Studies towards the development of an FIV DIVA vaccine.

By Matthew Harris

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Author's Declaration

I declare that the work submitted in this thesis is my own work unless otherwise referenced. The work contained in this thesis has not been submitted for any other degree at the University of Glasgow or any other institution.

Matthew Harris

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Abstract

The introduction of Fel-O-Vax FIV in 2002 was the first commercially available lentiviral vaccine. Consisting of whole inactivated virus (WIV) and inactivated infected cells, vaccination lead to the production of a strong humoral and T-cell immune response. As FIV is a lentivirus, the viral genome must be integrated into the host genome and therefore leads to lifelong infection. For this reason, the detection of FIV specific antibodies has been used as a proxy for FIV infection and led to the development of several commercially available serology diagnosis kits. As vaccination leads to the development of FIV antibodies it is not possible to differentiate infected from vaccinated animals (DIVA) using conventional serology diagnosis assays. Several attempts have been made to DIVA FIV vaccinated cats, however no test can differentiate FIV vaccinated from infected animals and FIV uninfected and unvaccinated animals.

The data presented in this thesis demonstrates that it is possible to DIVA cats when vaccinated with a mutated chimeric virus representative of isolates circulating in the field. The identification and characterisation of a novel envelope glycoprotein, isolated from a client owned cat with broad neutralising antibodies to FIV, a unique potential N-linked glycosylation mutation at the apex of variable loop 2 revealed a neutralisation sensitive epitope. By swapping the *env* gene of a molecular clone of FIV-Glasgow8 with that of the novel field isolate, it was hypothesised that vaccinated with the chimaeric virus (FIV ΔV2) could elicit a broadly neutralising antibody response capable of protection against low does challenge with virulent UK primary isolate, FIV-Glasgow8. Additionally, by mutating the principal immunodominant (PID) of the transmembrane region of FIV ΔV2 from that of FIV to lion lentivirus subtype-E (LLV-E), it was proposed that the humoral immune response towards the wildtype sequence would be abolished, and recognise only LLV-E PID when plasma/sera were screened for reactivity to either PID sequence in a peptide ELISA format.

The pathogenesis of FIV ΔV2 revealed the rapid production of homologous neutralising antibodies that corresponded with a marked reduction in the proviral load. Although no heterologous neutralising antibodies were detected, it was thought that If the PID of the FIV ΔV2 virus were mutated to that of LLV-E (DIVA virus) and used as a whole inactivated

virus candidate vaccine in a prime boost regime, cats may elicit both homologous and heterologous neutralising antibodies whilst at the same time demonstrate sufficient reactivities to the two different PID sequences that it would be possible to DIVA cats solely by conventional serology techniques.

The DIVA virus did not afford protection against low dose challenge with FIV-Glasgow8 and appeared to enhance infection as all vaccinates became infected before controls. Additionally, no homologous or heterologous neutralising were produced, although the antibody response towards the PID sequences differed substantially so that DIVA could be performed on cats by conventional serology. Such was the utility of the PID peptide ELISA it was possible to differentiate infected from vaccinate cats and unvaccinated from uninfected cats, an observation that has never previously been reported.

Chapter 1 Introduction.

1.1 Discovery and origins

Feline immunodeficiency virus (FIV) was first isolated in 1986 from a group of sick cats housed in a cattery in Petaluma, California and was initially named feline T-lymphotropic lentivirus (Pedersen *et al*, 1987). Its classification as a lentivirus was based primarily on the viral tropism for primary and permanent T-lymphoblastoid cell lines, the Mg^{2+}- dependent reverse transcriptase as well as the protein structure and morphology (Yamamoto *et al*, 1988. Stephens *et al*, 1989).

1.2 Virus genome and structure.

Sequencing of the prototypic FIV isolate, FIV-Petaluma (FIV-PET) revealed that the genome comprised approximately 9400 base pairs (Talbot *et al*, 1989). Within the genome, three distinct open reading frames (ORF) encoding the major structural proteins (*gag*), polymerase (*pol*) and envelope (*env*) were identified. The genome is flanked at the 5' and 3' prime ends by long terminal repeats (LTRs) that have roles in promotor and enhancer activity (Figure 1-1).

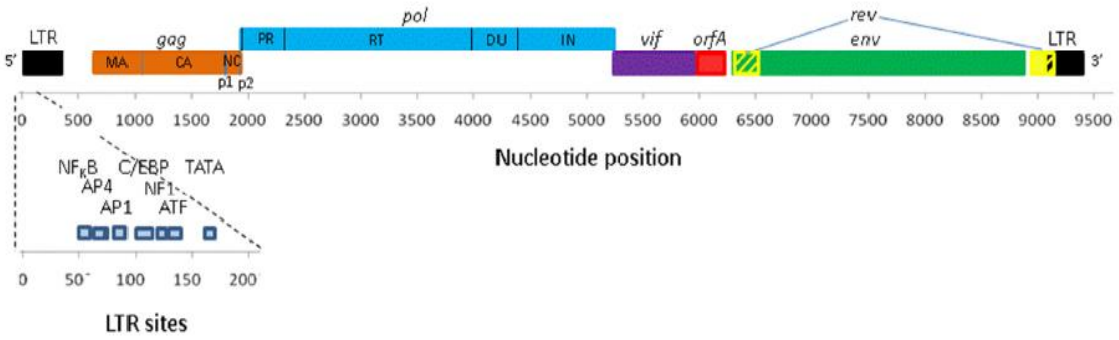


Figure 1-1. Genomic organisation of the FIV genome. The 3 major ORF are shown, indicating the proteins encoded. The transcriptional and enhancing binding sites are shown at the 5' LTR. Figure adapted from Kenyon and Lever, 2011.

The FIV virion is 105-125nm in diameter, with a spherical or ellipsoid shape (Pedersen *et al*, 1989). Characterisation of the protein content of mature virions revealed *gag*-derived proteins with estimated molecular weights of p24 (capsid, CA), p17 (matrix, MA), p10 (nucleocapsid, NC), and p55 (unprocessed Gag). A glycosylated protein with a molecular

weight of 130 kDa was shown to be processed into the envelope glycoprotein (Env) that comprises the surface unit (SU) protein (gp95) and the transmembrane domain (TM) (Steinman *et al*, 1990. Stephens *et al*, 1991. Verschoor *et al*, 1993). The structural morphology of FIV is represented in Figure 1-2. The *pol* gene encodes the reverse transcriptase (RT) enzyme as well as the protease (PR) and integrase (IN) proteins that are essential for virus replication and integration. Accessory proteins include OrfA, for which the exact role is unknown, the Vif protein that counteracts cellular restriction factors and Rev that facilitates nuclear export of spliced and unspliced viral RNA transcripts (Kenyon and Lever, 2011).

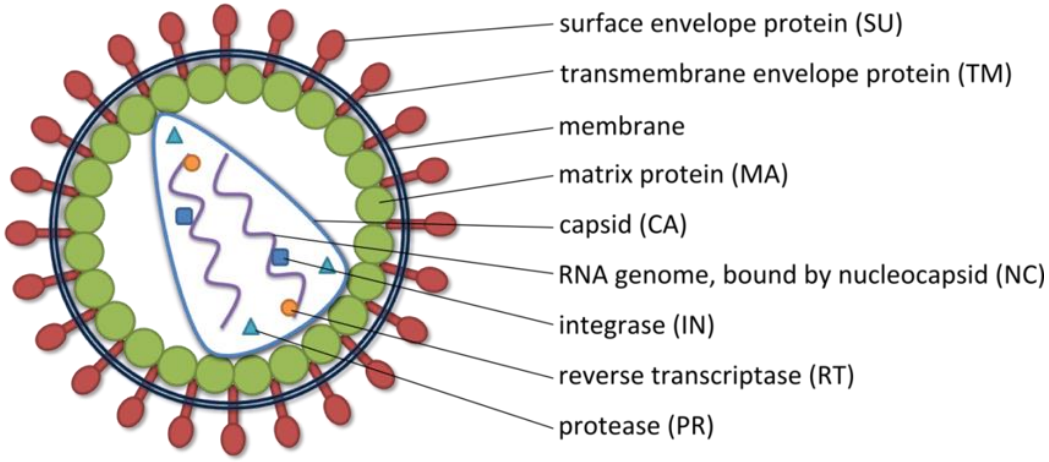


Figure 1-2. Structural morphology the FIV virion. The FIV virion contains 2 copies of the negative sense RNA genome. The SU and TM proteins facilitate virus entry in target cells where replication, integration, assembly and egress complete the virus life cycle (see below).

1.3 Virus Life cycle and replication.

FIV targets activated $CD4^+$ T lymphocytes as it utilises the T-cell activation marker CD134 as its primary receptor molecule (Shimojima *et al*, 2004). Upon binding of FIV Env, conformational changes occur within the Env protein that expose a cryptic epitope in the third hypervariable loop (V3) that facilitates binding with the co-receptor molecule CXCR4 (de Parseval *et al*, 2006). Fusion of the cellular and viral membranes allows the viral core to enter the cell where the initiation of reverse transcription begins. The reverse transcription is Mg^{2+} dependant and transcribes a positive DNA copy of the RNA genome

while it is still contained within the virion. The cDNA is then shuttled into the nucleus where it forms the pre-integration complex and becomes integrated into the host genome, a process facilitated by the *pol*-encoded IN protein. The integrated viral genome then acts as a template for the synthesis of the viral structural proteins. The Gag polyprotein, containing the MA, CA and NC proteins captures the viral genome through the signal sequence (Ψ) located in the NC (Lever, 2007) and is transported to the cellular membrane where MA facilitates the incorporation of Env into virions (Freed, 1998). Budding of the virus is facilitated by the unique FIV protein p2 (Luttge and Freed, 2010), after which maturation of the virion occurs. The virally encoded PR cleaves 9 separate proteins to give rise to a fully mature and infectious virus (Elder *et al*, 1993, Kohl *et al*, 1998).

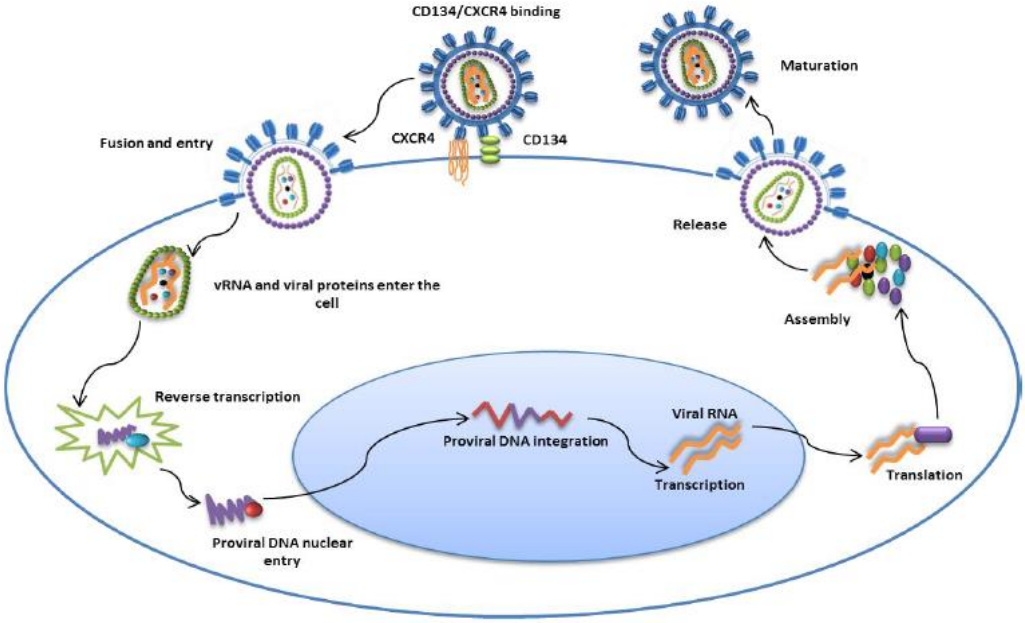


Figure 1-3. The lifecycle of FIV. A unique property of retroviruses is the integration of the viral genome in the host DNA resulting in latent, lifelong infection. These cells are virus DNA positive but viral RNA negative and so are invisible to immune surveillance (Murphy *et al*, 2012). Figure adapted from Beczkowski, 2012.

1.4 Epidemiology.

Based on the phylogeny of the *env* gene, FIV isolates can be classified into 5 distinct clades, A to E (Sodora *et al*, 1994. Kakinuma *et al*, 1995. Pecorara *et al*, 1996), with a sixth subtype F being isolated in New Zealand (Haywood *et al*, 2004). A distinct phylogenetic cluster has also been identified in Texas, which branches off from the clade B isolates (Weaver *et al*,

2004), however this appears to be restricted to Texas. Sero-epidemiological studies have revealed a wide range in the FIV prevalence around the world, from 2.5% in the US (Levy *et al*, 2006), 5.5% in Canada (Ravi *et al*, 2010), 23.3% in Japan (Nakamura *et al*, 2010), 8% in Australia (Norris *et al*, 2007), 12.7% in the UK (Hosie *et al*, 1989) and 3.2% in Germany (Gleich *et al*, 2009). Sick adult cats, male cats and cats with outdoor access are more likely to be infected, (Yamamoto *et al*, 1989, Ishida *et al,* 1989, Levy *et al*, 2006, Hosie *et al*, 1989), however free-roaming cats (especially stray or feral cats) are significantly more likely to be infected with FIV (Levy *et al*, 2006). In these studies a proportion of FIV infected cats displayed no clinical signs and appeared healthy. FIV has been isolated in every continent, with the exception of Antarctica, and the major clade distribution is shown in Figure 1-4.

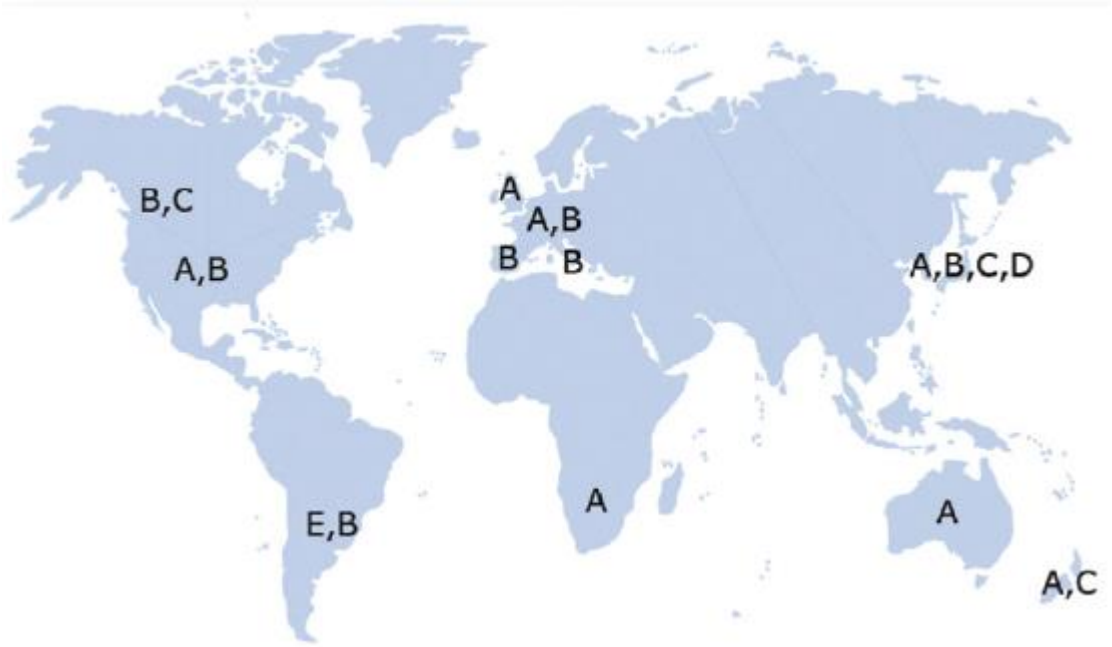


Figure 1-4. Distribution of the major FIV clades. Figure reproduced from Hosie *et al*, 2009. Clade A isolates are the most common circulating isolates of FIV and include, FIV-GL8, FIV-Pet, FIV-Dixon, FIV -AM19 and FIV-19k1 which make up a significant proportion of isolates used in pathogenesis and vaccine trials. Of note is the absence of FIV from large parts of Asia, however this is due to the lack of available data and not the absence of virus.

1.5 Pathogenesis.

Early studies indicated that the clinical signs associated with FIV infection included gingivitis/stomatitis, respiratory complications, emaciation and wasting as well as general lymphadenopathy, neurological signs and skin complaints (Hosie *et al*, 1989, Pedersen *et*

al, 1988 and 1989, Sparger *et al*, 1989, Ravi et al, 2010, Yamamoto *et al*, 1989, Muirden 2002, Ishida *et al*, 1989). Many of the clinical signs reported in FIV infection arise from secondary opportunistic infections, as infection with FIV alone does not induce severe clinical signs, with the rare exception of neurological disorders (Hosie *et al*, 2009. Hartmann, 2012). The primary or acute stage of infection with FIV typically results in sero-conversion within 4-6 weeks (Figure 1-5) and a peak in viraemia approximately 5 – 10 weeks post infection (Hosie *et al*, 2002 & 2005, Dunham *et al*, 2006). Infection leads to a gradual depletion of $CD4^+$ T cells (Hofmann-Lehman *et al*, 1996) as the virus targets cells expressing the T cell activation marker CD134 (Shimojima *et al*, 2004). This gradually erodes the foundation of the adaptive arm of the immune system and, as the numbers of $CD4^+$ T cells decrease, infected cats enter the silent or asymptomatic stage of infection (Figure 1-5). Cats may appear healthy during the asymptomatic stage, with the only indicators of infection being the persistence of FIV antibodies and the continuing gradual decline in $CD4^+$ T cell numbers. During the asymptomatic stage of infection, the $CD8^+$ T cell compartment expands, resulting in an inversion of the $CD4^+$:$CD8^+$ T cell ratio (Willett *et al*, 1996). Cats can remain in the asymptomatic stage of infection for weeks or years, depending on the virulence of the infecting strain of FIV (Diehl *et al*, 1995, Addie *et al*, 2000).

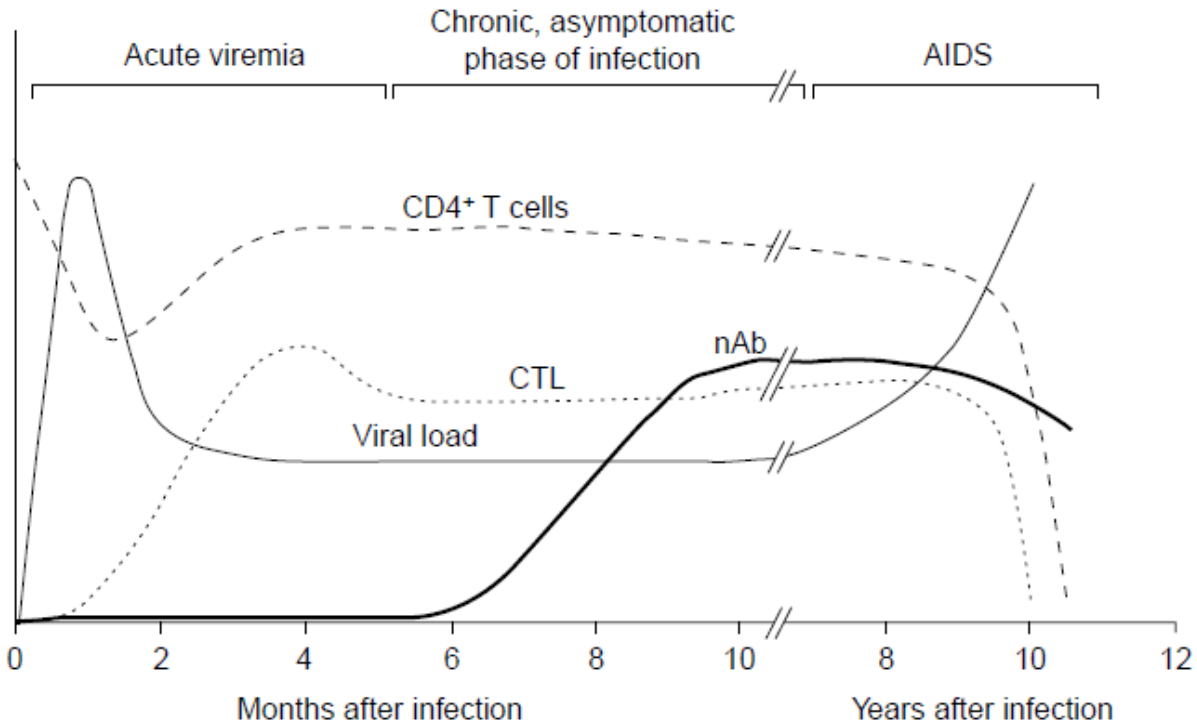


Figure 1-5. Pathogenesis of FIV. The initial peak viral load declines to the set point, coinciding with an increase in the cellular immune response. The development of a neutralising antibody (nAb) response is shown, however the time course for the development of nAb varies between different isolates. Most cats will develop nAb, although it has been demonstrated experimentally that some cats do not produce a significant nAb response. Figure from Ferrantelli and Ruprecht 2002.

The secondary phase of infection is characterised by pronounced immune dysfunction, including a significant reduction in the level of T cell proliferative responses to stimulation (Torten *et al*, 1991) and polyclonal B-cell activation leading to hypergammaglobulinemia (Flynn *et al*, 1994). The pronounced immunodeficiency induced by FIV predisposes cats to secondary opportunistic infections that can be fatal (de Rozieres *et al*, 2004. Diehl *et al*, 1995. Pedersen *et al*, 1987). Although one would expect infection with FIV to substantially decrease the life expectancy of a cat, longitudinal studies have demonstrated that FIV infection does not significantly shorten a cats' survival time (Addie *et al*, (2000). Ravi *et al,* (2010). Beczkowski *et al*, (2015).

The pathogenesis and tropism of FIV is also regulated by the interaction of the viral spike (gp120) with the primary receptor of FIV, CD134. As infection progresses, FIV is able to

infect diverse cell types including B-cells and $CD8^+$ T cells (English *et al*, 1993. Dean *et al*, 1996). As the primary receptor for FIV is CD134, this change in tropism must be facilitated by altered usage of the primary receptor by the viral spike. Willett *et al*, (2006) was able to demonstrate that at least two different domains within the CD134 receptor facilitate CDR-2 independent or CDR-2 dependant entry into the cell (Cysteine rich domain) (Figure 1-6).

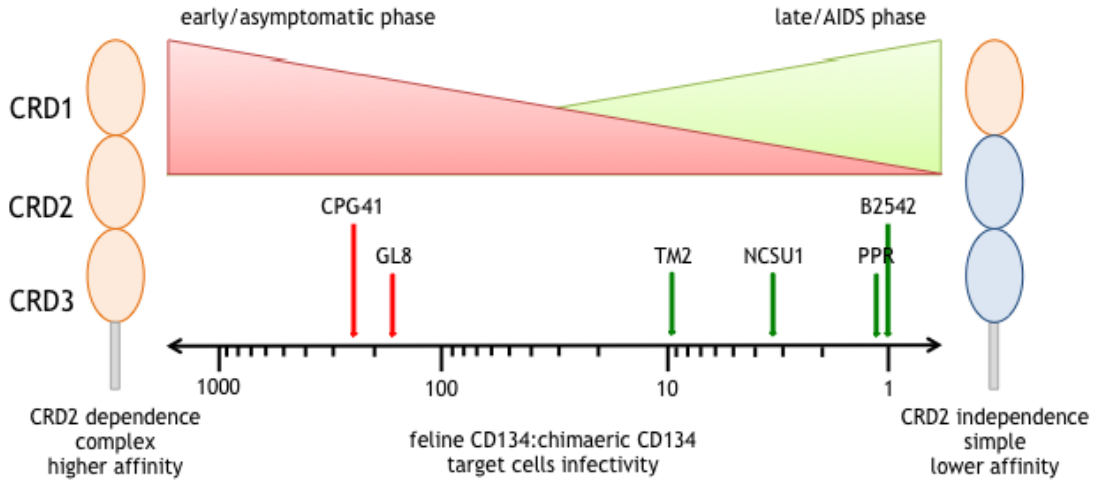


Figure 1-6. Differential utilisation of CD134 as FIV infection progresses. Oval shapes represent cysteine rich domain 1 to 3 (red feline, blue human) used in the studies performed by Willett *et al* (2006) and Beczkowski *et al* (2014) to decipher the receptor utilisation of various FIV strains. Dependence on CDR-2 was determined by comparing the infectivity of FIV isolates on 2 different cell lines, expressing either feline CD134 (Orange, left) or chimaeric (human/feline CD134, right). The x axis represents the infectivity ratios of the 2 cell lines where CDR-2 independent isolates (CPG41, GL8) cannot infect cells expressing chimaeric CD134, resulting in a higher ratio (red arrows). Isolates that are CDR-2 independent are able to infect both cell lines equally well, resulting in a lower infectious ratio. The green and orange triangles represent the theoretical quantities of CDR-2 dependant and independent variants throughout the clinical course of FIV infection. Figure from Hosie *et al* (2017).

During the early stages of infection, the predominant circulating virus requires a strict interaction with both CDR-1 and CDR2 domains of CD134 for entry into the cell (CDR-2 dependant), whereas the predominant circulating viruses present during the late stages of infection require only CDR-1 for cell entry (CDR-2 independent). The pathogenesis of such relaxed receptor utilisation was further characterised by the discovery that CDR-2

independent strains of virus were predominantly associated with cats that had deceased since the last time of sampling or were defined as clinically healthy or sick based on a $CD4^+$ T-cell of <350 cells/µl respectively. CDR-2 independent strains of virus were almost exclusively isolated from cats that had deceased or were classed as clinically sick since the last time of sampling (Beczkowski *et al*, 2014). Statistical analysis showed that the emergence of CDR-2 independent strains correlated with a $CD4^+$ T-cell count >350 cells/µl and demonstrates a correlation between the emergence of CDR-2 variants and declining clinical status. Whether the emergence of CDR-2 independent isolates of FIV is a cause or consequence of disease progression or immune system pressure remains to be established.

1.6 Diagnosis.

Since FIV integrates into the host genome, infection with FIV is persistent. Infection with FIV elicits strong cellular and humoral immune responses and the detection of FIV specific antibodies are used to diagnose FIV infection. Except for the research setting, detection of virus is rarely used, because virus isolation requires extensive expertise/materials, is time consuming and is not suited to large-scale screening of cat samples (Hosie *et al*, 2009. Crawford *et al*, 2007). Other problems that can arise when utilising virus isolation as a primary screening assay include the quality and type of sample and the requirement for a second confirmatory assay to be conducted. However, anti-FIV antibodies can be detected in plasma throughout infection. A complete description of the serological and molecular methods used for the diagnosis of FIV infection follows.

1.6.1 Measurements of diagnostic accuracy

1.6.1.1 Sensitivity and specificity

A common measure of the accuracy of a diagnostic assay is the sensitivity (proportion of true positive samples correctly identified) and specificity (proportion of true negative samples correctly identified). These values are calculated by the formula detailed below and the figures expressed as either a percentage or a proportion ranging from zero to 1.

Sensitivity: Number of positive samples detected/ total number of true positive samples

Specificity: Number of negative samples detected/ total number of true negative samples

When candidate diagnostic assays are evaluated it is important to generate a parallel data set using an accepted reference assay or 'reference standard'; for FIV diagnosis either immunostaining or virus isolation have been used as reference standard tests should identify all true positive and negative samples within a population (Hosie *et al*, 2009, Hartmann *et al*, 2007, Levy *et al*, 2004, Crawford *et al*, 2005). Since no method is 100% accurate, an accepted method is utilised that detects a high percentage of both negative and positive samples. Therefore, if a diagnostic assay has a sensitivity of 99% (or 0.99), it would be expected that 1 out of every 100 samples tested would result in a false negative result. If the same assay has a specificity of 99%, then 1 out of every 100 samples tested would result in a false positive result. The interpretation of such analyses should take into consideration the prevalence of FIV in the population/local area since this impacts the positive predictive value. In areas where the prevalence is low, for example 1%, then 1/100 samples would test positive (true positive). If the test specificity is 99%, then another 1/100 samples will test positive (false positive), so that the positive predictive value of a positive result is only 50%. Hence positive results from areas of low prevalence or from cats not at risk of FIV infection should always be confirmed using a confirmatory assay (Hosie *et al*, 2009). As different research groups utilise different reference standards, methodologies and cut-off values for diagnostic assays, it is often difficult to compare values for sensitivity and specificity from different data sets (Reitsma *et al*, 2005).

1.6.1.2 Receiver operator curve (ROC) analysis.

ROC analysis is used to compare the sensitivity and specificity data derived from studies where different reference standards or methodologies have been employed. ROC analysis converts each pair of sensitivity and specificity figures into a single measure of accuracy, the odds ratio (Reitsma *et al,* 2005. Lalkhen and McCluskey, 2008). The odds ratio calculates the odds of a sample testing positive if it had been exposed compared to the odds of the same result occurring if the sample had not been exposed (Lalkhen and McCluskey, 2008). A disadvantage of using ROC analysis to determine the odds ratio is that estimates of specificity and sensitivity are not directly available (Reitsma *et al*, 2005).

1.7 Serological diagnosis.

1.7.1 Immunofluorescence antibody assay (IFA)

The IFA assay utilises fixed virus infected cells as the source of antigen recognised by anti-FIV antibodies, visualised using a fluorescently labelled anti-cat Ig secondary antibody. Variations in the assay include the cell line used to propagate the virus, the isolate of virus used to infect the cells, the ratio of virus infected cells to uninfected virus cells, the fluorophore conjugated to the secondary antibody and the dilution of plasma sample tested. Although the sensitivities of IFA primary screening assays are typically high (94.7-100%), the observed rate of false positive results has led to lower specificities (90-100% (Levy *et al*, 2004. Reid *et al*, 1992). As the assay utilises fixed, infected cells, it is hypothesised that non-specific, cross-reactive antibodies could bind cellular antigens either on the cell membrane or incorporated into the viral envelope. Although IFA is routinely used as a screening test, few studies have compared the performance of IFA as a diagnostic tool against a reference standard test, such as virus isolation or immunostain analysis. However, it was possible to examine the performance of IFA from studies in which samples had undergone preliminary screened by IFA (Verschoor *et al*, 1993. Furuya *et al*, 1992). It appears that IFA is a useful screening test to identify samples that are clearly seropositive or seronegative, with the caveat that samples from cats in either the acute or terminal stages of FIV infection might be mis-diagnosed. Cats in the terminal stages of infection might test seronegative as a result of either B-cell exhaustion or a lack of available antibody following antigen-antibody complex formation in cats with high virus loads. In the early, acute stage of infection, delayed sero-conversion might also result in false negative results. Indeed, 14/206 samples (6.8%) tested inconclusive using IFA (Verschoor *et al,* 1993) and a similar percentage of inconclusive samples was reported in a study that compared IFA and immunostain analysis for the diagnosis of HIV-1 in people (73/999 samples (7.3%), Carlson *et al*, 1987). This demonstrates that non-specific, cross-reactive antibodies are not restricted to feline plasma or the isolates and cell lines used in FIV IFA.

1.7.2 Immunostain analysis.

Although immunostain analysis has been reported to have 98% sensitivity and specificity (Levy *et al*, 2004), problems can arise when using immunostain analysis for sero-diagnosis. The lack of a visible SU protein band on immunostain, despite the presence of anti-SU antibodies has been reported (Hosie and Jarrett, 1990, Verschoor *et al*, 1993). It was concluded that the FIV Env might be mechanically torn from the virion during the ultracentrifugation step of virus purification. This problem was circumvented by using infected cell lysates (Hosie and Jarrett, 1990), although this resulted in non-specific staining by antibodies cross-reacting with cellular proteins, requiring samples to be screened also against a lysate of uninfected cells (O'Connor *et al*, 1989). Nevertheless, immunostain analysis is often utilised as a confirmatory test and as a "reference standard" reference test (together with virus isolation) for assessing the sensitivity and specificity of serological diagnostic assays (Hosie *et al,* 2009. Crawford *et al*, 2005. Levy *et al*, 2004. Andersen and Tyrrell, 2004).

1.7.3 Enzyme linked immunoabsorbent assay (ELISA)

The first commercially available FIV ELISA was the PetCheck Feline T-Lymphotropic Lentivirus Antibody Test Kit (IDEXX Corporation, Portland, Maine, USA). This ELISA was developed by O'Connor et al (1989) and utilised disrupted virus, grown in Crandell feline kidney cells (Crfk cells) as the capture antigen. The ELISA assay identified all 141 sero-positive samples tested and the results were confirmed by immunostain analysis (antibodies recognising both p24 and p17 required for confirmation) and radioimmunoprecipitation (RIPA) assays to detect antibodies recognising gp120. Only 3/141 discrepant results were identified, with samples testing ELISA positive but inconclusive on immunostain analysis; these samples were defined as positive, based on the precipitation of anti-gp120 antibodies in a RIPA assay. However, when FIV sero-positive plasma samples were tested by both immunostain analysis and PetCheck ELISA, false positive rates of 26.5% (Hosie and Jarrett, 1990) and 10% (Reid *et al*, 1991) were reported. It was hypothesised that these false positive results might have been associated with the detection of antibodies that cross-reacted with cellular antigens derived from the Crfk cell line used to propagate the virus. Furthermore, the use of whole virus as the capture antigen

might have led to a reduction in the assay sensitivity, compared to the use of highly purified viral proteins in immunostain analysis (Rimmelzwaan *et al*, 1994).

The use of viral proteins purified from bacterial or insect expression systems consistently outperformed disrupted whole virus as a source of capture antigen in ELISA tests, reducing the numbers of false positive samples compared to the PetCheck ELISA (Verschoor *et al*, 1993. Furuya *et al*, 1992. Rimmelzwaan *et al*, 1994. Reid *et al*, 1991). However, it was recognised that the selection and processing of the viral proteins must be carefully considered. Expression of FIV r-Gag in Sf9 *Spodoptera frugiperda* cells produced a protein of approximately 50kDa, analogous to the native unprocessed Gag protein. When used as the source of capture antigen in an ELISA, only 2/93 samples reacted to the protein, although all samples had tested positive by immunostain analysis and contained antibodies recognising the core structural proteins (Verschoor *et al*, 1993). Conversely, FIV Gag protein expressed in *Escherichia coli* cells could be used to detect antibodies in 14/17 (Rimmelzwaan *et al*, 1994), 61/65 (Furuya *et al*, 1992) or 78/78 (Reid *et al*, 1991) FIV sero-positive samples. The highly sensitive ELISA reported by Reid *et al* utilised the pGEX-FIV p24 expression vector containing the ORF of p24 encoded by the *gag* gene. However, anti-p24 antibodies might not be detectable from samples collected during the early or terminal stages of FIV infection (Furuya *et al*, 1991) and the positive FIV sero-status of samples could be confirmed by the detection of anti-Env (gp120) antibodies by radioimmunoprecipitation with [^{35}S] L-methionine and [^{35}S] L-cysteine labelled antigen in the absence of p24 antibodies, a finding confirmed by Rimmelzwaan *et al* (1994). It was concluded that anti-Env antibodies appeared early in infection, and the delayed production of anti-p24 antibodies could lead to false negative results (Verschoor *et al*, 1993. Rimmelzwaan *et al*, 1994. Furuya *et al*, 1991). Reports by Reid *et al* (1991) and Verschoor *et al* (1993) demonstrated that the incorporation of two different viral antigens as the source of capture antigen increased both the sensitivity and specificity. However, the high variable Env amino acid sequence between FIV clades suggested that it might be difficult to identify a conserved Env epitope for inclusion (Bachmann *et al*, 1997).

Since anti-Env antibodies could be detected before anti-p24 antibodies, attempts were made to include a conserved, antigenic Env epitope in serological diagnostic assays. By screening fragmented Env libraries with FIV sero-positive samples, an immunodominant

domain within the transmembrane (TM) region of Env was identified (Pancino *et al*, 1992). FIV sero-positive plasma samples raised against 5 different isolates of FIV contained antibodies that recognised the immunodominant epitope, comprising 5 amino acids flanked by 2 cysteine residues. This epitope was structurally constrained and an unlikely site of non-synonymous substitution (Pancino *et al*, 1992), findings that were independently reproduced, in part, by de Ronde *et al* (1993). Two independent studies assessed the suitability of the immunodominant domain as a candidate capture antigen for the sero-diagnosis of FIV. The first study demonstrated that FIV sero-positive plasma samples contained high titres of antibodies recognising the immunodominant epitope that developed as early as 2 weeks post infection, with titres reaching a peak at 6-8 weeks post infection and being maintained for at least 97 weeks (Fontenot *et al*, 1992). This study also demonstrated a lack of non-specific antibody binding in samples from transiently viraemic and persistently viraemic FeLV infected cats, demonstrating the virus-specific nature of the epitope (Fontenot *et al*, 1992). In a follow-up study, the synthetic peptide CNQNQFFCK, representing the immunodominant epitope, was used in ELISA tests to examine FIV sero-positive plasma samples raised against at least 11 different FIV isolates from 4 geographically distinct regions of Europe. In this study, 100% of plasma samples collected from cats naturally and experimentally infected with FIV recognised the synthetic TM peptide, with higher titres than had been observed against any other individual or combined Env antigen(s) (Avrameas *et al*, 1993). This TM peptide ELISA accurately determined the true FIV sero-status of 47 inconclusive samples and outperformed the commercially available PetCheck ELISA when testing inconclusive samples. Use of the immunodominant TM epitope peptide allowed the detection of FIV antibodies, on average, 7 days sooner than the PetCheck ELISA (Avrameas *et al*, 1993). The sensitivity of the ELISA was further increased by extending the sequence of the immunodominant peptide from 9 to 17 amino acids in length (CNQNQFFCK vs QELGCNQNQFFCKVPSA). These changes increased the assay sensitivity to 99.5% and the specificity to 100% (Sibille *et al*, 1995).

Therefore, the characterisation of the major antigenic determinants of FIV greatly reduced the number of false positive results. This is important because, although the identification of FIV infection does not justify euthanasia (Hosie *et al*, 2009), in situations where resources are limited, such decisions could potentially be based solely on initial screening profiles (Crawford *et al*, 2005. Ravi *et al*, 2010). Where specificities of 57-100% (O'Connor *et al*,

1989, Levy *et al*, 2004, Reid *et al*, 1992, Hosie and Jarrett, 1990) were observed in early screening studies utilising whole virus as the source of capture antigen for ELISAs, the lower limit of specificity was greatly increased to values >95% by incorporating FIV recombinant proteins and the immunodominant TM peptide into ELISA tests (Sibille *et al*, 1995. Verschoor *et al*, 1993). This increased the sensitivity and specificity of diagnostic tests and also provided the tools necessary to study the development of the serological response against specific viral proteins in future vaccine and pathogenesis studies.

1.8 Molecular diagnosis of FIV.

The use of molecular techniques to diagnose FIV infection is recommended in cases where sero-diagnosis has been unsuccessful, or the use of serological assays is confounded, as in samples from vaccinated cats (Uhl *et al*, 2002). Molecular diagnostic tests for FIV infection were developed to aid the interpretation of serological tests and to overcome the limitations of the detection of antibodies in kittens (Crawford and Levy, 2007. Crawford *et al*, 2005. Nichols *et al*, 2016).

1.8.1 PCR and RT-PCR

The strict requirement for the proviral genome to integrate into the host genome leads to lifelong infection with FIV. Therefore, using the correct combination of primers/primers and probe, PCR and RT-PCR are useful and reliable diagnostic tools, having a high degree of specificity from complementary primers and probes and a relatively high assay throughput (Leutenegger *et al*, 1999). Several studies have evaluated PCR for use as a diagnostic assay in comparison to ELISAs and point of care (POC) kits. Initial results displayed considerable disparity between PCR results and those achieved with ELISAs/POC kits. Bienzle *et al* (2004) reported the outcomes of PCR testing when DNA from 10 mutually exclusive FIV sero-positive cats, as determined by ELISA and POC kits, was sent to 3 different laboratories. One laboratory (a research laboratory) detected FIV provirus in 10/10 samples, whereas two commercial laboratories detected FIV provirus in only 50% and 80% of the samples. A similar result was obtained when DNA samples from 10 concordant FIV sero-negative samples were tested by the same three laboratories (10/10

samples were classified as FIV negative by the research laboratory but only 70% and 90% of the samples were classified as FIV negative by the 2 commercial laboratories). The lack of sensitivity and specificity was further highlighted in this study by the detection of FIV provirus is three DNA samples obtained from healthy dogs, living in cat-free houses.

