLIDTCGE TQNVTGANPV DCTMYANRMY NCSLQDGFTM KVDDLIMHFN
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171358      AWIEFGCHRN KSKLHSEARF RIRCRWNVGD NTSLIDTCGE TQHVSGANPV DCTMYANKMY NCSLQDGFTM KVDDLIMHFN
171536      AWIEFGCHRN KSKFHSEARF RIRCRWNMGD NASLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
171700      AWIEFGCHRN KSRFHTEARF RIRCRWNVGD NASLIDTCGE TRNVSGANPV DCTMYANKMY NCSLQNGFTM KIDDLIMHFN
171781      AWIEFGCHRN KSKFHTEARF RIRCRWNVGD NISLIDTCGE TQNVTGANPV DCTMYANKMY NCSLQNGFTM KVDDLVMHFN
171810      AWIEFGCHRN KSRLQSEARF RIRCRWNVGD NTSLIDTCGA TKNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLVMHFN
171812      AWIEFGCHRK KSKLHSEARF RIRCRWNVGD NTSLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQDGFTM RVDDLIMHFN
171838      AWIEFGCNRN KSKFHSAARF RIRCRWNVGE NTSLIDTCGK TQNVSGANPV DCTMYANSMY NCSLQNGFTM KVDDLIMHFN
172325      AWIEFGCHRN KSKLHSEARF RIRCRWDVGD NTSLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQDGFTM KVDDLIMHFN
172458      AWIEFGCHRN KSLRHTEARF RIRCRWNTGN NASLIDTCGE NQNVSGANPV DCTMYANKIY NCSLQEGFTM KVDDLIMHFN
172506      AWIEFGCHRN KSKHYTEARF RIRCLWNKGN NTSLIDTCGE TKNVSGANPV DCTMYANRMY NCSLQDGFTM KVDDLIMHFN
172527      AWIEFGCHRN KSKLHSEARF RIRCRWDVGD NTSLIDTCGK NPNVSGANPV DCTMYANKMY NCSLQNGFTM KVDDLIMHFN
172532      AWIEFGCHRN KSRLQSEARF RIRSRWNVGD NTSLTDTCGE TKNVSGANPV DCTMYANRMY NCSLQTGFTM KVDDLVMHFN
178586      AWIEFGCHRN KSKQHTAARF RIRCRWNIGD NTSLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQNGFTM KVDDLIMHFN
178721      AWIEFGCHRN KSKMHSEARF RIRCRWNVGD NTSLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
179200      AWIEFGCHRK KTQFHDEARF RIRCRWNVGS NTSLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQDGFTM KVEDLIMHFN
179288      AWIEFGCHRN KSKLHSEARF RIRCRWNVGD NTSLIDTCGE TKNVTGANPV DCTMYANKMY NCSLQDGFTL KVEDLIMHFN
179297      AWIEFGCHRK KTQLHNEARF RIRCRWNVGG NTSFIDTCGE TQNVSGANPV DCTMYANKMY NCSSQDGFTM KVEDLIMHFN
179369      AWIEFGCHRN KSKFHTEARF RIRCRWNEGD NASLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQDGFTM KVDDLIMHFN
179466      AWIEFGCHRN KSKLHSEARF RIRCRWNVGD NTSLIDTCGE TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
180115      AWIEFGCHRN KSKLLTEARF RIRCRWNVGD NTSLIDTCGK TQNVSGANPV DCTMYTNRMY NCSLQNGFTM KIDDLIMHFN
180140      AWIEFGCHRN KSKLLTEARF RIRCRWNVGD NTSLIDTCGK TQNVSGANPV DCTMYTNRMY NCSLQNGFTM KIDDLIMHFN
180260      AWIEFGCHRN KSKLHSEARF RIRCRWNTGD NTSLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQNGFTM KVDDLIMHFN
180638      AWIEFGCHRN KSKLYDGARF RIRCRWNIGD NTSLIDTCGE TQNVSGANPV DCTMYANKMY NCSLQNGFTM KVDDLIMHFN
182309      AWIEFGCHRN KSKLHDEARF RIRCRWNVGE NTSLIDTCGN TPNVSGANPV DCTMYTNKMY NCSLQSGFTM KIDDLIMHFN
182455      AWIEFGCHRN KSKLHSEARF RIRCRWNVGD NTSLIDTCGE TKNVTGANPV DCTMYANKMY NCSLQDGFTL KVEDLIMHFN
206394      AWIEFGCHRK KSNFHTETRF RIRCRWNVGD NTSLIDTCGK TQNVSGANPV DCTMYANRMY NCSLQNGFTM KVDDLIMHFN
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               165        175        185        195        205        215
170003      MTKAVEMYNI AGNWSCTSDL PHGWGYMNCN CTNSSSS--- ----G-NKMA CPRTQGI
170141      MTKAVEVYNI AGNWSCTSDL PPTWGYMNCN CTNGSSS--- ---SGSNKMA CPRSQGI
170186      MTKAVEMYNI AGNWSCTSDL PSTWGYMNCN CTNG-TNS-- -GTYSGNKMA CPAKQGI
170305      MTKAVEMYNI AGNWSCTSDL PNTWGYMNCN CTNVTGEN-- -------KMA CPRNQGI
170338      MTKAVEMYNI AGNWSCTSDL PPTWGYMKCN CTNGSSNGI- ---NGINKMA CPKNEGI
170415      MTKAVEMYNI AGNWSCTSDL PPTWGYMKCN CTNSTSTL-- ---DTSNKMA CPSNHGI
170418      MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSSSS-- ---YSMQKMA CPRNQGI
170488      MTKAVEMYNI AGNWCCASDL PQGWGCINCS CTNSSDS--- ---DEIDKMA CPKSQGI
170617      MTKAVEMYNI AGNWSCTSDL PPTWGYMKCN CTNSTGN--- ---N---KMA CPRDHGI
170719      MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSTN---- ----DKSKMA CPRIQGI
171025      MTKAVEMYNI AGNWSCTSDL PSTWGYMNCN CTNSTSSS-- --GSNGAKMA CPRNKGI
171069      MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNR------ -SNNDNNKMA CPKGQGI
171070      MTKAVEMYDI AGNWSCKSDI PKEWGYMNCN CTNSTS---- ---NTNDKMA CPDTTGI
171101      MTRAVEMYNI AGNWSCTSDL PNTWGYMNCN CTSTNSDSY- ----GGTKMA CPGTQGI
171163      MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSVS--- ---DN-TKMA CPSNQGI
171169      MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNGSSHNN- ----Q--QMA CPKNQGI
171170      MTKAVEMYNI AGNWSCVSDL PPTWGYMNCN CTNGSSN--- ---VGSTKMA CPGKQGI
171175      MTKAVEMYNI AGNWSCTSDL PKDWGYMNCN CTNNT----- --GSN--KMA CPGNQGI
171265      MTKAVEMYNI AGNWSCTSDL PQEWGYMNCN CTNST----- --GSN--KMA CPKNQGI
171270      MTKAVEMYNI AGNWSCTSDL PPTWGYINCN CTNGSSS--- ----G-NKMA CPGNQGI
171303      MTKAVEMYNI AGNWSCTSDL PKTWGYMNCN CTNSTG---- ----GNNKMA CPTNQGI
171358      MTRAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNNSDSN-- ------NKMA CPRNQGI
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171536       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNR------ -SNNDNNKMA CPKGQGI
171700       MTKAVEMYNI AGNWSCMSDL PSTWGYMNCN CTNSTI---- -SGGTSNKMA CPKNQGI
171781       MTKAVEMYNI AGNWSCTSDL PSTWGYMNCN CTNST----- ---NQVNKMA CPKNQGI
171810       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSGDS-- --GGVNNKMA CPTKRGI
171812       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTN-STNS-- ---MGSNKMA CPRIQGI
171838       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNKN----- ---NDSDKMA CPNNQGI
172325       MTKAVEMYNI AGNWSCTSDL PQEWGYMNCN CTNST----- --GNN--KMA CPRNQGI
172458       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSKN--- -----HNKMA CPGNQGI
172506       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSSN--- ----DNNKMA CPRSQGI
172527       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSST---- ----DSNKMA CPTNQGI
172532       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSGDSG- ---GVNNKMA CPTKRGI
178586       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSS---- ----NQGKMA CPSTQGI
178721       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSTISSN SSTISSKKMA CPRNKGI
179200       MTKAVEMYNI AGNWSCMSDL PQEWGYMNCN CTNSTDS--- ---GSNNRMA CPRERGI
179288       MTKAVEMYNI AGNWSCTSDL PPTWGYMKCN CTNGTNSS-- ---DGKDKMA CPGNQGI
179297       MTKAVEMYNI AGNWSCMFDL PQEWGYMNCN CTNSSDY--- ---GSNNRMA CPRERGI
179369       MTKAVEMYNI AGNWSCTSDL PQEWGYMNCN CTNNTE---- ---TNYNKMA CPRNQGI
179466       MTKAVEMYNI AGNWSCTSDL PSTWGYMNCN CTNSTSSS-- --GSNGAKMA CPRNKGI
180115       MTKAVEMYNI AGNWSCTSDL PPTWGYMKCN CTNST----- -SQGTSNKMV CPESQGI
180140       MTKAVEMYNI AGNWSCTSDL PPTWGYMKCN CTNST----- -SQGTSNKMV CPESQGI
180260       MTKAVEMYNI AGNWSCTSDL PSTWGYMNCN CTNGTSN--- ---DGINKMA CPNREGI
180638       MTKAVEMYNI AGNWSCTSDL PSNWGYMNCN CTN------- -STNSINQMA CPKEQGI
182309       MTKAVEMYNI AGNWSCMSDL PPTWGYMNCN CSNST----- ----GNNKMA CPSKQGI
182455       MTKAVEMYNI AGNWSCTSDL PPTWGYMKCN CTNGTNSSD- ----GKDKMA CPGNQGI
206394       MTKAVEMYNI AGNWSCTSDL PPTWGYMNCN CTNSSGNS-- ------NKMA CPRNQGI
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Appendix 2. Details of the naturally FIV-infected cats included in the project.

Sample #	Age (years)	Sex	Breed	Condition	Clinical Signs / Notes	% neutralisation (GL8)
169267	10	Male	DLH	Sick	severe stomatitis/gingivitis	25
169244	8	Male	DSH	Healthy	sore mouth	15
169248	7	Female	DSH	Healthy	cat tested FIV+ several years ago but still healthy - has a bout of allergic skin disease	92
169364	17	Male	Birmese	NA	previously snap test positive for FIV	47
169421	6	Male	DMS	Sick	jaundice	43
169422	9	Male	DSH	Sick	gingivitis	92
169425	<1	Female	DSH	Healthy	mother tested FIV+	38
169426	<1	Male	DSH	Healthy	mother tested FIV+	40
169429	NA	Male	DLH	Healthy	no apparent lesion	46
169431	3	Male	DSH	NA	NA	52
169433	NA	Male	DSH	Healthy	no apparent lesion	25
169467	11	Male	DSH	NA	anaemia - sneezing	19
169468	11	Male	DSH	Sick	damaged tail	37
169485	14	Male	DSH	Sick	left eye ulceration	34
136590	NA	Male	DSH	NA	recurrent URT symptoms	22
169591	12	Male	DSH	NA	sore mouth - dullness - anorexia	43
169543	12	Male	DSH	Sick	recurrent gingivitis - slight weight loss	59
169630	12	Female	DSH	Sick	gingivitis	92
169649	3	Male	DSH	Healthy	stray cat	20
169671	NA	Male	DSH	Healthy	iritis and luxated lens	64
169729	5	Male	DSH	NA	NA	51
169762	NA	Male	DLH	NA	stray cat	34
169768	NA	Female	DSH	Sick	enlarged lymph nodes	39
169904	8	Male	DSH	NA	ulceration around ear & nose	69
169918	NA	Male	DSH	Healthy	dental problems	75
169920	13	Male	DSH	NA	NA	67
169926	8	Male	DSH	Sick	severe gingivitis	19