The commercially available PCR, FIV RealPCR™ Test (IDEXX Laboratories Inc), performed better; of 168 samples testing positive by POC ELISA (SNAP® FIV/FeLV Combo Test, IDEXX laboratories Inc) a 93% concordancy (156/168 results) was observed, with a calculated Kappa value of 0.87 (Nichols *et al*, 2016). Comparing the PCR results to virus isolation as the reference standard, the FIV RealPCR™ test gave 4 false positive results and one false negative result (Nichols *et al*, 2016).

RT-PCR has been reported to be more sensitive and specific than PCR for proviral DNA. The measurement of the fluorescence signal generated by the displacement of the hybridised probe essentially removes the inclusion of non-specific PCR product in data analysis. However, the use of 3 complementary hybridising sequences raises the possibility of generating false negative results if the target sequence is not complementary to the primer and probe (Nichols *et al*, 2016). In two studies that assessed the performance of RT-PCR, sensitivity estimates of 92.3% and 79.3% were reported, with specificity estimates of 99% or 100% respectively (Pinches *et al*, 2007. Ammersbach *et al*, 2013). However, the reference standard assays used in these studies were not those generally accepted as reference standards (virus isolation or immunostaining) (Hosie *et al*, 2009, Hartmann *et al*, 2007, Levy *et al*, 2004, Crawford *et al*, 2005) and so the results should be interpreted with caution. In a study comparing conventional PCR with RT-PCR, with all results confirmed by virus isolation (Crawford and Slater 2005), samples from 124 cats were tested (102 SPF and 22 naturally infected cats, infected with a range of isolates from FIV clades A, B and C) to evaluate the performance of three laboratories offering PCR as a commercial diagnostic test. The agreement between the PCR results and virus isolation ranged from 58-90%, with the PCR assay performing better in regards to specificity than sensitivity.

Theoretically, PCR testing should offer the ideal balance of sensitivity and specificity. The requirement for primer/template complementarity required for hybridisation and subsequent rounds of template amplification should eliminate false positive results. The

ability to optimise PCR assays and the inclusion of degenerate primers capable of hybridising with FIV isolates from all clades (Wang *et al*, 2010) should increase the test sensitivity. However, the FIV genome displays high levels of plasticity and the divergence within and between clades has been reported to be as high as 26% when based on *env* and *gag* phylogeny (Steinrigl *et al*, 2003, Bachmann *et al*, 1997). Good primer and probe design should counteract, to some degree, the divergence reported in the FIV *gag* genome when screening samples using RT-PCR. However, without confirmatory sequencing when screening samples by conventional PCR, there is a risk of false positive results occurring by non-specific hybridisation and amplification, with results susceptible to operator error and bias when FIV status is determined by visually sizing the amplified PCR product (Crawford and Slater, 2005). Additionally, RT-PCR/PCR assays have not achieved the levels of sensitivity demonstrated with serological diagnostic assays (Sibille *et al*, 1995. Hartmann *et al*, 2007), with the sole licensed diagnostic PCR assay for FIV (FIV RealPCR™, IDEXX) having a sensitivity of 80.5% and specificity of 99.9% (http://www.idexx.com.au/pdf/en_au/smallanimal/education/realpcr-test-for-fiv.pdf). Recently it was reported that samples that tested negative for FIV provirus using this licensed PCR assay tested positive for FIV antibody using three commercially available POC kits (Westman *et al*, 2015). Although re-sampling and re-testing of the animals resulted in positive PCR results, these data demonstrates the reliability of current serological assays.

1.9 Point of care test kits.

Point of care (POC) test kits combine ELISA and lateral flow technology (Koivunen and Krogsrud, 2006). Generally referred to as lateral flow immunoassays, the detection and recognition of the analyte is exclusively facilitated by antibody, whether monoclonal or polyclonal (Koczula and Gallotta, 2016). Many POC kits have been developed for the serological diagnosis of FIV infection over recent years (Hartmann *et al*, 2007). Although the POC test kits have been individually evaluated, few independent studies have compared the performance of several POC test kits using the same sample set, and utilising a robust confirmatory reference standard assay. Hartmann *et al* (2007) evaluated the performance of seven POC kits that utilise a combination of FIV antigens and methodologies. Sensitivity values ranged from 92.6-100% and specificity values ranged

from 99-100% (Table 1-1), indicating that the POC test kits are suitable for in-house diagnosis of FIV. Both of the lateral flow ELISAs (SNAP® Combo Plus and PetChek® Plus anti-FIV) performed better than other POC kits that utilised immunochromatography (Griessmayr *et al*, 2007).

	Witness®	SNAP® Combo Plus‡	FASTest®	DUO Speed®	Virachek®	PetChek® Plus anti-FIV
Assay type*	LFIC	LF ELISA	LFIC	LFIC	LFIC	LF ELISA
Antigen used†	TM peptide	TM peptide, r-p17,r-p24	TM peptide	TM peptide	p24 peptide	r-p17,r-p24
Number of samples/invalid result	535/2	535/6	535/3	535/6	535/3	535/1
Calculated number of samples.	533	529	532	529	532	534
Number of test positives/true positives	55/52	55/52	57/53	57/52	51/50	52/52
Number of test negatives/true negatives	478/475	474/474	475/473	472/470	481/477	482/479
Number of false positives/negatives	3/3	3/0	4/2	5/2	1/4	0/3
Sensitivity/specificity (%)	94.5/99.4	100/99.4	96.4/99.2	96.3/99.0	92.6/99.8	94.5/100
PPV/NPV (%)	94.5/99.4	94.5/100	93.0/99.6	91.2/99.6	96.1/99.2	100/99.4

Table 1-1. Comparison of performance of POC kits. * LFIC = Lateral flow immunochromatography, LF ELISA= Lateral flow enzyme linked immunosorbent assay. † TM peptide= Immunodominant domain of TM. Most will have additional residues added 5' or 3' to the nano-peptide CNQNQFFCK, r-p17 = recombinant p17, r-p24 =recombinant p24, p24 peptide = propriety peptide carrying an immunodominant epitope located with the p24 protein. ‡ = SNAP Combo plus is sold only in Europe whilst the SNAP Combo is sold in Australia, New Zealand and America. The SNAP Combo contains only TM peptide and r-p24. Squares in red highlight the lowest performance figures whilst green squares highlight the highest performance figures. Figure adapted from Griessmayr *et al/*Hartmann *et al*, 2007.

POC kits have been shown to be reliable and reproducible, with tests using lateral flow technology having sensitivity values ranging from 91.2-100%, and specificity values ranging from 99.2-100%. It is theoretically possible to achieve highly accurate FIV sero-diagnosis by testing samples using two different kits (eg SNAP Combo Plus and PetChek Plus anti-FIV). Given the low cost of development, ease of use and production and the high reproducibility of the results of such tests (Koczula and Gallotta, 2016), POC kits are used widely for the in-house diagnosis of FIV infection.

1.10 Vaccination

A number of vaccine trials have been conducted to evaluate efficacy to protect cats against FIV infection. Different vaccine strategies, including recombinant vaccines, DNA vaccination, immune stimulating complexes (ISCOMS), subunit vaccines, whole inactivated virus (WIV) and infected inactivated cell vaccines (ICV) have shown variable results, from enhancement of infection to complete protection against both homologous and heterologous challenge (table 1-2 to table 1-8). Little success has been achieved with conventional vaccine strategies, although limited success has been achieved against homologous challenge using DNA vaccination, albeit inferior to WIV (Hosie *et al*, 1998 & 2000). Protection against homologous challenge, that is challenge with the same virus isolate as the vaccine strain, has consistently been shown to be easier to achieve than challenge with heterologous isolates that differ from the vaccine strain (Yamaoto *et al*, 1991. Hosie *et al*, 1995. Pu *et al*, 2001. Matteucci *et al*, 1996. Bishop *et al*, 1996). One of the main obstacles in eliciting heterologous protection against FIV infection is the divergent nature of the FIV envelope glycoprotein. Based on phylogeny of the *env* gene, FIV can be classified into 5 distinct clades (Figure 1-7), displaying considerable envelope amino acid sequence divergence (Sodora *et al*, 1994. Kakinuma *et al*, 1995. Pecorara *et al*, 1996). FIV therefore displays isolate specific neutralisation epitopes explaining, in part, the difficulty in achieving heterologous protection.

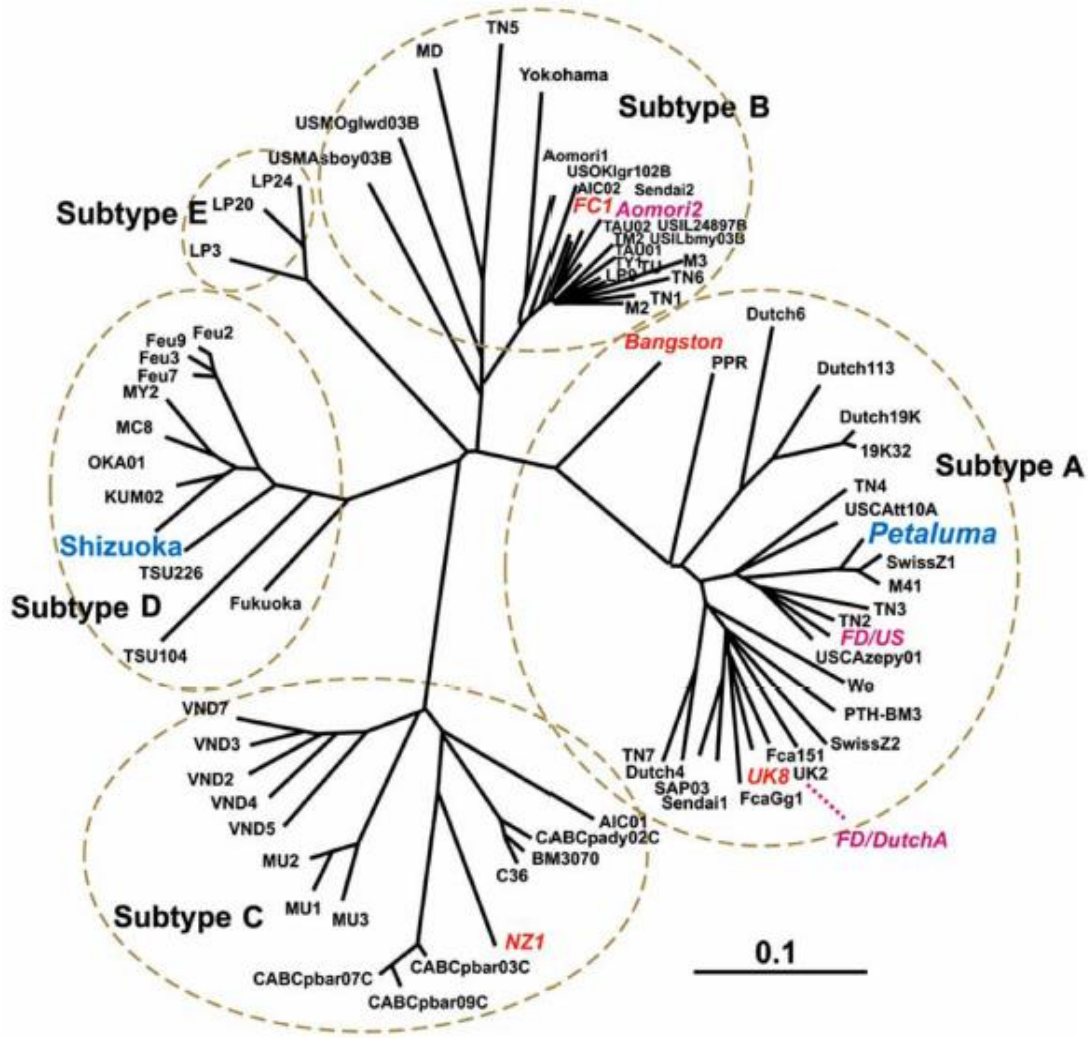


Figure 1-7. V3-V5 env phylogeny of 80 FIV isolates including vaccine and challenge strains. The FIV isolates that constitute the FIV vaccine (Fel-O-Vax FIV) are shown in blue, whilst challenge viruses are depicted in red and lavender. Any isolate depicted in italics has been utilised as a challenge virus during clinical trials. Isolates highlighted in lavender were used as challenge virus in trials funded by the NIH whilst isolates highlighted in red were used as challenge virus in trials funded by commercial companies. FIV isolates can be classified in to one of 5 clades (A-E) and thus display significant genetic diversity. Taking into account the global distribution of FIV isolates (figure 1-4), the identification of a single isolate that can protect against all circulating strains and recombinants is challenging. Figure from Yamamoto *et al*, 2010.

The most successful strategy utilised WIV derived from long term progressor cats as the candidate immunogens (Yamamoto *et al*, 2010). The prototype of the commercially available Fel-O-Vax FIV (Pu *et al*, 2001) demonstrated protection against low dose

homologous challenge, low dose intraclade heterologous challenge as well as protection against a low dose challenge with a distinct, heterologous isolate (Pu *et al*, 2001 & 2004. Yamamoto *et al*, 1993. Kusuhara *et* al, 2005). Similar results have been obtained using ICV, although protection against heterologous challenge was only shown following vaccination with a dual subtype vaccine containing immunogens derived from two diverse strains of FIV (Matteucci *et al*, 1996 & 1997, Bishop *et al*, 1996, Hohdatsu *et al*, 1997). After the use of 5000 laboratory cats over 14 years of vaccine testing, a commercial FIV vaccine (Fel-O-Vax FIV) was released in the US in 2002, based on two, yearlong efficacy studies using a clade A challenge virus that differed to the two vaccine strains by 9 and 20 % at the Env amino acid sequence level (Pu *et al*, 2004). Consisting of 50µg FIV-Petaluma and FIV-Shizuoka WIV and 1.5 – 2.5 x 10^7 inactivated infected Fet-J cells, supplemented with 5µg of human recombinant IL-12, (Omori *et al*, 2004. Coleman *et al*, 2014), the vaccine has demonstrated 84% efficacy against heterologous challenge and 71% efficacy against heterologous challenge one year after the last vaccination. Over 1.8 million doses were sold in the US within the first 4 years of licencing of the vaccine (Huang *et al*, 2004. Huang *et al*, 2010. Yamamoto *et al*, 2007), however a recent study published by Westman and colleagues (2016) suggested that the vaccine showed a protective rate of only 56% under field conditions.

Author.	Year.	Vaccine type.	Inoculum/dose/clade.	Immunisation schudual (Weeks)/route.	Challenge strain/dose/clade	Challenge week/route/source	Protection rate.	Control Protection.
Hosie *et al*	1992	FIV ISCOMS	1/ FIVGL8 ISCOMS/ 10µg/A	0,5,18/S.C.	FIV-GL8/20 CID_{50}/A/	20/I.P./TCF	0/4	1/4
		r-p24 ISCOMS	2/ r-p24 ISCOMS/ 50µg/A	0,3,5,7/S.C.		9/I.P./TCF	0/4	1/5
		inactivated, infected cell vaccine	$2x10^6$ FIVGL8 infected cells/A	0,3,6,9,12,15/S.C.		21/I.P./TCF	0/5	
Siebelink *et al*	1995	Recombinant vaccinia virus with vGR657 (Native Env)	vGR657 FIVAM19 Env ISCOMS/10µg protein/	0,4,10/S.C.	FIV-AM19/20 CID_{50}/A	12/i.m./TCF	0/6	
		Recombinant vaccinia virus with vGR657 x 15 (lacking SU/TM cleavage site)	vGR657 x 15 FIVAM19 Env ISCOMS/10µg protein/				0/6	
		vGR657x15	vGR657 x 15 FIVAM19 Env ISCOMS/10µg protein/				2/6	
		β-Gal-FIV Env fusion protein.	FIVAM19 Env ISCOMS/10µg protein/				0/6	
		SIV Env ISCOMS	SIVmac32H 10µg/dose				0/6	
		Phosphate buffered saline	Controls					0/6

Table 1-2. Tabulation of FIV vaccine trials that have enhanced.

Author.	Year.	Vaccine type.	Inoculum/dose/clade.	Immunisation schudual (Weeks)/route.	Challenge strain/dose/clade	Challenge week/route/source	Protection rate.	Control Protection.
Flynn *et al*	1997	V3- PID-p24 linear peptide. Subunit vaccine	200µg peptide	0,3,6,15,19/S.C	FIV-Pet/25 CID_{50}/A	35/I.P./TCF	0/7	
			Adjuvant (controls)					0/3
Huisman *et al*	1998	FIV ISCOMs	FIV AM6c/10µg Gag protein dose	0,4,10/S.C.	FIV-AM19/20 CID_{50}/A	12/i.m./TCF	0/6	
		FIV ISCOMs + vGR657x15 ISCOMs	FIV AM19 10µg Env/dose - FIV AM6c 10µg Gag/dose				0/6	
		vGR657x15 ISCOMS + FIV Gag ISCOMs	FIV AM19 10µg Env/dose - FIV 19k1 10µg Gag/dose				0/5	
		Crfk ISCOMS	10µg Crfk protein/dose					0/6
		SIV Env ISCOMS	SIVmac32H 10µg/dose					0/6
		PBS						0/6
Richardson *et al*	1997	DNA	FIV 34TF10 w/t env/400µg/A	0,2,4/I.M.	FIV-Pet/10 CID_{50}/A	6/I.P/TCF	0/7	
			FIV 34TF10 n14 env/400µg/A				0/7	
			FIV 34TF10 n92 env/400µg/A				0/7	
			pUC DNA/400µg/NA					0/7
Karlas *et al*	1999	ICV	FIV-19K1/5 X 10^6 cells/ A	0,2,4,6,8,16/I.V.	FIV-19K1/ 10 CID_{50}/A	18/I.M/TCF	0/3	0/2
			5 X 10^6 uninfected cells (controls)					

Table 1-3. Tabulation of FIV vaccine trials that have enhanced.

Author.	Year.	Vaccine type.	Inoculum/dose/clade.	Immunisation schudual (Weeks)/route.	Challenge strain/dose/clade	Challenge week/route/source	Protection rate.	Control Protection.
Yamaoto *et al study 1*	1991	WIV	FIVPet/200µg/A	0,2,4,6,8/S.C.	FIV-Pet/ 10 ID_{50}/A	10/I.P./TCF	3/3	
			FIVPet/200µg/A + 1X10^7 inactivated FET1 cells				2/3	
			Adjuvant (controls)					0/3
Yamaoto *et al study 2*		ICV	Inactivated FL4 cells/1 x10^7 cells/A	0,2,4,6,8,10,18/S.C.	FIV-Pet/ 10 ID_{50}/A	10/I.P./TCF	2/4	
			Inactivated FET1 cells(FIVpet)/1 x10^7 cells/A				4/5	
			inactivated FET1 cells/1X10^7 cells (controls)					0/5
			Adjuvant (controls)					0/5
Yamamoto *et al. Study 1*	1993	WIV	FIVPet/250µg/A	0,2,5/S.C.	FIV-Pet/ 10 ID_{50}/A	8/I.P./TFC	13/15	0/10
		ICV	FIVPet/2.5 x 10^7 cells/A				15/15	
Yamamoto *et al. Study 1*	1993	WIV	FIVPet/250µg/A	0,2,5,38/S.C.	FIV-Dixon/10 ID_{50}/A/	45/I.P./TCF	13/13	
		ICV	FIVPet/250µg/A				14/15	
Hosie *et al study 1*	1995	WIV	FIVPet/250µg/A	0,2,4,7,10,17/I.P.	FIV-Pet/ 10 CID_{50}/A (FeT-1)	20/I.P./TCF	5/6	0/6
					FIV-GL8/ 5 CID_{50}/A (Q201)		0/5	0/5
Hosie *et al study 1*			FIVPet/250µg/A	0,3,6/I.P.	FIV-Pet/ 10 CID^{50}/A (FeT-1)	9/I.P./TCF	4/5	0/5
					FIV-GL8/ 10 CID_{50}/A (Q201)		1/5	0/5
Pu *et al study 1*	2001	WIV	FIVPet+FIVshi/250µg +250µg/A+D	0,3,6/?	FIV-Bang/ 10 CID_{50}/B	9/I.V./in vivo	4/5	0/4
Pu *et a study 2*		WIV	FIVPet+FIVshi/250µg +250µg/A+D	0,3,6/?	FIV-Bang/100 CID_{50}/B	9/I.V./in vivo	2/5	0/5
Pu *et al study 3*		WIV	FIVPet+FIVshi/250µg +250µg/A+D. Boosted FIVBang	0,3,6,29	FIV-Pet/ 50 CID_{50}/A	31/i.v/in vivo	4/4	0/5
Pu *et al study 4*		WIV	FIVShi/500µg/D	0,3,6	FIV-shi/50 CID_{50}/D	9/i.v/in vivo	1/3	0/3
Pu *et al study 5*		WIV	FIVPet+FIVshi/250µg +250µg/A+D	3 or 6 immunosations	FIV-Pet/ 20-25 CID_{50}/A	3 weeks after last boost/i.v/in vivo	6/6	0/9

Table 1-4. Tabulation of predominantly whole inactivated virus FIV vaccine trials.

Author.	Year.	Vaccine type.	Inoculum/dose/clade.	Immunisation schudual (Weeks)/route.	Challenge strain/dose/clade	Challenge week/route/source	Protection rate.	Control Protection.
Pu *et al study 1*	2005	WIV/ICV	FIVPet + FIVShi (Fel-O-Vax). 3 doses as per manfacturers instructions/ A+D	0,3,6/S.C	FIV-FC1/15 CID_{50}/B	9/I.V./in vivo	4/4	
			inactivated FeT-j/?/NA (controls)					0/2
			PBS/NA/NA (controls)					0/4
Pu *et al study 2*		WIV/ICV	FIVPet + FIVShi (Fel-O-Vax). 3 doses as per manfacturers instructions/ A+D	0,3,6/S.C	FIV-FC1/15 CID_{50}/B	9/I.V./in vivo	4/4	
			PBS/NA/NA (controls)					0/3
Huang *et al*	2004	WIV/ICV	FIVPet + FIVShi (Fel-O-Vax). 3 doses as per manfacturers instructions, boosted after 1 year/ A+D	0,3,6/S.C.	Undescribed challenge virus. 11% amino acid divergencey at the Envelope level/ 11 ID_{50}/undescribed clade.	52/I.M/TCF	19/25	2/19
Kusuhara *et al.*	2005	WIV/ICV	FIVPet + FIVShi (Fel-O-Vax). 3 doses as per manfacturers instructions, boosted after 1 year/ A+D	0,3,6,58 /S.C	FIV-Aomori-2/contact challenge/B		6/6	
			Unvaccinated	Untreated	Untreated			5/8
			Challenge group	FIV-Aomori-2/10000 $TCID_{50}$/B	Untreated			0/10
Hosie *et al*	2005	WIV	$FIVGL8^{YI}$/250µg/A	0,3,7/S.C.	FIV-GL8/10 CID_{50}/A	10/I.P./TCF	0/4	
			0.5mls PBS/0.5mls adjuvant					0/4

Table 1-5. Tabulation of Fel-O-Vax FIV vaccine trials.

Author.	Year.	Vaccine type.	Inoculum/dose/clade.	Immunisation schudual (Weeks)/route.	Challenge strain/dose/clade	Challenge week/route/source	Protection rate.	Control Protection.
Dunham *et al.*	2006	WIV/ICV	FIVPet + FIVShi (Fel-O-Vax). 3 doses as per manfacturers instructions/ A+D	0,3,6/S.C.	FIVGL8/ 10 CID_{50}/A	10/I.M/TCF	0/6	
			1ml PBS (controls)	0,3,6/S.C.				0/5
Huang *et al*	2010	WIV/ICV	FIVPet + FIVShi (Fel-O-Vax). 3 doses as per manfacturers instructions, boosted after 1 year/ A+D	0,3,6/S.C	FIV-FC1/1000 infected PBMCs/B	54/I.V./in vivo	10/14	0/5
Coleman *et al*	2014	WIV/ICV	FIVPet + FIVShi (Fel-O-Vax). 3 doses as per manfacturers instructions, boosted after 1 year/ A+D	0,3,6/S.C.	FIVBangstrom/?/A/B	10/I.V/*in vivo*	1/4	
					FIVFC1/?/B	10/I.V/infected PBMC	8/8	
					FIVNZ1/?/F/C	10/I.V/infected PBMC	2/5	

Table 1-6. Tabulation of predominantly whole inactivated virus FIV vaccine trials that have protected.

Author.	Year.	Vaccine type.	Inoculum/dose/clade.	Immunisation schudual (Weeks)/route.	Challenge strain/dose/clade	Challenge week/route/source	Protection rate.	Control Protection.
Matteucci *et al*.	1996	ICV	Inactivated FIVM2 infected MBM cells/ $3X10^7$ cells/B	0,3,6,9,21/S.C.	FIV-M2/ 10 CID_{50}/B	37/i.v./plasma	5/6	
			Inactivated MBM cells/ $3X10^7$ cells (controls)					0/9
Bishop *et al study 1*	1996	ICV	Inactivated FL4 cells/$2.5X10^7$ cells/A	0,2,4,7,10,22/S.C.	FIV-Pet/ 10 CID_{50}/A	24/I.P./TCF	4/4	
			Inactivated FL4 cells/$2.5X10^7$ cells/A		FIV-GL8/ 10 CID_{50}/A		3/4	
			Q201 cells/2.5 x 10^7/NA		FIV-GL8/ 10 CID_{50}/A		1/3	
			Adjuvant (controls)		FIV-GL8/ 10 CID_{50}/A			0/7
			Adjuvant (controls)		FIV-Pet/ 10 CID_{50}/A			
Bishop *et al study2*		ICV	Inactivated FL4 cells/$2.5X10^7$ cells/A	0,2,5,10/S.C.	FIV-Pet/ 10 CID_{50}/A	13/I.P./TCF	1/8	
			Inactivated FL4 cells/$2.5X10^7$ cells/A		FIV-GL8/ 10 CID_{50}/A		1/6	
			FeTJ cells/2.5 x 10^7/NA		FIV-Pet/ 10 CID_{50}/A		0/6	
			Adjuvant (controls)		FIV-Pet/ 10 CID_{50}/A			0/8
			Adjuvant (controls)		FIV-GL8/ 10 CID_{50}/A			

Table 1-7. Tabulation of inactivated cell FIV vaccine trials.

Author.	Year.	Vaccine type.	Inoculum/dose/clade.	Immunisation schudual (Weeks)/route.	Challenge strain/dose/clade	Challenge week/route/source	Protection rate.	Control Protection.
Hohdatsu et al study 1	1997	ICV	FIVPet + FIVShi/2.5 x 10^7 cells/ A+D	0,4,8/S.C.	FIV-Pet /10 CID_{50}/A	10/I.P./TCF	4/4	
			FIVPet/2.5 x 10^7 cells/A				4/4	
			Adjuvant (controls)					0/8
Hohdatsu *et al study 2*		ICV	FIVPet + FIVShi/2.5 x 10^7 cells/ A+D	0,4,8/S.C.	FIV-shi/10 CID_{50}/D		4/4	
			FIVPet/2.5 x 10^7 cells/A				1/4	
			Adjuvant (controls)					0/4
Matteucci *et al*.	1997	ICV	Fixed FIVM2 infected MBM cells/ 3X10^7 cells/B	0,3,6,9,21/S.C.	PBMC FIVM2 infected/ 3X10^7 fixed, infected MBM cells/B	73/i.v./Plasma	2/3	
					FIVM2/ 10 CID50/B		0/3	
			Inactivated MBM cells/ 3X10^7 cells/controls	0,3,6,9,21/S.C.	PBMC FIVM2 infected/ 3X10^7 fixed, infected MBM cells/B			0/3
					FIVM2/ 10 CID50/B			0/3

Table 1-8. Tabulation of inactivated cell FIV vaccine trials.

1.11 Correlates of immunity

Initial trials where protection had been elicited correlated the titre of virus neutralising antibody (VNA) with protection (Yamamoto *et al*, 1993. Hosie *et al*, 1995), however some cats displayed low VNA titres and yet were still protected. However, the protection afforded by vaccination with IWV or ICV could be passively transferred and cats were protected against low dose homologous challenge (Hohdatsu *et al*, 1993). The protection could be attributed to the nAb by challenge of kittens, derived from vaccinated queens, with low dose homologous virus. The protection observed could only be afforded by maternally derived antibodies obtained from colostrum (Pu et al, 1995). Passive transfer of antibody mediated immunity could not be demonstrated against heterologous challenge, using highly purified antibody preparations derived from the blood of Fel-O-Vax FIV vaccinated cats (Coleman *et al*, 2014), although protection against homologous challenge could be demonstrated. Many vaccination studies using WIV or ICV (FIV-Petaluma) could detect high titres of nAb against FIV-Petaluma but low titres or no heterologous nAb (Pu *et al*, 2001. Hosie *et al*, 1995. Coleman *et al*, 2014). The importance of nAb may have been over stated by the methodology used to originally quantitate the nAb titres induced by vaccination. Originally the presence of nAb was determined using a focus reduction assay that utilised culture adapted FIV-Petaluma and the CD134 negative cell line Cfrk. Culture adapted strains of FIV can infect cells independently of CD134 by directly binding the FIV co-receptor CXCR4 via the V3 region of Env (Seibelink *et al*, 1995). The V3 is known to be an immunodominant epitope (Avrameas *et al*, 1992) and a neutralisation epitope (Lombardi *et al,* 1993). It was demonstrated by Verschoor and colleagues (1995) that Crfk adaptation was the result of a glutamic acid to lysine mutation at amino acid position 407 (E407K). The E407K mutation led to an increased net charge of the V3 loop and enhanced the neutralisation of culture adapted strains of FIV by antibodies targeting V3 (Baldinotti *et al*, 1994).

Initial vaccine trials failed to assess the immunity afforded by cellular immunity. Cytotoxic T-lymphocytes (CTL) were demonstrated in lymphoid tissues 50 weeks after vaccination with FIV-Petaluma WIV that were Env-specific (Flynn *et al*, 1996). Importantly, it was shown that vaccinated protected cats developed Env-specific CTL responses whereas unprotected

(naturally infected) cats developed Gag-specific CTL responses. The levels of CTL activity were comparable to those observed during the initial stages of infection (Tellier *et al*, 1997) and adoptive transfer of T-cells to matched MHC recipients could protect 80% of cats against low dose homologous (FIV-Petaluma) challenge and 50% of cats against heterologous (FIV-FC1) challenge (Aranyos *et al*, 2014). Although limited protection was demonstrated by adoptive transfer of only $CD4^+$ or $CD8^+$ T-cells, optimal protection was observed when mixed T-lymphocytes were infused. No protection was observed when T-cell depleted lymphocytes (i.e. B-cells alone) were infused. A defined Th1 immune response was observed in protected vaccines with increased levels of IFNγ, IL-2, and CTL associated cytotoxins and cytolysin (Aranyos *et al*, 2014).

1.12 Broadly neutralising antibodies (BnAb).

The importance of virus neutralising antibodies (VNA) has been demonstrated for both HIV and FIV through the use of passive transfer studies of sera derived from vaccinated animals. Passive transfer of immunity was first demonstrated by Hohdatsu *et al*, (1993) where by cats passively immunised with sera from cats that were experimentally vaccinated with either an inactivated infected cell vaccine or a whole inactivated virus vaccine where protected. This finding was further examined by the low dose challenge of kittens with FIVpet born to queens that were either experimentally infected or vaccinated with FIVpet. The authors were able to demonstrate protection to kittens born to queens that were not vaccinated or infected but received milk from infected or vaccinated queens demonstrating that the immunity transferred was mediated solely by maternally derived antibody. This study also revealed a relationship between the titre of passively transferred antibody and the level of protection observed. Kittens that received a high titre of antibody remained completely protected, whilst kittens receiving a medium titre where not completely protected (Pu *et al*, 1995). Similar observations have been reported for HIV, where humanised mice passively transferred with a cocktail of broadly neutralising antibodies were able to control and suppress viremia to undetectable levels (Klein *et al*, 2012). Sterilising immunity has been achieved using the humanised mouse model, where mice received a single intramuscular injection of self-complementary Adeno-associated virus

vectors that express the full length BnAb, 4E10 (Balazs et al, 2011). Mice were completely protected against HIV challenge, even when intravenously challenged with high dose HIV.

Antibodies to infection with FIV has been well characterised, and passive immunity has been demonstrated, the development of nAb or BnAb is less well understood. The development of homologous nAb was studied by Beczkowski *et al*, (2015), comparing the levels of neutralisation (both homologous and heterologous) in cats with different degrees of disease progression and survival outcomes. Cats that developed strong homologous nAb showed no difference in the $CD4^+$ T-cell decline compared to cats that did not mount a nAb response. This study also revealed that there was no correlation between the development of BnAb and time infected, or survival time. In this study, only 13% of the cats developed BnAb, as assessed by the ability to neutralise HIV(FIV) pseudotypes bearing Envs from the reference strains FIV-Glasgow8 (clade A) and FIV-B2452 (clade B). In a separate study by Hosie *et al*, (2011) the development of homologous nAb was examined in cats challenged with an infectious molecular clone of the virulent UK field isolate FIV-Glasgow8. Over the observation period (4 years), only 1/3 cats developed homologous nAb that strongly (>80% neutralisation) neutralised FIV-Glasgow8, whilst the remaining two cats only moderately neutralised FIV-Glasgow8 (50-60% neutralisation). The frequency of cats developing BnAb following natural infection has been reported to be as low as 0.9% (2/345). Although nAb could be detected in 8.7% of the plasma samples tested for neutralisation against FIV-Glasgow8, only two displayed broad neutralisation against a panel of FIV pseudotypes bearing Envs derived from clades A, B and C (Samman, 2010, Hosie *et al*, 2011). The development of BnAb is believed to correlate with the time spent infected and might reflect the poor antigenicity of FIV Env. As BnAb require extensive somatic hypermutation, the antigenicity of the Env is crucial and the majority of B-cell epitopes have been shown to be immunologically silent for FIV (Liao *et al*, 2013. McLellan *et al*, 2011. Haynes *et al*, 2012. Massi *et al*, 1997). Adding to this problem is the fact that BnAb have been shown to be poly/auto-reactive (Flynn *et al*, 1994) and thus many potential B-cells that may produce BnAb are deleted at the fist self-tolerance check point. Taken together, the literature demonstrates the importance of BnAb and thus they are believed to form a crucial component of any further efficacious lentiviral vaccine. The difficulty in eliciting BnAb remains a challenge of vaccinology in general.

1.13 Differentiation of infected from vaccinated animals

In 2002, the release of the Fel-O-Vax FIV vaccine led to concerns regarding the sero-diagnosis of FIV. The dual subtype vaccine is composed of whole inactivated virus (WIV) and inactivated infected cells (Pu *et al*, 2001) and elicits strong cellular and humoral immune responses (Pu *et al*, 2001. Hohdatsu *et al*, 1997, Huang *et al*, 2010). As the antibodies elicited by vaccination are indistinguishable from those produced following natural infection, it is not possible to differentiate infected from vaccinated animals (DIVA) using serology assays (Uhl *et al*, 2002). In an independent study, samples from FIV infected, FIV uninfected and FIV vaccinated and uninfected cats were screened for FIV antibodies using a range of serological assays (Levy *et al*, 2004) as shown in Table 1-9.

	Sensitivity	**Specificity**	
Assay	Infected cats (n=41)	Unvaccinated cats (n=42)	Vaccinated cats (n=41)
Lateral-flow ELISA[1]	100	100	0
Microwell plate ELISA[2]	100	100	0
Western blot	98	98	54
Immunofluorescence antibody assay.	100	90	22

Table 1-9. The sensitivities and specificities of 4 serological assays used to screen samples from FIV infected, FIV negative and FIV vaccinated cats. All assays demonstrated sensitivities ranging from 98-100% and specificities ranging from 90-100% when used to screen plasma samples from FIV infected and uninfected cats. However, when screening samples from vaccinated cats, the specificity rate ranged from 0-54% demonstrating that these assays detecting FIV antibody could not differentiate infected from vaccinated animals. [1]SNAP FIV/FeLV Combo, [2]PetChek FIV antibody test. Figure adapted from Levy *et al*, 2004.

As conventional serological assays could not DIVA, alternative diagnostic tests were required. Virus isolation is not routinely used outside of FIV research as it is costly, highly specialised, time consuming and not suitable for large-scale screening. If samples have been in transit for several days, the viability of the infected PBMCs may be significantly

reduced, leading to false negative results. Heparinised whole blood is required for virus isolation as the anti-coagulant EDTA is toxic to the cells used in co-cultivations with PBMCs to propagate FIV. Furthermore, some primary strains of FIV do not induce large syncytia and so a confirmatory assay must be performed to detect virus in culture fluids, such as an ELISA for FIV p24 or an assay for RT.

The first attempts to develop a serological DIVA assay to DIVA were made by Kusuhara *et al* (2007), testing a range of FIV antigens. By comparing the antibody binding of samples screened against different FIV antigens and employing discriminant analysis, it was possible to differentiate infected from vaccinated animals using a combination of formalin inactivated whole virus and a peptide corresponding to an immunodominant epitope on TM. Using this method, 134/138 samples were correctly identified as FIV infected or uninfected, producing a discriminating variable of 97.1%. As samples from vaccinated/uninfected cats did not exceed the linear discriminant function, such samples tested FIV antibody negative. However, in this study no samples from FIV uninfected and unvaccinated cats were tested and therefore there was no evidence that the ELISA could be used to differentiate samples from FIV negative cats from samples collected from FIV vaccinated cats. Using the same antigens, Levy *et al* (2008) extended this study and demonstrated a sensitivity of 97.1% and specificity of 100%; however, the ELISA did not differentiate samples from either FIV negative cats or FIV vaccinated cats, presumably because of non- specific antibody reactivity with the formalin treated whole virus antigen.

More recent studies have utilised existing assays/technologies as DIVA tests. The use of several POC kits has been examined as a potentially cost-effective method of differentiating infected from vaccinated animals. The use of the Witness FeLV/FIV test (which contains TM as the capture antigen) in conjunction with the Anigen Rapid FeLV/FIV test (which contains p24 as well as TM as capture antigens) allows FIV negative cats (both uninfected and unvaccinated) to be differentiated from FIV positive cats. (Westman *et al*, 2015). However, it was not possible to differentiate FIV negative cats from those that were vaccinated. The testing regime and definition of a positive FIV test, together with the small numbers of FIV infected cats included in the study, raises questions about evaluating the serological status of cats on the basis of the results of testing using these two-lateral flow immunochromatography POC kits. The results obtained in this study must be viewed in

light of the technology employed in the selected POC kits. Of the 358 samples screened in this study, 119 samples were from fully vaccinated cats, 21 samples were from FIV infected cats and 218 FIV from uninfected, unvaccinated cats. The Witness FeLV/FIV and Antigen Rapid FeLV/FIV kits correctly identified 351/358 samples; all of the vaccinated cats tested negative, all of the FIV infected cats tested positive and all FIV negative cats tested negative. In contrast, using the SNAP FIV/FeLV Combo test all vaccinated cats tested positive. It is not yet clear if these results reflected the different capture antigens or the technology coupled to each test. The use of fluorescence-activated cell sorting (FACS) analysis was recently evaluated as a DIVA assay. The identification of weak florescence from the β chain of the CD8αβ glycoprotein is more commonly observed in FIV infected cats, leading to increased levels of $CD8^{low}$ expression (Shimojima *et al*, 1998). By examining PBMCs for expression of the T-lymphocyte receptors CD4 and $CD8^{low}$, Litster *et al* (2014) differentiated infected from vaccinated cats with a sensitivity of 98% and a specificity of 84%. Comparing the CD4:$CD8^{low}$ ratios, the majority of vaccinated cats tested negative for FIV infection, however there were no statistically significant differences observed between any of the FIV uninfected groups. To date, none of these assays are licensed as FIV DIVA tests and only PCR testing is not confounded by vaccination.