169944	NA	Male	DSH	Healthy	entire male	26
170003	3	Male	DLH	NA	no apparent lesion	48
170014	12	Male	DSH	NA	NA	48
170091	6	Female	DSH	NA	positive snap combo	6
170122	10	Female	DSH	Sick	off colour - went missing for 2 weeks - anaemia - off food	36
170131	7	Male	DLH	NA	NA	30
170141	12	Male	Tonkinese	Sick	coughing - lung worms - off food - weight loss – loss of voice	93
170152	9	Female	DSH	Healthy	no apparent lesion	35
170186	15	Male	DSH	Sick	anemia	9
170226	10	Male	DSH	Sick	skin disease	19
170227	NA	Male	DSH	NA	feral cat in garden	44
170282	6	Male	DSH	Sick	severe gingivitis	65
170305	NA	Female	Domestic	NA	NA	83
170335	1	Female	DSH	Sick	poor growth - widespread crusting skin lesions on face and neck	83
170338	11	Male	DSH	Sick	NA	85
170396	12	Male	DSH	Healthy	FIV positive on in-house test	75
170415	NA	Male	DSH	NA	NA	69
170418	1	Male	DSH	NA	NA	72
170420	NA	Male	DSH	Sick	chronic gingivitis	61
170437	11	Female	NA	NA	NA	80
170488	4	Male	DSH	NA	pale - poor condition	58
170617	NA	Female	DLH	Sick	NA	80
170630	1	Male	DSH	Healthy	stray cat	78
170688	NA	NA	NA	Healthy	NA	75
170719	9	Male	DSH	Sick	anaemia - dehydration	68
170764	12	Male	DSH	Sick	stomatitis	81
170773	11	Male	DLH	Sick	weight loss	72
170777	13	Male	DLH	NA	NA	84
170875	11	Female	DSH	Sick	persistent pyrexia (>40^{o}C)	78
170901	6	Male	DSH	Healthy	no apparent lesion	79

170959	2	Male	DSH	Healthy	stray cat	75
170962	8	Male	DSH	NA	NA	81
170973	NA	Male	DSH	NA	Gingivitis	89
171025	3	Male	DSH	NA	NA	75
171037	NA	Male	DSH	Sick	oral hyperplasia - poor condition - weight loss	49
171069	NA	Male	DSH	Healthy	no apparent lesion	47
171070	8	Male	DSH	Healthy	ulcerative pharyngitis	61
171086	7	Male	DLH	Sick	PCV: 19% - pale mucous membranes - lethargy - weight loss - previous history of fibrosarcoma	54
171101	<1	Male	DSH	Sick	Alopecia	67
171163	<1	Female	DSH	Sick	cyclical pyrexia (104^{o}F)	58
171167	11	Female	DSH	NA	NA	66
171169	2	Female	DSH	Healthy	slight conjunctivitis	41
171170	NA	Male	DSH	Healthy	ex- stray	54
171175	16	Female	DSH	Sick	upper respiratory tract signs	67
171265	NA	Male	DLH	Healthy	abscess on head	63
171267	NA	Male	DSH	NA	Stray cat	35
171270	3	Male	DSH	Healthy	no apparent lesion	14
171271	NA	Male	DSH	NA	stray cat - large abscess left face	40
171272	13	Female	DSH	NA	Dermatitis	38
171302	13	Male	DSH	Healthy	no apparent lesion	47
171303	NA	Male	DSH	Sick	NA	45
171358	16	Male	DLH	Sick	persistent non-regenerative anaemia – leucopenia	53
171437	NA	Female	DSH	Sick	upper respiratory tract signs	34
171440	4	Male	DSH	Healthy	no apparent lesion	44
171505	4	Male	Siamese	Sick	recurrent bouts pyrexia - has been a fighter	40
171512	NA	Male	DSH	NA	Stray cat	41
171532	5	Male	DSH	Sick	recurrent gingivitis/stomatitis	37
171555	NA	Male	DSH	NA	NA	70
171640	NA	Male	DSH	NA	NA	67
171653	NA	Female	DSH	Healthy	no apparent lesion	66
171697	10	Male	DSH	NA	NA	74

171700	NA	Male	DLH	NA	NA	-4
171753	10	Male	DSH	NA	NA	63
171781	10	Male	DSH	Healthy	anorexia - recurrent gingivitis	39
171804	NA	Male	DLH	Sick	enlarged lymph nodes	63
171806	8	Male	DSH	Healthy	Stray	87
171807	4	Male	DSH	Healthy	outdoor cat - has been a fighter	28
171810	NA	Female	DLH	Sick	feral - eating fine - change of coat colour	24
171812	5	Male	DSH	Healthy	no apparent lesion	24
171838	2	Female	DLH	Sick	dullness - off food	90
171851	NA	Female	DSH	Healthy	no apparent lesion	39
171877	NA	Male	DLH	Healthy	no apparent lesion	49
171885	10	Male	DSH	Sick	jaundice - weight loss – anorexia	65
171985	NA	Male	DSH	Sick	slow healing abscess - has multiple fights in past	59
172010	NA	Male	DSH	Healthy	no apparent lesion	54
172025	NA	NA	DSH	NA	NA	20
172260	NA	Male	DSH	Healthy	stray	16
172264	6	Male	DLH	Sick	anorexia - leathery - not passing urine - high respiration rate	30
172279	12	Male	DSH	Sick	tongue ulceration - laryngeal swelling	97
172300	9	Male	DSH	Healthy	no apparent lesion	48
172308	1	Male	DSH	Sick	oropharyngeal infection	67
172320	NA	NA	NA	Healthy	no apparent lesion	48
172323	NA	Male	DSH	NA	was free roaming - poor coat condition	66
172325	12	Female	DSH	NA	NA	65
172407	7	Male	DSH	NA	NA	8
172429	2	Male	DSH	Sick	severe uveitis - increased intraocular pressure	29
172458	4	Male	DSH	Sick	gingivitis - stomatitis	7
172471	2	Male	DSH	Healthy	was stray cat	30
172506	11	Male	DSH	Sick	NA	34
172527	9	Female	DSH	Sick	ataxia (4 legs)	39
172532	NA	Male	DSH	NA	free roaming	100
172610	11	Female	DSH	Healthy	NA	46
172695	10	Male	DSH	sick	gingivitis- off food - nasal discharge - sore mouth	79

172839	13	Male	DSH	Sick	severe gingivostomatitis	49
172840	9	Female	DSH	NA	NA	63
172843	5	Female	DSH	NA	lymphadenopathy	50
172844	NA	Male	DSH	Healthy	no apparent lesion	29
172851	9	Female	DSH	NA	NA	49
172863	9	Male	DSH	Sick	lethargy	7
172867	6	Male	DSH	NA	NA	43
172878	2	Male	DSH	Healthy	NA	13
178384	NA	Male	DSH	Sick	NA	25
178407	NA	Male	DSH	Healthy	Adopted stray	53
178410	2	Male	DSH	Healthy	no apparent lesion	30
178416	6	Male	DSH	Sick	gradual weight loss - reduced appetite - malaise - poor body & coat condition - HR 220	41
178423	NA	Male	DSH	NA	NA	52
178424	NA	Female	DSH	NA	NA	39
178539	8	Male	DSH	Sick	NA	46
178564	4	Male	DSH	Sick	NA	35
178571	NA	NA	NA	Healthy	no apparent lesion	43
178586	5	Female	DSH	Sick	severe gingivitis	41
178635	NA	NA	NA	Sick	NA	57
178636	2	Male	DSH	Healthy	severe stomatitis	50
178637	11	Female	DSH	Healthy	gingivitis	48
178639	10	Male	DSH	Sick	stray	99
178706	NA	Female	DSH	Healthy	no apparent lesion	14
178717	5	Male	DSH	Sick	dullness - dehydration - URT infections	48
178721	5	Male	DSH	Sick	large abscess on side of the face	45
178753	6	Male	DSH	Sick	NA	56
178780	4	Male	DSH	Healthy	no apparent lesion	1
178784	NA	Male	DSH	NA	NA	68
178837	3	Male	DSH	Healthy	no apparent lesion	72
178898	5	Female	DSH	Healthy	off colour	47
178911	NA	Male	X breed	Healthy	chronic gingivitis/stomatitis	62
178982	NA	Female	DLH	NA	NA	40

179028	11	Male	DSH	Sick	chronic gingivitis	28
179035	NA	Male	DSH	Sick	poor coat condition - granulation tissue at back of mouth	92
179056	11	Male	DSH	Sick	poor condition - weight loss	-2
179059	2	Male	DSH	Healthy	no apparent lesion	11
179105	3	Male	DSH	NA	NA	37
179114	10	Male	DSH	NA	NA	53
179117	1	Male	DLH	Healthy	no apparent lesion	-37
179118	8	Male	DSH	Sick	Weight loss	8
179120	NA	Male	DSH	Healthy	no apparent lesion	25
179176	NA	Male	DSH	Healthy	no apparent lesion	48
179181	3	Male	DSH	Sick	NA	25
179200	10	Male	DSH	Sick	weight loss - lethargy - pyrexia	41
179288	3	Male	DSH	Sick	mild gingivitis - neck lesions	31
179297	8	Male	DSH	Healthy	no apparent lesion	24
179323	11	Male	DSH	sick	Raised liver enzymes + abdominal fluids	7
179369	NA	Male	DSH	NA	NA	31
179383	NA	NA	DSH	NA	NA	24
179466	5	Male	NA	NA	NA	39
179481	14	Male	DSH	Healthy	previously tested FIV+	38
179550	2	Female	DSH	Healthy	no apparent lesion	32
179582	2	Male	DSH	Healthy	Halitosis - severe gingivitis - dental caries - slightly muffled heart sounds	54
179638	8	Male	DSH	Healthy	no apparent lesion	50
179795	9	Male	DSH	sick	intermittent vomiting - weight loss - pale mucous membranes	60
179837	2	NA	Persian	NA	NA	64
179845	12	Male	DLH	sick	gingivitis - pancytopaenia - hypoalbuminaemia - hyperglobulinaemia	58
179850	13	Female	DSH	NA	NA	28
179876	10	Female	DSH	sick	generalised malaise - leukopaenia (neutropaenia)	60
179898	NA	Female	DSH	NA	NA	56
180111	12	Male	DSH	Healthy	gingivitis - previously tested FIV+	15

180115	12	Female	DSH	sick	mucopurlent ocular and nasal discharges - stomatitis - bilateral conjunctivitis	1
180116	8	Male	DSH	sick	lethargy - anorexia - off colour	10
180136	12	Male	DSH	sick	NA	0
180138	NA	Male	DSH	NA	NA	3
180140	NA	Male	DSH	Healthy	no apparent lesion	63
180223	8	Male	DSH	Healthy	no apparent lesion	58
180229	10	Male	DSH	sick	anorexia - pyrexia	50
180255	10	Male	DSH	sick	weight loss - unhealed wounds - behavioural change	63
180260	10	Male	DSH	NA	NA	23
180302	NA	NA	NA	NA	NA	51
180313	4	Male	DSH	NA	gingivitis - all teeth removed	54
180354	11	Male	DSH	sick	chronic gingivostomatitis	67
180370	1	Female	DLH	sick	slightly off colour - persistent mild pyrexia - mild lymphomegaly	49
180389	9	Male	DSH	sick	diabetes - influenza-like symptoms	12
180392	7	Male	DSH	Healthy	no apparent lesion	51
180458	<1	NA	DSH	Healthy	no apparent lesion	27
180498	8	Female	DSH	sick	chronic gingivitis	23
180536	11	Male	British	sick	Ulcerative glossitis	-31
180638	NA	Male	DSH	sick	severe periodontitis - all teeth lost - swollen and lysed jaws bones - thickened bowel loops	-13
180644	9	Male	DSH	NA	NA	85
180647	NA	Male	DSH	sick	NA	2
180652	4	Male	DSH	NA	NA	24
180653	12	Male	NA	NA	NA	32
180659	13	Female	Siamese	NA	NA	49
180662	1	Female	DSH	Healthy	no apparent lesion	36
180724	10	Male	DSH	NA	9 months history of gingivitis and dental problems	5
180779	NA	Male	DSH	sick	Bilateral epitasis - NAD on biochemistry and X-rays	84

180795	NA	Male	DSH	sick	tongue ulcerations - purulent discharges	14
180800	NA	Male	DSH	NA	NA	47
180801	5	Male	DSH	Healthy	no apparent lesion	8
180850	NA	Male	DSH	Healthy	no apparent lesion	17
180871	1	Male	DSH	sick	pyrexia - inappetance+O179 - lethargy	86
180911	2	Female	DSH	Healthy	no apparent lesion	29
180914	5	Male	DSH	sick	lethargy - mild dyspnea for 2-3 months - with history of fights	-6
180949	NA	Male	DSH	NA	NA	5
180956	14	Male	DSH	Healthy	chronic gingivitis	-3
180961	5	Male	DSH	NA	NA	22
180966	7	Male	DSH	sick	lethargy - vomiting	35
181091	2	Female	DSH	Healthy	no apparent lesion	-2
181143	10	Male	Persian	Healthy	no apparent lesion	-4
181146	<1	Male	DSH	NA	NA	6
181215	12	Male	DSH	sick	weight loss - tongue ulcerations	21
181218	NA	NA	DSH	Healthy	no apparent lesion	43
181255	2	Male	DSH	NA	NA	27
181256	13	Male	DSH	sick	general malaise - recurrent drooling lingual ulcers - halitosis - marginal anaemia	24
181259	2	Male	DSH	Healthy	stray cat – fighter	55
181291	3	Male	DSH	Healthy	no apparent lesion	47
181294	5	Male	DSH	Healthy	scars from previous fight	52
181369	5	Male	DSH	Healthy	no apparent lesion	27
181380	8	Male	DSH	sick	NA	95
181423	6	Female	DSH	Healthy	no apparent lesion	34
181439	4	Male	DSH	sick	pyrexia	62
181440	NA	Male	DSH	Healthy	no apparent lesion	48
181459	5	Male	DSH	sick	off food - poor condition - enlarged lymph nodes - chronic stomatitis/gingivitis	70
181466	11	Female	Siamese	sick	mouth breathing - inappetance - nasal discharge	75
181516	NA	Male	DSH	sick	conjunctivitis	85
181574	7	Male	DSH	Healthy	NA	71