Studies evaluating the performance of RT-PCR/PCR as a potential DIVA assay have shown variable results (Westman *et al*, 2015, Crawford and Slater, 2005). As none of the available PCR based assays were licenced at the time of publication, assays were not standardised and used various proprietary formulations of primers, probe and reagents. Crawford and Slater, 2005 (table 1-10), reported sensitivity values of 41-93% and specificity values for uninfected, unvaccinated animals of 81-100%, although it was not possible to identify a single laboratory that achieved performance figures that would justify the use of a commercial PCR test to screen cats for FIV infection. Specificity values for uninfected, vaccinated animals ranged from 44-95% with the results of one laboratory achieving acceptable specificity figures for uninfected cats and vaccinated, uninfected cats. Tests performed in this laboratory did, however, have a poor sensitivity value of 76%, so that 24 of every 100 positive samples tested would be reported falsely as negative.

FIV status	Number of cats	PCR1*	PCR2†	PCR3Θ	PCR4‡
Infected (Sensitivity)	41	76	93	51	41
Uninfected, unvaccinated (Specificity)	42	100	81	81	81
Uninfected, vaccinated (Specificity)	41	95	66	44	51

Table 1-10. Tabulation of comparative PCR sensitivities and specificities for three diagnostic laboratories. Two types of PCR were available from 3 different diagnostic laboratories where one offers PCR based on whole blood a dried blood smear. * = Laboratory 1, uses RT-PCR, † = Laboratory 2, uses a nested conventional PCR. Θ= Laboratory 3, uses a conventional PCR. ‡ =Laboratory 3, uses a conventional PCR on template derived from a dried blood smear. Squares in red highlight the lowest performance figures whilst green squares highlight the highest performance figures. Figure adapted from Crawford and Slater, 2005.

A subsequent study evaluating the usefulness of a commercially available PCR (FIV RealPCR™, IDEXX) to differentiate infected from vaccinated animals demonstrated improved test sensitivity and specificity (table 1-11). These figures were obtained following the testing of 358 independently obtained samples from Australia. The sensitivity of the PCR assay was reported to be higher than stated by the manufacturer, although this may be related to there being fewer FIV infected cats included in the sample population, or that samples used in the Westman *et al* (2015) study had previously been screened for FIV antibodies using three POC kits, biasing the PCR results. Specificities for both uninfected and vaccinated animals closely matched the values quoted by the manufacturer, making the FIV PCR a reliable molecular diagnostic tool for detecting FIV infection and a potentially useful DIVA assay. Four false positive samples (1 originating from an FIV vaccinate and three from FIV negative, unvaccinated cats) and two false negative samples (both originating from FIV vaccinates) were identified.

	FIV RealPCR™			
	Westman *et al*, 2015		IDEXX diagnostic update, 2011*	
FIV status	Number of cats	Diagnostic performance	Number of cats	Diagnostic performance
Infected (Sensitivity)	26†	93	36	80.5
Uninfected, unvaccinated (Specificity)	218	98.6	96	99.9
Uninfected, vaccinated (Specificity)	114	99.1	92	99.9

Table 1-11. Independent validation of FIV RealPCR, utilising FIV infected, FIV uninfected and FIV vaccinated samples. † = 21 samples that were initially thought to be FIV negative were found to be FIV provirus positive and FIV antibody positive by three different POC kits. A further 5 samples that were derived from FIV vaccinated cats were shown to be FIV provirus and virus isolation positive (likely vaccine breakthrough cases).

*(http://www.idexx.com.au/pdf/en_au/smallanimal/education/realpcr-test-for-fiv.pdf

None of the DIVA assays described in this chapter are commercially licensed for the sero-diagnosis of FIV. The IDEXX FIV RealPCR™, is licensed, has good specificity but lacks sensitivity as the primers used in the PCR reaction might not be optimal to detect the circulating clades of FIV in the region. The data demonstrated that all the assays could be used to differentiate infected from uninfected cats (all samples from vaccinated cats tested FIV negative) but it was not possible to differentiate vaccinated from uninfected, unvaccinated cats. The ideal DIVA assay should allow the identification of samples from (a). vaccinated, uninfected, (b). vaccinated infected, (c). unvaccinated, uninfected and (d). unvaccinated, infected cats. When utilising assays that specifically titrate sensitivity against specificity so as to avoid the detection of antibodies elicited by vaccination, there is a significant risk of false negative results. Cats in the initial stages of FIV infection with low titres of FIV antibody would test antibody negative, as would cats in the terminal stages of FIV infection with B-cell exhaustion or high viral loads that form complexes with any antibody so that no unbound antibody remains available for detection. The POC kit comparisons using different capture antigens indicate that kits containing the epitope from

TM display the highest specificities, with values of 99-100% (Sibille *et al*, 1995, Hartmann *et al*, 2007). This raises the question: can a DIVA test be developed based solely on the immunodominant epitope within TM?

One hypothesis is that mutation of a conserved yet highly antigenic B-cell epitope within the virus could potentially differentiate infected from vaccinated cats. The principal immunodominant domain (PID) of TM has been shown to be highly conserved and antigenic; indeed, the epitope is a major constituent in many POC kits including the Witness®, SNAP® Combo Plus, FASTest® and DUO speed®. Therefore, if this epitope was able to withstand mutation, whilst maintaining virological function, and included in a WIV vaccine candidate, the humoral immune response towards the wildtype PID sequence would be abolished. If a peptide ELISA were to be developed utilising peptides that corresponded to the wildtype and mutated PID sequences as capture antigen, it would be possible to screen cat plasma for reactivity to wild type sequence (indicative of infection) or the mutated PID sequence (indicative of vaccination). Samples responding to neither peptide would be considered uninfected and unvaccinated, whilst samples responding to both peptides would be classed a vaccinated and infected. All retroviruses contain their own unique PID sequence, and have been shown to be able to withstand significant mutation. Therefore, the PID of FIV is an attractive DIVA epitope and could be mutated to that of another retrovirus (Pancino *et al*, 1995. Pancino and Songio, 1997. Broche-Pierre *et al*, 2005). A prime candidate for this is the PID sequence of FIV derived from lions (FIVple). More specifically subtype E FIVple (termed lion lentivirus subtype E [LLV-E]), shares sequence homology with that of FIV derived from the domestic cat (FIVfca), however the 5' end of the PID sequence (Figure 1-8) displays sufficient divergency so that reactivity against the wildtype PID sequence should be abolished. Therefore, the rational of mutating the PID of FIV to that of LLV-E may allow for the differentiation of infected from vaccinated animals.

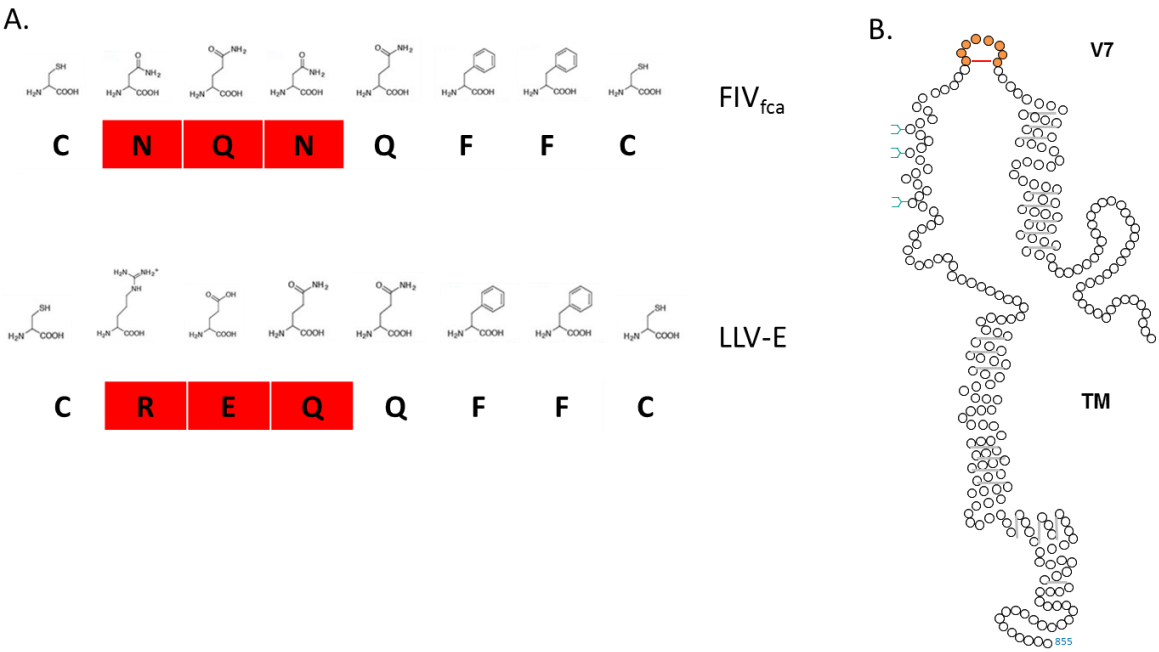


Figure 1-8. The difference in PID amino acid sequence between FIVfca and LLV-E (A) and the orientation of the PID with TM (B). A, amino acids shown in red highlight the difference between the FIVfca and LLV-E PID sequences. B, a cartoon representation of the transmembrane domain of the envelope glycoprotein with the location of the PID highlighted in orange. The PID forms a loop at the apex of variable loop 7 by the formation of disulphide bonds between the two terminal cysteines. The PID sequence of LLV-E differs by only 3 amino acids when compared to FIVfca (A), but it is hoped that this is enough sequence heterogeneity from the wild type sequence to abolish plasma reactivity if the wildtype PID sequence was mutated to that of LLV-E (B) and included in a candidate WIV vaccine.

1.14 Aims.

1. Determine the pathogenesis of a chimaeric FIV isolate containing the gag and pol proteins of FIV-Glasgow8 contained within a novel field isolate envelope (designated FIV ΔV2).
2. The development of a DIVA peptide ELISA that is capable of differentiating infected from vaccinated animals.
3. Mutation of the PID sequence of FIV ΔV2 to that of LLV-E (DIVA virus).
4. Perform a DIVA WIV vaccine trial utilising the DIVA virus to determine of protection can be afforded whilst simultaneously performing DIVA.

Chapter 2 General materials and methods

2.1 Molecular biology techniques.

2.1.1 Primers and probes

Primers and probes were designed using DNADynamo blue tractor software and ordered from the IDT website (Integrated DNA technologies, Interleuvenlaan, Belgium). Lyophilized primers were reconstituted with TE buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA) to prepare stocks at the final concentration of 100pmol/µl. Secondary primer stocks at 10pmol/µl were subsequently prepared in TE buffer for use in PCR assays. All primer stocks were stored at -20^{o}C until needed.

Probes were ordered using the custom probe design tool and labelled at the 5' end with the reporter dye Fluorescein (FAM) and at the 3' end with the quencher TAMRA. Lyophilized probes were reconstituted to 100pmol/µl with probe dilution buffer supplied by the manufacturer, aliquoted into 10µl amounts and stored at -20^{o}C until required. Prior to use, probes were thawed at room temperature in the dark and diluted to the final concentration (5pmol/µl) using deionised water.

2.1.2 Polymerase chain reaction (PCR)

Proviral DNA encoding the FIV envelope gene (*env*) was amplified using a nested PCR method. First, gDNA from *ex vivo* derived PBMCs or cells cultured *in vitro* was extracted using the QIAmp DNA mini extraction kit (Qiagen, Manchester, UK), as described in section 2.1.8. First round PCR products were amplified using the degenerate primers IF4 and XR2 (Appendix 2) with the Phusion® high fidelity DNA polymerase kit (Hitchin, Hertfordshire, UK) using the volumes shown in Table 2-1.

Reagent	Volume (µl)
Phusion high fidelity DNA polymerase (2000u/ml)	0.5
GC buffer	10
dNTPs (8µM)	1
Forward primer (10pmol/µl)	1
Reverse primer (10pmol/µl)	1
DMSO	2
Template	2
H_20	32.5

Table 2-1. Phusion PCR reactants and volumes.

Selected templates were amplified on the GeneAmp 9700 PCR systems thermo cycler (Applied Biosystems) using the cycling conditions detailed in Table 2-2.

Temperature (^oC)	Time (mm:ss)	Number of cycles	Stage of reaction
98	03:00	1	Initial denaturation
98	00:10	35	Denaturation
62.5	00:30		Annealing
72	01:15		Elongation
72	10:00	1	Final Elongation
4	∞	∞	

Table 2-2. Phusion PCR cycling conditions

PCR products were separated by agarose gel electrophoresis, extracted and purified as described in 2.1.4. Second round PCR were again performed with the Phusion® high fidelity DNA polymerase kit using GL8 Sal and GL8 Not1 specific primers (Appendix 1). GL8 Sal and GL8 Not1 primers anneal at 69^{o}C. All other cycling conditions remained the same.

2.1.3 Agarose gel electrophoresis and PCR product purification.

Amplified PCR products were separated using a 0.7 - 0.8% agarose gel and visualised under ultra violet (UV) light. PCR products were mixed with X10 loading dye before electrophoresis using an agarose gel containing ethidium bromide at 250ng/ml (Sigma Aldrich, Dorest, UK) for 1.5 hours at 100V in TBE buffer. Bands were visualised under UV light and excised using a sterile scalpel.

Amplified DNA was extracted from the agarose gel using the QIAquick gel extraction kit (Qiagen, Manchester, UK), as per the manufacturer's protocol. Briefly, the DNA and agarose were incubated with QG buffer at 52^{o}C until the agarose had completely dissolved. The mixture was then centrifuged through a QIAquick spin column in which the low pH (≤7.5pH) and high chaotropic salt concentration allowed binding of DNA to the silica membrane. Residual agarose, ethidium bromide and other impurities were washed through the membrane using PE wash buffer. The addition of a small volume of EB buffer (≥7.5pH) eluted the purified DNA.

2.1.4 Quantitative polymerase chain reaction (qPCR)

QPCR was performed to amplify and quantify the FIV proviral genome sequences from tissues. The relative proviral load was estimated by normalising the sample data against a housekeeping gene and extrapolating this value to a standard curve.

The proviral FIV *gag* gene was detected by PCR amplification using the reagents listed in Table 2-3. Reactions of 20µl were added to a MicroAmp fast optical 96 well reaction plate. Each plate contained a set of standards containing 10^0-10^7 copies of the GL8-414 molecular clone of FIV in the plasmid pBR328. The *gag* gene was amplified using the cycling conditions listed in Table 2-4. Fluorescence was measured using the 7500 fast system sequence detection software v1.4 on the ABI 7500 fast PCR machine (Leicestershire, UK)

Reagent	Volume (µl)	Supplier
x2 Taqman universal master mix	10	Applied Biosystems
Forward primer (FIV-1360F)	1	IDT
Reverse primer (FIV-1437R)	1	IDT
FIV Gag probe	1	IDT
template	1	N/A
Denionised water	6	Gibco, Life Technologies

Table 2-3. FIV gag qPCR reactants and volumes.

Temperature (^{o}C)	Time (mm:ss)	Number of cycles
50	02:00	1
95	10:00	1
95	00:15	40
60	01:00	

Table 2-4. QPCR cycling conditions.

Sample values were normalised against the ribosomal DNA housekeeping gene. PCR reactions were set up using the reagents listed in Table 2-5 in total reaction volumes of 20µl in the wells of MicroAmp fast optical 96 well reaction plates. Each normalisation plate contained a set of rDNA standards derived from the genomic DNA of the feline lymphoblastoid cell line, Mya-1. Five-fold serial dilutions of genomic DNA were prepared, from 800ng/µl to 1.02ng/µl. Amplifications were performed using the cycling conditions shown in Table 2-4 and fluorescence was detected using the 7500 fast system sequence detection software v1.4 on the ABI 7500 fast PCR machine (Leicestershire, UK).

Reagent	Volume (µl)	Supplier
x2 Taqman universal master mix	10	Applied Biosystems
Forward primer (rDNA 343F)	1	IDT
Reverse primer (rDNA 409R)	1	IDT
rDNA 370 probe	1	IDT
gDNA template	1	N/A
Denionised water	6	Gibco, Life Technologies

Table 2-5. rDNA qPCR reactants and volumes

Sequence detection data was expressed as *ct* values and the mean values for three replicates were calculated. Proviral loads were calculated by dividing the number of copies of FIV *gag* by the corresponding amount of DNA present in the normalisation wells. This value was then multiplied by 5000 to estimate the number of copies of FIV genome per 10^6 cells (Leutenegger *et al*, 1999).

2.1.5 FIV TM peptide ELISA

A peptide ELISA was used to detect antibodies recognising an immunodominant TM epitope of FIV (Avrameas *et al,* 1993). The wells of 96-well microtitre plate (Immulon 2 HB) were coated with 250ng/well of FIV TM peptide (AltaBioscience, Birmingham, UK) in sodium carbonate bicarbonate binding buffer (0.2M anhydrous sodium carbonate, 0.2M sodium carbonate and deionised water at a ratio of 1:11.5:4 respectively). The plate was incubated at 4^{o}C overnight whilst being agitated at 30rpm. The next day, the wells were aspirated and washed 5 times with 200µl of phosphate buffered saline supplemented with 0.1% Tween (PBST). Unabsorbed sites were blocked following incubation with 200µl of 2% low fat milk powder PBST (block) for 1 hour at room temperature. The wells were then aspirated and washed 5 times with 200µl of PBST and 100µl of plasma/sera were added to wells at a dilution of 1/200 in block. One hundred microliters of positive and negative

controls were also added to the plate at a dilution of 1/200 in block. The positive control consisted of plasma derived from an experimentally infected SPF cat, whilst the negative control consisted of an SPF plasma from a control cat that was not experimentally infected. Both samples had previously undergone virus isolation to confirm their FIV status. The plates were sealed and incubated at room temperature for 1 hour before being washed 5 times with 200µl of PBST, after which 100µl of biotinylated goat anti-cat secondary antibody (Vector laboratories, Peterborough, UK) were added to each well at a dilution of 1/1000 (1-1.5µg/µl) in block. The plates were sealed and incubated at room temperature for 1 hour. Wells were then aspirated and washed 5 times with 200µl of PBST and 100µl of horseradish peroxidise conjugated to streptavidin [Vector laboratories, Peterbouough, UK (1-1.5µg/µl)] were added per well at a dilution of 1/1000 in block. The plates were sealed and incubated at room temperature for 20 minutes, aspirated and washed 5 times with 200µl of PBST and then 100ul of 3',3',5'5'-Tetramethylbenzidine liquid (TMB super slow, Sigma Aldrich) were added to each well. Plates were again sealed and incubated at room temperature for 30 minutes before being read at 650nm on the MultiSkan ascent spectrophometer (MTX labsystems, Virginia, USA).

To determine the sero-status of a give sample, the optical density of the plasma sample was divided by 2X the optimal density of the negative control. If this value, termed negative fold, was less than 2, the sample was considered a true negative. If the negative fold was between 2 and 3.9, the sample was considered inconclusive. If the negative fold was equal to or greater than 4, the sample was considered a true positive.

2.1.6 DIVA ELISA.

The DIVA ELISA was performed exactly as described for the FIV TM ELISA apart from the capture antigen used. As well as using a peptide that corresponds to the principal immunodominant domain of FIV, the plate was also coated with a peptide corresponding the principal immunodominant domain of LLV-E. More specifically one half of the plate (4 X 12 wells) were coated with the FIV TM peptide whilst the other half were coated with LLV-E TM peptide in Sodium carbonate bicarbonate binding buffer as described in 2.1.6. All other reagents, concentrations, dilutions and suppliers are the same as those listed in 2.1.6.

The FIV positive control consisted of plasma derived from an experimentally infected SPF cat, whilst the negative control consisted of an SPF plasma from a control cat that was not experimentally infected. Both samples had previously undergone virus isolation to confirm their FIV status. The LLV-E positive control was derived from an 8-year-old male African lion from the Moremi game reserve in the Okavango delta (McEwan *et al*, 2008). Again, virus isolation confirmed the true status of this sample. As there was no difference in the optical density between an FIV or LLV sero-negative sample, and considering that African lion plasma is an expensive and limiting resource, it was deemed unnecessary to include an LLV negative control.

To determine the vaccination status of a given sample, first the optical density of the plasma sample was divided by 2X the optimal density of the negative control. The negative fold of the optical density against the LLV-E peptide was divided by the negative fold of the optical density against the FIV TM peptide. If this value, termed absorbance ratio, was equal to or less than 3, the sample was considered unvaccinated. If the absorbance ratio was greater than 3, the sample was considered vaccinated. The absorbance ratio was calculated using plasma from cats that had been experimentally vaccinated with a genetically altered chimeric FIV virus that carried the PID amino acid sequence of LLV-E instead of FIV.

In summary, the negative fold is used to determine the FIV sero-status of a given sample and the absorbance ratio is used to differentiate infected from vaccinated animals.

2.1.7 Genomic DNA (gDNA) extraction.

The QIAamp DNA mini kit (Qiagen, Manchester, UK) was used to extract gDNA from cultured cells, Ficoll purified peripheral blood mononuclear cells (PBMCs) and whole blood according to the manufacturer's instructions. Briefly, up to $5x10^6$ cells were resuspended in 200µl of PBS containing 20µl of proteinase K. Next, 200µl of AL lysis buffer was added and the mixture incubated at 56^{o}C for 10mins to ensure complete lysis of the cells. Two-hundred microlitres of 96-100% ethanol was added and the lysate mixed thoroughly to precipitate the DNA after which it was passed through a QIAamp mini spin column by centrifugation at 8609*g* for 1 minute. Centrifugation steps were performed using the eppendorf 5418 centrifuge (Eppendorf UK Limited, Stevenage, UK). The bound DNA was

then washed in 500µl of AW1 buffer by centrifugation at 8609*g* for 1 minute. Another wash step was performed using 500µl of AW2 buffer by centrifugation at 16873*g* for 3 minutes. A dry spin was performed at 16873*g* for 1 minute to remove residual contaminants after which the DNA was eluted in 200µl of buffer AE.

2.2 Tissue culture

2.2.1 Recovery of cells frozen in liquid nitrogen.

Cells were recovered from liquid nitrogen storage and rapidly thawed in a humidified incubator at 37^{o}C. The cells were then resuspended in 10mls of PBS and centrifuged at 159*g* for 5 minutes in order to pellet the cells and remove the DMSO in the freezing medium. All cells were centrifuged using the eppendorf 5810 centrifuge (Eppendorf UK Limited, Stevenage, UK). Pelleted cells were then resuspended in 5mls of culture medium and cultures were set up in T25 flasks (Corning, Warrington, UK). Cultures were inspected daily and the medium was replaced, and/or the cells subcultured as required.

2.2.2 Suspension cells

Suspension cells were maintained in RPMI 1640 media (Gibco, Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Thermo Scientific), 100u/ml of penicillin, 100mg/ml of streptomycin, 2mM L-glutamine, 0.11mg/ml sodium pyruvate and 50µM 2-β-mercaptoethanol ("complete RPMI"). Cells were initially cultured in T25 culture flasks, examined daily and subcultured to maintain the cell concentration between 5 x 10^5 per ml and 5 x 10^6 per ml. Culture media were changed every 4 or 5 days by centrifugation at 159*g* for 5 minutes and the cells were subcultured in fresh complete RPMI. Genetically modified cells were selected by the supplementing medium with 400µg/ml G418 (G418 sulphate, Promega, Southampton, UK).

2.2.3 Adherent cells

Adherent cells were maintained in Dulbecco's modified eagle medium (DMEM, Gibco, Life Technologies, Paisley, UK) supplemented with 10% heat inactivated foetal bovine serum (Hyclone, Thermo Scientific), 100 IU/ml of penicillin, 100µg/ml of streptomycin and 2mM L-glutamine ("complete DMEM"). Cells were initially cultured in T25 culture flasks, examined daily and subcultured when confluent. Culture media were changed every 4 to 5 days. Briefly, the medium was aspirated and 5mls of trypsin were added to cell monolayers for 5 minutes at 37^{o}C. Trypsinised cells were centrifuged at 159*g* for 5 minutes and new cultures were set up in fresh complete DMEM media. Genetically modified cells were selected by supplementing the media with 400µg/ml G418 (G418 sulphate, Promega, Southampton, UK).

2.2.4 Cells

2.2.4.1 Feline lymphoblastoid cells (Mya-1)

The feline lymphoblastoid cell line, designated Mya-1, has been shown to be highly susceptible to FIV infection (Miyazawa *et al*, 1989). The cell line was developed in 1989 by Miyazawa *et al*, following the inoculation of a 5-month-old specific pathogen free (SPF) cat with blood from a male cat testing seropositive for FIV and FeSFV. PBMCs collected from the SPF cat were shown to be free of exogenous FIV, FeFSV and FeLV at 9 and 11 months post inoculation.

Mya-1 cells were maintained in complete RPMI 1640 medium supplemented with 100IU/ml recombinant human interlukin-2 (rhIL-2). Mya-1 cells express both the primary receptor of FIV, CD134, and the co-receptor, CXCR4, with surface expression >90% (our unpublished data).

2.2.4.2 CLL-OX40

CLL-OX40 cells were derived from canine lymphocytic leukaemic cells; an immortalised clone was obtained by extensive *in vitro* passage of the $CD3^{+}$ $CD4^{-}$ $CD8^{-}$ cells. The resulting

cell line was then transduced with a lentiviral vector containing feline OX40 and selected with G418 (Willett *et al*, 2006). Stably transduced cells were maintained in complete RPMI supplemented with G418. During these studies, surface expression of feline OX40 was >95% and the expression of canine CXCR4 was also >95%. The expression of the FIV primary and secondary receptors renders CLL-OX40 cells highly susceptible to FIV infection.

2.2.4.3 Human embryonic kidney cells [HEK(293T)]

293T cells are transformed human embryonic kidney (HEK) cells that express the 'T' antigen of adenovirus type 5 (Ad5). Developed by Graham *et al* in 1977, 293T cells were produced by calcium phosphate transfection of HEK cells with sheared Ad5 DNA. After extensive passage (~550 days) a stable, immortalised cell line was obtained that harboured integrated Ad5 'T' antigen DNA. Due to the relative ease with which 293T cells can be cultured and manipulated, they are commonly used for the translation of genetically modified DNA transcripts.

293T cells were maintained in complete DMEM and sub cultured into fresh medium, containing G418, every 4-5 days.

2.2.5 Separation of plasma and PBMC from whole blood

Whole blood collected into heparin or ethylenediaminetetraacetic acid (EDTA) anticoagulant was centrifuged at 635*g* for 10 minutes to separate the cellular fraction from the plasma which was aliquoted into 200-300µl volumes and stored at -80^{o}C until required. The cellular fraction was resuspended in 5mls of sterile PBS and layered over 5mls of Ficoll (GE healthcare, Amersham, Buckinghamshire, UK) in a 15ml falcon tube. The tubes were then centrifuged at 635*g* for 30 minutes with no brake and the buffy coats were collected into fresh 15ml falcon tubes containing 10mls of PBS. The PBMCs were then and centrifuged at 159*g* for 5 minutes to remove the Ficoll and the washed cells were stored as two cell pellets at -80^{o}C.

2.2.6 Isolation of FIV

Virus isolation was performed as a confirmatory test as it is considered to be the 'reference standard' for FIV diagnosis. Co-cultivation of PBMCs with an FIV susceptible cell line leads to viral replication and the appearance of syncytia or cell death. The presence of FIV in culture fluids is confirmed using an ELISA detecting FIV p24 antigen.

Cultures containing 1 x 10^6/ml Mya-1 cells in 5mls were co-cultivated with PBMC in complete RPMI 1640 media containing 100IU/ml rhIL-2 and monitored for 21 days. Cultures were inspected daily for the appearance of syncitium formation, 'ballooning' cells and cell death, typical of FIV infection. Culture fluids were sampled on day 7 when the cells were resuspended in 10mls of fresh medium and transferred to T75 flasks (Corning, Warrington, UK). On day 14, the cells were resuspended in fresh culture media following centrifugation at 102*g* for 10 minutes with no brake. On day 21 the culture fluids were clarified and screened for FIV p24 by ELISA.

After 21 days in culture, cells were harvested, washed in PBS for 10 minutes and cell pellets were stored at -80^{o}C. Culture fluids were filtered using a 0.45µm filter (Ministart, Sartorius Stedim Biotech, Goettingen, Germany) and stored at -80^{o}C in 1ml aliquots.

2.2.7 Titration of FIV on Mya-1 cells

Serial fivefold dilutions of each virus stock were made in complete RPMI. In triplicate, 50µl volumes of each dilution of virus were incubated with 250µl of Mya-1 cells at 8x10^5/ml in BD FACS tube (Corning, Flintshire, UK) at 37^{o}C for 1 hour in a humidified incubator. Cells were then washed twice in 4mls of sterile PBS by centrifugation at 800rpm with no brake and the PBS was removed by aspiration using a Vacusafe bench top aspirator (Integra biosciences AG, Zizers, Switzerland). After washing, the cell pellets were resuspended in 500µl of complete RPMI containing rhIL-2 and cultured in the wells of a 48 well plate. Cultures were then incubated for 14 days at 37^{o}C in a humified incubator. On day 7 post infection, 250µl of the culture fluids were removed and replaced with fresh medium containing rhIL-2. On day 14, 200µl of culture fluid were collected and tested for reverse transcriptase activity or FIV p24 and the cells were pelleted and stored. The infectious virus

titre was calculated using the Karber formula and expressed as tissue culture infectious doses 50% ($TCID_{50}$).

2.3 Analysis of viral proteins

2.3.1 Cell lysate production.

Cell samples (1 x 10^6 of FIV-infected or –uninfected cells) were collected and centrifuged at 2152*g* for 2 minutes and the pelleted cells were then washed in 1ml of sterile PBS. The cell pellet was lysed by resuspension in 75µl of NP-40 buffer containing Roche Complete ULTRA protease inhibitor cocktail tablets (Roche diagnostics GmbH, Mannheim, Germany) followed by incubation on ice for 30 minutes. To each cell lysate, 25µl of X4 reducing loading dye (125mM tris-HCL, pH 6.8, 40% glycerol, 4% SDS, 0.1% bromophenol blue, 5% β-mercaptoethanol) was added before sonication at 4Hz for 30 seconds to mechanically disrupt large cellular proteins. The samples were then heated to 90^{o}C for 10 minutes and centrifuged at 16873*g* for 10 minutes to pellet nuclei, chromatin and other cellular debris. The resulting samples of cell lysate were separated and either analysed immediately or stored at -20^{o}C.

2.3.2 Viral lysate production.

1ml of cell free culture fluid was layered over 400µl of 20% sucrose in PBS and centrifuged at 16873*g* for 90 minutes at 4^{o}C. The viral pellet was then washed with 1ml of sterile PBS and centrifuged again at 16873*g* for 40 minutes at 4^{o}C. The supernatant was aspirated and discarded and the viral pellet was lysed in 25µl of x4 reducing loading dye and heated to 90^{o}C for 10 minutes. The mixture was then centrifuged at 16873*g* for 10 minutes to remove viral nucleotides and residual cellular debris before the lysate was either analysed by SDS-PAGE immediately or stored at -20^{o}C.

2.3.3 Reverse Transcriptase (RT) activity assay.

Lentiviral reverse transcriptase (RT) activity was measured quantitatively in culture fluids using the Cavidi Lenti RT kit (Uppsala, Sweden). Based on a direct ELISA format, the quantity of RT present is determined by comparing the optical densities of sample wells with those of known lenti RT standards. If RT is present in a sample, the enzyme synthesises DNA, using Brd labelled nucleotides, from the RNA template. The DNA product is then detected using an anti-DNA antibody conjugated to alkaline phosphatase, giving a colorimetric change upon the addition of substrate.

Poly-A wells were first incubated with 200µl of RT reaction mix and incubated at 33^{o}C for 1 hour. Ten microlitres of cell free culture fluid was added to the wells along with 10µl of Lenti-RT standard, and the plate was sealed and incubated at 33^{o}C for 24 hours. Wells were then washed 5 times with 200µl of wash buffer (0.75% TritonX-100, deionised water and 0.25% plate wash concentrate) and 100µl of tracer (anti-bromo-deoxyribouridine monophosphate) were added to each well. Plates were sealed and incubated at 33^{o}C for 90 minutes. The wells were then washed again 5 times with 200µl of wash buffer and 125µl of pNPP (paranitrophenylphosphate disodium) substrate were added to each well. The plates were sealed and incubated at room temperature for 2 hours. Plates were read at 405nm on the MultiSkan ascent spectrophometer (MTX labsystems, Virginia, USA) at 30 minutes and 2 hours after the addition of substrate.

2.3.4 FIV p24 antigen ELISA

The Vpg62 (Weijer *et al*, 1995) monoclonal antibody raised against FIV p24 was used in an antigen capture ELISA to detect the Capsid protein, p24, in culture fluids to confirm the presence of FIV.

Immulon 2 HB plates (Thermo scientific, UK) were coated with 100µl of Vpg62 at a dilution of 1/1000 (2ng/µl) in serum free DMEM. Plates were incubated at 4^{o}C overnight whist being agitated at 30rpm. Post adsorption, the coating solution was aspirated and the wells washed 5 times in PBST. Unadsorbed sites were blocked with 200µl of blocking solution and plates were incubated at room temperature for 1 hour, after which the blocking solution was aspirated and wells washed 5 times in PBST. Next, 10µl of sample treatment

solution (30mM Tris/HCL pH 7.2, 450mM NaCl, 1.5% Triton X-100, 1.5% sodium deoxycholic acid, 0.3% SDS, 10mM EDTA and 0.02% sodium azide: Schupbach *et al*, 2006) was added to each well followed by 100µl of cell free tissue culture fluid. The plate was incubated at room temperature for 1 hour before being washed 5 times with PBST. Fifty microlitres of αp24 monoclonal antibody conjugated to horseradish peroxidase (IDEXX laboratories, Maine, USA) was added to each well and the plate incubated at 37^{o}C for 1 hour. The secondary antibody was then removed and the plate washed 5 times in PBST before 50µl of 3',3',5'5'-Tetramethylbenzidine liquid (TMB super slow, Sigma Aldrich) were added to each well. After incubation at room temperature for 10 minutes, the plates were read at 650nm on the MultiSkan ascent spectrophometer (MTX labsystems, Virginia, USA). Positive samples were defined as having optical density values greater or equal to 3 times the optical density of the negative control well.

2.3.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Immunostain analysis.

To analyse the protein content of selected samples, reduced cell lysates or purified virus were loaded into a reducing polyacrylamide gel for electrophoresis, before the proteins were transferred to a PDVF membrane and visualised by staining using a combination of primary antibodies, species-specific antibodies conjugated to biotin, streptavidin and a chromogenic substrate. The molecular weights of proteins were estimated by comparison to known standards (Seeblue plus2 pre-stained protein marker, Invitrogen, Paisley, UK) and specific proteins identified using monoclonal antibodies.

2.3.6.1 SDS-PAGE.

Gradient (4-12%) BisTris gels (Novex, Invitrogen, Paisley) were first washed in x1 NuPage MES running buffer (Novex, Invitrogen, Paisley) to remove any unpolymerised acrylamide and allowed to equilibrate for 5 minutes in 850mls of x1 NuPage wash buffer in a Novex mini-cell (Invitrogen, Paisley). Cell or viral lysates, along with protein standards, were loaded into the wells and the lysate was allowed to settle for 5 minutes. Next, current was applied to the mini-cell (100 V) and the lysates were allowed to migrate for ~3 hours. The proteins were then transferred to a Trans-blot turbo Midi PVDF membrane (Bio-rad, Hemel

hempstead, UK) and transferred on the Bio-rad turbo blot using the turbo midi programme (25 V for 7 minutes). Unbound sites on the membrane were then blocked in X1 casein solution (Vector Laboratories, Peterborough, UK) overnight at 4^{o}C whilst being agitated at 30rpm.

2.3.6.2 Chromogenic staining.

Membranes were first washed 3 times for 5 minutes in 5mls of PBST. Membranes were then probed with primary antibody in X1 casein solution for 1 hour at room temperature whilst being agitated at 30rpm. The membrane was then washed in 5mls of PBST 4 times for 15 minutes each, whilst being agitated at 30rpm. A biotinylated species-specific secondary antibody was then used to probe the membrane at a dilution of 1/1000 [(1-1.5µg/µl) Vector Laboratories, Peterborough, UK] in X1 casein solution. Membrane and antibody were incubated for 1 hour at room temperature whilst being agitated at 30rpm. The membrane was again washed in PBST 4 times for 15 minutes. The membrane was next incubated with 20µl of both biotinlyated alkaline phosphatase and streptavidin from the Vectastain ABC-Amp kit (Vector Laboratories, Peterborough, UK) at a dilution of 1/500 in X1 casein solution. The membrane was incubated for 15 minutes at room temperature whilst being agitated at 30rpm after which it was once again washed in PBST 3 times for 10 minutes at room temperature whilst being agitated at 30rpm. Protein bands were resolved using 5'-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrate (BCIP/NBT substrate kit, Vector, Laboratories, Peterborough, UK) as per the manufacturer's instructions. Briefly, 1 drop of solutions 1, 2 and 3 were added to every 2.5mls of substrate buffer (0.1M TRIS/HCL pH 9.5) and incubated with the membrane for 1-30 mins at room temperature whilst being agitated at 40rpm. Upon resolution of the protein bands, the membrane was rinsed and then washed in 5mls PBST for 5 minutes at room temperature whilst being agitated at 40rpm to stop the enzymatic reaction.

2.3.7 Analysis of the serological response by immunostaining

To determine the true FIV sero-status of a given sample, a PDVF membrane containing known viral antigens was prepared. This membrane was then cut into strips which were then probed with serum or plasma samples from individual cats. Following

immunostaining, it was then possible to determine which viral proteins were recognised by antibodies within the test samples.

Purified virus was prepared by harvesting culture fluids from FIV infected Mya-1 cells. Briefly, cultures were centrifuged at 102*g* for 10 minutes without no brake. Culture fluid was then aspirated, clarified using a 0.45µm filter and layered over 5mls of 20% sucrose (that had also been 0.45µm filtered) in thin walled, ultraclear™ SW28 tubes (Beckman Coulter, High Wycombe, UK) and centrifuged on the Sovall WX100 ultra-centrifuge for 2 hours at 116000*g* at 4^{o}C using the Surespin 360 SW28 rotor (Kendro, Connecticut, USA). Ultrafiltrates were poured off and the remaining viral pellets were pooled and washed in 1ml of sterile PBS at 16873*g* at 4^{o}C for 1 hour. The PBS was removed and 750µl of NP40 plus protease inhibitor cocktail tablets were mixed with the viral pellet. The mixture was incubated on ice for 30 minutes to ensure thorough lysis of all virions before 250µl of X4 reducing loading dye was added to the viral lysate. The mixture was heated to 90^{o}C for 10 minutes and then the lysate was centrifuged at 16873*g* for 10 minutes. The resulting clarified lysate was aspirated and the optimal loading volume determined for separation using a 4-12% Bis-Tris 2D polyacrlyamide gel (Novex, Invitrogen, Paisley) as described in 2.3.6.1. After the protein transfer and blocking of the membrane, the membrane was cut into ~30 strips that were used to detect anti-FIV antibodies using the chromogenic staining method described in 2.2.6.2.

2.3.8 Estimated total protein concentration using the Bradfords Coomassie assay.

Eleven protein standards of BSA (New England Biolabs) were prepared from a 2mg/ml stock solution, ranging from 0ng/µl to 2µg/ul. Standards increased in concentration by 200ng/µl and the vaccine immunogen was assayed at dilutions of neat, 0.5 and 0.25 in deionised water. Ten micro-litres of standard and analyte were mixed with 100µl of Pierce™ Coomassie G-250 protein stain in a 96 well plate. The plate was incubated at room temperature for 5 minutes and then read at 595nm on the MultiSkan ascent spectrophometer (MTX labsystems, Virginia, USA). The total protein concentration was estimated by extrapolating the optical density of the analyte against the protein standards.

2.4 *In silico* analysis

2.4.1 Sequence alignments.

Sequence data were downloaded from the GATC biotech website and analysed using DNADynamo sequence analysis software. Sequence reads were analysed for mis-called nucleotides and aligned using the CustaL alignment option where complete sequences were saved in the .cow format.

2.4.2 Data analysis

General numerical data analysis was performed using Microsoft Excel 2007 (Microsoft, Redmond, Washington, US).

2.4.3 Graphs and statistical analysis

Statistical analysis and graph generation were performed using the GraphPad Prism 5 software (La Jolla, California, US). In Chapters three and five non-parametric tests were utilised. Although a large number of samples were used in generating the data for chapter three (540 samples in total), this was ordinal data collected into categories (Sero-positive, sero-negative, male, female) and so non-parametric analysis was performed. Additionally, the low optical densities of FIV sero-negative samples and the high optical densities of FIV sero-positive samples indicated that statistical analysis was best performed based on analysis of the median and not the mean values. In chapter five, the low study number (8) warranted the used of non-parametric statistical analysis. Significant outliers were present in this chapter than could not be excluded from analysis and, as such, the data could not fit a normal distribution. Therefore, non-parametric analysis was utilised.

2.4.4 QPCR data analysis

QPCR data were analysed using the 7500 Fast System sequence detection software v1.4 (Applied biosystems, Thermo scientific).

Chapter 3.

Serological diagnosis of FIV infection using TM peptide ELISA.

3.1 Introduction.

The identification of cats that are infected with FIV is confounded by the lack of pathognomonic clinical signs displayed by infected cats. Early studies demonstrated that clinical signs associated with FIV infection included gingivitis/stomatitis, respiratory complications, emaciation and wasting, as well as general lymphadenopathy, neurological signs and skin problems (Hosie *et al*, 1989. Pedersen *et al*, 1988 and 1989. Sparger *et al*, 1989. Ravi et al, 2010. Yamamoto *et al*, 1989. Muirden, 2002. Ishida *et al*, 1989). To date, no study has examined the relationship between FIV infection and the prevalence of FIV associated opportunistic infections. Many epidemiological studies have highlighted an increase in degenerative/inflammation disorders in FIV infected cats mostly associated with the mouth (gingivitis, stomatitis) although this is also common in FIV negative cats (Hosie *et al*, 1989. Muirden, 2002. Addie *et al*, 2000). Additionally, there appears to be an increase in neoplasia in FIV infected cats (Beczkowski *et al*, 2015. Hartmann, 2012). This is believed to be as a result of deceased immune surveillance due to immunodeficiency, however two studies have independently shown that FIV infected cats can live as long as FIV uninfected cats (Addie *et al*, 2000. Ravi *et al*, 2010). The observed neoplasia in FIV infected cats may well be explained by old age rather than FIV infection. Risk factors associated with FIV infection include age, sex, neutered status, outdoor access and domestication status (Levy *et al*, 2006. Muirden, 2002. Yamamoto *et al*, 1989. Gleich *et al*, 2009. Hosie and Jarrett, 1989). The picture that emerged was that older male cats that were feral, or had unrestricted outdoor access and were known to fight were more likely to be infected with FIV than younger cats with no outdoor access. In these studies, FIV infected cats that displayed no clinical signs and appeared healthy made up a significant proportion of the study group and therefore, outside an experimental setting, these cats would not normally have been diagnosed as FIV infected. As infection with FIV is lifelong

and associated with non-specific clinical signs, the diagnosis of FIV infection is based on the detection of anti-FIV antibodies.

3.1.1 Plasticity of the immunodominant domain of TM.

As has been documented previously, the immunodominant domain of TM is an important epitope for the sero-diagnosis of FIV, with ELISA tests that detect antibodies recognising this epitope having sensitivity and specificity values >99%. Furthermore, the inclusion of the TM epitope in serological assays permitted detection of anti-FIV antibodies on average 7 days earlier compared to an ELISA using whole virus alone (Sibille *et al*, 1995). In a comparative study in which TM, SU and p24 proteins were assessed in diagnostic ELISA tests, TM performed significantly better as a capture antigen, displaying the lowest pre-inoculation titres and detecting FIV antibodies at higher titres sooner compared to the other capture antigens (Calzolari *et al*, 1995). Antibodies recognising the TM peptide have been detected as early as 2 weeks post infection, with titres persisting for over 97 weeks (Fontenot *et al*, 1992).

Structural studies of the principal immunodominant domain (PID) of the FIV TM have shown that the PID can withstand substantial mutation within the region contained within the two proximal cysteine residues, including mutation to the sequence of a lentivirus of another species, such as HIV-1. The progeny of the mutant virus displayed altered fusogenic properties, however, following transfection of Crfk cells; RT activity could be detected for up to 9 days and syncytia could be observed up to 72 hours post transfection (Pancino *et al*, 1995). Other viral kinetics were similar to the wild type virus, albeit attenuated. Virus in which the entire PID was mutated did not productively infect cells, but the virus retained infectivity when mutations occured only within the N-terminal domain of the PID. However, FIV positive sera no longer recognised the mutated PID when used as an ELISA capture antigen (Pancino *et al*, 1995. Pancino and Songio, 1997).

In this chapter, a DIVA ELISA measuring FIV antibodies was developed and validated, using a peptide corresponding to the PID of the subtype E lion lentivirus (LLV-E). The hypothesis that was tested was that samples of serum or plasma collected from cats infected with FIV would not recognise a peptide corresponding to the PID of LLV-E. Our rationale was that a whole inactivated virus (WIV) vaccine based on an infectious molecular clone of FIV-GL8

bearing a mutation that replaced the PID of FIV with that of LLV-E could form the basis of a DIVA vaccine. Cats vaccinated with such a vaccine could be tested for FIV infection by detecting antibodies recognising the FIV PID; all vaccinated cats would develop antibodies recognising the LLV-E PID, whereas only infected cats would recognise the FIV PID. Hence, by utilising the unique characteristics of the PID, it was proposed that it might be possible to differentiate infected from vaccinated cats, as well as uninfected from infected, vaccinated cats.

3.2 Materials and Methods.

3.2.1 UK Samples of known FIV sero-status

(Appendix 3i, Immunostains 1-15. Signalment tables 1a and 1b).

A total of 175 feline blood samples were tested in this study to optimise an ELISA to detect antibodies recognising a peptide representing the FIV TM PID. The samples had been submitted to the Veterinary Diagnostic Service (VDS), School of Veterinary Medicine, University of Glasgow by UK veterinary clinics and comprised plasma or serum collected from client-owned, stray or shelter cats. The samples had been submitted for FIV diagnostics and had already tested positive for FIV antibodies by immunofluorescence testing conducted by VDS prior to this study. Details of age, sex, breed and clinical signs were recorded from the sample submission forms, but all information was not always available, particularly for stray or shelter cats.

3.2.2 Samples of indeterminant FIV sero-status

(Appendix 3ii, Immunostains 1-16. Signalment tables 2a and 2b).

When inconclusive results were obtained using the IFA for FIV antibodies, further testing was conducted as part of this study. These samples had been submitted by veterinarians who had suspected FIV infection in cats, the cats had been sampled and tested antibody positive using a POC kit, but had then tested inconclusive by IFA and immunostain when tested by the commercial VDS laboratory. A total of 93 samples from 77 cats (62 individual

and 31 sequential samples) were tested using the TM peptide ELISA as well as immunostaining. As a further confirmatory test, virus isolation was conducted when heparinised whole blood samples were available, with virus being detected by reverse transcriptase activity or the production of FIV p24, as described in Chapter 2 (Section 2.3.4 and 2.3.5 respectively).

3.2.3 Criteria for FIV serostatus as determined by IFA.

Plasma or serum samples were screened for anti- FIV antibodies by VDS, using IFA. Briefly, methanol-fixed, FIV infected CrFK cells were incubated with test samples at tenfold dilutions from 1 in 10 to 1 in 10 000 in PBS, followed by a second incubation with fluorescein-conjugated goat anti-cat Ig and then examined by fluorescence microscopy to determine the titre of anti-FIV antibodies in each sample. Samples testing positive underwent confirmatory testing by immunostain analysis, incubating samples with a membrane prepared from a lysate of FIV infected CrFK cells to visualise antibodies recognising the FIV p24 (CA) and p17 (MA) proteins. Samples with anti-FIV titres of ≥ 1000 that demonstrated antibodies against both p17 and p24 were reported as positive. Samples with anti-FIV titres of 10-100 were classified as inconclusive. If such samples demonstrated antibodies recognising both p17 and p24, they were confirmed as positive, whereas samples with no antibodies recognising p17 or p24 were confirmed as negative. Samples with no antibodies detectable by either IFA or immunostaining were classified as negative.

3.2.4 Fel-O-Vax vaccinated samples collected in Australia

(Appendix 3iii, Immunostains 1-9. Signalment tables 3a, 3c and 3d).

The samples from FIV vaccinated cats that were examined in this study had been analysed in a previous study by Westman *et al* (2015). Samples had been collected between 2013-14 from client-owned cats in south eastern Australia and then shipped to the University of Glasgow for further testing. Samples were classified as “vaccinated” if the cat had received the primary immunisation, as per the manufacturer’s recommendations, and had subsequently received 3 annual booster immunisations (n=114). Cats that had never been vaccinated against FIV and tested FIV antibody negative (using a commercially available

test kit) comprised the unvaccinated and uninfected group (n=113). Samples that had tested positive for FIV proviral DNA, using a commercially available PCR (FIV RealPCR™, IDEXX), were considered to be infected and hence were predicted to be sero-positive (n=27). Since the FIV RealPCR™ is not 100% accurate and produced false positive results (Nichols *et al*, 2016) it was imperative to be able to accurately designate the correct sero-status to the vaccinated, unvaccinated group and uninfected and infected groups. Failure to accurately determine this would result in a bias sensitivity and specificity calculation. As such, the cats' clinical history was extensively interrogated whilst plasma samples underwent additional screening using three point of care test kits to ensure the recruitment criteria was met. Any ambiguous results underwent virus isolation for confirmation.

3.2.5 Fel-O-Vax vaccinated samples collected in US

(Appendix 3iii, Immunostain 10. Signalment table 3b).

Whole blood was collected in heparin from eighteen client-owned cats during October 2012 (comprising part of the cohort described by Litster *et al* 2014) and shipped to the University of Glasgow for analysis. These samples had been collected from cats that had tested negative for FIV antibodies prior to vaccination and had received all 3 primary immunisations. The FIV infection status of these cats was determined using virus isolation, as described in Materials and Methods section 2.2.6.

3.2.6 Genetically modified Crandell feline kidney cells [CrFK (FeFab)]

FeFab cells are genetically modified Crandell feline kidney cells (CrFK) that are susceptible to feline syncytium forming virus (FeSFV). FeFab cells were developed by Zemba and colleagues in 2000 specifically to titrate FeSFV (Zemba *et al*, 2000). The U3 region of the LTR is spliced together, in frame, with the reporter gene β-galactosidase. Upon infection with FeSFV, the *bel1* gene is expressed and binds to an internal promoter, increasing expression of Bel*1*, as well as binding the U3 region of the 5' LTR. This leads to increased transcription of the integrated viral genome in the FeFab cells and increased β-galactosidase expression, which is visualised by staining with X-gal solution (3mM $K_4[Fe(CN)_6]$ (II)/3mM/ $K_3[Fe(CN)_6]$ (III)/ 0.02% X-Gal/1.3mM $MgCl_2$).

FeFab cells were maintained in complete DMEM and sub cultured into fresh medium containing G418 every 4-5 days.

3.2.7 Product enhanced reverse transcription assay

The product enhanced reverse transcription (PERT) assay is a highly sensitive method used to detect and quantify the amount of reverse transcriptase (RT) in a test sample. Based on the assay developed by Arnold *et al* in 1998 (Arnold *et al*, 1998), the RT within a test sample synthesises cDNA from the input RNA transcript. This cDNA is then amplified using PCR and the product is detected using a fluorescently labelled probe. Since no additional RT is added to the reaction, the only source of RT is that contained within the test sample.

Cell free tissue culture fluids were prepared and diluted 1 in 10 in deionised water (Gibco, Life Technologies) in sterile eppendorf tubes. Five microlitres of each sample were added to wells of a MicroAmp fast optical 96-well reaction plate (Applied biosystems, Thermo scientific, Paisley, UK), together with the reagents listed in Table 3-1.

Reagent	Volume (µl)	Supplier
Lysis buffer (50mM KCl, 100mM Tris HCl pH 7.4, 40% glycerol, 0.25% Triton X-100)	5	Various
BSA (10mg/ml)	0.2	New England biolabs
Rnase inhibitor(40U/µl)	0.1	Promega
MS2 Forward primer (10pmol/µl)	0.1	IDT
MS2 Reverse primer (10pmol/µl)	0.1	IDT
Ms2 Probe (5pmol/µl)	0.05	IDT
MS2 RNA template (0.8µg/µl)	0.02	Roche diagnostics
Taqman universal master mix	10	Applied biosystems

Table 3-1. PERT reactants and volumes.

Reverse transcription of the input RNA transcript was followed by PCR amplification of the cDNA template using the cycling conditions listed in Table 3-2, using the ABI 7500 fast PCR machine (ABI, Leicestershire, UK).

Temperature (°C)	Time (mm:ss)	Number of cycles	Stage of reaction
48	30:00:00	1	Reverse transcription
95	10:00	1	RT inactivation.
95	00:15	40	cDNA melting
60	01:00		Annealing & amplification

Table 3-2. PERT reverse transcription and amplification cycling conditions.

PERT data were analysed using Applied Biosystems sequence detection software, 7500 fast system SDS software v1.4. Samples were scored positive or negative by comparing the mean test value (each test sample was tested in triplicate) to the mean values obtained for triplicate tests of a negative control and a range of known RT standards.

3.2.8 ELISA to detect antibodies against FeLV-A SU-Fc

Samples that tested negative for FIV infection but tested positive using the PERT assay were assessed for exposure to FeLV using an ELISA to detect antibodies against an FeLV SU-Fc fusion protein that had been developed by Ms Yasmin Parr, University of Glasgow. The SU protein of FeLV-A had been fused in fame with the conserved region of human IgG (Fc) and cloned into the retroviral vector pTORSTEN and then stably expressed in 293 cells. Cultures were selected in 200µg/ml of Hygromycin (Sigma-Aldrich UK, Gillingham, Dorset) and expanded into five T150 culture flasks before culture fluid was harvested every 3-4 days by centrifuging at 159*g* for 5 minutes and then clarifying the culture fluids by filtration through a 0.45µM and then a 0.2µM filter (Millipore UK LTD, Bedfont Cross, Feltham). The FeLV SU-Fc fusion protein was then purified from a litre of culture fluid using a protein A column (Sigma-Aldrich UK, Gillingham, Dorset). The total protein concentration of the retentate was estimated using the Bradford's Coomassie assay. One hundred nanograms per well of FeLV SuFc fusion protein were absorbed onto a 96 well microtiter plate (Immulon 2 HB) in sodium carbonate bicarbonate binding buffer and incubated at 4^{o}C overnight whilst being agitated at 30rpm. Wells were then aspirated and washed 5 times with 200µl of phosphate buffered saline supplemented with 0.1% Tween (PBST). Unabsorbed sites were blocked with 200µl of 2% low fat milk powder PBST (MPBST, block) for 1 hour at room temperature. Wells were then aspirated and washed 5 times with 200µl of PBST and 100µl of each plasma or serum sample were added to triplicate wells, diluted 1 in 200 in block. Plates were sealed

and incubated at room temperature for 1 hour, wells were aspirated and washed 5 times with 200µl of PBST and then 100µl of a biotinylated anti-cat Ig secondary antibody (Vector laboratories, Peterborough, UK) were added to wells at a dilution of 1 in 1000 (1-1.5µg/µl) in block. Plates were sealed and incubated at room temperature for a further hour, wells were aspirated and washed 5 times with 200µl of PBST and then 100µl of horseradish peroxidise conjugated to streptavidin [Vector laboratories, Peterborough, UK] were added to wells, diluted 1 in 1000 (1-1.5µg/µl) in block. Plates were sealed and incubated at room temperature for 20 minutes before the wells were aspirated and washed 5 times with 200µl of PBST and then 100 µl of 3',3',5'5'-tetramethylbenzidine liquid (TMB super slow, Sigma Aldrich) were added to each well, the plates were sealed and then incubated at room temperature for 30 minutes before the absorbance values were read at 650nm on the MultiSkan ascent spectrophotometer (MTX labsystems, Virginia, USA).

3.3 Results

3.3.1 Samples of known FIV infection status

3.3.2 Study population

In this study, 175 samples that had been tested for anti-FIV antibodies by VDS were tested; 62% of the samples had been collected from male cats (46 entire and 62 neutered male cats) 33% had been collected from female cats (29 entire and 29 neutered female cats) and 5% (9 samples) had been collected from cats with no gender recorded on the sample submission form. A higher prevalence of FIV was observed in male cats compared to female cats, as shown in Figure 3-1 and neutering did not appear to be a risk factor for FIV infection. As shown in Figure 3-2, cats testing FIV antibody positive were generally older than the cats testing negative and this difference was statistically significant. These data supported previous findings that older male cats were more likely to be FIV infected compared to female cats. Of the 175 samples tested, 93 tested positive and 82 tested negative for FIV by IFA and immunostain analysis.

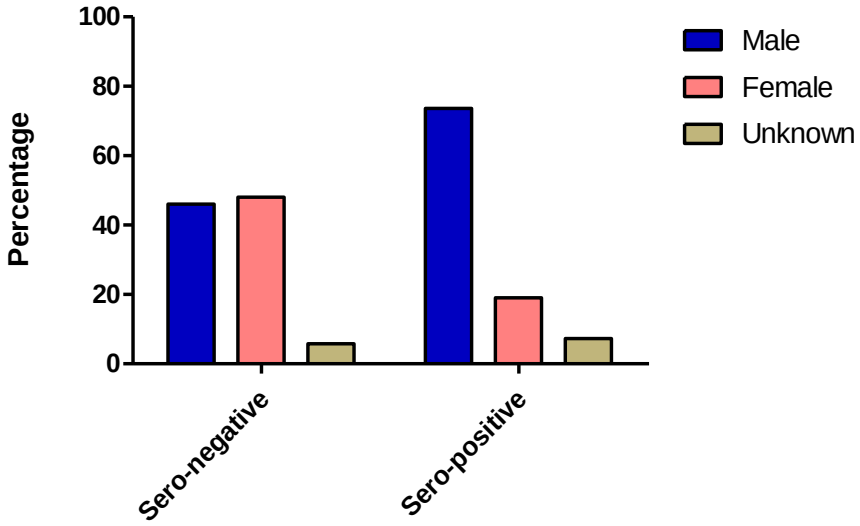


Figure 3-1. Gender distribution in cats testing positive or negative for FIV antibodies within the known FIV status group. Male and female cases were equally represented in the antibody negative cats (46% male versus 48% female cats), whereas the FIV antibody positive group was more likely to contain male than female cats (73% male versus 19% female).

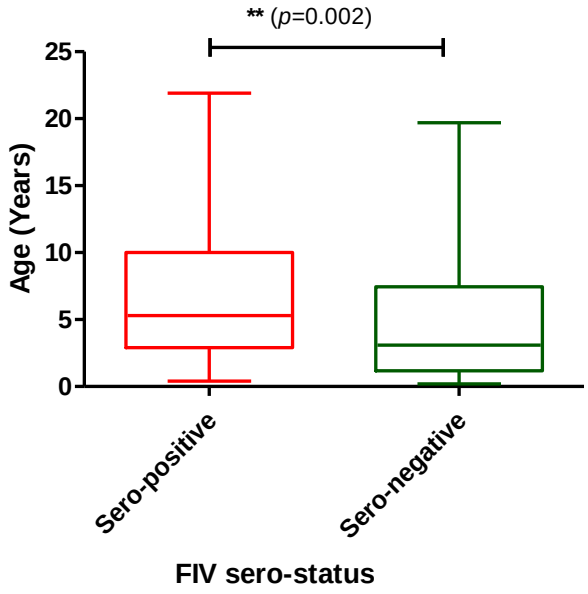


Figure 3-2. Age distribution of cats testing positive and negative for FIV antibodies within the known FIV status group. The interquartile range of the sero-positive group was higher at the 25th and 75th percentiles compared to the sero-negative group (25th percentile of 2.9 versus 1.2 years and 75th percentile of 10 versus 7.4 years of age for sero-positive and sero-negative groups respectively). The difference between the median values of the 2

groups (5.3 years for sero-positive cats versus 3.1 years for sero-negative cats) was statistically significant (p=0.002, Mann-Whitney test).

The clinical signs reported by the veterinarian on the sample submission forms were tabulated and examined for associations between FIV sero-status and clinical signs. As shown in Table 3-3, a lack of clinical signs within the sero-positive group was commonly reported, followed by gingivitis, weight loss and non-specific signs of sickness, lethargy, malaise or pyrexia. These signs closely matched their rank within the sero-negative group, although overall more of the cats in the seronegative group showed gingivitis or weight loss that had been observed in the sero-positive cats. It was noted that 55.5% (10/19) of cats displaying gingivitis and/or stomatitis in the sero-negative group had tested positive for feline calcivirus (FCV), as well as 66.7% (6/9) of cats in the sero-positive group. Similar findings have been reported previously (Hartmann, 2011), indicating that gingivitis and/or stomatitis is a common clinical sign reported in FIV-infected cats, often associated with coinfection with FCV.

Sero-negative samples		
Clinical Sign	**%**	**Number**
Gingivitis	17.8	13
Weight loss/emaciation	16.4	12
Gingivostomatitis	5.5	4
Pyrexia	5.5	4
Lethargy	5.5	4
Dysponea	2.7	2
Stomatitis	2.7	2
Neutropenia	2.7	2
Dental Disease	2.7	2
Seizures	2.7	2
Disorientation	2.7	2
URT	2.7	2
Plasma cell podermatitis	2.7	2
Vomiting	1.4	1
Ataxia	4.1	3

Sero-positive samples		
Clinical Sign	**%**	**Number**
Gingivitis	12.0	9
Weight loss/emaciation	8.0	6
Gingivostomatitis	1.3	1
Pyrexia	4.0	3
lethargy	6.7	5
Dysponea	1.3	1
Healthy	13.3	10
Sick	6.7	5
Lingual ulcers	5.3	4
Abscess	4.0	3
Malaise	4.0	3
Inappentance	2.7	2
Conjunctivitis	2.7	2
Anaemia	2.7	2
Periodontis	1.3	1

Table 3-3. Tabulated and top 15 ranked clinical signs recorded for cats in the known FIV status population. Of the 82 sero-negative samples examined, 73 clinical signs had been reported for 56 samples, while 26 samples had been submitted to VDS for either routine or confirmatory FIV screening or for reasons not disclosed. Of the 98 sero-positive samples examined, 75 clinical signs had been reported for 46 samples, while 47 samples had been submitted for either routine or confirmatory screening or for reasons unknown.

3.3.3 The utility of an ELISA test to detect antibodies against FIV TM peptide

The panel of 175 samples of known FIV antibody status were screened using the TM peptide ELISA to determine whether the ELISA could be used as a diagnostic test. As shown in table 3-4, the TM peptide ELISA correctly identified 92/93 sero-positive samples and 82/82 negative samples (Figure 3-3); the one sample with an inconclusive result tested positive by immunostain analysis. The negative fold values were calculated and used to differentiate between sero-positive (values 4.59-20.56), sero-negative (values 0.34-1.77) and the one inconclusive sample with a value of 3.85.

	IFA	TM ELISA	Reference standard (Immunostain)
Positive	93	92	93
Inconclusive	0	1	0
Negative	82	82	82
Total	175	175	175

Table 3-4. Comparison of IFA and the TM peptide ELISA for the detection of anti-FIV antibodies. Immunostain analysis (appendix 3i, immunostains 1-15) was used as the reference standard as both IFA and the peptide TM ELISA were serological assays and PBMC were not available for virus isolation. On this basis, IFA showed specificity and sensitivity values of 100%, while the TM peptide ELISA showed a sensitivity of 98.9% and a specificity of 100%.

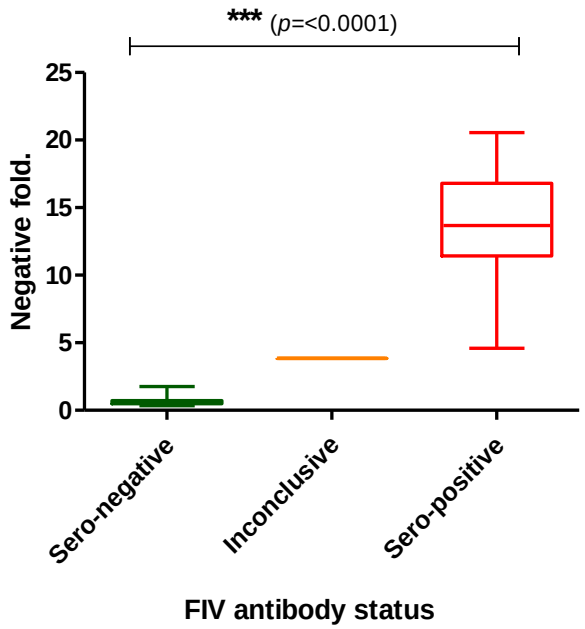


Figure 3-3. Negative fold values for the 175 samples of known FIV status tested, grouped according to FIV sero-status. The negative fold values were used to assign samples to three groups: sero-negative (n=82), sero-positive (n=93) and inconclusive (n=1). The differences between the median negative fold values of the sero-positive and negative samples were statistically significant (*p*=<0.0001. Kruskal-Wallis test).

3.4 Inconclusive samples.

3.4.1 Sampling criteria and sample population.

In total, 93 samples from 77 cats were examined, comprising both single samples from individual cats as well as sequential samples as shown in Figure 3-4.

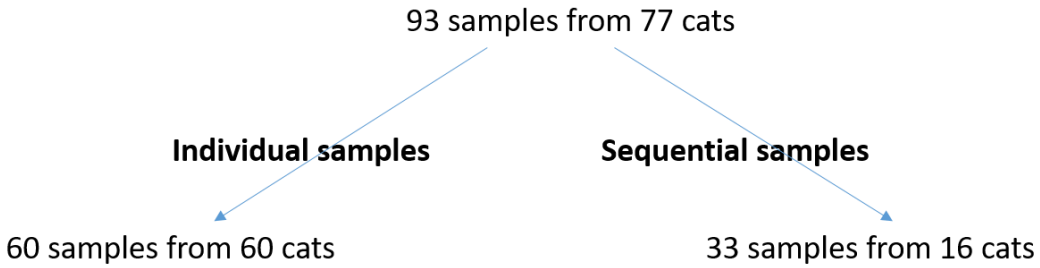


Figure 3-4. The FIV inconclusive sample population. Individual samples comprised 64.5% and sequential samples comprised 35.5% of the sample population. Sequential samples were tested to confirm the results of previous tests, as cats that had been in the initial

stages of infection when first tested could be shown to have seroconverted at the time of later samplings.

As these samples had tested inconclusive by IFA, a panel of additional tests was conducted to investigate the true FIV status of each sample. The additional tests that were performed depended on the sample type submitted for testing, as shown in Table 3-5.

Sample type	TM ELISA	VI	p24 Ag ELISA	PERT	Immunostain
Serum	+	N/A	N/A	N/A	+
Heparinised blood	+	+	+	+	+
EDTA blood	+	+	+	+	+

Table 3-5. Additional validation assays performed to confirm TM ELISA results. As most of the serum samples were over 7 days old or had been submitted in serum separating tubes, it was not possible to purify PBMC for virus isolation. At the start of the study, it was thought that virus isolation could be performed only on PBMC derived from whole blood collected in heparin and not EDTA, since EDTA is toxic for Mya-1 cells. However, it was demonstrated that extensive washing of the PBMC in PBS removed residual EDTA so that virus isolation could be performed on these samples, with no evidence of EDTA toxicity.

3.4.2 Sample population and clinical signs.

In the population of 77 cats examined in this study, 54.5% were male (23 entire males and 19 neutered males) and 36.4% were female (14 entire females and 14 neutered females). For 7 cats (9.1%), this information was not available. As shown in Figure 3-5, FIV infection was more prevalent in male cats, whereas similar numbers of male and female cats were observed in the uninfected group. This gender distribution reflected that of the samples of known FIV status, confirming that gender is a strong risk factor for FIV infection, regardless of neutering status. As shown in Figure 3-6, the age distributions of both infected and uninfected cats were similar; the median ages of the two groups were not statistically significantly different. This was most probably a result of the small numbers of positive samples within the tested population.

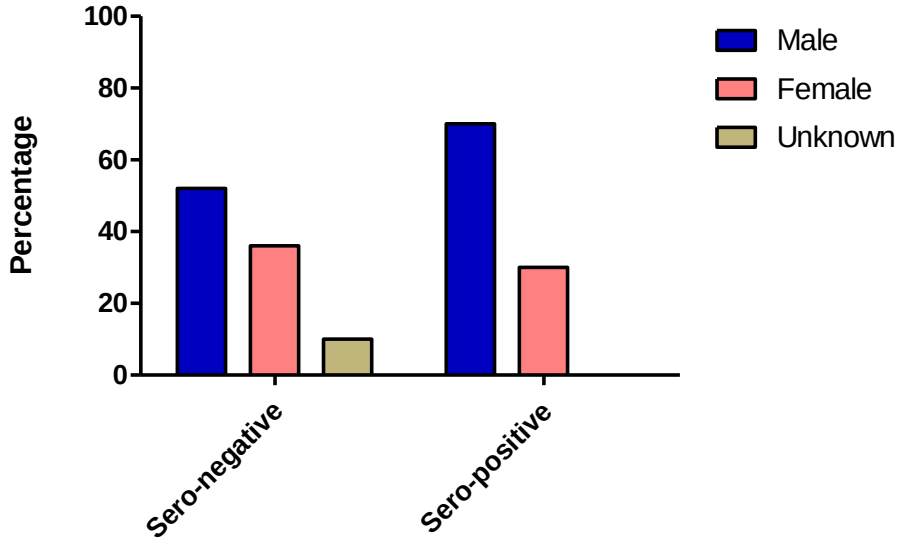


Figure 3-5. Gender distribution of the FIV inconclusive sample population. More male than female cats were observed in the sero-negative group (52% males versus 36% females) and can most likely be explained by the increased likelihood of male cats being tested for FIV infection. There were significantly more samples from male than female cats in the sero-positive group (70% males versus 30% females).

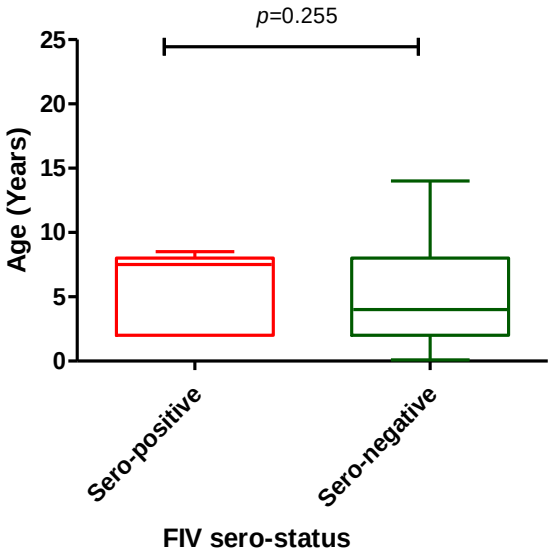


Figure 3-6. Age distribution of infected and uninfected cats from the FIV inconclusive population. Both groups displayed similar 25th percentile and 75th percentile values (2 and 8 years respectively) and the difference between the median ages (7.5 years for sero-

positive cats versus 4 years for sero-negative cats) was not statistically significant (p=0.255, Mann-Whitney test).

When the clinical signs were tabulated and ranked, as shown in Table 3-6, weight loss and gingivitis ranked highest; however, gingivitis and fight-related abscesses were more closely associated with FIV infection than weight loss. Samples that had been sent to VDS for confirmatory testing had tested positive for FIV using one of the commercially available point of care (POC) tests (Snap, Witness or Snap duo). Of the 40 samples submitted for confirmatory testing, only 3/40 samples were confirmed as positive by immunostaining, raising concerns about how the POC tests had been conducted, since independent evaluation demonstrated that these tests were highly sensitivity and also highly specific (Hartmann *et al*, 2007).

Clinical sign	%	Total number	Number positive
Weight loss	11.9	5	1/5
Gingivitis	7.1	3	3/3
Pyrexia	7.1	3	-
Gingivo-stomatitis	4.8	2	-
Stomatitis	4.8	2	-
Anaemia	4.8	2	-
Fight abcesses	4.8	2	2/2
Chelitis	2.4	1	1/1
Mass in mouth	2.4	1	1/1
Healthy	2.4	1	1/1
Jaundice	2.4	1	-
Incontinence	2.4	1	-
Ulcerated wound	2.4	1	-
Renal disease	2.4	1	-
Puritis	2.4	1	-

Table 3-6. Top 15 ranking clinical signs reported from the FIV indeterminant population. For the 77 cats tested in this study, 30 clinical signs had been recorded on the sample submission forms. However, many samples had been submitted for confirmatory testing following positive POC test results.

3.4.3 The TM ELISA can be used to confirm IFA inconclusive samples.

Of the 77 samples for which IFA results were inconclusive, 67 (87%) tested negative, 8 (10.4%) tested positive and 2 (2.6%) gave inconclusive results using the TM peptide ELISA.

One of the samples that gave an inconclusive result had been collected from a cat that had been sampled sequentially (2 samples); this cat was shown to have seroconverted by the time of the second sampling. The negative fold values obtained from the TM peptide ELISA were examined for samples with inconclusive IFA results and it was demonstrated that the TM peptide ELISA could be used to accurately identify the IFA inconclusive samples as either sero-positive or sero-negative, with similar statistical power as was demonstrated previously for samples of known serological status (Figure 3-7).

	Number of animals	Number of samples
Sero-negative	67	83
Sero-positive	8	8
Inconclusive	2	2
Total	77	93

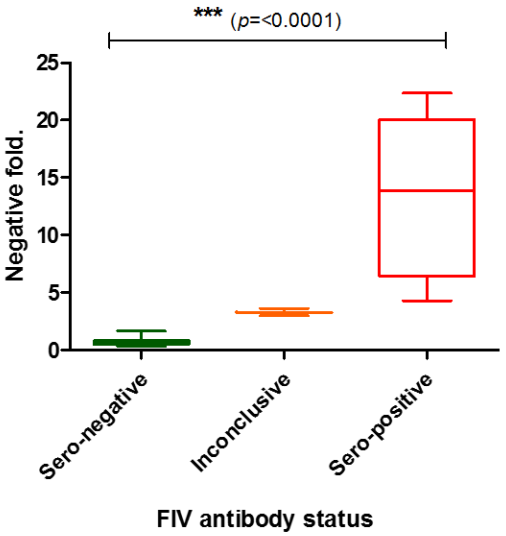


Figure 3-7. Summary of the number of animals and samples and negative fold values (determined by TM ELISA) for samples according to FIV antibody status. The negative fold values calculated from the sample absorbance results could be used to confirm the FIV sample status. The difference between the median negative fold values for the positive and negative samples was statistically significant (p=<0.0001. Kruskal-Wallis test).

3.4.5 Validation of the TM ELISA result.

Of the 93 samples received, 66 had been collected in heparin or EDTA and 27 had been submitted to the laboratory as sera. The number of samples undergoing further testing are shown in Table 3-7.

	Serology		Virus detection		
Sample type	TM ELISA	Immunostain	VI	p24 antigen ELISA	PERT
Sera (n=27)	27	27	N/A	N/A	N/A
Plasma (n=66)	66	66	65	65	53

Table 3-7. Numbers of samples for which additional tests were performed to validate the TM ELISA results. To confirm the TM ELISA results, immunostain tests were performed. When PBMC were available, attempts were made to isolate FIV following co-cultivation with Mya-1 cells, using either a retrovirus specific assay (PERT) or an FIV specific assay (p24 antigen ELISA) to monitor FIV infection.

Immunostaining is the reference standard for FIV diagnosis (Wang *et al*, 2010. Hartmann *et al*, 2007, Hosie *et al*, 2009). In this study, the results of immunostaining were classified using the following criteria: antibodies recognising at least two FIV core proteins (p55, p24, p17, p10) or gp95/gp41 were required for a sample to be confirmed as positive. When the two samples that had tested inconclusive using the TM ELISA (and 8 samples that had tested positive) were examined, all samples tested positive by immunostain analysis using these criteria (Figure 3-8).

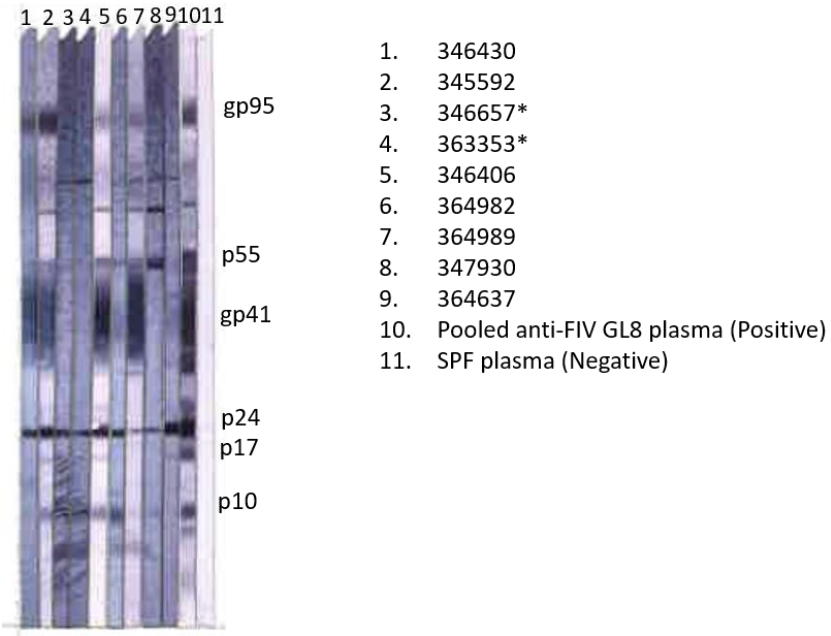


Figure 3-8. Immunostain analysis of samples testing positive or inconclusive using the TM peptide ELISA from the inconclusive sample population. All samples demonstrated strong antibody reactivity to at least two specific viral proteins during the optimisation procedure. These additional immunostain results are shown in Appendix 3ii. * samples that tested inconclusive by TM ELISA.

3.4.6 Detection of virus.

As a further step to validate the serological testing of the inconclusive samples, attempts were made to isolate virus from all plasma samples (whether collected in heparin or EDTA). Virus isolation was conducted on 65/66 available samples and cultures were monitored for the production of FIV p24 by ELISA (65/65) or RT activity using PERT (53/65), with the results shown in table 3-8.

	p24 Ag ELISA	PERT
FIV sero-status	Number positive/Number tested	Number positive/Number tested
Sero-positive (n=8)	7/65	8/53
Sero-negative (n=56)	0/65	5/53
Inconclusive (n=1)	0/1	0/1

Table 3-8. Detection of FIV in the culture fluids of the FIV inconclusive samples. Culture fluids were screened for the presence of FIV p24 by ELISA, or for the presence of reverse transcriptase by PERT.

As PERT has been reported to be more sensitive for virus detection compared to an ELISA detecting FIV p24 (Arnold *et al*, 1998)**,** the five sero-negative samples that displayed RT activity were examined further. Since the serological tests and virus isolation tests had detected 8 positive and 2 inconclusive samples, it was possible that the RT activity detected in cultures from the FIV sero-negative samples might have been associated with infection with a feline retrovirus other than FIV. Alternatively, the TM ELISA might have resulted in a false negative result. As 3/5 samples that showed RT activity in cultures had been collected from cats that had been sampled sequentially, additional testing was conducted on these follow-up samples.