181581	12	Male	DSH	sick	lethargy - inappetance	74
181606	<1	Female	DSH	Healthy	NA	73
181650	8	Male	DSH	NA	NA	81
181670	NA	Male	DSH	Healthy	very old - poor condition	87
181725	NA	NA	DSH	NA	NA	66
181793	12	Male	DSH	sick	recurrent episodes of gingivitis - pain – inappetance - anorexia	97
181809	10	Male	DSH	sick	lethargy - pyrexia - inappetance - slight ataxia	68
181831	8	Male	DSH	sick	marked gingivostomatitis - halitosis - off food - dehydration	76
181834	NA	Male	DSH	sick	chronic recurrent gingivitis	59
181838	11	Male	DSH	sick	NA	77
181882	5	Female	DSH	NA	NA	67
181940	14	Male	Burmese	sick	gingivitis - stomatitis	79
181946	10	Male	DSH	Healthy	no apparent lesion	50
181948	5	Male	DSH	sick	chronic weight loss	61
182000	12	Male	DSH	Healthy	no apparent lesion	69
182189	11	Male	DSH	sick	lethargy - weight loss	23
182193	NA	Male	DSH	Healthy	stray	42
182246	9	Male	DSH	sick	severe gingivitis	-15
182250	18	Female	DSH	sick	anorexia	11
182263	17	Male	DSH	sick	pyrexia - dullness - lethargy - severe ulcerative stomatitis	32
182304	2	Male	DSH	sick	off food - mild dehydration - lameness	44
182309	10	Male	DSH	NA	inappetance - halitosis	34
182312	8	Male	DSH	sick	severe depression - normal TPR - maintaining weight	18
182313	7	Male	DSH	sick	inappetance – lethargy - ocular discharges	37
182337	4	Male	DSH	Healthy	stray cat - uncontrollable bleeding during castration	42
182342	7	Male	DSH	Healthy	no apparent lesion	56
182349	10	Male	DSH	sick	URT infection - cough - enlarged sub nasal LN - mild anemia - PCV 26%	42

182387	3	Male	DSH	Healthy	no apparent lesion	72
182421	5	Male	DSH	sick	severe trauma on back (very pruritic) - mild gingivitis	49
182426	4	Male	DSH	Healthy	no apparent lesion	51
182427	7	Male	DSH	Healthy	no apparent lesion	54
182428	7	Male	DSH	Healthy	no apparent lesion	30
182453	7	Male	DSH	sick	chronic lethargy - inappetance - mild anemia & lymphopenia	32
182454	1	Male	DSH	Healthy	no apparent lesion	58
182455	NA	Female	DSH	NA	NA	37
182457	<1	Male	DSH	NA	NA	60
182461	12	Male	DSH	sick	NA	28
182470	9	Male	DSH	sick	nervous signs	46
182511	6	Male	DSH	NA	NA	39
182517	NA	Female	DSH	NA	NA	39
182591	NA	Male	DSH	Healthy	no apparent lesion	45
182631	9	Male	DSH	sick	lumbo-sacral pain - amorphous mass within bone	41
182640	7	Male	DSH	sick	intermittent pyrexia - gingivitis	42
182789	2	Male	Siamese	Healthy	was stray in Portugal	50
182795	8	Male	DSH	Healthy	gingivitis	57
182842	2	Female	COSH	Healthy	no apparent lesion	66
182942	NA	Male	NA	sick	stray	57
182943	11	Male	DLH	Healthy	Gallop rhythm - weight loss	76
182945	10	Female	DSH	sick	NA	52
182970	15	Male	DSH	sick	NA	65
183039	6	Male	DSH	sick	keratitis- anterior uveitis	68
183045	2	Female	DSH	NA	NA	78
183053	NA	Female	DSH	Healthy	previous tested FIV+	81

Appendix 3. Raw luciferase activity counts.