In total, 8 samples collected from 6 cats were screened for the presence of the other feline retroviruses, FeSFV and FeLV. Upon X-Gal staining of FeFab cells that had been cultured with the original virus isolation culture fluid, 0/8 samples showed blue staining, indicating that 8/8 samples tested negative for FeSFV. Next, the samples were tested by ELISA to detect antibodies recognising the SU (gp70) protein of FeLV-A. As shown in Figure 3-9, 6/11 samples tested displayed antibodies recognising the FeLV SU recombinant protein (4/6 cats). Cats represented by the purple, orange and yellow columns showed antibody binding to the recombinant FeLV SU, whereas the binding observed in sample 347266 and 347445 (from a single cat, green columns) was likely non-specific. However, when sample 347266 was screened on the IDEXX p27 ELISA, a marginal increase in absorbance was observed, although the value was below the threshold for a positive result. Samples from the cat represented by the yellow columns displayed a dramatic decrease in antibody titre between the two samplings. It was not possible to conduct virus isolation for FeLV on the

first sample received from this cat (346170), but the second sample (347929) was borderline positive on the PERT assay (2/3 replicates were RT positive). It is tempting to speculate that this cat might have been sampled as it was recovering from FeLV infection, having developed nAb, although this was not investigated further. The results of the FeLV SU ELISA were confirmed by immunostaining (Figure 3-10).

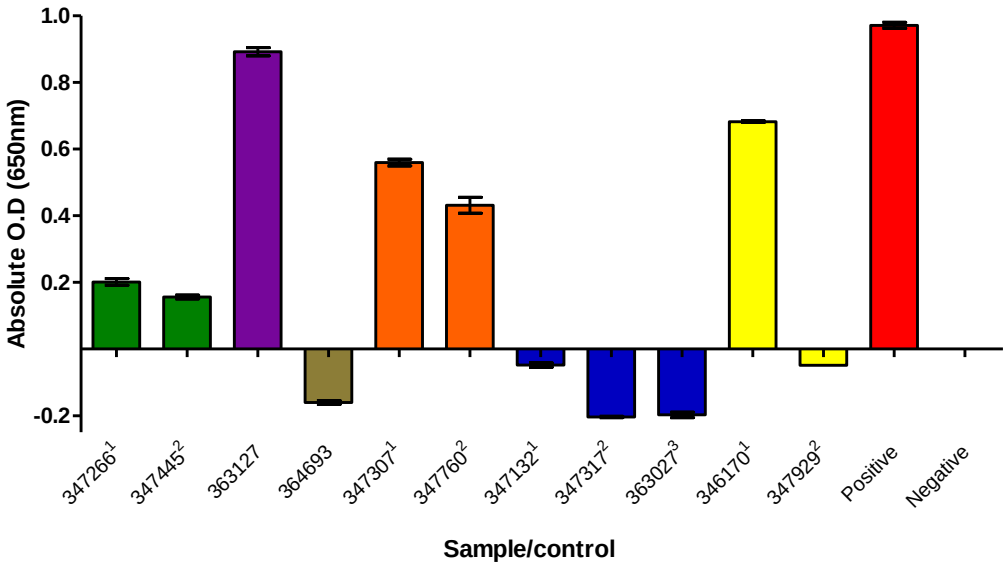
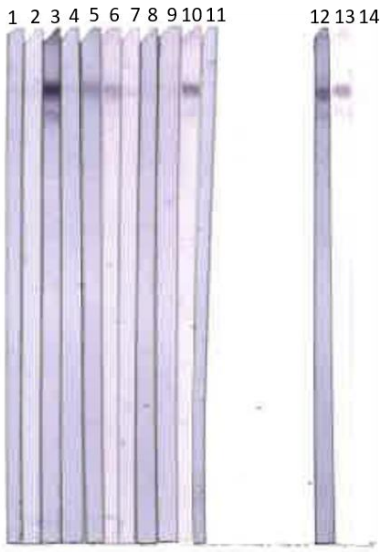


Figure 3-9. FIV sero-negative, RT positive samples reactivity to recombinant FeLV SU. FeLV antibody status was determined using an empirical cut-off value; the absorbance of the negative control was subtracted from the sample absorbance to give the absolute absorbance. Samples represented in the same colour were collected from the same cat, with superscript numbers (*x*-axis) used to denote the order of sampling.



1. 347226[1]
2. 347445[2]
3. 363127
4. 364693
5. 347307[1]
6. 347760[2]
7. 347132[1]
8. 347317[2]
9. 363027[3]
10. 346170[1]
11. 347929[2]
12. Positive (FeLV vaccinated plasma)
13. Anti-gp70 monoclonal
14. Negative (FIV vaccinate plasma)

Figure 3-10. Immunostain analysis to detect antibodies recognising recombinant FeLV SU protein. Strips were probed with sera or plasma samples diluted 1 in 400 in X1 casein solution. Antibody binding to the recombinant FeLV SU protein detected in samples 3, 5, 6 and 10, confirming that these cats had been exposed to FeLV infection. Plasma collected from a cat vaccinated against FeLV was used as the positive control, as well as an anti-FeLV gp70 monoclonal antibody to confirm that the FeLV SU was detected. The negative control sample was collected from an FIV vaccinated cat.

These data demonstrated that the TM ELISA could be used to identify FIV sero-positive samples as the results were confirmed and validated by immunostain and virus isolation. The FIV sero-negative samples that displayed RT activity were shown to have been exposed to FeLV (Table 3-9), confirming that the TM ELISA did not yield false negative results and it appeared that FIV testing could be performed using the TM ELISA to test old, sub-optimal samples in poor condition.

Sample ID[a]	Re-sample date	Ct value[b]	SuFc FeLV A ELISA absolute O.D.	SuFc FeLV A immunoblot	FeSFV Titre (FFU/ml)
347226 [1]	15/09/2014	>40	0.201	-	0
347445 [2]	25/09/2014	37.935*	0.156	-	0
363127		32.993	0.892	+	0
346639		36.764*	-0.160	-	0
347307[1]	17/09/2014	34.434**	0.560	+	0
347760[2]	13/10/2014	n/a	0.431	+	n/a
347132 [1]	10/09/2014	n/a	-0.047	+/-	n/a
347317 [2]	17/09/2014	38.956*	-0.204	-	0
363027 [3]	22/10/2014	>40	-0.197	-	0
346170 [1]	17/07/2014	n/a	0.682	+	n/a
347929 [2]	16/10/2014	39.261**	0.049	-	0

Table 3-9. Results for FIV sero-negative, RT positive samples. [a], individual samples shown in green and sequential samples highlighted in purple. The order of sampling is denoted in superscript. [b], the sensitivity limit of the PERT assay was 40 cycles. The number of asterisks beside the Ct values indicates the number of triplicate wells in which RT was detected. If no asterisk is present, 3/3 wells tested RT positive; n/a, not applicable (serum samples); +/-, faint band.

3.4.7 TM ELISA performed better than IFA.

The results of this study demonstrated that IFA could not be used to reliably determine the sero-status of any of the 93 samples sent for sero-diagnosis, whereas the TM ELISA performed well, as illustrated in Figure 3-11. It was evident that the IFA assay produced both false negative and false positive results when used to screen many samples collected over a 12-month period.

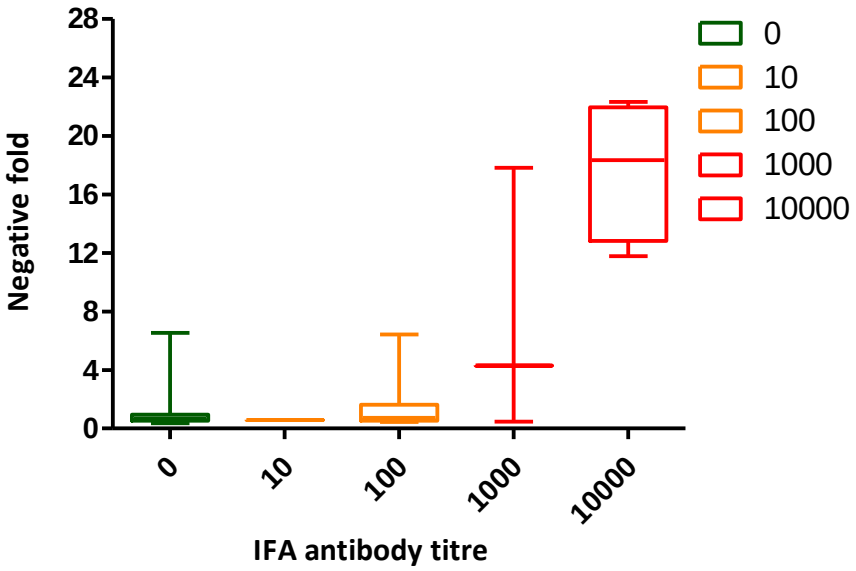


Figure 3-11. Comparison of IFA antibody titres to the negative fold of the sample absorbance obtained using the TM ELISA. It was evident that some samples with IFA titres of 0 tested positive by TM ELISA (false negative by IFA), while some samples with IFA titres of 1000 tested negative by TM ELISA (false positive by IFA). In contrast, there was close agreement between the two tests in samples with IFA titres $\geq$ 1000. False positive results likely resulted from antibodies in the sample recognising cellular proteins, while false negative results might have been obtained with samples from cats in either the initial or terminal stages of FIV infection.

As the samples in the known sero-status group had previously had their FIV status determined, the sampling of this population was biased and was not representative of the true prevalence of FIV in the UK. It was therefore not feasible to calculate positive or negative predictive values for either IFA or the TM ELISA in this study. A more appropriate calculation was to determine the specificity and sensitivity of IFA and the TM ELISA comparing the results to immunostaining as the 'reference standard' test (table 3-10).

IFA	
True positives =103	False positives =16
False negatives= 3	True negative= 149

Sensitivity = (103/103) + 16 x 100 = 86.6%
Specificity = (149/149) + 3 x 100 = 98.0%

TM ELISA	
True positives =103	False positives = 0
False negatives= 3	True negative= 149

Sensitivity = (103/103) + 0 x 100 = 100.0%
Specificity = (149/149) + 3 x 100 = 98.0%

Table 3-10. Comparison of the specificity and sensitivity of IFA and the TM ELISA. To ensure that only the test result is considered in the calculations (and not the methodology that determines the category of infection status), any samples giving an IFA titre greater than 0 were deemed positive and any sample giving a negative fold of less than 4 were considered negative on ELISA. This essentially removes the redundancy from both test and forces the assay to give a clear-cut positive or negative result. When both populations of samples were examined together (samples of a known sero-status and inconclusive samples), both diagnostic test displayed identical levels of specificity at 98%, however the TM ELISA has substantially better sensitivity at 100% compared to 86.6% for IFA.

The TM ELISA demonstrates greater specificity than IFA, a result that confirms Figure 3-11. The better rate of specificity can be explained by the simplicity of the TM ELISA. The TM ELISA uses a single 11 amino acid peptide that corresponds to the PID of the transmembrane region of the envelope, whereas IFA uses infected, inactivated Crfk cells. By utilising a single epitope in the TM ELISA, the vast majority of cross reactive antibodies are rendered useless as there is very little for the antibodies to bind to, whereas they have an unlimited array of conformational and linear epitopes to cross react with when used in the IFA platform.

3.5 Use of the TM peptide as a DIVA epitope.

A recent report suggested that the serological response to the TM epitope could be used to differentiate infected from vaccinated animals (Westman *et al*, 2015). In this published study that was conducted in Australia, plasma samples from vaccinated cats were screened using three FIV POC antibody test kits to detect antibodies recognising viral proteins (Figure 3-12)

Figure 3-12. Viral antigens contained within 3 point of care test kits used to differentiate infected from vaccinated animals. Figure reproduced from Westman *et al* 2015.

Although the SNAP FIV/FeLV Combo test detected antibodies against p15 and p24 in 119/119 samples tested, the Witness FeLV/FIV and Anigen rapid FIV/FeLV test kits detected FIV specific antibodies in only 11/119 (9.25) and 5/119 (4.2%) of the samples respectively. As TM antigen is a major component of both the Witness and Antigen rapid tests, it was hypothesised that the humoral immune response against the TM epitope would permit differentiation of infected from vaccinated animals.

In the present study, the same samples that had been tested in the study of Westman and colleagues were screened for anti-TM antibodies using the TM ELISA. In total 256 Australian samples were screened comprising 114 vaccinated/uninfected samples, 115 unvaccinated/uninfected samples and 27 infected/unvaccinated samples were screened (Appendix 3iii, immunostains 1-9, signalment 3a, 3c and 3d). Another 18 samples from cats in the USA that had been vaccinated with the Fel-O-Vax FIV vaccine and were uninfected were tested in parallel (Appendix 3iii, immunostain 10, table 3b). As demonstrated in Figure 3-13, the ELISA appeared to differentiate infected from vaccinated cats to some extent, with the differences between the median values for each category being statistically significant. However, there was substantial cross-over between the highest negative fold values observed in one category and the minimum negative fold value of another category (e.g. highest negative fold value of the vaccinated group overlaps with the minimum negative fold of the infected group).

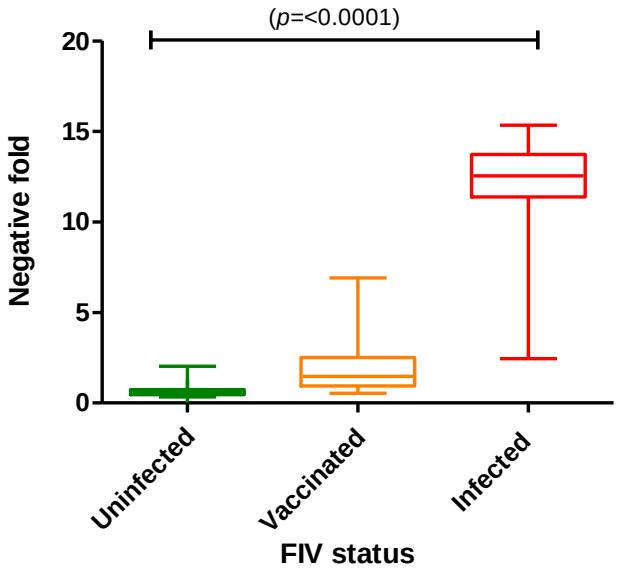


Figure 3-13. The relationship between FIV status and negative fold. Although the TM ELISA showed some differentiation between the three groups, based on the difference between the median values, there was substantial cross-over between the maximum and 75th percentile values of the vaccinated group (6.9-2.5 respectively) with the minimum value (2.4) of the infected group. (p=<0.0001. Kruskal-Wallis test).

When FIV status was cross-referenced with the FIV sero-status for each sample, as shown in Table 3-11, it was apparent that the TM ELISA alone could not be used to differentiate infected from vaccinated cats. The TM ELISA could be used to differentiate uninfected and infected cats, but 67.4% of the vaccinates (89/132) were classified as sero-negative, 22.7% as inconclusive and 9.8% as sero-positive using the TM ELISA.

		FIV antibody status			
		Positive	Inconclusive	Negative	Total
FIV status	Uninfected	0	1	112	113
	Vaccinated	13	30	89	132
	Infected	26	1	0	27
	Total	39	32	201	

Table 3-11. Relationship between FIV vaccination or infection status and FIV sero-status as determined using TM ELISA.

It has been stated that vaccination with the Fel-O-Vax FIV vaccine induces antibodies that interfere with the sero-diagnosis of FIV (Kusuhara *et al*, 2006, Crawford and Levy, 2004,

Hartmann *et al*, 2007, Andersen and Tyrrell, 2004). As most samples from vaccinated cats that were tested in this study were shown to test negative using the TM ELISA, the humoral immune response to vaccination with Fel-O-Vax FIV was examined further. As the TM ELISA is based on a single, highly conserved epitope representing the PID of the FIV Env, immunostain analysis was performed to determine whether antibodies were induced by vaccination to FIV epitopes other than TM. As shown in Figure 3-14, the major difference between the responses of vaccinated and infected cats was the lack of antibodies recognising the Env proteins (gp95 and gp41) detected by immunostaining. Similar levels of reactivity were observed against the core structural proteins (p55, p24, p17, p10) between the vaccinated and infected cats. The lack of antibodies elicited against the Env proteins was surprising, since it has been suggested that vaccination with Fel-O-Vax FIV induces broadly neutralising antibodies against Env (Coleman *et al*, 2014. Pu *et al* 2004, Pu *et al*, 2001). Based on the criteria for immunostaining outlined in Materials and Methods, 43/132 (32.6%) of the samples from vaccinated cats were classified as sero-positive and 89/132 (67.4%) were sero-negative.

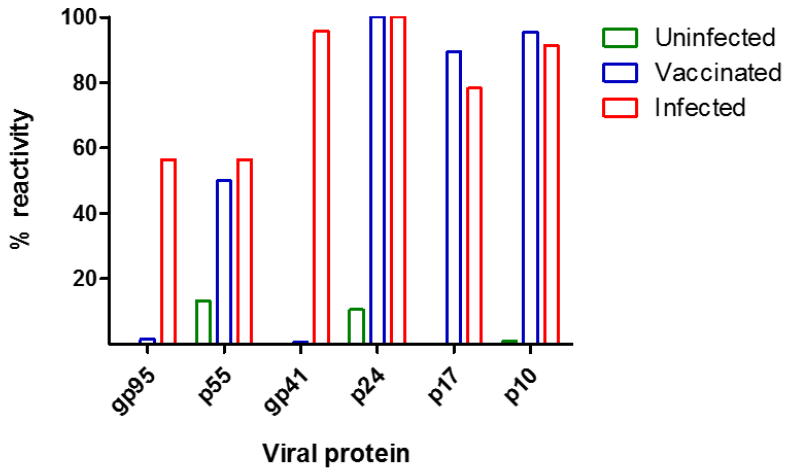
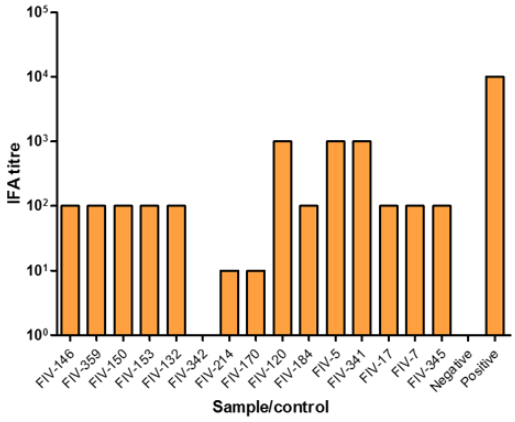


Figure 3-14. The percentage of each FIV group reacting to viral proteins by immunostaining. A viral lysate of the vaccine strain of FIV (FIV-FL4) was prepared and probed with plasma samples from each of the FIV groups.

Selected samples from vaccinated cats were also screened for antibodies against FIV by IFA to determine whether this method would detect more sero-positive samples. When the IFA titres of the vaccinated samples were examined (Figure 3-15), all but one sample tested sero-positive.

A.



B.

Sample I.D.	SU	p55	TM	p24	p17	p10
FIV-146	-	+	-	+	+	+
FIV-359	-	+	-	+	+	+
FIV-150	-	+	-	+	+	+
FIV-153	-	+	-	+	+	+
FIV-132	-	-	-	+	+	+
FIV-342	-	-	-	+	+	+
FIV-214	-	-	-	+	+	+
FIV-170	-	+	-	+	+	+
FIV-120	-	-	-	+	+	+
FIV-184	-	+	-	+	+	+
FIV-5	-	+	-	+	+	+
FIV-341	-	-	-	+	+	+
FIV-17	-	+	-	+	+	+
FIV-7	-	+	-	+	+	+
FIV-345	+	+	-	+	+	+

Figure 3-15. Detection of anti-FIV antibodies by IFA titre (A) and immunostain (B). Three samples were sero-positive based on IFA titre alone (A), while the remaining samples (except FIV-342) were sero-positive by immunostaining (B). Green shading denotes samples testing negative by TM ELISA; Yellow, inconclusive; Pink, positive.

These results demonstrated that the TM ELISA could not be used to differentiate infected from vaccinated animals. It has been shown that vaccination with Fel-O-Vax FIV does induce antibodies against major structural FIV proteins, however only few vaccinated cats in this study population produced anti-Env antibodies. It was also shown that IFA detected anti-FIV antibodies in samples from vaccinated animals, since 14/15 samples from vaccinates tested by IFA would have been submitted for further examination by immunostaining for confirmation and would then have tested positive. It was concluded that conventional serological diagnostics using the methods and antigens/epitopes described above could not be used to differentiate vaccinated from infected cats.

3.5.1 Differentiation of infected from vaccinated animals (DIVA)

To determine whether the chimaeric virus expressing the PID of LLV-E could be used as the basis of a DIVA vaccine, plasma samples were screened against both the FIV and LLV-E TM

peptides on the same ELISA plates (DIVA ELISA). Samples were screened against both capture antigens at the same time to reduce the variability in absorbance values that can arise between ELISA plates and normalised absorbance ratio values were calculated as described in 2.1.7. In total, 268 samples were screened; 175 samples were of known FIV sero-status (determined by IFA and confirmed by TM ELISA and immunostain). These samples constituted the 'known' population and were either sero-negative or sero-positive. Neither of these groups had received any FIV vaccination. An additional 93 samples with inconclusive FIV sero-status by IFA were also tested. This group comprised the 'inconclusive' group and were either sero-negative or sero-positive. Neither of these groups had received any FIV vaccination. Table 3-12 summarises the number of FIV sero-positive and negative samples derived from each group.

	Known	Inconclusive
Sero-positive	93*	10**
Sero-negative	82	83
Total	175	93

Table 3-12. Summary of the FIV sero-status of the samples derived from both the known and inconclusive populations. * Samples were tested FIV sero-positive by IFA, TM ELISA and immunostain. ** Samples tested sero-positive by TM ELISA, immunostaining and virus isolation. Although virus could not be unequivocally isolated from 2/10 of these samples, they were classified as sero-positive because they displayed negative fold values greater than 2 and demonstrated reactivity against at least 2 specific viral bands by immunostaining.

3.5.2 DIVA of known samples

Of the 175 samples from the 'known' group, the DIVA ELISA was able to differentiate all 175 samples as illustrated in Figure 3-16.

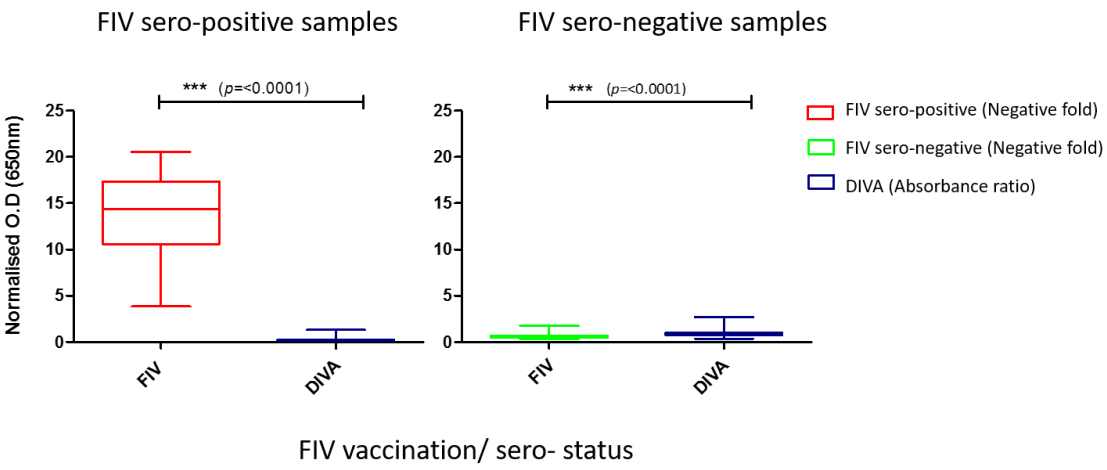


Figure 3-16. Normalised absorbance ratio values of samples derived from the known group. As the plasma samples used in this study had been collected from domestic cats exposed to FIV, one would predict little or no cross-reactivity between the two capture antigens. This is highlighted by the statistically significant difference between the medians of the samples tested against the FIV and DIVA peptides. (*p*=0.0001, Mann-Whitney U-test)

3.5.3 DIVA of inconclusive samples.

Of the 93 samples from the 'inconclusive' group, the DIVA ELISA could be used to differentiate all 93 samples as illustrated in Figure 3-17.

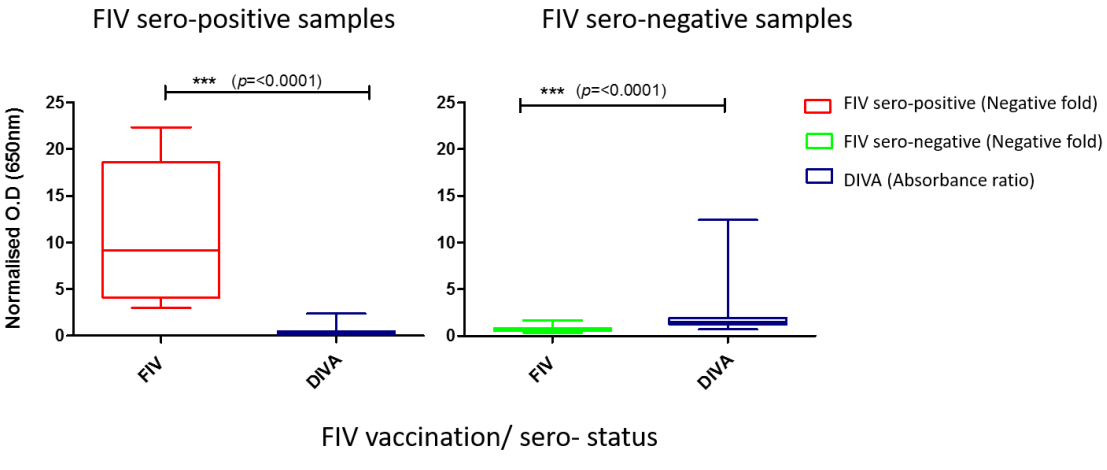


Figure 3-17. Normalised absorbance ratio values of samples derived from the inconclusive group. Differentiation of FIV sero-positive samples is evident but FIV sero-negative samples showed false positive results. The differences between the median values of the groups were statistically significant (*p*=0.0001, Mann-Whitney U-Test).

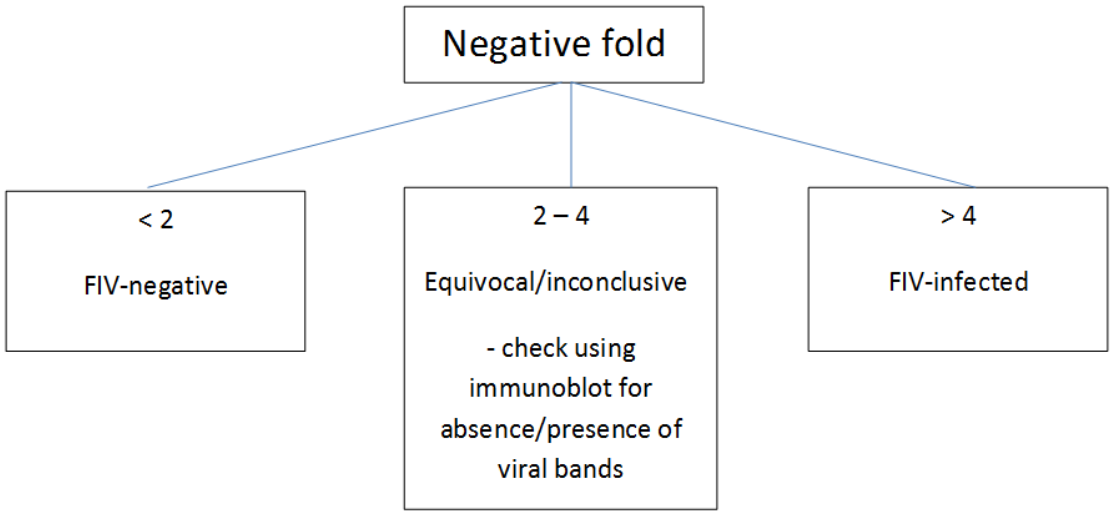
Although the DIVA ELISA could be used to differentiate all FIV sero-positive samples, the FIV sero-negative samples within this group gave false positive results. As none of the samples used in this study had been collected from cats that had been exposed to the LLV-E TM PID, it was concluded that the false positive results could be attributed to cross-reactive antibodies. To identify any causative agent/condition that might have accounted for false positive results, the clinical information for these 10 samples (collected from 8 cats) was examined and 7/8 of these cat samples had been submitted for a confirmatory test following a positive FIV result using a POC test (Table 3-13). These results were shown to be false positive on subsequent laboratory serological testing. As no further information could be obtained about these cats, it was concluded that the DIVA positive result likely arose from cross-reactive antibodies, elicited by an unknown infectious agent or condition. Evidence in support of this conclusion could be found by examining the cats that had been sampled sequentially. Two of the 3 sequentially sampled cats were sampled 4 and 8 weeks apart, however both samples tested positive for antibodies against the LLV-E TM peptide. A third cat was sequentially sampled on two occasions just 6 days apart and clearly demonstrated different reactivity compared to the original sampling (the first sample 364503 tested LLV-E sero-positive and FIV sero-negative while 6 days later, the second sample 364637 tested LLV-E sero-negative but FIV sero-positive). It appeared therefore that the production of anti-TM antibodies early in FIV infection was associated with reactivity against the conformational epitope of the PID. As the antibodies matured and underwent class switching/somatic hypermutation, the affinity of the antibody was focused towards the linear component of the PID. The fact that no FIV sero-positive sample cross reacted with the LLV-E peptide, and that FIV sero-negative, LLV-E cross-reactive samples maintained a similar reactivity profile for 4-8 weeks, suggested that the LLV-E sero-positive samples contained cross-reactive antibodies.

						Immunoblot$^{\Phi}$							TM ELISA	DIVA ELISA
Identification number*	**Re-sample date**	**Species**	**Clinical symptoms/history**	**IFA^{+}**	**p24 antigen**	**SU + TM**	**SU**	**p55**	**TM**	**p24**	**p17**	**p10**	**Negative fold**	**Absorbance ratio**
346793		*Felis catus*	Peripheral lymphadenopathy	100ns	-	-	-	-	-	-	-	-	0.575	5.461
346823		*Felis catus*	Sent for confirmation	0	-	-	-	-	-	(+)	-	-	0.349	3.990
347621		*Felis catus*	Sent for confirmation	0 toxic	-	-	-	-	-	+	-	-	0.717	4.189
347309		*Felis catus*	Sent for confirmation. Weight loss	0	-	-	-	-	-	+	-	-	0.396	3.591
367547		*Felis catus*	Sent for confirmation	0	n/a	-	-	-	-	(+)	-	-	0.525	4.627
345089 [1]	08/05/2014	*Felis catus*	Sent for confirmation. Healthy	0	n/a	-	-	-	-	(+)	-	-	1.067	4.199
345547 [2]	09/06/2014			0 ns	-	-	-	-	-	(+)	-	-	1.033	3.692
345855 [1]	27/06/2014	*Felis catus*	Sent for confirmation	0 ns	-	-	-	-	-	(+)	-	-	0.715	12.430
346573 [2]	12/08/2014			0ns	-	-	-	-	-	(+)	-	-	0.456	10.365
364503[1]	14/01/2015	*Felis catus*	Sent for confirmation	0	-	-	-	-	-	+	-	-	1.022	5.385
364637[2]	20/01/2015		Sent for confirmation. Previous VDS inconclusive	0	-	-	-	-	(+)	+	(+)	-	3.625	2.383

Table 3-13. Summary of the serological profiles of samples producing false positive absorbance ratios. Sample 364637 is included as the first sampling from this cat was contained anti-LLV-E TM antibodies, while the second sampling was shown to contain only anti-FIV TM antibodies. As this cat originated from a shelter that was keen establish the FIV status of the animal to re-home it, a second sample was taken 6 days after the first. *, samples shaded in purple collected from the same cat, with the superscript number denoting the order in which the samples were collected. $^{+}$, immunofluorescence antibody assay; ns, non-specific fluorescence; $^{\Phi}$ +, strong band visualisation; -, no band visualisation; (+), weak band visualisation.

These results indicated that the TM ELISA and DIVA ELISA tests are highly sensitive and specific for the detection of anti-FIV TM and anti-LLV-E TM antibodies. The TM ELISA displayed a sensitivity of 100% and a specificity of 98%, while the DIVA ELISA displayed a specificity of 94.9%. The 0% sensitivity figure regarding the DIVA ELISA resulted because none of the samples tested during the optimisation of the ELISAs had been raised against LLV-E or the chimaeric vaccine virus which contain the PID of LLV-E. Based on the results presented in this chapter, the following diagnostic algorithm is proposed to establish whether a test sample is FIV positive or negative, from a cat that has been vaccinated or unvaccinated, or from a cat that has been infected and has been vaccinated.

FIV diagnostic algorithm (irrespective of FIV vaccination status)



Vaccination diagnostic algorithm (irrespective of FIV status)

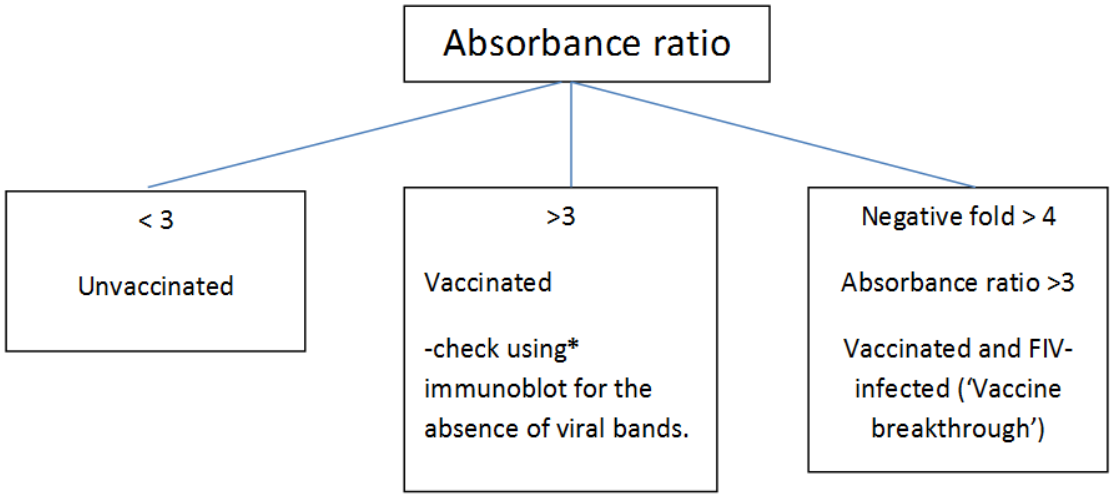


Figure 3-18. Diagnostic algorithm for determining the FIV infection and vaccination status of cats. *As some FIV sero-negative plasma samples (3.2 %) were shown to contain antibodies that cross-reacted with the LLV-E peptide, immunostaining is used to confirm the absence of antibodies recognising FIV (checking for a false positive vaccination result). The absence of virus specific bands would indicate that the cat had not been vaccinated and therefore cross-reactive antibodies had likely been elicited by other infectious agents another condition.

3.6 Conclusions.

Infection with FIV elicits high titres of antibodies recognising the PID of FIV. Although clade A is the predominant subtype of FIV found in the UK (Samman *et al*, 2011), in this study it was demonstrated that plasma raised against subtype C isolates also recognises the PID; plasma raised against the highly pathogenic FIV-CPG41 isolate (Diehl *et al*, 1995, De Rozieres *et al*, 2004) tested positive using the TM ELISA. The reactivity of plasma samples raised against diverse stains of FIV confirms the conserved, immunodominant nature of the PID, supporting the results of Avrameas *et al* (1992 and 1993) and Kania *et al* (1997). Additionally, the sensitivity and specificity values displayed by the TM ELISA (100 % and 98% respectively) are similar to those reported by others (Avrameas *et al*, 1993. Kania *et al*, 1997). The TM ELISA produced no false positive or false negative results and detected anti- FIV TM antibodies in one sample that had been collected during the period of sero-conversion, early post infection.

The inclusion of FIV inconclusive samples in this study demonstrated the usefulness of the TM ELISA. However, it was possible to assign the FIV sero-status of such samples following testing using the TM ELISA. The 93 inconclusive samples (77 animals) represented the most challenging samples for FIV diagnosis that a large veterinary diagnostic laboratory would receive. The advantage of implementing the TM ELISA as a screening test is the removal of the cost of additional sampling and screening; in general, such costs are met by the laboratory when sample results are inconclusive. As the samples used in this study had been collected from companion animals, the samples contained antibodies to other infectious agents and yet no non-specific cross-reactivity was demonstrated. The results presented in this chapter demonstrated that the TM ELISA is a reliable and robust assay for the detection of antibodies to FIV, with a format that may easily be used for large-scale screening of samples.

The use of the TM peptide corresponding to the PID has been shown to useful in the sero-diagnosis FIV infection in the domestic cat (*Felis catus*), African lion (*Panthero leo*), puma (*Puma concolor*) and pallas cat (*felis manul*) (Avrameas *et al*, 1993, Fontenot *et al*, 1992, Kania *et al*, 1997, van Vuuren *et al*, 2003). Peptides corresponding to the PID have been

assessed also for the sero-diagnosis of HIV-1 (Gnann *et al*, 1987) and HIV-2 (Petrov *et al*, 1990. Shin *et al*, 1997). The specificities of ELISA using FIV PID as the capture antigen ranged from 96-100% with sensitivities ranging from 78.6-100%, depending on the species of FIV against which the serological response is being examined. Similar specificities and sensitivities have been reported for the detection of HIV-1 and HIV-2 PID antibodies (specificities of 99.8-100% and sensitives of 99.7-100%). The conserved nature of the PID, accompanied by the species-specific nature of the epitope not only make TM an attractive and useful target for the sero-diagnosis of lentiviral infection but also a potential candidate epitope for inclusion in a DIVA vaccine.

Previous studies have demonstrated that the PID can withstand substantial genetic mutations, especially at the 3' end of the cysteine loop, and that altering the amino acid sequence of the PID abolished immunogenicity to the wild type PID sequence (Richardson *et al*, 1997, Pancino *et al*, 1995, Pancino and Songio, 1997). For these reasons, the LLV-E PID sequence was incorporated into a FIV chimaera that could be tested as a vaccine candidate while assessing the potential for the PID mutation to permit differentiation of vaccinated from infected cats. The identification and characterisation of such an FIV vaccine candidate are described in Chapter 5, section 5.3 of this thesis.

Chapter 4

Pathogenesis of FIV ΔV2 chimeric virus.

4.1 Introduction

FIV was originally isolated from a sick cat with an immunodeficiency like syndrome (Pedersen *et al*, 1987). Originally named feline T-lymphotropic lentivirus, the virus was renamed feline immunodeficiency virus based on its morphological, genomic, protein and reverse transcriptase similarities to the human and simian immunodeficiency viruses. As FIV does not typically induce pathological clinical signs, rather the signs of infection displayed tend to be non-specific, the major determinants of FIV pathogenesis have been elucidated from experimental infection studies (Pedersen *et al*, 1989. Yamamoto *et al*, 1998. Ishida and Tomoda, 1990). From early transmission studies, it was shown that FIV could be isolated from the saliva of infected cats and this finding led to the hypothesis that FIV was transmitted mainly through biting, although experimental transmission was demonstrated via the oro-nasal route (Yamamoto *et al*, 1989*b.* Matteucci *et al*, 2000. Kusuhara *et al*, 2005). The primary or acute stage of infection with FIV typically results in sero-conversion to the major structural proteins, p24, p17 and p55 and the envelope glycoprotein (Env) by 4-6 weeks post infection (Figure 4-1). During this time, virus can be isolated from PBMC, although in some very rare cases virus can be isolated from sero-negative cats (Egberink *et al*, 1992. Yamamoto *et al*, 1988. Hopper *et al*, 1988). The isolation of virus from PBMC corresponds with a peak in viremia approximately 5 – 10 weeks post infection (Hosie *et al*, 2002 & 2005. Dunham *et al*, 2006). The major consequence of FIV infection is the gradual depletion of $CD4^+$ T cells (Hofmann-Lehman *et al*, 1996.) that ocurs through the virus targeting cells expressing the T cell activation marker CD134 (Shimojima *et al*, 2004). Initial reports suggested that the chemokine receptor CXCR4 was the primary receptor for FIV (Nakagaki *et al*, 2001), however it was later shown that CXCR4 is the co-receptor and that FIV displays a wide tropism, infecting T cells expressing $CD4^+$ or $CD8^+$, macrophages, $CD25^+CD4^+$ T_{reg} cells, B-cells and astrocytes (English *et al*, 1993. Vahlenkamp *et al*, 2004). FIV infection leads to a gradual depletion of cells at the foundation of the adaptive arm of the immune system that characterises the asymptomatic stage of infection

(Figure 4-1). Cats may appear relatively healthy during the asymptomatic stage, with the only indicators of infection being the persistence of anti-FIV antibodies and a gradual decline in the numbers of $CD4^+$ T-cells. During the asymptomatic stage of infection, the $CD8^+$ T-cell compartment expands, resulting in an inversion of the $CD4^+$:$CD8^+$ T cell ratio (Willett *et al*, 1996). Cats can remain in this asymptomatic stage of infection for weeks or years depending on the virulence of the infecting strain of FIV (Diehl *et al*, 1995. Addie *et al*, 2000). The secondary phase of infection is characterised by pronounced immune dysfunction, including a significant reduction in T cell proliferative responses (Torten *et al*, 1991) and polyclonal B cell activation leading to hypergammaglobulinemia (Flynn *et al*, 1994). The pronounced immunodeficiency caused by FIV predisposes cats to secondary opportunist infections that can be fatal (de Rozieres *et al*, 2004. Diehl *et al*, 1995. Pedersen *et al*, 1987).

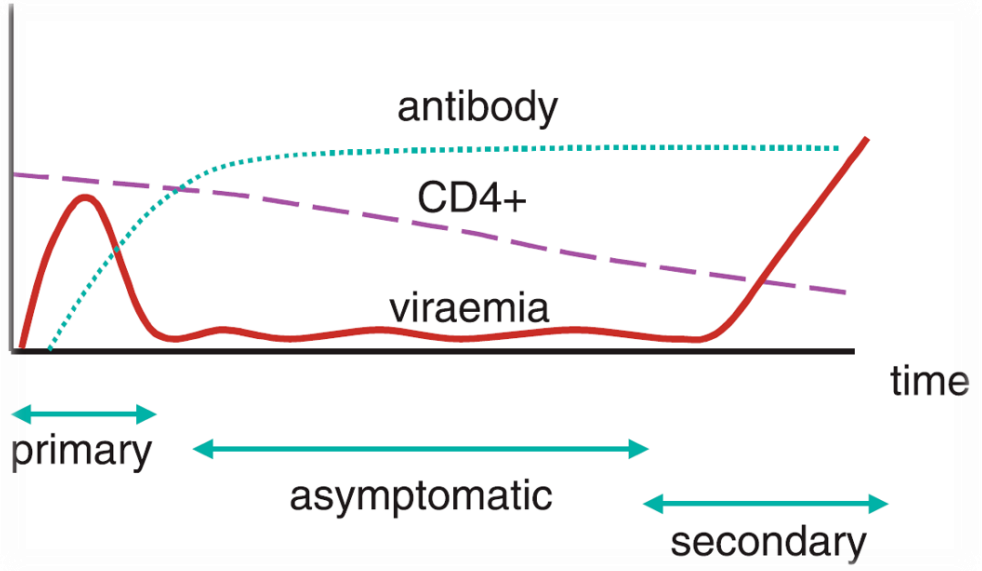


Figure 4-1. Schematic diagram summarising the pathogenesis of FIV. An Initial blip in viremia during the primary stage of infection is brought under control by both the humoral and cellular immune response resulting in the cat entering the asymptomatic stage. During this time the $CD4^+$ t-cell numbers decline whilst the $CD8^+$ t-cell numbers expand resulting in an inversion of the $CD4^+/CD8^+$ t-cell ratio. In the second stage of infection the immune system is no longer able to control infection and immunodeficiency develops.

Although the pathogenesis of FIV follows the general trend described above for the majority of infected individuals, the time between infection and death can vary greatly between cats. Because of this and the non-specific clinical signs displayed by FIV infection,

it is difficult to accurately stage infection (Hosie and Beatty, 2007). As shown in table 4-1, the virulence of different FIV isolates can vary. Experimental infection with FIV-Petaluma or FIV-M2 resulted in modest viral burden and negligible inversion of the $CD4^+$:$CD8^+$ ratio, while infection with *in vivo* passaged FIV-CPG41 resulted in a high viral burden, significant loss of $CD4^+$ T cells (with numbers decreasing to 52% of those in uninfected control cats) and death by 8 weeks post infection. Hence FIV infection can be difficult to stage and the differences in the pathogenicity of different isolates might explain the differences in vaccine protection that have been observed between challenge viruses that belong to the same clade and display similar degrees of Env amino acid sequence divergence from the vaccine virus.

Isolate	Clade	Proviral load	$CD4^+/CD8^+$ ratio change	Reference
CPG-41	C	++++	++++	Diehl *et al* , 1995. de Rozierers *et al* , 2004
FIV-GL8	A	+++	+++	Hosie *et al* , 2002. Hosie *et al* , 2000. Willett *et al* , 1993.
AM19	A	++	+	Hosie *et al* , 2000. Siebelink *et al* , 1995. Huisman *et al* , 1998.
FIV-M2	B	++	+	Matteucci *et al* , 1997. Matteucci *et al* , 1996.
FIV-Pet	A	+	+	Hosie *et al* , 2002. Hosie *et al* , 2000.

Table 4-1. Spectrum of pathogenicity of selected FIV strains observed following in vivo infection. The pathogenicity of isolates varies from clade to clade, with some evidence to suggest that clade B is less virulent than clade A. Extensive *in vivo* passage of the CPG-41 isolate led to the development of a super pathogenic isolate of FIV, capable of causing death within 8 weeks. + low/negligible, ++ moderate, +++ high/pronounced, ++++ very high/severe.

In this study the pathogenesis of a chimaeric FIV was studied. The chimaeric virus contained the Env of a field isolate that contained a unique mutation at the apex of the second hypervariable loop 2 [figure 4-2 (V2)] within a backbone derived from the FIV-Glasgow8 molecular clone. Characterisation of the novel field isolate Env, designated 206394 (Samman, 2010), identified an asparagine to serine mutation at position 298 (N298S) that ablated a potential N-linked glycosylation site (PNGS), revealing a neutralisation sensitive epitope. The homologous plasma collected from cat 206394 contained potent broadly neutralising antibodies (BnAb) that neutralised FIV isolates derived from clades A, B and C, as shown in table 4-2. This led us to test whether a chimaeric

FIV in which the 206394 Env (comprising both SU and TM) replaced the Env of the infectious FIV GL8 molecular clone, might elicit BnAb with the potential to neutralise a broad range of isolates, including FIV GL8.

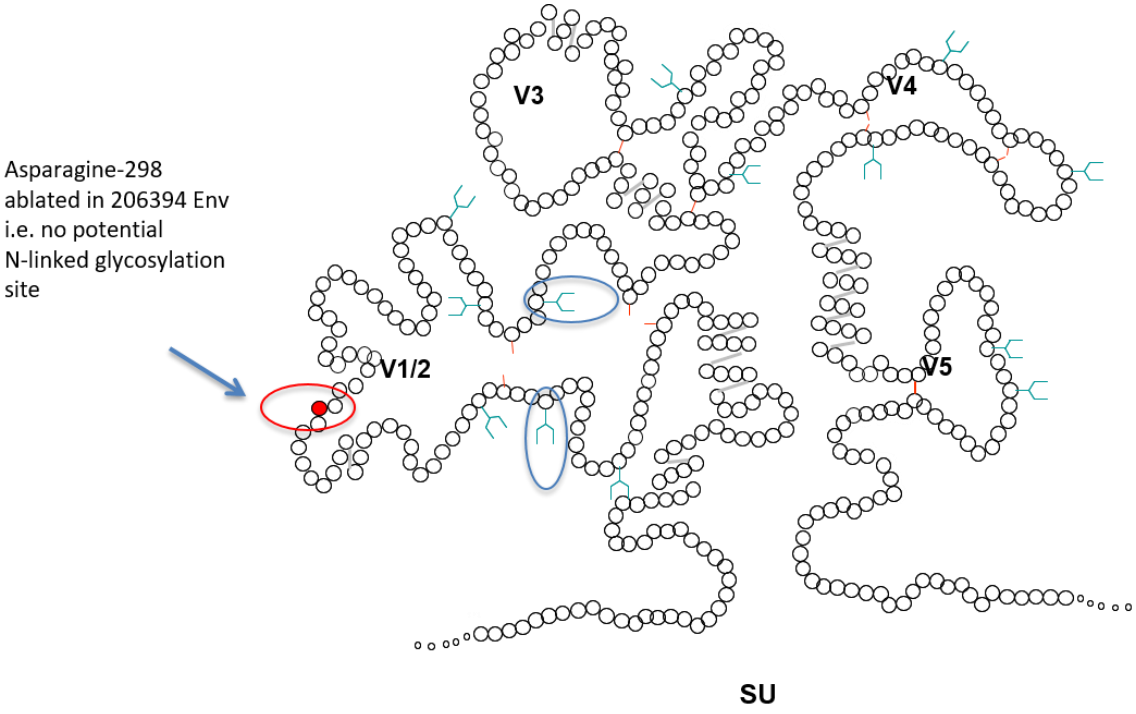


Figure 4-2. Schematic diagram of 206394 SU, showing the position of the ablated PNGS at the apex of the second hypervariable loop (V2). Blue forks represent PNGS; those highlighted in blue are predicted to be missing in FIV-Petaluma, the prototype FIV vaccine strain, and the PNGS highlighted in red indicates the unique mutation that was observed in Env 206394.

Virus	Origin	Subtype	Plasma 206394
GL8	UK	A	95
180638	UK	A	90
171838	UK	A	99
180260	UK	A	96
180140	UK	A	97
178721	UK	A	97
179200	UK	A	97
206394	UK	A	93
0425	UK	A	96
0827	UK	A	95
1419	UK	A	96
PPR	USA	A	99
B2452	USA	B	99
KNG2	Japan	B	99
TM2	Japan	B	99
Pisa M2	Italy	B	100
Leviano	Brasil	B	100
CPG41	USA	C	99
Poose	Sri Lanka	-	100
LLV-B	Tanzania	-	16

Table 4-2. Broad cross neutralisation by plasma from cat 206394, shown as percentage neutralisation. Cat 206394 was a 14-year-old cat with chronic, age-related renal problems but was otherwise healthy (Samman, 2010). Plasma from cat 206394 neutralised HIV(FIV) pseudotypes bearing Envs of FIV isolates from clades A, B and C (percentage neutralisation relative to a no plasma control ≥ 90%). Figure reproduced from Hosie *et al,* 2011.

4.2 Materials and Methods.

4.2.1 Construction of a chimaeric infectious molecular clone of FIV (FIV ΔV2)

The chimaeric infectious molecular clone of FIV GL8 containing the novel Env 206394 was constructed by Ms Nicola Logan of the Retrovirus Research Laboratory. Briefly, the *env* gene of 206394 was amplified from gDNA derived from virus isolation cultures. Cultures were centrifuged for 5 minutes at 1000rpm, the culture fluid discarded and the cell pellet washed by resuspending the cells in 10mls of PBS followed by centrifugation for 5 minutes at 1000rpm. The PBS was then aspirated and the gDNA from the pelleted cells was extracted as described in Materials and Methods (Chapter 2). First round PCR was performed using degenerate primers IF4/XR2, the PCR product was separated on a 0.7-0.8% agarose gel, excised, purified and then sent for sequencing to determine the sequence of the second-round PCR primers. Second round amplification was performed from the product of the first round of PCR using primers complementary to the 5' and 3' prime ends of the *env* gene. Mlu1 and Nde1 restriction endonuclease sites were engineered into the primers at the 5' and 3' ends respectively. Following the second round of PCR, the product was separated on a 0.7-0.8% agarose gel, excised, purified and eluted in 50µl of EB buffer and then stored at -20^{o}C until required.

4.2.2 Restriction digests

The concentration of DNA to be digested was first determined using the Nanodrop (Thermo-scientific). One microlitre of each of the Mlu1 and Nde1 restriction enzymes (New England biolabs, Hitchen, Hertsfordshire) were added to 1µg of purified PCR product/molecular clone and 5µl of cut smart restriction digest buffer. The reaction volume was made up to 50µl using deionised water and then incubated in a 37^{o}C water bath for 5 hours. The DNA was then purified using the Qiagen gel extraction kit as per the manufacturer's instructions. The product was eluted and stored at -20^{o}C until required.

4.2.3 Ligation

The vector (FIV GL8 molecular clone in the vector PBR328) and insert were mixed at a vector: insert ratio of 3:1with 4µl of ligation buffer and 1µl of T4 DNA ligase (Invitrogen, Paisley, UK). Ligation reaction volumes were made up to 20µl with deionised water and

incubated in a circulating water bath at 14°C overnight. Ligations were then used immediately to transform DH5α cells.

4.2.4 Transformation

Escherichia coli Max efficiency® DH5α™ competent cells [Invitrogen, Paisley, UK (phenotype: F- ϕ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1)] were transformed using the heat shock method (which relies on the porosity of the bacterial cell wall being governed, in part, by temperature), as per the manufacturer's instructions. Hence regulation of the reaction temperature controls the reactants entering the bacteria. Briefly, 3µl of ligated product were added to 30µl of DH5α max efficiency cells and then incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for 40 seconds. Cells were then incubated on ice for 2 minutes after which 400µl of SOC broth were added. The culture was agitated at 250rpm at 37°C for 1 hour to recover the transformed cells and 200µl of culture were spread onto ampicillin-selected LB agar plates and incubated at 30°C overnight.

4.2.5 Mini-prep DNA preparation

Colony screening was performed using the QIAprep® spin miniprep kit (Qiagen, Manchester, UK). The assay utilises alkaline lysis of bacteria that are then centrifuged through a silica membrane in a high salt environment, which facilities DNA binding. Residual RNA, proteins and cell debris are then washed away and bound DNA is eluted using a low salt, pH 7-8 elution buffer.

One millilitre of bacterial culture was centrifuged at 16873*g* for 5 minutes to pellet cells. The ultrafiltrate was aspirated and the cells resuspended in 250µl of buffer P1 followed by lysis in 250µl of P2 and incubated for 5 minutes at room temperature. Three hundred and fifty microliter of buffer N3 were added to neutralise the reaction and the solution was centrifuged at 16873*g* for 10 minutes, pelleting all of the precipitated cellular debris and leaving an aqueous fraction that contained the bacterial and plasmid DNA. The aqueous fraction was then passed through a QIAprep spin column at 14549*g* for 1 minute. The flow-through was discarded and the bound DNA was washed with 750µl of PE wash buffer at 14549*g* for 1 minute. The flow-through was again discarded and a dry spin at 16873*g* for 1

minute removed any residual wash buffer. The bound DNA was then eluted in 50µl EB elution buffer into a sterile 1.5ml eppendorf tube and stored at -20^{o}C until required.

Colonies expressing the FIV Env were screened by Mlu1/Nde1 double digest as described previously and visualised on a 0.7-0.8% ethidium bromide agarose gel. Positive clones were sequenced to confirm the sequence of the cloned *envs*. Colonies expressing the cloned novel FIV *env* gene 206394 were used in further studies, the virus and clones hereafter being referred to as FIV ΔV2.

4.2.6 Maxi-prep DNA preparation

Selected colonies were grown in bulk in 400mls of ampicillin LB broth. Cultures were agitated at 250rpm at 30^{o}C overnight and then centrifuged at 2540*g* for 10 minutes at 4^{o}C using the Beckman Coulter centrifuge. The ultrafiltrate was aspirated and the pelleted cells were then processed to obtain plasmid DNA.

Bacterial lysates were process using the PureLink™ Hipure plasmid filter purification kit (Invitrogen, Paisley, UK). Using this method, plasmid DNA was separated and purified from bacterial cell lysates by anion exchange chromatography. The DNA was washed and then eluted using a high salt elution buffer, de-salted and precipitated, resulting in the purified plasmid solution.

After centrifugation of the bacterial culture, the ultrafiltrate was aspirated and the cell pellet was resuspended in 10mls of buffer R3 and lysed by the addition of 10mls of L7 lysis buffer. Lysis was allowed to proceed for 5 minutes at room temperature. The bacterial lysate was then neutralised and salt-buffered following the addition of 10mls of N3 neutralisation buffer. The solution was then allowed to pass through an equilibrated filter column. The filter column allowed the aqueous fraction containing plasmid DNA to pass through whilst retaining precipitated cellular debris. The aqueous fraction then entered the anion exchange resin filter that bound the negatively charged phosphate group of the DNA backbone. Once the aqueous fraction has passed through the filter and the ion exchange column, the filter was discarded and the bound DNA was washed in 50mls of W8 wash buffer. The washed DNA was then eluted in 15mls of E4 elution buffer and 10.5mls of

isopropanol was then added to precipitate the DNA. The precipitate was then centrifuged at 2540*g* for 30 minutes at room temperature. The ultrafiltrate was then aspirated and the DNA pellet was resuspended in 1ml of 70% ethanol and centrifuged at 16873*g* for 5 minutes at room temperature. The ethanol was then aspirated and the resulting DNA pellet was allowed to air dry for 5-10 minutes, allowing residual ethanol to evaporate. The DNA pellet was then dissolved in 400µl of TE buffer and stored at -20^{o}C until required.

4.2.7 Pathogenesis trial of FIV ΔV2 chimaeric virus

The aim of the study was to examine the pathogenesis of the FIV ΔV2 chimaeric virus and to determine whether infection would elicit BnAb. Since BnAb have been shown to take up to months or years to develop (Hosie *et al*, 2011), the infected cats were monitored for 52 weeks. Four specific pathogen free (SPF) kittens of mixed European breed had been specifically bred for experimental use and were allowed to acclimatise to the study conditions at the trial facility (Charles River Laboratories LTD, Co Mayo, Ireland) for 2 weeks. All of the kittens were inoculated with 2000 $TCID_{50}$ of FIV ΔV2 that had been titrated *in vitro* using Mya-1 cells. Following infection with FIV ΔV2, the kittens were monitored for infection and for the development of antibodies, as detailed in Table 4-3. At the end of the trial, the cats were anaesthetised before they were euthanised, exsanguinated and examined post mortem. Blood and tissue samples were collected for further examination. Blood samples were collected into either lithium heparin or EDTA, packaged and shipped at 4^{o}C to the Retrovirus Research Laboratory at the University of Glasgow. Ethical approval for the study was obtained from the Charles River Laboratory Ethics Committee.

Week of study	Age (weeks)	Sample	Procedure	Experimental notes	[a]Assays performed.
-2	6	0.5ml EDTA, 0.5ml hep	Pre-bleed 1	Acclimatisation	1,2,3,4,5
0	8	1ml EDTA, 1ml hep	Pre-bleed 2/ day of challenge.	Acclimatisation/ 2000 $TCID_{50}$ FIV ΔV2	1,2,3,4,5
4	12	2ml EDTA, 2ml hep	Blood sample		1,2,3,4,5
8	16	2ml EDTA, 2ml hep	Blood sample		1,2,3,4,5
12	20	2ml EDTA, 2ml hep	Blood sample		1,2,3,4,5
16	24	2ml EDTA, 2ml hep	Blood sample		1,2,3,4,5
20	28	2ml EDTA, 2ml hep	Blood sample		1,2,3,4,5
24	32	2ml EDTA, 2ml hep	Blood sample		1,2,3,4,5
28	36	5ml EDTA, 5ml hep	Blood sample		1,2,3,4,5
32	40	5ml EDTA, 5ml hep	Blood sample		1,2,3,4,5
36	44	5ml EDTA, 5ml hep	Blood sample		1,2,3,4,5
40	48	5ml EDTA, 5ml hep	Blood sample		1,2,3,4,5
44	52	5ml EDTA, 5ml hep	Blood sample		1,2,3,4,5
48	56	5ml EDTA, 5ml hep	Blood sample		1,2,3,4,5
52	60	5ml EDTA, 5ml hep	PM examination	Exsanguation and post mortem examination. Tissue collection	1,2,3,4,5

Table 4-3. Schedule of the FIV ΔV2 pathogenesis study. [a]1. DIVA ELISA, 2. Gag qPCR, 3. Immunostain, 4. Neutralisation assay, 5. Virus isolation.

4.3 Results.

4.3.1 Inoculation of Cats

Cats were inoculated with 2000 $TCID_{50}$ (0.5mls clarified culture fluid) of FIV ΔV2 via the intramuscular route. None of the cats displayed any signs of swelling, erythema or pain at the inoculation sites. During the trial, none of the cat displayed evidence of haematoma at the sites of blood collection on any of the sampling days and all cats remained healthy until the end of the trial.

4.3.2 Recovery of virus from PBMC

Virus was recovered from PBMC, following virus isolation, by week 8 of the study and was repeatedly isolated at each time point when the cats were sampled (Figure 4-3)

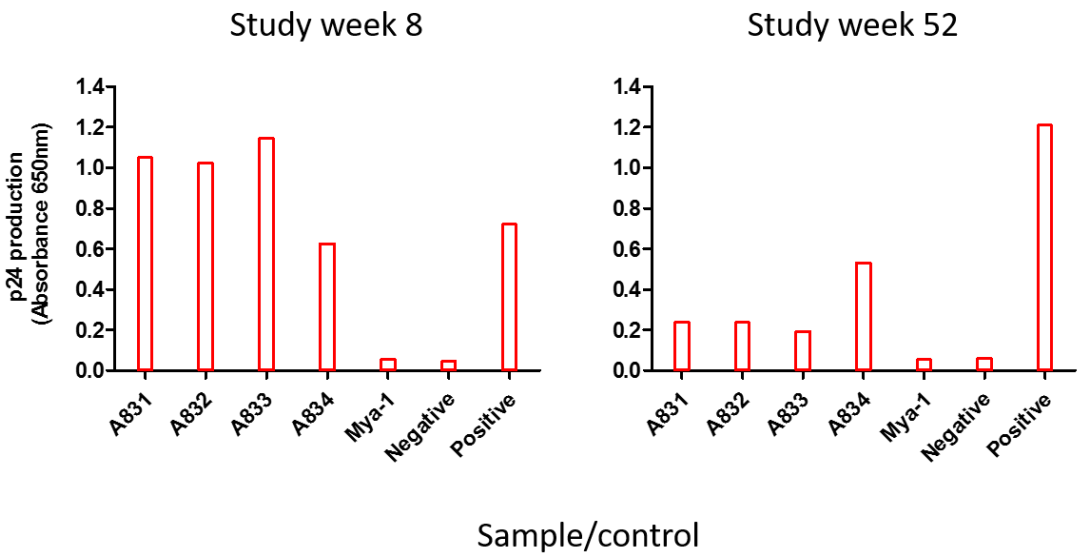


Figure 4-3. FIV p24 antigen production detected in the clarified culture fluid from co-cultivations of cats A831-4 at day 21. FIV p24 was first detected in cultures set up from PBMC collected from cats A831-834 at 8 weeks post infection and all co-cultivations tested positive thereafter. Mya-1 indicates uninfected cells that were not co-cultivated with PBMC; negative and positive controls were also included in the assay for FIV p24 detection. The lower absorbance values observed at week 52 could reflect the lower proviral loads that were observed in the cats at the end of the study. However, the method of virus isolation used in his study was not quantitative, and so this difference might indicate that fewer PBMCs had been available for co-cultivation at the later time point.

4.4 Serological response to infection with FIV ΔV2 chimaera

4.4.1 All cats developed anti-Env antibodies detectable by immunostain analysis

To examine the serological responses of the cats following infection with FIV ΔV2, we first determined the time to seroconversion for each of the viral proteins using immunostain analysis (Figure 4-4). By 12 weeks post infection, 3/4 cats (all cats except A834) had developed antibodies that recognised all of the major viral proteins. Seroconversion was detected as early as 4 weeks post infection, with antibodies recognising p24, p10 and TM being detected in 3/4 cats. In contrast, antibodies recognising Env (SU) were not detected until 8-12 weeks post infection. Cat A834 showed delayed sero-conversion compared to the other cats, with antibodies recognising TM, p24 and p10 detected 8 weeks post infection. Indeed, cat A834 did not appear to fully seroconvert until week 34, when a weak antibody response against SU was detected. These results confirmed that the FIV ΔV2 chimaera was fully immunogenic and elicited a defined humoral immune response. It was also demonstrated that the FIV ΔV2 chimaera was replication competent *in vivo* and that the novel Env was recognised in the context of the FIV GL8 backbone.

4.4.2 Demonstration of antibodies recognising the FIV TM peptide

To compare the antibody response to both the FIV and LLV-E TM peptides (as described in Chapter 2) during the early stage of FIV infection, plasma samples were tested for antibodies recognising the TM peptides of FIV and LLV-E (Figure 4-5). From 4 weeks post infection, anti FIV TM peptide antibodies were detected in 4/4 cats infected with the FIV ΔV2 chimaera. The antibody response against the FIV TM peptide increased until week 20 when a plateau was reached. The antibody response against the LLV-E peptide was less marked during the initial stages of infection, with absorbance values never exceeding 6 times the negative fold value of the negative control. When the negative fold and absorbance ratios were calculated (Figure 4-5), minimal cross reactivity with the LLV-E peptide was detected. This finding was consistent with the immunostain data, as anti-TM antibodies were detected at the same time points using two different methods. It was likely

that the discrepancy between the results of the DIVA ELISA and immunostain analysis for cat A834 reflected the different sensitivities of the two assays.

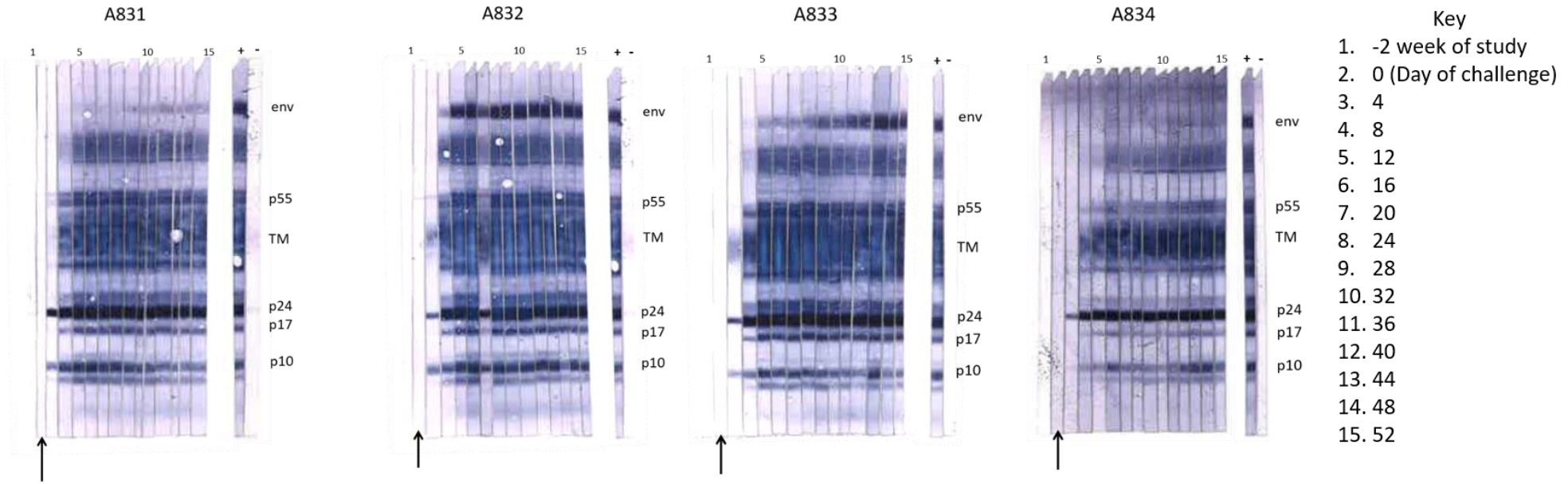


Figure 4-4. Serological responses of cats A831-4 infected with FIVΔV2 as determined by immunostaining. The black arrow indicates the day of challenge. Strong antibody responses against the core structural proteins p24, p17, p10 and the precursor p55 developed soon after challenge in 3/4 cats, as well as antibodies recognising the TM protein. Strong anti-Env (SU+TM) responses were observed 12 weeks post infection in cats A832 and A833, whereas the anti-Env response of cat A831 was much weaker (although anti-Env antibodies were detected at a similar time). Cat A834 did not develop detectable anti-Env antibodies until 32 weeks post infection.

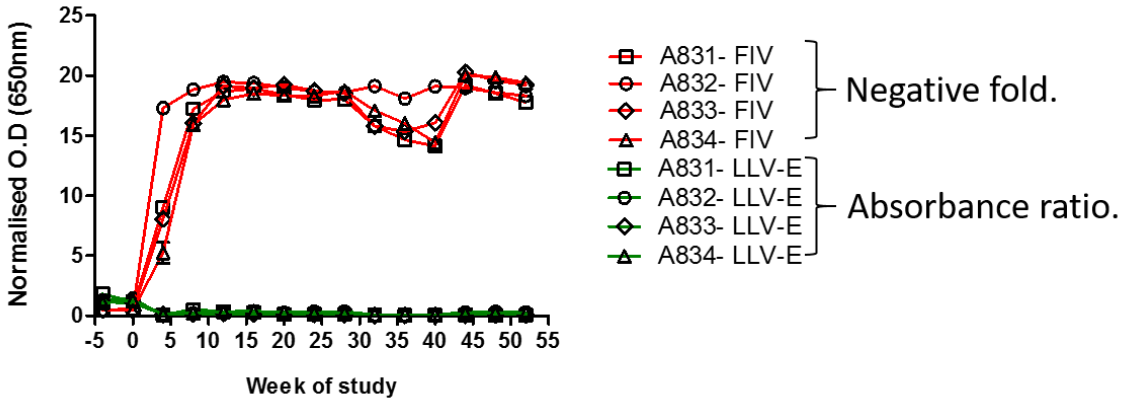


Figure 4-5. **Antibody response of cats A831-4 against the FIV and LLV-E TM peptides.** The normalised optical density value was used to compare the antibody responses to FIV TM (red symbols) and LLV-E TM (green symbols).

4.5 Pathogenesis of FIV ΔV2.

4.5.1 Induction of homologous neutralising antibodies.

To assess whether ablation of the PNGS at the apex of V2 resulted in the production of BnAb, neutralisation assays were performed using pseudotypes bearing the Envs of FIV isolates representative of clades A, B and C. The luciferase count results (Figure 4-6) demonstrated that homologous neutralising antibodies were detectable, since plasma samples collected from 3/4 infected cats neutralised pseudotypes bearing the homologous Env 206394 by 20 weeks post infection. Expressing the results as fold neutralisation (Figure 4-7) indicated that strong homologous neutralising antibody responses were produced in 3/4 cats by 28 weeks post infection, with >90% neutralisation of pseudotypes bearing the homologous 206394 Env. In contrast, cat A831 did not develop homologous neutralising antibodies until 40 weeks post infection.

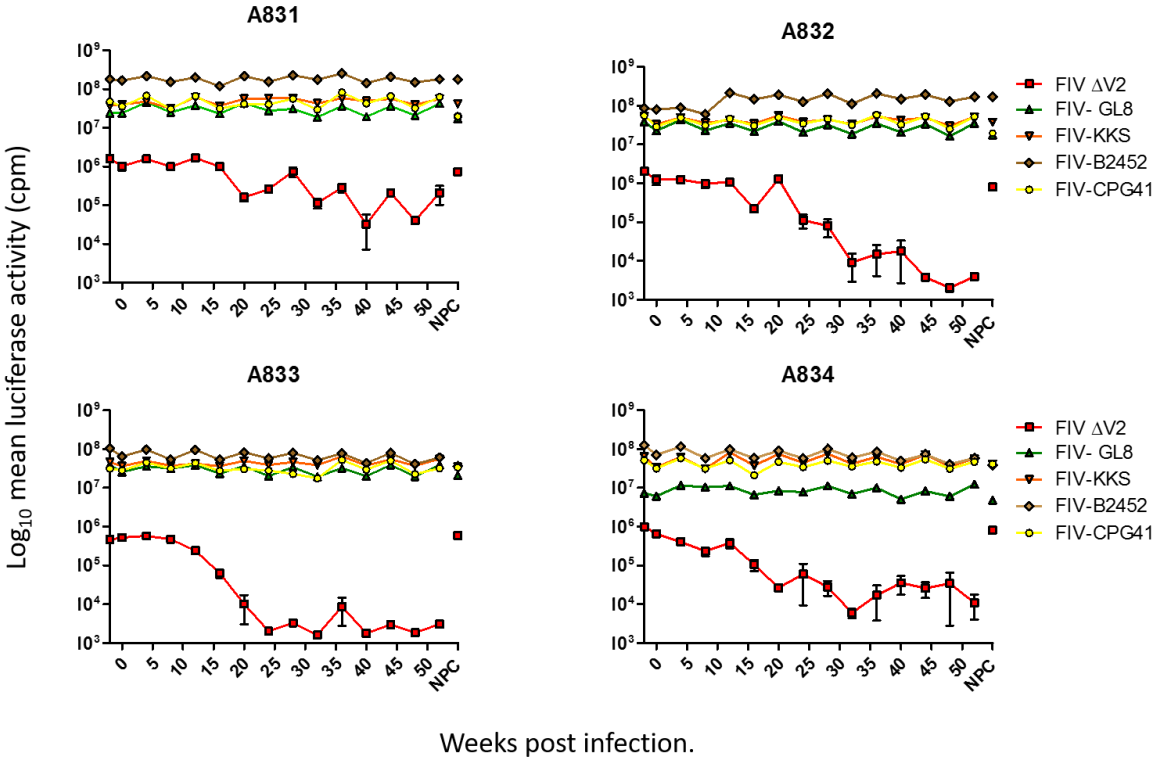


Figure 4-6. Homologous and heterologous virus neutralising antibody responses of cats A831-4. Neutralisation (indicated by decreased luciferase activity) responses of cats A831 to A834 following infection with the FIV ΔV2 chimaera, expressed as mean Log_{10} luciferase activity +/- SEM. Heparinised plasma samples were screened for neutralisation against the homologous ΔV2 pseudotype and the heterologous pseudotypes GL8 (Clade A), PET (KKS, Clade A), B2452 (Clade B) and CPG41(Clade C).

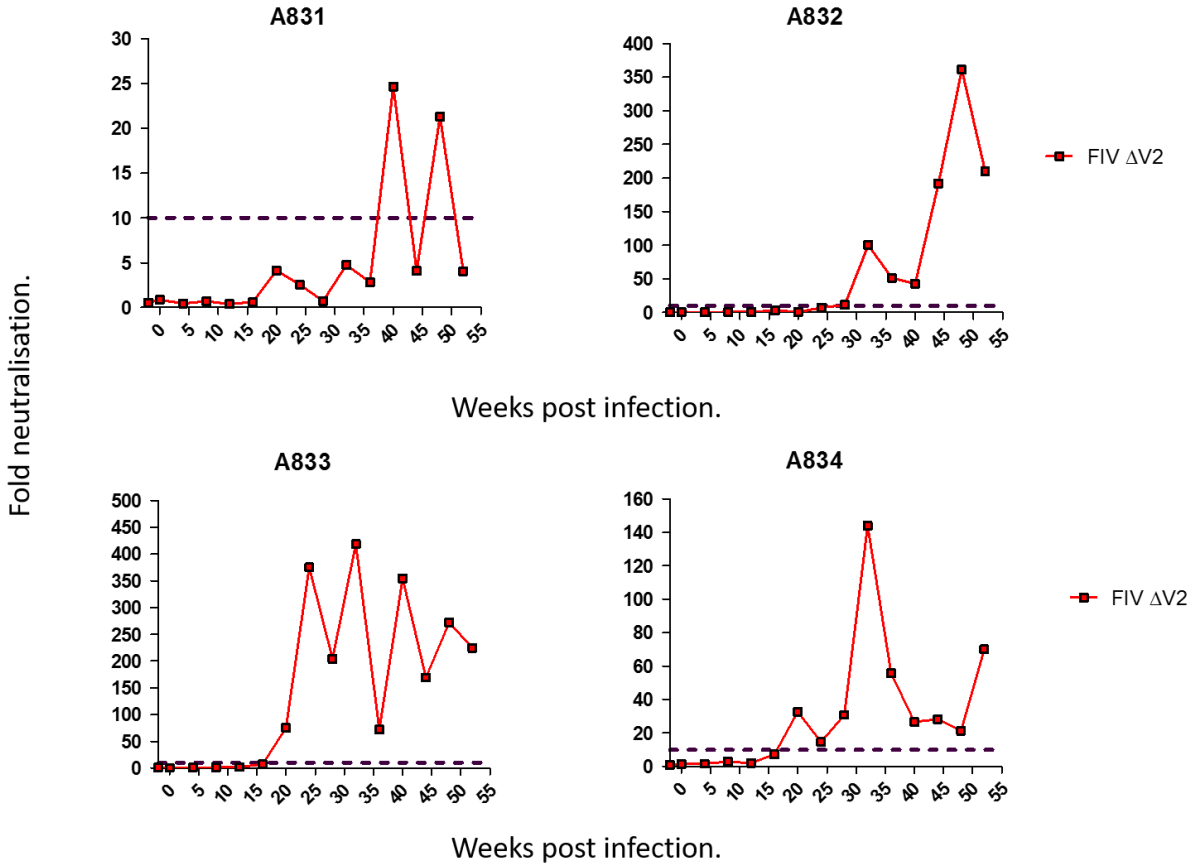


Figure 4-7. Neutralisation of homologous pseudotype 206394 by cats A831-4. Neutralisation expressed as fold neutralisation. Fold neutralisation values >10.0 (broken line) are equivalent to >90% neutralisation (Beczkowski, 2012).

No correlation was observed between neutralisation response and the development of anti-Env antibodies detected by immunostain (table 4-4). Anti-Env antibodies were detected approximately 4-8 weeks before neutralising antibodies were detected, with the exception of cat A834 that developed neutralising antibodies before antibodies recognising Env were detected by immunostain analysis. It appeared that cats that developed anti-Env antibodies earlier in infection developed stronger neutralising responses against the homologous pseudotype. On the other hand, cat A834 appeared to develop neutralising antibodies before anti-Env antibodies were detected by immunostaining, suggesting that neutralising antibodies might recognise conformational epitopes or might occur at low titres that were not detected by immunostaining. Three of four cats demonstrated maximal homologous neutralising titres at 40-48 weeks post infection, 28-40 weeks after anti-Env antibodies were detected by immunostain. In contrast, cat A834 demonstrated the maximum homologous neutralising titre and anti-Env antibodies were detected by immunostaining at 32 weeks post infection.

Cat I.D.	Development of env antibodies (Immunoblot).	Development of homologous nAb.	Greatest reduction in titre (%)/ week occurred.	Highest fold neutralisation/ week occurred.
A831	Wk 12	Wk 20	95.65/ Wk 40	24.64/ Wk 40
A832	Wk 8	Wk 16	99.75/ Wk 48	361.05/ Wk 48
A833	Wk 8	Wk 12	99.83/ Wk 40	418.71/ Wk32
A834	Wk 32	Wk 8	99.26/ Wk 32	143.95/ Wk 32

Table 4-4. Detection of anti-Env antibodies and nAb. Anti-Env antibodies were detectable by immunostain on average 15 weeks post infection, with homologous neutralising antibodies detected on average 14 weeks post infection. This discrepancy could be due to the sensitivities used to detect antibody/neutralisation, or cat A834 displaying neutralising antibody considerably sooner than antibodies were detected by immunostain.