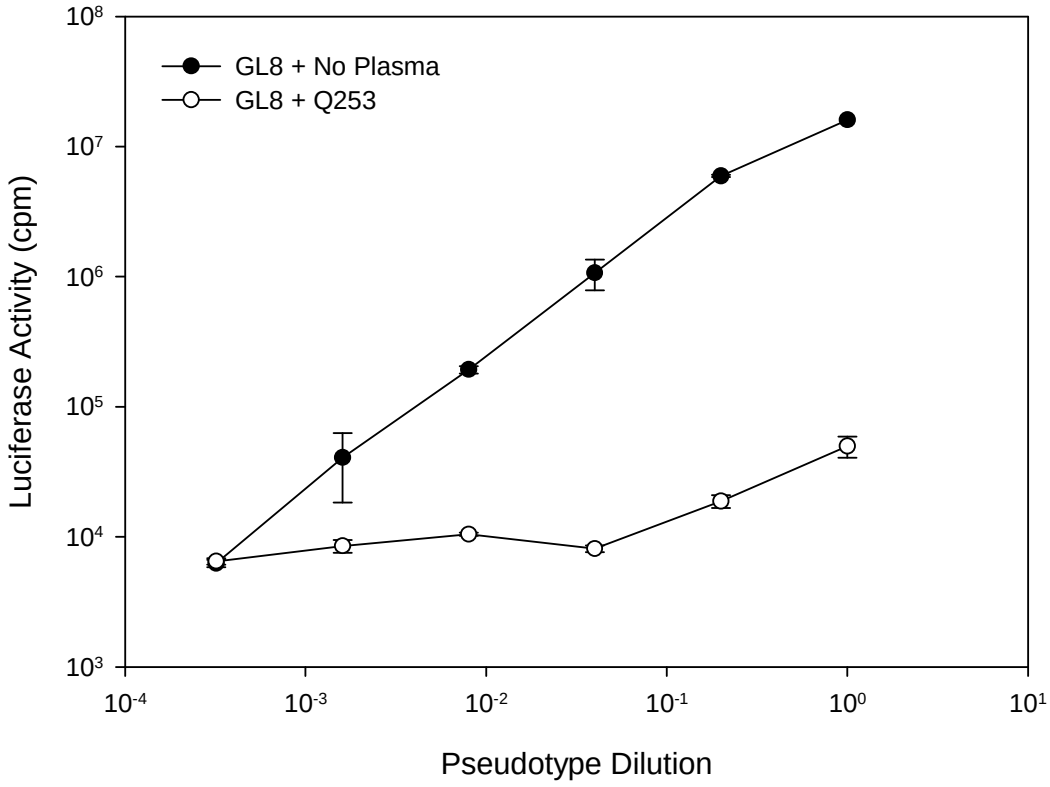

Figure 3.4

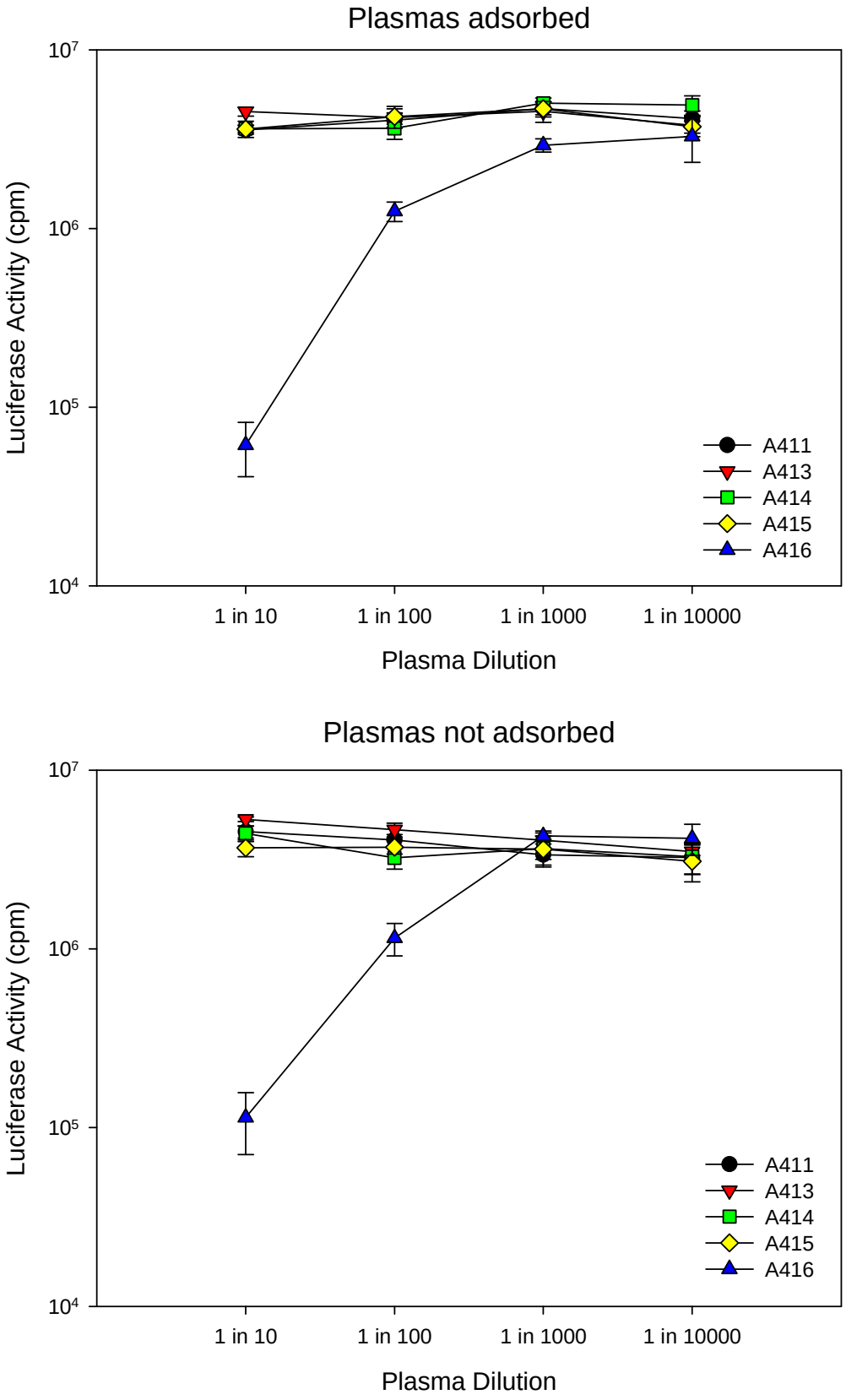
Plasmas adsorbed
Luciferase Activity (cpm)
10⁷
10⁶
10⁵
10⁴
1 in 10
1 in 100
1 in 1000
1 in 10000
Plasma Dilution
A411
A413
A414
A415
A416
Plasmas not adsorbed
Luciferase Activity (cpm)
10⁷
10⁶
10⁵
10⁴
1 in 10
1 in 100
1 in 1000
1 in 10000
Plasma Dilution
A411
A413
A414
A415
A416