These results demonstrated that infection with the FIV ΔV2 chimaeric virus induces homologous neutralising antibodies relatively soon post infection. The presence of anti-Env antibodies detectable by ELISA or immunostaining did not correlate with neutralisation, with low titres of anti-Env antibodies being potently neutralising in one cat, although these antibodies were not detectable by immunostaining.

4.5.2 Heterologous neutralisation.

As shown in Figure 4-8, no heterologous neutralising antibodies were observed in the plasma samples from the cats infected with FIV ΔV2. There was no reduction in luciferase activity for the heterologous pseudotypes tested and the values for fold neutralisation indicated the absence of heterologous neutralisation (Figure 4-7). Even when the fold neutralisation values against the heterologous strains were examined at the time points when the highest values for homologous neutralisation were observed for each cat (table 4-5), none of the cats showed any evidence of heterologous neutralisation.

Cat I.D.	Week of highest homologous fold neutralisation.	FIV ΔV2	GL8	KKS	B2452	CPG41
A831	40	24.64	0.84	0.86	1.28	0.41
A832	48	361.05	1.02	1.29	1.49	0.76
A833	32	418.71	1.03	0.91	0.70	1.17
A834	32	143.95	0.69	0.95	0.64	1.09

Table 4-5. Fold neutralisation of homologous and heterologous pseudotypes by cats A831-4 at the time point when the homologous neutralisation was at its highest in each cat. Red cells indicate >90% neutralisation whilst yellow cells indicate the absence of neutralisation.

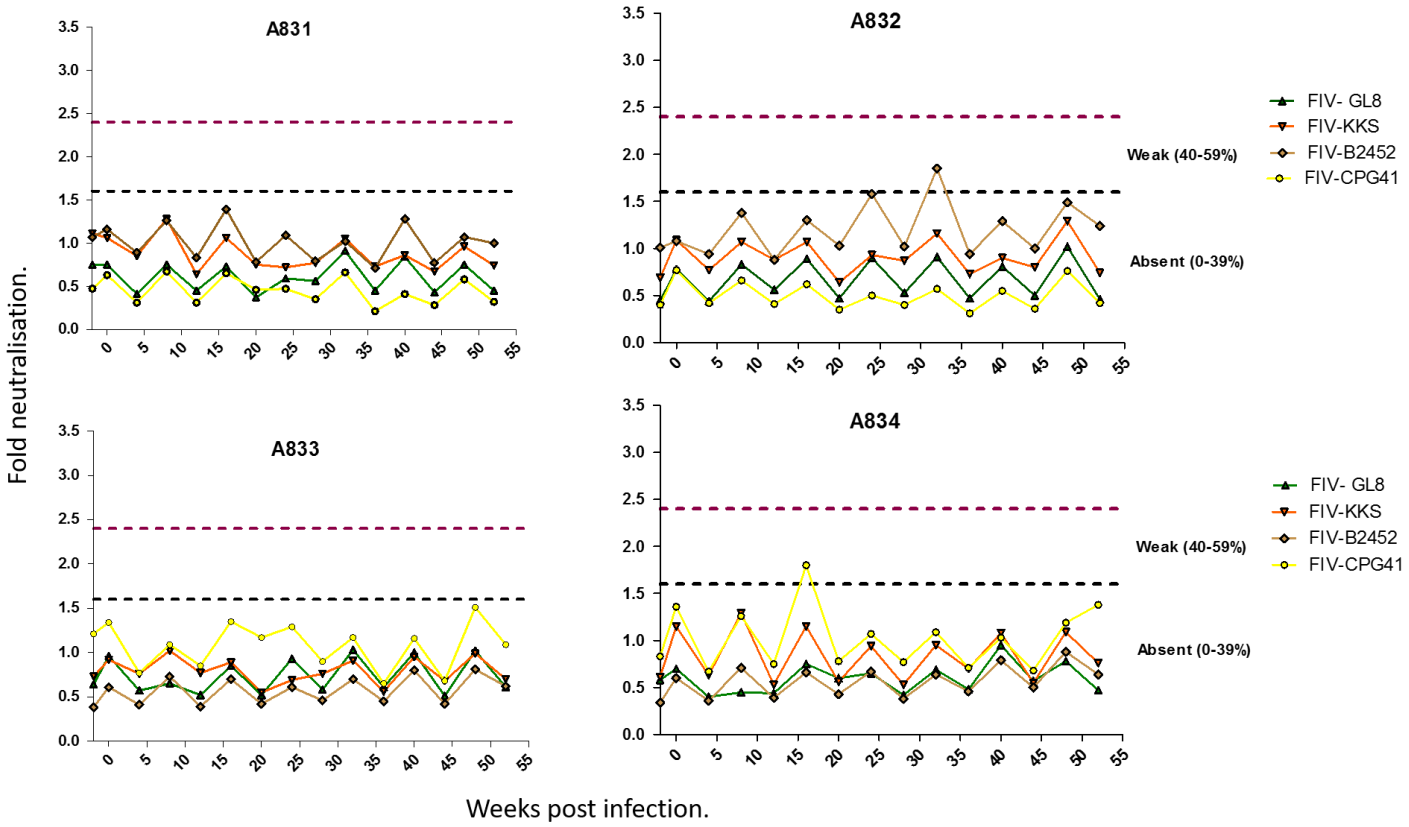


Figure 4-8. Heterologous neutralisation responses of cats A831-4 infected with FIV ΔV2. No neutralisation of pseudotypes bearing Envs of the heterologous clade isolates FIV-GL8 and FIV-PET (KKS) were observed, with fold neutralisation values lower than 1.6 (broken line). Cats A832 and A834 showed evidence of weak neutralisation of B2452 (A832 at week 32) and CPG41 (A834 at week 16) pseudotypes, but these responses were not sustained.

4.5.3 Lower proviral loads in cats that developed more rapid neutralisation responses

To examine the proviral loads that were established in the early phase of infection with FIV ΔV2, QPCR was performed on genomic DNA extracted from PBMC isolated at the time points shown in Table 4-3. For each cat, 10^6 PBMC were analysed and no more than 3% (30,000 cells) were infected with detectable virus (Figure 4-9). Indeed, only 0.5% of sampled PBMC contained virus in 3/4 cats. There was a peak in viral load 8 weeks post infection in all cats before the loads decreased to near undetectable levels by 28 weeks post infection in 3/4 cats, with cat A834 displaying a markedly reduced proviral load by 32 weeks post infection. In all cats the decrease in proviral load correlated with the development of strong homologous neutralising antibody responses, however the proviral loads appeared to increase gradually subsequently, in spite of the homologous neutralisation responses induced in each cat. Indeed, cat A834 displayed its peak proviral load 4 weeks after its highest neutralisation response. These data indicated that although homologous neutralising antibodies initially controlled virus replication and reduced the proviral burden to near undetectable levels, this suppression was overcome before the set proviral load was established.

In this study it appeared that the observed reduction in the proviral load correlated with the development of a strongly homologous neutralising antibody response and no evidence was found that the decreased proviral loads were associated with decreased numbers of target cells available for viral infection (Figure 4-10).

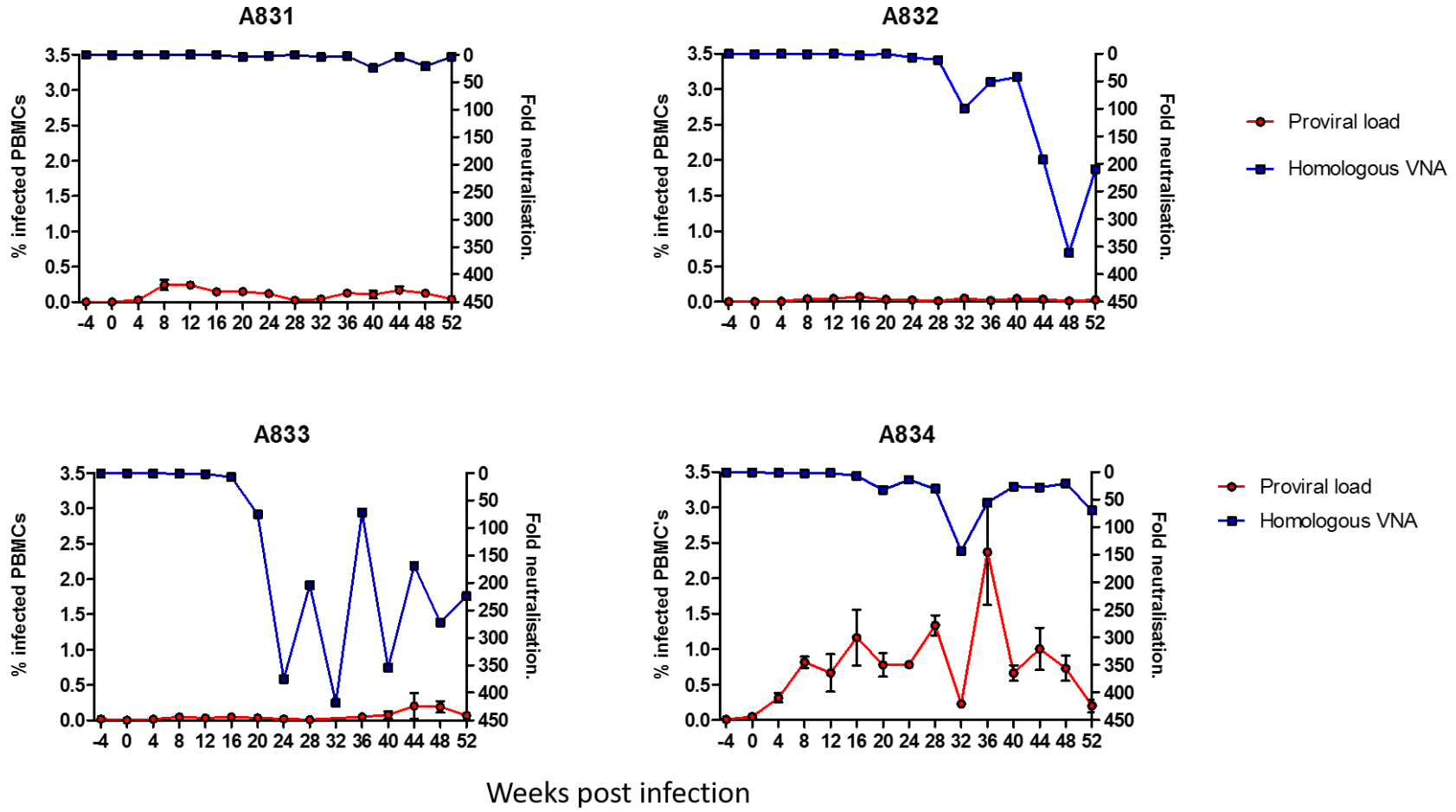


Figure 4-9. The development of the homologous neutralisation response in relation to the proviral load in cats A831-A834 following infection with FIV ΔV2. The blue line shows homologous neutralisation (fold neutralisation) whist the red line depicts the proviral load (percentage of infected PBMC).

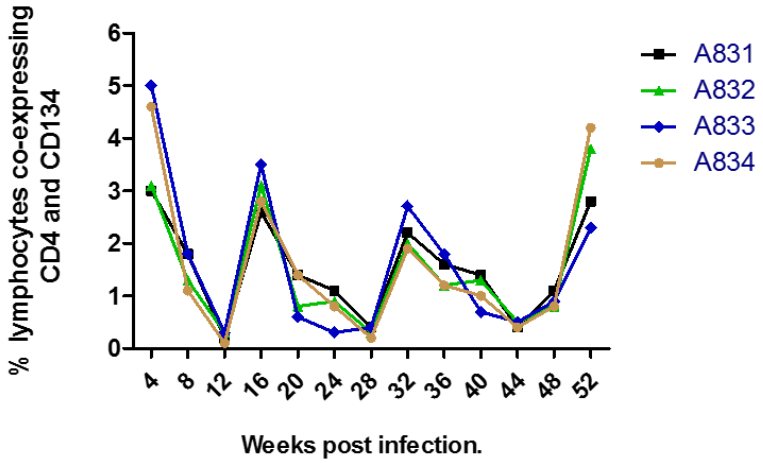


Figure 4-10. The percentage of activated T-lymphocytes expressing CD4 and CD134 for cats A831-4. As is evident in all four cats, a rhythmic cycle of expansion of the activated T-cell subset appears to occur every 16 weeks. Although a relevant observation, as this could potentially afford a larger target cell population to infect, the cause of this activated T-cell expansion was not further investigated.

4.5.4 CD4:CD8 lymphocyte ratio inversion in cats infected with FIV ΔV2.

To determine whether the chimaeric virus FIV ΔV2 was virulent or attenuated in cats, the $CD4^+$:$CD8^+$ T lymphocyte ratio was determined by FACS analysis (Figure 4-11), revealing a gradual inversion of the $CD4^+$:$CD8^+$ ratio that was most marked at 20 weeks post infection. As demonstrated in Figure 4-12, there was a marked $CD4^+$:$CD8^+$ inversion at 20 weeks' post infection, coinciding with the initial peak of viral replication, reflected by the high mean proviral load. It appears that $CD4^+$ lymphocyte numbers showed a significant gradual reduction (p=0.025) that correlated with time post infection. These data demonstrated that the FIV ΔV2 virus was virulent and targeted $CD4^+$ lymphocytes. Despite the chimaeric virus containing the *gag* and *pol* genes of FIV-GL8, the viral loads induced post infection were lower than had been observed following infection with a similar dose of the FIV-GL8 molecular clone (our unpublished data), suggesting that the novel *env* gene that was incorporated into the chimaeric virus was likely responsible for the decreased virulence of FIV ΔV2 relative to FIV-GL8.

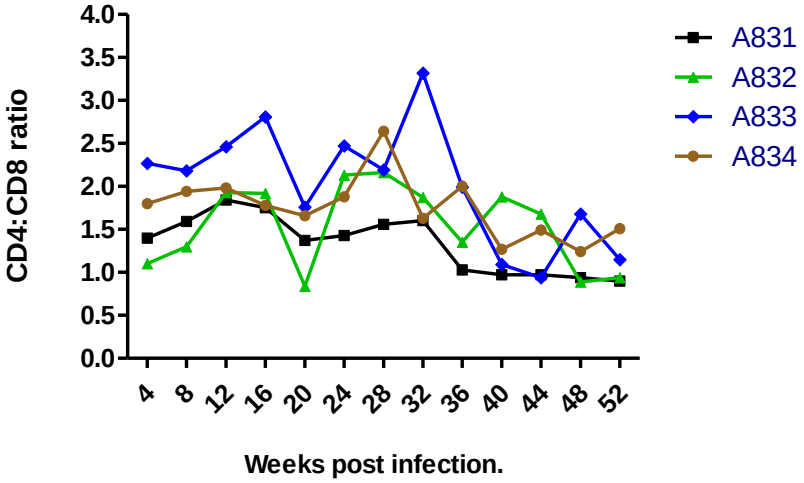


Figure 4-11. CD4⁺:CD8⁺ lymphocyte ratios of cats A831-A834. The gradual inversion of the CD4+/CD8+ t-cell ratio demonstrates that the chimaeric virus was indeed virulent and targeted the CD4+ t-cell compartment whilst expanding the CD8+ t-cell compartment. This observed pathogenesis is consistent with the published literature (see section 1.5, chapter 1).

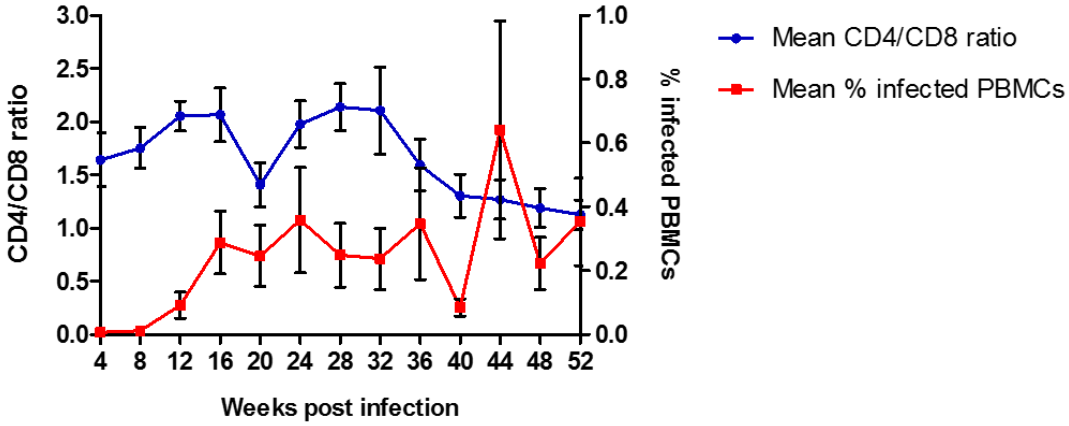


Figure 4-12. Mean CD4+:CD8+ ratios and proviral loads of cats A831-4. The negative correlation between time post infection and CD4⁺:CD8⁺ ratio was statistically significant (p= 0.025, R^2=0.378, Pearson r correlation).

4.5.5 Consensus sequence of FIV ΔV2 recovered from infected cats.

The stability of the unique PNGS was investigated, to determine if the potent humoral immune responses had resulted in mutations occurring in the PNGS at the apex of V2. The *env* gene was amplified and sequenced from genomic DNA extracted from the virus isolation cultures. The amino acid sequence of the predominant *env* gene appeared to be

stable, with the *env* genes from all of the infected cats retaining the same sequence as the original challenge virus, although the *env* gene did become more difficult to amplify from later cultures, indicating that either the primer annealing sites may have mutated or the viral copy number had decreased following the early stage of infection. However, this could not be investigated further because of time limitations. A comparison of the amino acid sequences of the V2 region of the amplified *env* genes (Figure 4-13) showed that the V2 PNGS mutation was present in all of the sequences derived from all of the infected cats at the time points examined. This demonstrated that the V2 PNGS mutation of the FIV Δ2 virus was stable for at least 52 weeks post infection.

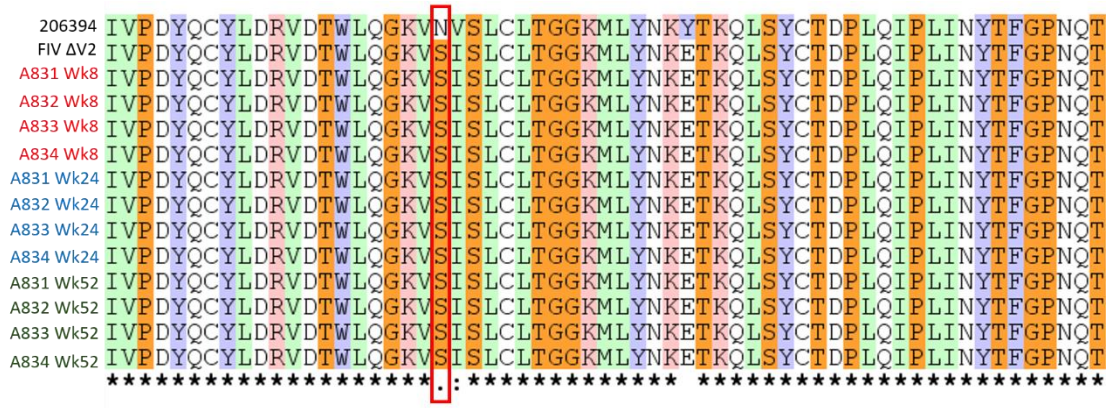


Figure 4-13. Consensus sequence of the major species of env gene amplified from cats A831-4. The sequences shown represent the apex of variable loop 2 with the N298S mutation boxed in red. Sequences from all 4 cats derived from week 8 (first virus isolation positive result, red) week 24 (midway through the study period, light blue) and week 52 (end of study, green) are aligned with the challenge virus, FIV ΔV2 and the original novel field isolate (206394) from which the FIV ΔV2 *env* was derived.

4.6 Discussion.

The ancestral virus that the FIV Δ2 envelope glycoprotein was derived from was isolated from a 14-year-old cat that displayed chronic age-related problems but was otherwise healthy. Although the length of time that the cat had been infected was unknown, since the cat was adopted from the feral population, it would be expected that the cat was infected for some time based on the known risk factors associated with FIV. This would therefore make this cat a long-term survivor, the same as the cats from which the two FIV vaccine strains were isolated from (Yamamoto *et al*, 2007). Vaccine efficacy appears to be greatest when attenuated strains of FIV, derived from long term survivors, are used as the immunogen as protection appears to be refectory to virulence (Yamamoto *et al*, 2007). Therefore, this makes the use of the FIV ΔV2 as a vaccine immunogen an attractive target. Infection with FIV ΔV2 elicited a strong humoral immune response detectable 4 weeks after infection and containing substantial homologous VNA. The initiation of homologous VNA appears substantially sooner than previously reported for FIV-Glasgow8 (Hosie *et al*, 2011) indicating that the inclusion of the envelope containing the V2 PNGS mutation can induce a neutralisation sensitive profile. Indeed, when the mutation was included into the envelope glycoprotein of HIV(FIV) pseudotyped FIV-Glasgow8, the pseudotyped virus was 5 times more sensitive to neutralisation. The PNGS mutation was under considerable selection pressure *in vivo,* as the glycosylation site had been repaired when the cat was sampled again, some 5 months after the original sampling (Samman 2010). This is consistent with the V2 PNGS playing a role in the development of a neutralisation resistant escape mutant. In this study the V2 mutation was stable for the duration of the study period (52 weeks). Although the method used to amplify and sequence the envelope in this study would only detect the major species of envelope glycoprotein circulating in PBMCs, it became evident that amplification and sequencing of the envelope became more difficult as the study progressed. In particular, the chromatograph of the sequence data become more difficult to read. This is indicative of mutations within the sequence primer binding sites and the amplification of more than 1 species of envelope glycoprotein resulting in 'dirty' reads. As the original PCR product is stored at -80^{o}C, it would be possible to more thoroughly re-examine the envelope glycoprotein amino acid sequence by cloning the envelope into the VR1012 expression vector and examining how many *env* variants can be obtained. This is a particularly pertinent point as the sequence data became more difficult

to generate from around week 32-36 of the study which coincides with a gradual increase in the proviral load seen in all 4 cats (to a greater or lesser extent), but also the development of especially potent homologous VNA.

Although the role of cell mediated immunity was not investigated in this study, the low proviral load observed in the study may have been to do, in part, cell mediated immunity. Cell mediated immunity was not measured in this study for several reasons. Primarily the main hypothesis being tested in this study was whether or not inoculation with the unique envelope of the FIV ΔV2 could elicit broadly neutralising antibodies, as *in vitro* mutagenesis studies had demonstrated that the V2 PNGS mutation to be a neutralising sensitive epitope when incorporated onto other envelopes from different FIV isolates, and the plasma from the cat from which the ancestral FIV isolate of FIV ΔV2 was derived from was shown to contain potent broadly neutralising antibody. Secondly, although inoculation with 'live' virus is known to elicit a strong cellular immune response, a more appropriate candidate immunogen for assessing the cellular immune response would have been immunisation with DNA or inactivated infected cell vaccine (Flynn *et al*, 1996. Flynn *et al*, 2000. Omori *et al*, 2004). Regardless of these facts the assessment of what type of cytotoxic T lymphocyte (CTL) response could inform on the possibility of protection using FIV ΔV2 as a candidate immunogen. It is known that vaccinated cats that are protected from FIV infection produce a specific type of CTL response. Protected cats produce a $CD8^+$ CTL response that are predominantly Env specific, can be detected in the blood during the initial stages of infection but then are detected almost solely in the lymphoid tissues. In contrast unprotected cats predominantly produce a Gag specific CTL response (Flynn *et al*, 1996. Flynn *et al*, 2000. Omori *et al*, 2004. Aranyos *et al*, 2016). By assessing the effector type of CTL response, an indication of the potential performance of the FIV ΔV2 as a vaccine could have been attained. Regardless of these facts, challenge with FIV ΔV2 elicited a potent homologous neutralising antibody response that did correlate with a decrease in proviral load.

Many BnAb have recently been identified that are able to efficiently neutralise a diverse range of HIV-1 isolates derived from tier 1, 2 and 3 viruses. Many of these antibodies target N-linked glycans at the apex of the V1/V2, including PG9 and PG6, CHO1 and CHO4, PGT141 and PGT145. The V1 and V2 regions of the envelope has been demonstrated as being a

global regulator of neutralisation sensitivity in HIV-1 (McLellan *et al*, 2011. O'Rourke *et al*, 2012. Rolland *et al*, 2012. Pinter *et al*, 2004). The majority of these papers describe mutations affecting PNGS either at the apex or the base of V1/V2 region, similar to the mutated PNGS described in this study and those seen in the FIV-Petaluma strain (Samman, 2010). However, the characterisation of neutralisation sensitive epitopes is restricted to the technology available to identify VNA or BnAb bound to its cognate epitope and precisely determine the neutralisation of the virus. A recent study has suggested the possibility of V1/V2 glycan shielding of neutralisation sensitive epitopes in V3 (Rusert *et al*, 2011). In this study the shielding of V3 by V1/V2 glycans was determined by the use of monoclonal antibodies (many of which have been shown to be broadly neutralising) and V1/V2 deleted virus. The authors propose the model depicted in Figure 4-14, where by the glycosylation of the V1 and V2 loop shields the neutralisation sensitive V3 loop, in a trimer dependant manner.

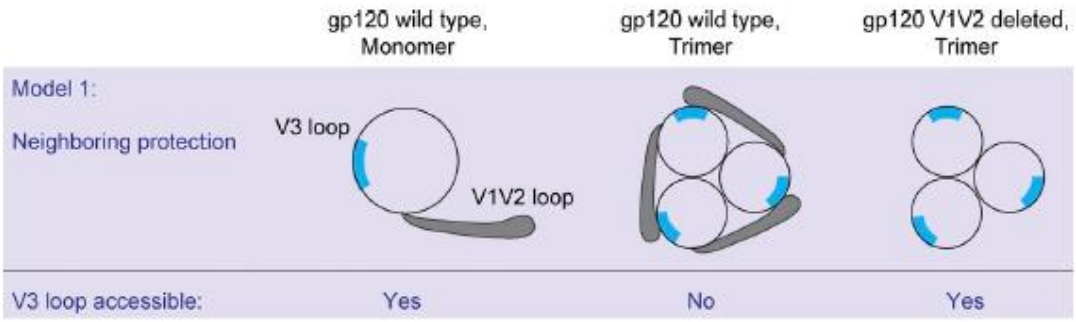


Figure 4-14. Proposed model of V3 protection by neighbouring V1/V2 glycosylation. The neighbouring protection model suggest that the glycosylation of the V2 loop of one monomer of gp120, shields neutralisation sensitive epitopes (V3) on a neighbouring gp120 monomer. This is consistent with mutations affecting PNGS within the V1/V2 loops resulting in neutralisation sensitive virus. Figure adapted from Rusert *et al*, 2011.

Therefore, the characterisation of an apparent neutralisation sensitive epitope located at the apex of V2, may actually expose the hypervariable loop 3. Hypervariable loop 3 of FIV has been shown to be an immunodominant domain of the FIV SU (Avrameas *et al*, 1992), to contain a neutralisation sensitive region (Lombardi *et al*, 1993) and to interact with FIV co-receptor, CXCR4 (de Parseval *et al*, 2006), yet vaccine trials using a peptide derived from the V3 neutralisation sensitive region failed to protect against infection but induce neutralising antibodies (Lombardi *et al*, 1994).

Although a neutralisation sensitive epitope has been discovered, and that epitope is able to elicit strong homologous VNA, the development of BnAb may require the co-evolution of the viral epitope and the reactive B-cell. Broad neutralisation has been correlated with time spent infected and BnAb are believed to require extensive somatic hypermutation driven by long periods of viral replication (Liao *et al*, 2013). The majority of BnAb characterised in HIV-1 infection harbour unusual features including long third complementary determining region, polyreactivity to none viral antigens, and the inability of the unmutated common ancestral B-cell (that BnAb eventually are derived from) to bind Env with sufficient affinity (Liao *et al*, 2013. McLellan *et al*, 2011. Haynes *et al*, 2012). The priming of a broadly neutralising antibody response to HIV has been attempted by utilising an immunogen that targets the activation of rare B-cell receptor gene combinations (Jardine *et al*, 2015). The aim of the study was to elicit the VRC01 class of BnAb (which targets the CD4 binding site) by immunising with a self-assembling nanopeptide that corresponds to the core of gp120. Activation of the VRC01 class of genes was achieved that created a pool of memory B-cells that could likely be boosted by a more native like immunogen (Jardin *et al*, 2015). It is thought that the generation of BnAb will require the germline targeted priming and subsequent selective boosting using antigenically related, but progressively more native immunogens (Jardine *et al*, 2015. Liao *et al*, 2013. Haynes *et al*, 2012).

As this mutated PNGS had been observed in patients with HIV-1 where BnAb have been isolated, it would appear that the V1/V2 homologue is also a global regulator of virus neutralisation in FIV as well as HIV-1. (Sanchez-Merino *et al*, 2016. McLellan *et al*, 2011. O'Rourke et al, 2012. Rolland *et al*, 2012. Pinter *et al*, 2004.)

Taken together it has been shown that infection with FIV ΔV2 elicits a strong humoral immune response that contains substantial homologous VNA. The virus has been shown to be pathogenic, as determined by the $CD4^+/CD8^+$ ratio inversion, however no BnAb were elicited. Had the trial been conducted for longer the appearance of BnAb may have occurred. The induction of such a strong homologous VNA appeared to correlate with the reduction in proviral load and thus qualifies the FIV ΔV2 virus as a candidate immunogen. The nature of the VNA response needs to be further characterised to determine if the

neutralisation target is the V1/V2 homologue or is facilitated by the unmasking of V3 neutralisation sensitive epitopes by V1/V2 de-glycosylation.

Chapter 5

Differentiation of infected from vaccinated cats in a trial testing a vaccine based on a chimaeric molecular clone of FIV

5.1 Introduction

The relatively lower cost of housing cats compared to non-human primates and the similarities between FIV and HIV-1 have allowed studies of FIV pathogenesis and the investigation of correlates of immunity to inform future research to develop an efficacious HIV vaccine. Prior to the licensing and release of the commercially available FIV vaccine Fel-O-Vax FIV, a plethora of vaccine studies had tested different viral proteins presented in a variety of ways to stimulate different arms of the immune system with variable outcomes. To date, the most success has been achieved with whole inactivated virus (WIV) and/or inactivated infected cell vaccines (ICV), however some of the first FIV vaccine trials utilised recombinant technology to produce subunit vaccines.

5.1.2 Subunit vaccines.

Subunit vaccines typically use viral proteins as the source of immune stimulation. Proteins that have been evaluated for their protective effect include recombinant p24, p17, TM and SU incorporated in to immune stimulating complexes (ISCOMs). Due to the purity of the candidate immunogen, vaccinates seroconvert to only the viral proteins contained within the vaccine prior to challenge. Although several FIV vaccine trials utilising subunit vaccines have been performed, none have resulted in significant protection and some have even shown enhancement of infection (Hosie *et al*, 1992. Siebelink *et al*, 1995. Huisman *et al*, 1998. Richardson *et al*, 1997). Siebelink and colleagues (1995) analysed the observed enhancement and demonstrated that the enhancement effect could be passively transferred to SPF cats. This indicated that the cause of enhancement was likely to be antibody mediated, however this was not replicated in a later study (Huisman *et al*, 1998).

5.1.3 Recombinant vaccines.

The use of DNA as a recombinant vaccine has yielded variable results. The viral genes incorporated into candidate recombinant vaccines include whole provirus harbouring essential deletions in the replication machinery, the entire *env* gene or fragments of the SU gene (Dunham *et al*, 2006. Hosie *et al* ,1998 & 2000. Richardson *et al,* 1997 & 1998. Broche-Pierre *et al*, 2005. Gupta *et al*, 2007). Theoretically, recombinant vaccination using FIV provirus containing deletions allows expression of the native form of the viral proteins devoid of inactivation artefacts. Accompanied to this is the ability to use biological adjuvants instead of conventional adjuvants. Homologous protection was demonstrated using this method when cats were challenged with the homologous FIV-Petaluma isolate, and decreased viral burdens were observed in vaccinated cats (Hosie *et al*, 1998). However, depending on the strain of challenge virus used, the results were inconsistent and levels of protection were consistently lower than using WIV or ICV as immunogens.

5.1.4 WIV and ICV vaccines and the development of Fel-O-Vax FIV.

Protection against FIV infection was first demonstrated following vaccination with WIV and ICV by Yamamoto *et al* (1991). In both cases the FIV-Petaluma isolate was used to elicit protection to low dose homologous challenge. The titre of virus neutralising antibodies induced by vaccination correlated with protection, however higher virus neutralising antibody titres were achieved with ICV compared to WIV, as were T-cell proliferation responses. The protection afforded by vaccination with FIV-Petaluma WIV and ICV was shown to extend to low dose, intra-clade heterologous challenge with FIV-Dixon, an isolate with an Env amino acid sequence that differed by 11% compared to the vaccine strain (Yamamoto *et el*, 1993). However, the Env divergency of the challenge virus is not a measure of virulence. The authors highlight that the amino acid sequence divergency observed between FIV-Petaluma and FIV-Dixon is similar to that observed between FIV-Petaluma and both FIV-Glasgow8 and FIV-PPR. Vaccination with FIV-Petaluma WIV did not afford protection to challenge with low dose FIV-Glasgow8 (Hosie *et al*, 1995), demonstrating a disparity between Env amino acid sequence divergence and virulence. It was confirmed that the homologous protection reported by Yamamoto *et al*, (1991, 1993) could be reproduced (Hosie *et al*, 1995) and the titre of anti-Env antibodies correlated with

protection, as did the titre of neutralising antibodies (nAb). In a series of experiments in which FIV-Petaluma WIV or ICV were assessed for protection against distinct heterologous challenge virus strains, only 1/10 cats were protected against FIV-Shizuoka challenge. The protected cat had been vaccinated with ICV and it was concluded that protection could not be extended to challenge with a virus that differed significantly (21% amino acid divergence) in the Env sequence (Johnson *et al*, 1994). In an effort to broaden immunity to heterologous challenge, a dual subtype ICV was developed, consisting of the chronically FIV-Petaluma-infected cell line FL4 and FeT1 cells infected with FIV-Shizuoka. FL4 cells are IL-2 independent and were derived from FeT1 cells by the gradual depletion of IL-2 from infected cultures (Yamamoto *et al*, 1991). Hohdatsu and colleagues (1997) demonstrated no heterologous protection against FIV-Shizuoka following vaccination with a single type ICV (FIV-Petaluma) but cats were protected against FIV-Shizuoka challenge when vaccinated with a dual subtype vaccine. Unlike previous trials, in which protection was associated with the titre of anti-Env antibodies and the titre of nAb, no nAb were detected against the FIV-Shizuoka strain of the vaccine, suggesting that protection was mediated by cytotoxic T-lymphocytes or other immune effector mechanisms (Hohdatsu *et al*, 1997). However, it is important to note that since the vaccine contained two subtypes of FIV, derived from clades A (Petaluma) and D (Shizuoka), the efficacy demonstrated in this study constitutes homologous protection. Deviations of this study (dual subtype vaccination of FIV-Petaluma and FIV-Shizuoka WIV) showed that 80% of cats could be protected against low dose challenge using the distinctly heterologous subtype A/B recombinant virus FIV-Bangston (Pu *et al*, 2001). Until this study, the majority of challenge viruses had been derived from minimally passaged *in vivo* derived virus. However, this study used pooled plasma or PBMC rather than tissue culture fluid (TCF) as this was thought to represent a more realistic challenge to mimic natural infection (Pu *et al*, 2001. Matteucci *et al*, 1996).

The dual subtype vaccine was licensed for commercial use in 2002 in the USA based on two year-long efficacy studies against challenge with a subtype A virus that differed by 9% and 20% at the Env sequence compared to the vaccine strains (Pu *et al*, 2004). The commercial vaccine contains 50µg FIV-Petaluma and FIV-Shizuoka WIV and 1.5 – 2.5 x 10^7 inactivated infected FeT-J cells, supplemented with human recombinant IL-12 at 5µg per dose (Omori *et al*, 2004. Coleman *et al*, 2014). As the commercial vaccine differed from the prototype vaccine, further studies were performed to assess protection against low dose subtype B

challenge. Protection was demonstrated against FIV-FC1 (Pu *et al*, 2005), FIV-Amori2 (Kusuhara *et al*, 2005) and the heterologous protection was shown to last for a least one year after the final immunisation (Huang *et al*, 2010). However, in a recent study the overall protection rate of Fel-O-Vax FIV was 70% when vaccinated cats were challenged with a range of isolates that included FIV-Petaluma (Clade A), FIV-Glasgow8 (Clade A), FIV-Bangston (Clade A/B), FIV-FC1 (Clade B) and FIV-NZ1 (Clade F/C). This protection rate was marginally better than that afforded by immunisation with the prototype WIV vaccine and, importantly, showed efficacy (40% protection) against challenge with FIV-Glasgow8 (Coleman *et al*, 2014). This finding contrasted with the results of a study performed by Dunham *et al* (2006) in which enhancement was observed following low dose FIV-Glasgow8 challenge. In addition, enhancement and increased susceptibility to infection were also reported following vaccination of cats with Fel-O-Vax FIV (Berlinski *et al*, 2003).

Due to the concerns raised about interference with the sero-diagnosis of FIV and the lack of protection against FIV field isolates, the Fel-O-Vax FIV vaccine has not been licenced in Europe. In countries where Fel-O-Vax FIV is licensed, veterinary practitioners do not routinely vaccinate against FIV and Fel-O-Vax FIV is not considered to be a core vaccine. Indeed, the vaccine is no longer manufactured in North America (Nichols *et al*, 2016), whereas vaccination against FIV remains high in Australia (Westman *et al*, 2016).

The aim of the study described in this chapter was to evaluate the use of a chimeric WIV candidate vaccine containing a mutation in the PID of the transmembrane (TM) region of Env. By mutating the PID of the FIV ΔV2 virus to that of lion lentivirus subtype E (LLV-E), it was predicted that nAb could be elicited and perhaps afford protection against a low dose challenge with the virulent isolate FIV-Glasgow8. In addition, by mutating the PID to that of LLV-E it was proposed that vaccination with the FIV ΔV2 PID mutant would permit differentiation of infected from vaccinated animals.