Figure 3.5

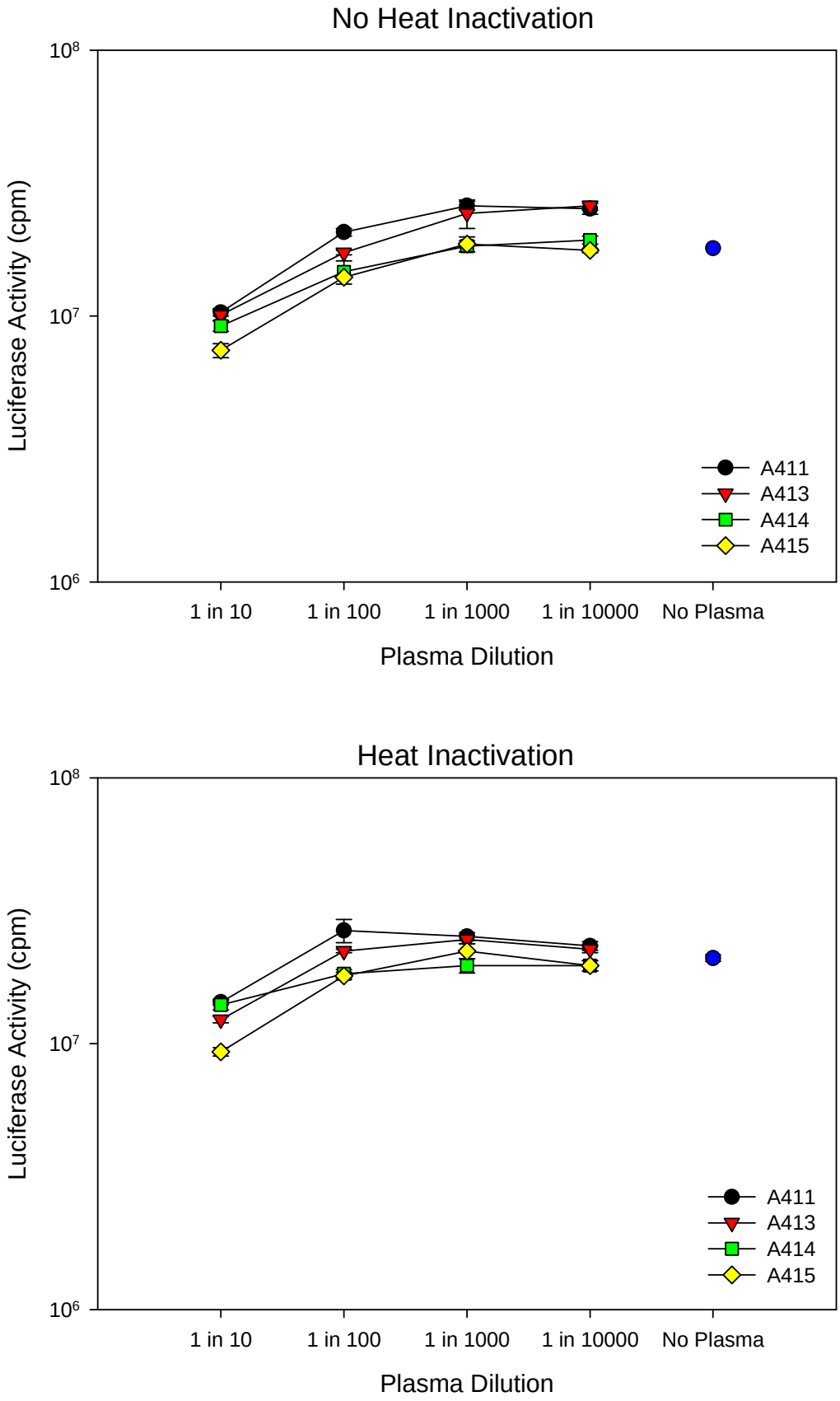
No Heat Inactivation
Luciferase Activity (cpm)
10^8
10^7
10^6
1 in 10
1 in 100
1 in 1000
1 in 10000
No Plasma
Plasma Dilution
A411
A413
A414
A415
Heat Inactivation
Luciferase Activity (cpm)
10^8
10^7
10^6
1 in 10
1 in 100
1 in 1000
1 in 10000
No Plasma
Plasma Dilution
A411
A413
A414
A415

Figure 3.6

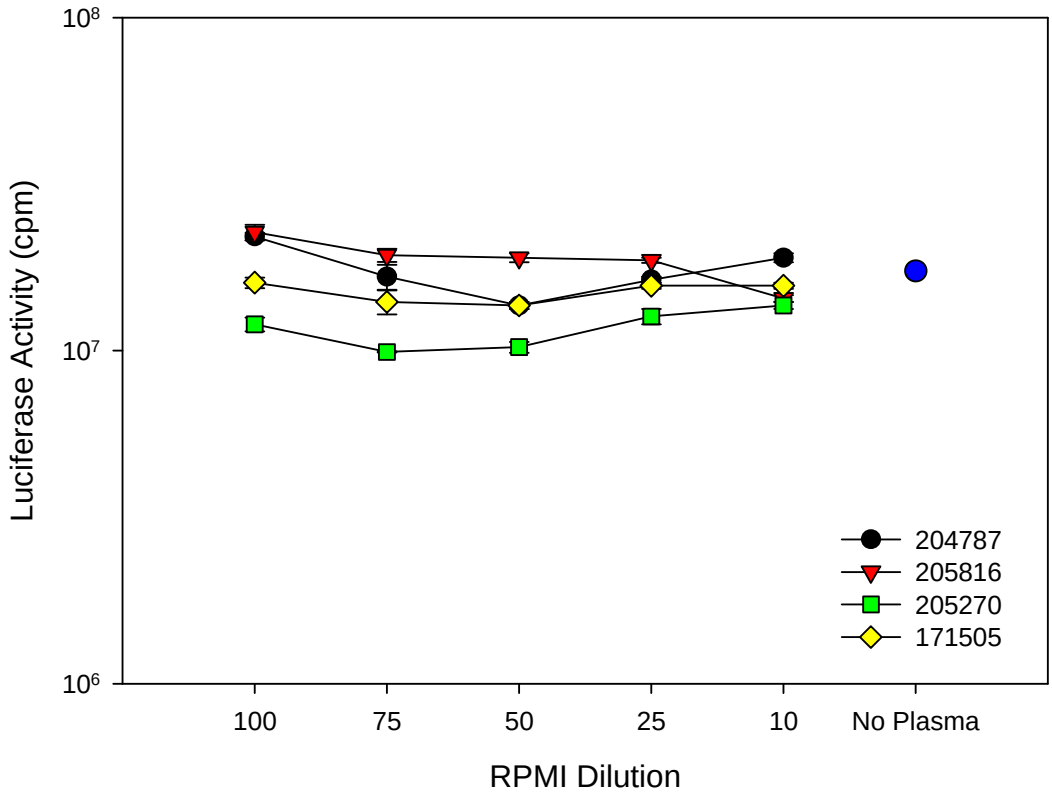

Figure 3.7

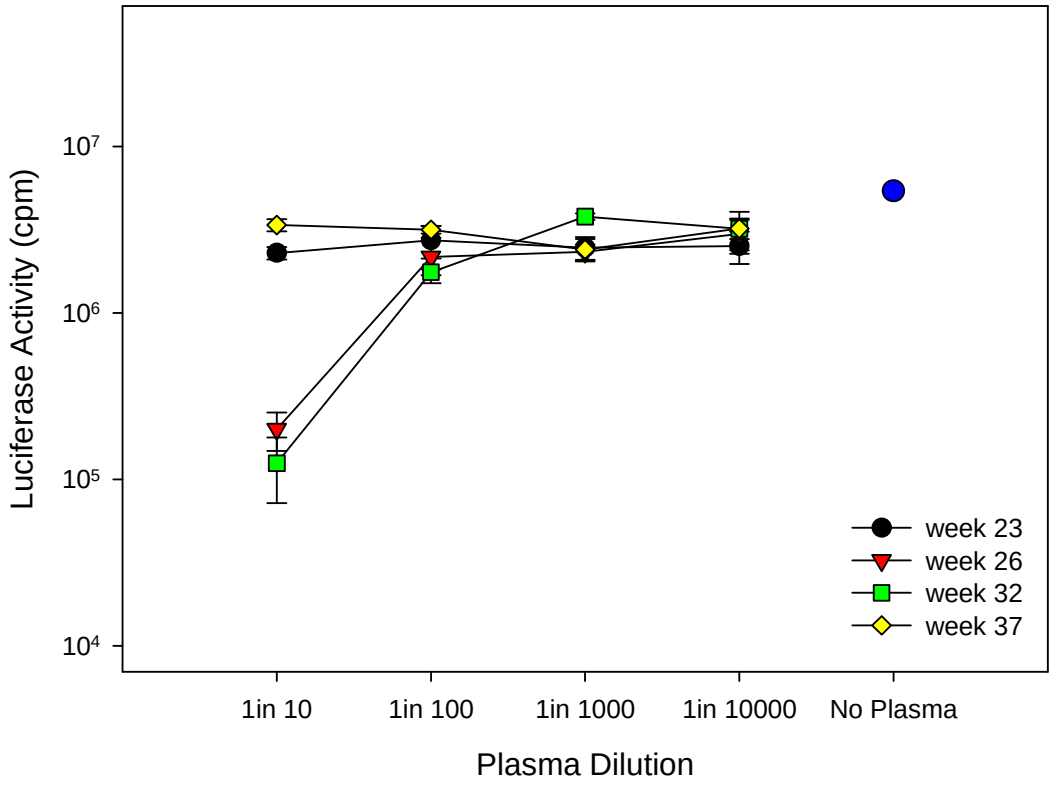

Figure 3.8

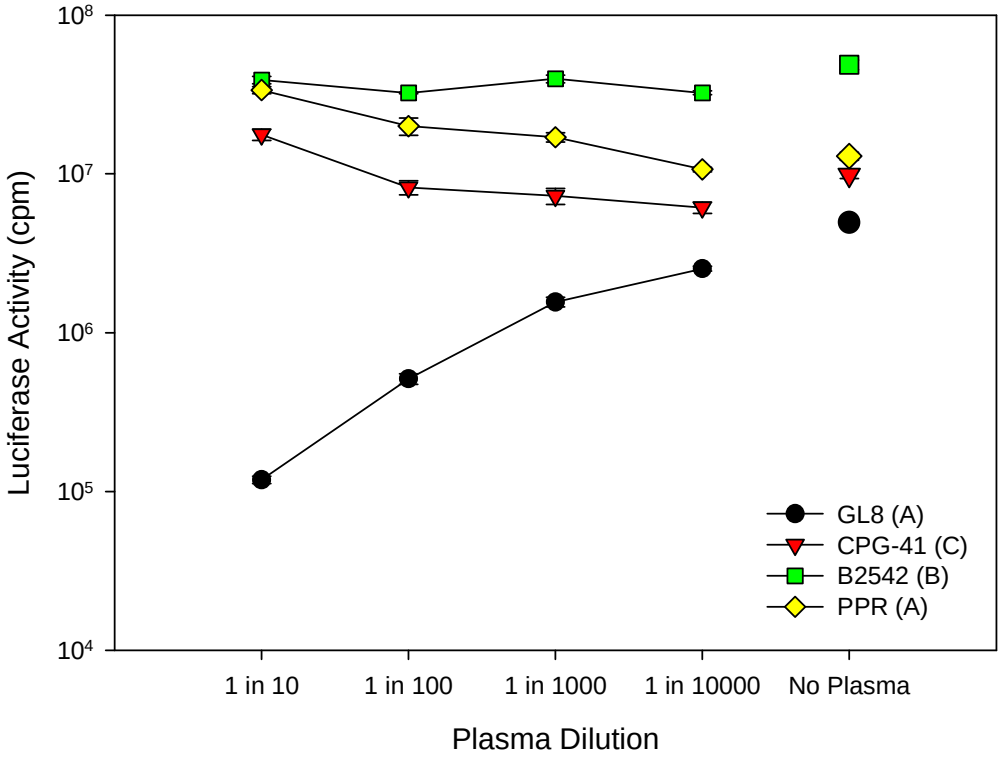
Luciferase Activity (cpm)
10^8
10^7
10^6
10^5
10^4
1 in 10
1 in 100
1 in 1000
1 in 10000
No Plasma
Plasma Dilution
GL8 (A)
CPG-41 (C)
B2542 (B)
PPR (A)

Figure 3.9

Appendix 4. A list of plasma/serum samples and pseudotypes used in this project

A) Plasma/serum samples

A411-A413: cats experimentally infected with molecularly cloned FIV-PET.
A414-A416: cats experimentally infected with molecularly cloned FIV-GL8.
Q253: a cat experimentally infected with molecularly cloned FIV-GL8.
169267-210101: cats naturally infected with FIV in the UK.
KNG1, KNG2, NG4, TOI1 and TOT1: cats naturally infected with FIV in Japan.

B) Pseudotypes

180638	primary subtype A pseudotype prepared in this project (UK)
171838	primary subtype A pseudotype prepared in this project (UK)
180260	primary subtype A pseudotype prepared in this project (UK)
182309	primary subtype A pseudotype prepared in this project (UK)
180140	primary subtype A pseudotype prepared in this project (UK)
178721	primary subtype A pseudotype prepared in this project (UK)
179200	primary subtype A pseudotype prepared in this project (UK)
206394	primary subtype A pseudotype prepared in this project (UK)
KNG1	primary subtype B pseudotype, previously prepared (Japan)
KNG2	primary subtype B pseudotype, previously prepared (Japan)
NG4	primary subtype B pseudotype, previously prepared (Japan)
TOI1	primary subtype B pseudotype, previously prepared (Japan)
TOT1	primary subtype B pseudotype, previously prepared (Japan)
TM2	subtype B pseudotype, previously prepared in the lab (Japan)
GL8	subtype A pseudotype, previously prepared in the lab (UK)
B2542	subtype B pseudotype, previously prepared in the lab (USA)
CPG-41	subtype C pseudotype, previously prepared in the lab (USA)
PPR	subtype A pseudotype, previously prepared in the lab (USA)
PET-KKS	subtype A pseudotype, previously prepared in the lab (USA)
0425	primary subtype A pseudotype, previously prepared (UK)
1419	primary subtype A pseudotype, previously prepared (UK)
0556	primary subtype A pseudotype, previously prepared (UK)
0827	primary subtype A pseudotype, previously prepared (UK)
M2	primary subtype B pseudotype, previously prepared (Italy)
Leviano	primary subtype B pseudotype, previously prepared (Brazil)

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