5.2 Method and materials

5.2.1 Principal immunodominant domain (PID) mutation.

The FIV ΔV2 virus was further genetically manipulated so that the PID of this virus was mutated to that of FIVple- subtype E. Briefly, the FIV ΔV2 molecular clone was used as a template to amplify a segment comprising approximately 500 base pairs of the envelope gene. The sequence of the reverse primer was 5'- AAT GGA TTC A**TA TG**A CAC ATC TTC CTC-3' and contained the restriction site for the endonuclease Nde1 (highlighted in red). The sequence of the forward primer was 5'- GAG CAT CAA GTA **CTA G**TA ATA GGA TTA AAA GTA GAA GCT ATG GAA AAA TTC TTA TAT ACT GCT TTC GCT ATG CAA GAA TTG GGA TGT **AGA GAA CAA** CAA **TTT** TTC TGT AAA GTC CCT TTT-3' and contained the restriction site for the endonuclease Spe1 (highlighted in red). This primer also contained the sequence corresponding to the FIVple-E PID (highlighted in bold and underlined). PCR reactions contained 25µl of 2X Roche Master mix, 1µl of forward and reverse primers at 100pmol/µl, 1µl of FIV ΔV2 molecular clone at 100ng/µl and 22µl of deionised water. The target sequence was amplified using the following cycling conditions: 94°C for 5 minutes, then 35 cycles of 94°C for 30 secs, 55°C for 30 secs, 72°C for 1 minute, followed by a final extension of 72°C for 10 minutes. The amplicon was visualised on a 0.8% agarose gel containing ethidium bromine and then extracted, purified and double digested using Nde1 and Spe1(New England Biolabs, Hitchin, UK) at 37°C for 3 hours. The digested ends of the PCR product were removed by purifying the amplicon through a QIAquick gel extraction column and eluting in 50µl of EB buffer. The mutated and digested amplicon was then ligated back into the FIV ΔV2 molecular clone (which had also been double digested and purified) by incubating the molecular clone and PCR product at a ratio of 1:3 with 1µl of T4 DNA ligase at 5U/µl in a total of 20µl (Invitrogen, Paisley, UK). The reaction was then incubated at 14°C overnight, after which DH5α Maximum efficiency cells Genotype: F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk^-, mk^+) *pho*A *sup*E44 λ^- *thi*$^-$1 *gyr*A96 *rel*A1(Invitrogen, Paisley, UK) were transformed using the ligation reaction. Briefly, 3µl of ligated DNA were incubated with 30µl of DH5α cells at 4°C for 30 minutes. The cells were then heat shocked at 42°C for 40 seconds and allowed to cool on ice for 2 minutes. Four hundred microliters of SOC broth were added to the cells and the culture was incubated at 37°C for 1 hour. The cultures were then added to kanamycin selection plates (200µl of culture per plate) and incubated

overnight at 30°C. Colonies were picked and re-cultured into ~5mls of kanamycin selection LB broth and incubated at 30°C overnight whilst being agitated at 250rpm. Plasmid DNA was then extracted from the cultures using the Qiagen miniprep kit and positive colonies were identified by double digestion of the plasmid DNA with Nde1/Spe1. Colonies that were positive had their entire envelope sequenced to ensure that the V2 PNGS mutation was still intact and that the PID had been mutated to that of FIVple-E. DNA from colonies that retained the same envelope sequence as the FIV ΔV2 envelope but carried the PID sequence of FIVple-E was used. Fifteen micrograms of this DNA were used to transfect 293T cells, as described in Materials and Methods (Chapter 2) and the resulting virus was named the DIVA virus. Initial stocks of DIVA virus were grown in Mya-1 cells, after which the culture fluids were clarified through a 0.45µM filter, aliquoted and stored at -80°C until needed.

5.2.2 FIV envelope DNA sequencing

DNA sequencing was performed to obtain the consensus nucleotide sequence of either amplified or cloned DNA. First, 5µl of amplified DNA (20-80ng/µl) or 5µl of plasmid DNA (80-100ng/µl) were mixed with 5µl of primer at 5pmol/µl in an eppendorf tube. The nucleotide sequences of the primers used in sequencing reactions are shown in Appendix 1. Eppendorf tubes were labelled with barcodes and shipped to the GATC Biotech European Custom Sequencing Centre in Cologne, Germany. Using the Lightrun sequencing service, DNA was sequenced using the Sanger method. Sequence data were downloaded from the GATC Biotech website (www.GACT-biotech.com) in the .ab1 format and analysed using DNADynamo (Bluetractor software).

5.2.3 HIV(FIV) luciferase pseudotype production.

HIV(FIV) pseudotypes carrying the firefly luciferase reporter gene (HIV(FIV)-luc) were prepared using the HIV pNL4-3 construct kindly provided by Dr N Landau. This construct contains the firefly luciferase gene in place of the deleted *nef gene* (pNL4-3.Luc.R^-.E^-). Figure 5-1 briefly illustrates the methodology for pseudotype production. For each pseudotype, a 10cm^2 petri dish (Corning, Warrington, UK) was seeded with 2x10^6 293T cells in 10mls of complete DMEM and incubated overnight at 37°C in a humidified incubator to allow the cells to adhere. The following day, 7.5µg of both pNL4-3.Luc.R^-.E and the VR1012

vector containing full-length FIV *env* were mixed with 300µl of serum free DMEM. The DNA was precipitated into complexes following the addition of 60µl of polyethylenimine (PEI). The reactants were briefly vortexed and centrifuged and the DNA allowed to precipitate at room temperature for 20 minutes. The transfectants were added to the 10cm petri dish and incubated at 37°C for 72 hours in a humidified incubator. The culture fluids containing the pseudotype was then centrifuged at 1000rpm for 5 minutes, filtered using a 0.45µm filter, aliquoted and stored at -80°C.

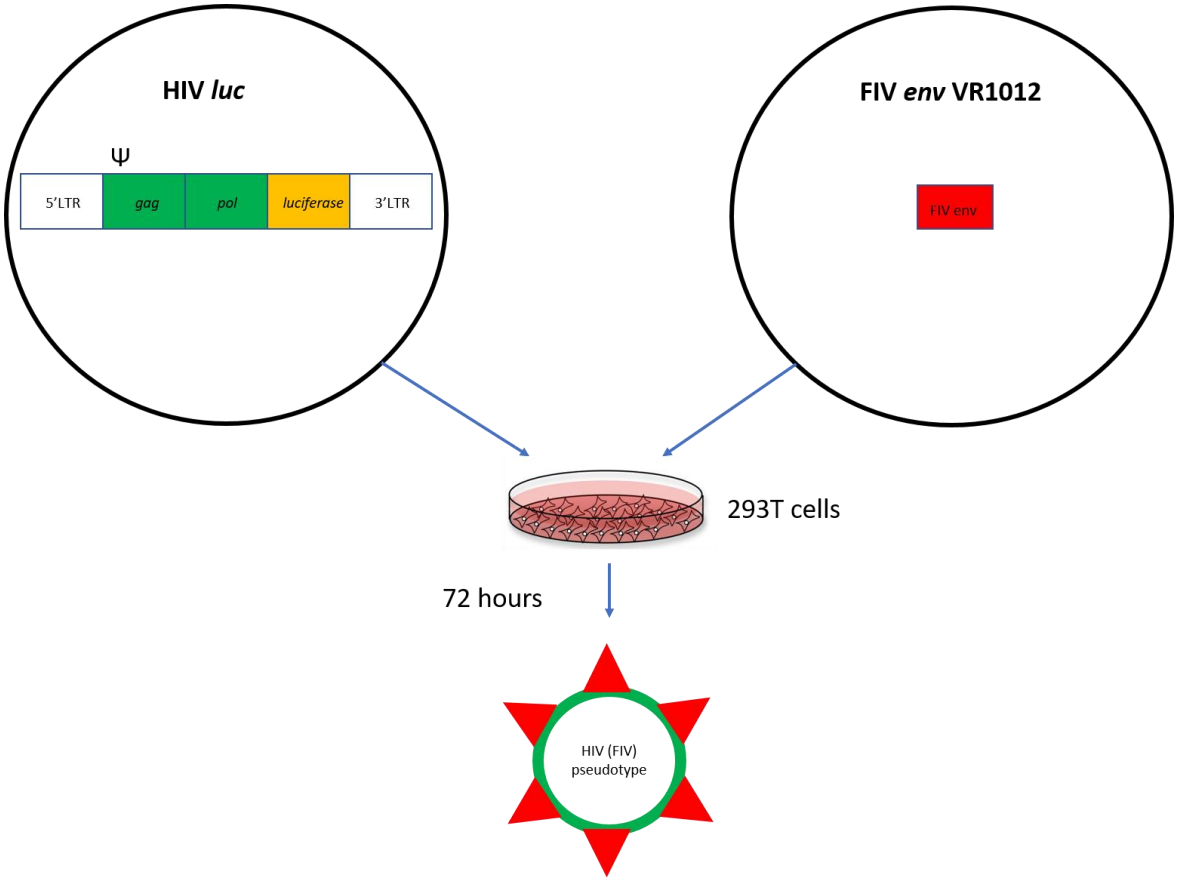


Figure 5-1. A schematic representation of HIV(FIV) pseudotype production. After co-transfection of both plasmids, translation produces the structural and replication apparatus of HIV. The HIV gag protein facilitates formation of immature, naked virions containing two copies of the HIV genome captured by the signal sequence (Ψ). Upon budding, FIV envelope glycoproteins are incorporated onto the virion surface. This allows for the creation of a pseudotype particle that is capable of entry into FIV susceptible cells, but are restricted to one round of infection, since the genome contains no FIV *env* gene.

5.2.4 Neutralisation assay

Samples of plasma collected in heparin or as sera were screened for FIV neutralising antibodies (nAb) using the HIV(FIV) luciferase pseudotype assay that has been described previously (Beczkowski *et al,* 2015). The work flow and principle of the assay is illustrated in figure 5-2.

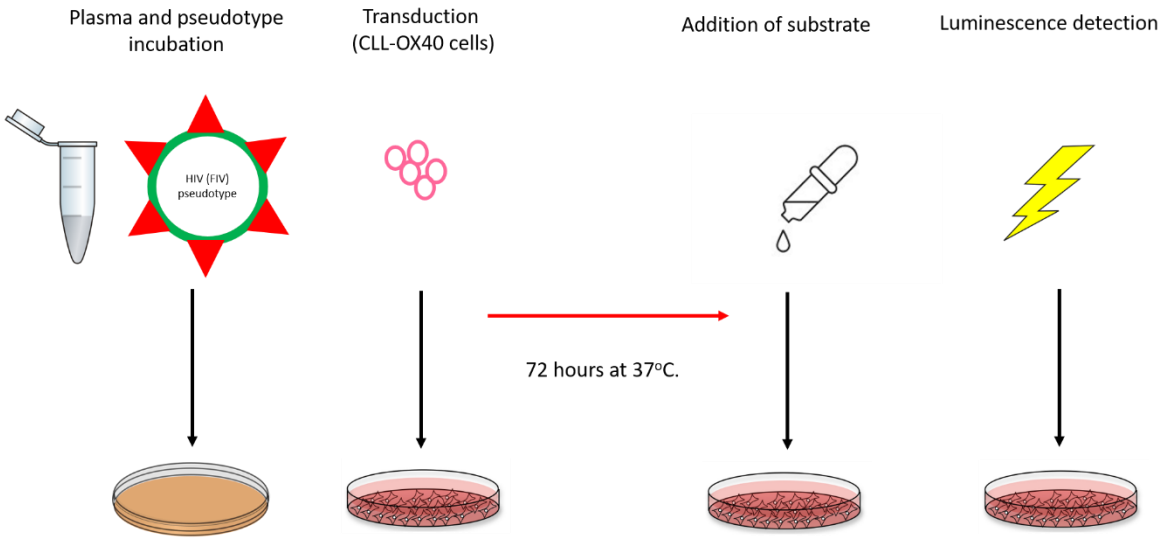


Figure 5-2. A schematic representation of the neutralisation assays work flow. Plasma containing FIV antibodies are incubated with pseudotype for one hour before the addition of FIV susceptible cells. After 72 hours, substrate is added and luminescence is detected by the 1460 microbeta liquid scintillation and luminescence counter. The principle of the assays is based on the incubation of the pseudotyped virus with plasma containing FIV antibodies that will inhibit cell entry should virus neutralising antibodies be present in the plasma. As the pseudotyped virus contains two copies of the HIV genome, that is *nef* deleted and carries the firefly luciferase gene in its place, translation of the luciferase gene produces the enzyme luciferase. Upon addition of luciferin substrate, luminescence is produced that can then be measured. Should the plasma sample contain virus neutralising antibodies, the pseudotype is prevented from cell entry and no luciferase luminescence is produced (neutralisation). Should the plasma sample contain no virus neutralising antibodies, the pseudotyped virus can enter the cell and large amounts of luciferase enzyme are produced, resulting in a large amount of luminescence being produced upon addition of luciferin substrate. As the pseudotyped virus contains only FIV envelope glycoproteins, since it was translated from a pure plasmid preparation, the assay is highly

specific for FIV virus neutralising antibodies. Additionally, due to the 72-hour incubation time, any pseudotype that is not neutralised and enters the cell is able to produce large amounts of luciferase that is readily detected upon substrate addition.

Firstly, plasma samples were heat inactivated for 30 minutes at 56^{o}C to inactivate complement before 4 serial 10 fold dilutions of the plasma were prepared in complete RMPI media. Plasma dilutions (25µl) were added to the wells of 96 well opaque culture plates (Perkin Elmer, Seer Green, UK) and incubated with 25µl of HIV(FIV) pseudotype for 1 hour at 37^{o}C in a humid incubator. After incubation, 50µl of CLL-OX40 cells at 5 x 10^{5}/ml were added to each well and the plates were incubated at 37^{o}C for 72 hours in a humid incubator. Next, 100µl of Steadylite HTS substrate (Perkin Elmer, Seer Green, UK) were added to each well, the plates were sealed (Perkin Elmer optical plate seals, Perkin Elmer, Seer Green, UK) and the reactions allowed to develop for 10 minutes. Luminescence was measured on the 1460 Microbeta liquid scintillation and luminescence counter (Microbeta, California, USA).

The neutralising activity of each sample was determined by calculating the percentage and fold neutralisation using the following equations:

$$\text{Percentage neutralisation} = \frac{\text{mPS-mNPC}}{\text{mNPC}} \times 100$$

Where mPS = mean luciferase count of sample wells in counts per minute (cpm)

mNPC = mean luciferase count of the no plasma control wells in cpm

$$\text{Fold neutralisation} = \text{mNPC/mPS}$$

5.2.5 Immunogen production.

DIVA virus was grown to large volumes in CLL-OX40 cells as detailed in table 5-1. After the virus in the culture fluids had been inactivated using 0.5% (w/v) paraformaldehyde, the fluids (approximately 1L in volume) were concentrated approximately 5-fold using the Centramate™ tangial flow concentrator with the Omega 300kDa medium screen channel

(Pall life sciences, Portsmouth, UK). The retentate was layered over 5mls of 0.45µm-filtered, 20% sucrose solution in thin walled, ultraclear™ SW28 tubes (Beckman Coulter, High Wycombe, UK) and centrifuged on the Sovall WX100 ultra-centrifuge for 2 hours at 107000*g* at 4^{o}C using the TH-641 SW28 rotor (Thermoscientific). The ultrafiltrate was aspirated and discarded and the viral pellets were resuspended in 6mls of sterile PBS and loaded into thinwalled, ultraclear™ SW41 tubes (Beckman Coulter, High Wycombe, UK) and centrifuged on the Sovall WX100 ultra-centrifuge for 90 minutes at 116000*g* at 4^{o}C using the Surespin 630 SW41 rotor (Kendro, Connecticut, USA). The ultrafiltrate was aspirated and the pelletted virus was resuspended in 2mls of sterile PBS and dialysed extensively against 10 changes of 2L sterile PBS at 4^{o}C. Post dialysis, the total protein concentration of the whole inactivated virus (WIV) immunogen was estimated by the Bradfords Coomassie assay and stored frozen at -80^{o}C until immunisation was due to take place.

5.2.6 Experimental vaccination using the DIVA virus.

For logistical reasons, the DIVA WIV was combined with Versifel FeLV vaccine that contained a commercial adjuvant with proven safety and efficacy in cats (Zoetis, Brabant Wallon, Belgium). Briefly, 4 doses of Versifel FeLV were pooled in a 15ml falcon tube using a sterile 22G needle. Four doses of DIVA WIV (250µg per dose) were added to the pooled Versifel FeLV vaccine and mixed before the di-valent vaccine was replaced in the Versifel FeLV vials using a 22G needle (approximately 1.2mls per vial). This process was repeated with another 4 doses of Versifel FeLV using sterile PBS instead of DIVA WIV to prepare the control vaccine.

Eight specified pathogen free (SPF) kittens of 8 weeks of age were divided into two groups of 4 kittens. This allocation was based on an arbitrary equal distribution of males in both groups (in case castration had any effect upon challenge) and that both groups had roughly the same average age, again to remove any bias. Following a week of acclimatisation, the kittens in Group 1 (vaccinates) were vaccinated subcutaneously at study weeks 0, 3 and 6 (at 15, 18 and 21 weeks of age respectively) with 1.2ml of the test FIV vaccine. At the same time, the kittens in Group 2 (controls) received the control vaccine containing PBS alone. At weeks -6 and 1 of the study (9 and 16 weeks of age respectively), both groups received additional vaccinations with PureVax-RCP plus FeLV to protect against feline herpesvirus,

feline calicivirus and feline panleukopaenia virus, since the cattery had a history of feline herpesvirus infection. Ten weeks after the final test FIV vaccination, all of the kittens were challenged with 20 cat infectious doses 50% (ID_{50}) of the infectious molecular clone FIVGL8-414 that had previously been titrated *in vivo*. FIVGL8-414 is a highly pathogenic primary UK isolate of FIV that is thought to be representative of isolates that are transmitted in the field. Vaccinates and controls were bled according to the schedule shown in Table 5-2 from the jugular or cephalic vein using 20-22G needles. Blood samples collected in heparin and EDTA were shipped overnight from the trial facility (Charles Rivers Laboratories, Co Mayo, Ireland) to the Retrovirus Research Laboratory at the University of Glasgow by courier and were processed within 24 hours of collection.

Days post infection.	Action.
0	5mls of CLL-OX40 cells at 1 x 10^6/ml were infected with 2000 $TCID_{50}$ of DIVA virus ($TCID_{50}$ calculated following titration on Mya-1 cells). Cells were incubated in a T25 culture flask at 37°C overnight in a humidified incubator.
1	The cells were centrifuged at 1000rpm for 5 minutes and a culture was set up in 5mls of fresh complete RPMI. The infected cells were incubated in a humidified incubator at 37°C.
4	The cells were added to 10mls of complete RPMI and transferred to a T75 culture flask. The flask was incubated at 37°C in a humidified incubator.
7	The infected culture was subcultured (cells and SN) and added to 30mls of 1 x10^6/ml of fresh CCL-OX40 cells in 2xT150 culture flasks. Flasks were incubated at 37°C in a humidified incubator.
11	The cultures were centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and clarified through a 0.45µm filter and stored in a sterile glass bottle at 4°C. The cell pellet was counted and cells were recultured at 1x 10^6/ml in fresh complete RPMI in 4 T150 flasks. Fresh cells were added to maintain the cell density at 1 x 10^6/ml if required. Cultures were incubated at 37°C in a humidified incubator.
15	The infected cultures were subcultured 1 in 2 (cells and SN) and added to 20mls of 2 x10^6/ml of fresh CCL-OX40 cells in 8xT150 culture flasks. Flasks were incubated at 37°C in a humidified incubator
18	20mls of culture (cells + SN) were added to 20mls of fresh complete RMPI and set up in 16xT150 culture flasks. Flasks were incubated at 37°C in a humidified incubator
21	Cultures were centrifuged at 1000rpm for 10 minutes to pellet the cells. The supernatant was aspirated and pooled with the supernatant that had been collected on day 11. The pooled supernatant was twice clarified through a 0.45µm filter and treated with 0.5% formaldehyde for 24 hours at 4°C to inactivate the virus. The culture fluids were agitated using a magnetic stirring rod to ensure thorough inactivation of the virus.

Table 5-1. Production of DIVA virus immunogen in CLL-OX40 cells.

Week of study	Age of subjects (weeks)	Sample	Procedure	[a]Experimental notes	[b]Assays performed.
-7	8	0.5ml EDTA, 0.5ml hep	Blood sample		1,2,3
-6	9	None	PureVax-RCP + FeLV	#	
-3	12	0.5ml EDTA, 0.5ml hep	Blood sample		1,2,3
0	15	0.5ml EDTA, 0.5ml hep	FIV Immunisation 1		1,2,3
1	16	None	PureVax-RCP + FeLV	#	
3	18	2.0ml EDTA, 5.0ml hep	FIV Immunisation 2		1,2,3
6	21	2.0ml EDTA, 5.0ml hep	FIV Immunisation 3		1,2,3
10	25	2.0ml EDTA, 5.0ml hep	Blood sample		1,2,3
13	28	2.0ml EDTA, 5.0ml hep	Blood sample		1,2,3
16	31	2.0ml EDTA, 5.0ml hep	Day of challenge	20 ID_{50} FIV-GL8 414	1,2,3,4,5
19	34	2.0ml EDTA, 5.0ml hep	3wpc		1,2,3,5
22	37	2.0ml EDTA, 5.0ml hep	6wpc		1,2,3,5
25	40	2.0ml EDTA, 5.0ml hep	9wpc		1,2,3
28	43	2.0ml EDTA, 5.0ml hep	12wpc		1,2,3
28	43	Exsanguination	12wpc	Exsanguination and post mortem examination. Tissue collection.	1,2,3

Table 5-2. Immunisation, sampling and analysis schedule of the experimental DIVA FIV vaccine trial. [a]#, Vaccination with PureVax-RCP and FeLV (inactivated feline herpesvirus (FHV) strain F2, inactivated feline calcivirus (FCV) strain 431/G1, attenuated feline panleukopaenia virus (FPV), FeLV recombinant canarypox virus (solvent) and 23µg gentamicin. [b], 1. DIVA ELISA, 2. Gag qPCR, 3. Immunostain analysis, 4. Neutralisation assay, 5. Virus isolation.

5.3 Results.

5.3.1 Immunogen production.

To determine the optimal time to harvest the DIVA virus, time course experiments were performed to monitor the growth of both the DIVA and FIV ΔV2 viruses. Initial virus stocks were grown on the primary feline lymphoblastoid Mya-1 cells. However, Mya-1 cells are an unattractive target cell for the large-scale production of vaccine immunogen because of their slow rate of growth, sensitive nature and dependence on IL-2. In contrast, the CLL-OX40 cell line that stably expresses feline OX40 is IL-2 independent, grows rapidly and is more robust than Mya-1 cells. For this reason, we compared the growth kinetics of both viruses in the CLL-OX40 cell line to determine whether it would be a suitable cell line for the production of large quantities of DIVA vaccine virus.

5.3.2 Growth of FIV ΔV2 and DIVA viruses in CLL-OX40 cells

CLL-OX40 cells were infected with 2000 $TCID_{50}$ of either FIV ΔV2 or the FIV DIVA virus and the cultures were monitored by p24 ELISA and by immunostaining of 1ml samples of purified culture fluid. As shown in Figure 5-3, the CLL-OX40 cells produced more DIVA virus than FIV ΔV2 in the early stages of infection. This difference is likely attributable to the PID mutation, since both viruses are otherwise identical. It is possible that the PID mutation facilitates a more rapid/secure binding of the fusion peptide with the cell membrane, increasing viral entry; however, this was not investigated further. The levels of p24 continued to increase until day 18 when there was a dramatic decrease in virus production. To investigate this further, the cell counts of the infections were analysed (Figure 5-4) to assess whether the reduction in p24 production could be explained by the lack of viable cells. However, the cell numbers did not explain the dramatic reduction in p24 levels as the cell counts increased from day 21. FACS analysis of the cells from both infections (Figure 5-5) revealed that the number of target cells decreased by approximately 60% at 29 days' post infection and by almost 95% by day 36 post infection. This finding explained the dramatic reduction in p24 levels and the increase in CLL-OX40 cell numbers. Although the cells were engineered to stably express feline OX40, the virus growth selected for cells that did not express feline OX40. This resulted in an expansion of the wild type CLL cells that

were not susceptible to virus infection and so the cell count was maintained or even increased while the level of virus production decreased.

It was concluded that CLL-OX40 cells were suitable for the large-scale production of vaccine virus and the immunogen production method described in Table 5-1 was developed.

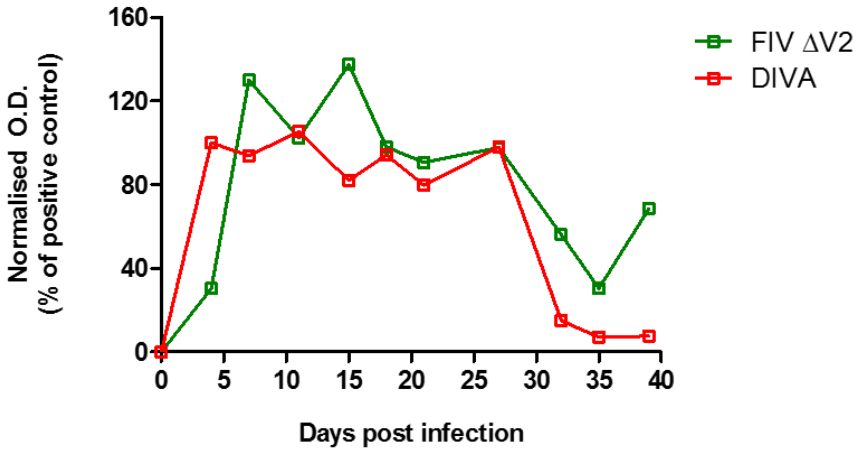


Figure 5-3. Growth kinetics of FIV ΔV2 and DIVA viruses in CLL-OX40 cells. Peak virus production, as determined by FIV p24 production, was observed at day 18 of the infection. Therefore, culture fluids were collected on day 21 for the production of the vaccine immunogen.

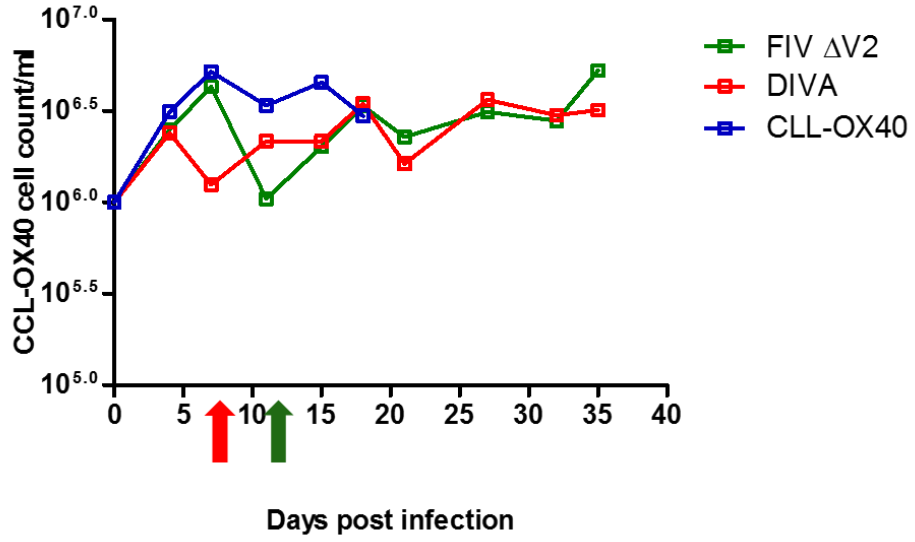


Figure 5-4. CLL-OX40 cell growth during the production of FIV ΔV2 and DIVA viruses. Green arrow indicates that fresh cells were added to the FIV ΔV2 infection to maintain a cell density of 1 x 10^6/ml. The red arrow indicates that fresh cells were added to the DIVA virus infection to maintain a similar cell density

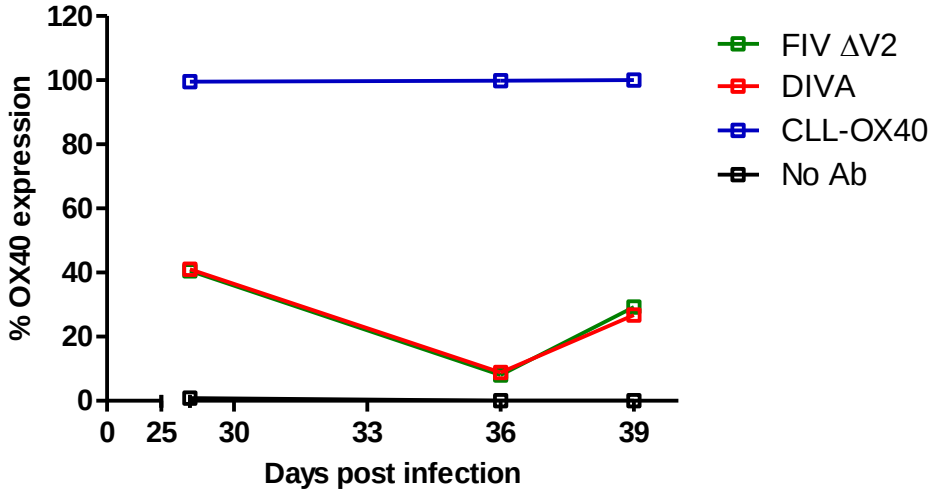


Figure 5-5. OX40 expression on CLL-OX40 cells during the propagation of FIV ΔV2 and DIVA viruses. The surface expression of OX40 was markedly reduced in both infections by day 36 post infection. Infected cultures were supplemented with fresh CLL-OX40 cells after sampling on day 36. This accounted for the increase in OX40 expression observed between days 36 and 39. CLL-OX40 = Uninfected CLL-OX40 cells stained for feline OX40, No Ab = Unstained, uninfected CLL-OX40 cells.

5.3.3 Immunostain analysis

Immunostain analysis revealed that the DIVA virus grew more rapidly than FIVΔV2 in CLL-OX40 cells. Four days' post inoculation, immunostaining of viral lysates using a pooled polyclonal FIV anti-serum revealed all of the major viral bands from the DIVA virus infection, whereas a strong response to p24 but only weak responses to p55 and p10 were evident in the FIVΔV2 infection (Figure 5-6)

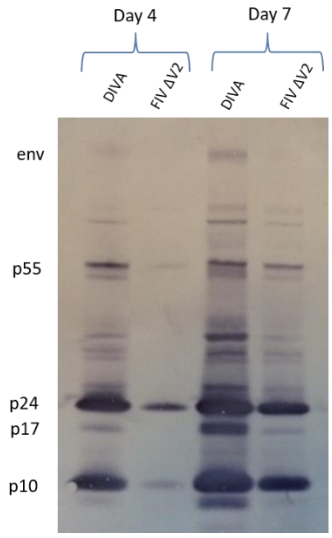


Figure 5-6. Immunostain analysis of virus collected on days 4 and 7 from cultures of CLL-OX40 cells infected with the FIV ΔV2 and DIVA viruses. Although only semi-quantitative, it appeared that more DIVA than FIV ΔV2 virus was released into the culture fluid early in infection. This finding was consistent with the p24 production observed during the early stages of infection.

The appearance of both the DIVA and FIVΔV2 envelope glycoprotein (Env) on immunostain was similar to that previously observed for the FL4 vaccine strain of FIV (Figure 5-7). The predicted amino acid sequence of the Env of FIV derived from FL4 cells suggests that two potential N-linked glycosylation sites (PNGS) at the base of the V2 loop are absent (Samman, 2010). Similarly, the DIVA virus has a single PNGS that is missing at the apex of the V2 loop (Samman, 2010). The lack of the single PNGS within the DIVA virus Env would not account for the appearance of the Env observed by immunostain, as the amino acid sequence of the DIVA virus Env showed no additional mutations of PNGS had occurred during mutation and cloning of the DIVA virus. To investigate this observation further, we compared the Env of both the FIV ΔV2 and DIVA viruses when grown in either Mya-1 or

CLL-OX40 cells. As can be seen in Figure 5-5, both viruses displayed Envs of lower molecular weight when grown in CLL-OX40 cells compared to Mya-1 cells. The 'sharpness' of the bands also indicated that the Envs were either less heavily glycosylated or had different glycosylation as a result of having been grown in canine cells rather than feline cells. This might imply that the Env would be less shielded by glycosylation and hence be more accessible to neutralising antibody if the DIVA virus was grown in CLL-OX40 cells.

Based on all these findings, it was decided to grow the DIVA virus in CLL-OX40 cells for 21 days. The rapid virus growth kinetics, robustness, IL-2 independence and high levels of Env produced by CLL-OX40 cells made them an ideal candidate for vaccine virus production.

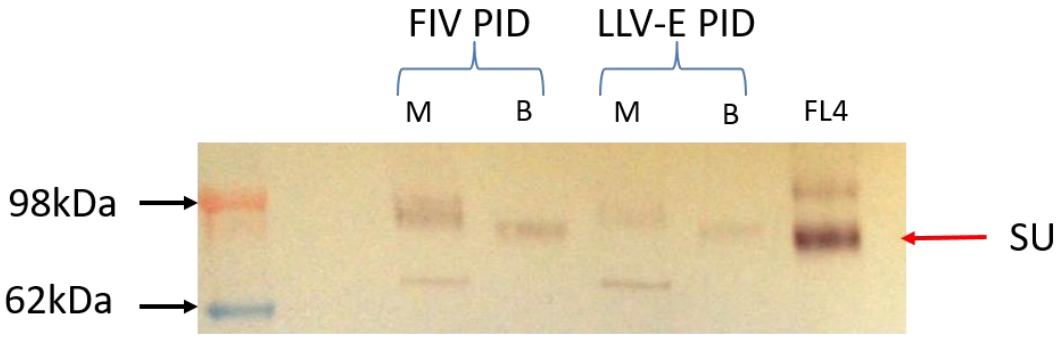


Figure 5-7. The envelope phenotype of FIV ΔV2 and DIVA virus grown in both CLL-OX40 and Mya-1 cells compared to FL4 vaccine virus. The altered phenotype of the viral envelope when grown in CLL-OX40 cells is predicted to expose more of SU potentially aiding in the development of VNA. M = Mya-1, B = CLL-OX40. Proteins stained with the monoclonal antibody Vpg71.2 recognising FIV envelope.

5.3.4 Virus inactivation.

To ensure that the vaccine preparation had been completely inactivated, a single dose of purified and dialysed virus (250µg) was incubated with Mya-1 cells and FIV p24 levels in the culture fluids were monitored, following the procedure outlined in Table 5-3.

Days post inoculation	Action	O.D. 650nm
-1	250µg inactivated DIVA virus cultured with 5mls of Mya-1 cells at 1 x 10^6/ml	N/A
0	Cells washed with 3x 13mls PBS	N/A
3	Sampled- Washed with 3x 13mls PBS	0.220
6	Sampled	0.053
9	Sampled - Cultured in T75	0.040
12	Sampled and media changed	0.044
15	Sampled	0.041
18	Sampled	0.049
20	Sampled	0.039
Mya-1	N/A	0.041
DIVA (positive)	N/A	0.898
Negative	N/A	0.044

Table 5-3. The FIV p24 levels of the cultured inactivated DIVA virus. Although FIV p24 could be detected in culture fluid three days post inoculation, it could not be detected six days post inoculation once another wash step had been performed. FIV p24 could not be detected at any time point after day six of inoculation, indicating that the virus was indeed inactivated.

When residual inactivated DIVA virus had been thoroughly washed away, the levels of FIV p24 detected in the sampled culture fluid were similar to those of the negative control. This confirmed that the DIVA virus was not actively replicating. Importantly this process confirmed the effectiveness of the dialysis step as Mya-1 cells are particularly sensitive to culture additives. The Mya-1 cells remained confluent and healthy throughout the 20-day experiment, indicating that all of the paraformaldehyde inactivating agent had been removed. On this basis, it was concluded that the DIVA virus had been inactivated completely and was safe to be administered to the study animals.

5.4 Vaccination of cats using the DIVA virus.

5.4.1 Immunisation of cats with DIVA WIV.

All cats received the experimental FIV vaccine (vaccinates) or control vaccine (controls) according to the immunisation schedule in table 5-2. There were no reported adverse side effects at the sites of injection or as a result of the immunisations. At -6 week of the study,

1 cat (A842) displayed clinical signs of mild oedema of the conjunctiva of the left eye and mild epiphora. This kitten was treated with 10mg of oral Veraflox once daily for 7 days, and the remaining 7 kittens received prophylactic treatment with the same drug at the same dose. No further clinical signs were reported and all kittens were healthy on the day of the first immunisations.

5.5 Serological analyses

5.5.1 Immunostain analysis.

Of the 4 cats that received the FIV vaccine, all developed antibodies recognising the structural proteins p55, p24 and p17 by week 3 of the study (Figure 5-8). By week 10 of the study, when all 3 immunisations had been administered, all of the cats had fully seroconverted to all of the major structural viral proteins. This response was maintained in 3/4 vaccinated cats until the day of challenge. No antibody response to the SU protein was detected in cat A842 until week 10 of the study and this response appeared to be transient, as no anti-SU response was subsequently detected until after the day of challenge. Since the antigen on the PVDF membrane was purified FIV ΔV2 (containing the wild type PID) and the vaccinates had been immunised with the DIVA virus containing the mutated PID, it might be expected that the antibody response to the TM protein would be absent or diminished. This was indeed evident in all vaccinates until week 19 of the study (Appendix 5) when vaccinates developed antibodies recognising the wildtype PID, 3 weeks after challenge. None of the 4 unvaccinated control cats showed evidence of seroconversion until week 22 of the study; all had sero-converted to all the major structural viral proteins by the end of the study at week 28.

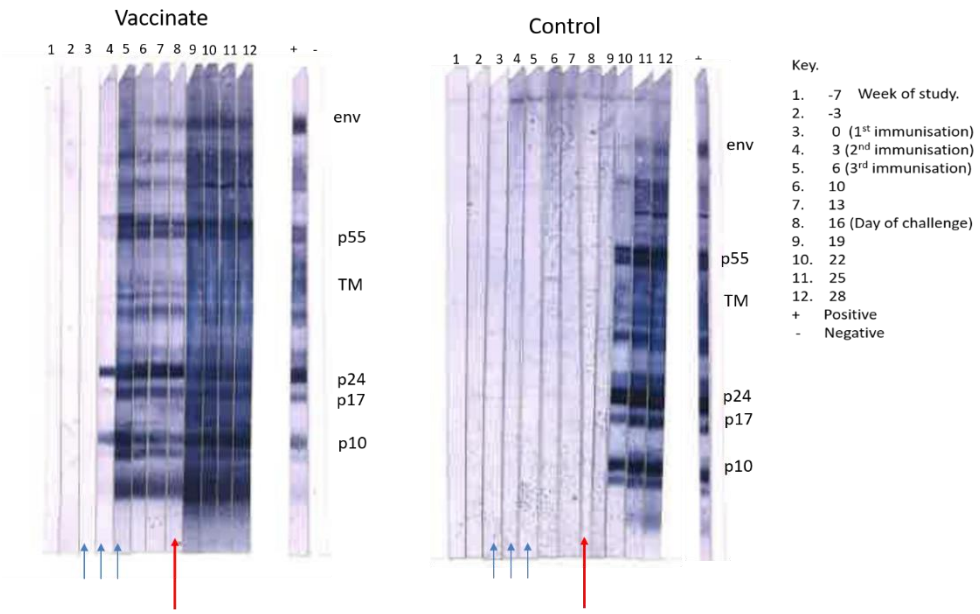


Figure 5-8. Serological responses of representative vaccinated and control cats. Results are shown for vaccinate A843 and control A847. (Responses of all cats shown in Appendix 5). Sero-conversion to viral proteins was evident at 3 weeks' post immunisation in the vaccinated cat. There was no antibody response to FIV in the control cat prior to challenge. Small blue arrows indicate timings of immunisations, whilst the large red arrow indicates the day of challenge.

5.5.2 DIVA ELISA

All 4 vaccinated cats responded strongly to the LLV-E peptide and maintained a strong reaction to it until the day of challenge (Figure 5-9). Very little cross reactivity could be demonstrated against the FIV peptide, although one cat (A841) exhibited marginal cross reactivity. Following challenge, a dramatic increase in reactivity against both the LLV-E and FIV peptides was observed. The finding that the LLV-E reactivity was boosted post challenge indicated that immunological memory might have been induced by vaccination with the DIVA virus. However, the dramatic prolonged increase in reactivity to the FIV peptide observed post challenge suggested that the challenge virus actively replicated in the kittens and that the vaccinates were not protected against the FIV-GL8 414 challenge. The 4 control cats did not demonstrate an antibody response to either peptide until week 19 of the study (3 weeks post challenge). This finding confirmed the specificity of the peptide encoding the FIV PID (since the control cats have been vaccinated against the retrovirus FeLV). The cross reactivity between the 2 peptides, post challenge, was substantially less in

the controls than the vaccinates and can be explained as minimal cross reactivity as observed in the pathogenesis trial (Chapter 4). Despite the concerns regarding protection against challenge, immunisation with the DIVA virus allowed the differentiation of infected from vaccinated animals.

5.5.3 Virus neutralising antibody responses

To determine whether the SU specific antibody response induced following immunisation included the production of virus neutralising antibodies (nAb), neutralisation assays were performed using the heparinised plasma samples collected from each cat on the day of challenge. No significant decrease in the luciferase activity was observed compared to the no plasma control (Figure 5-10) indicting that no nAb were detected. Based on these finding alone it was concluded that immunisation with the DIVA vaccine elicited envelope specific antibodies, however these were not neutralising.

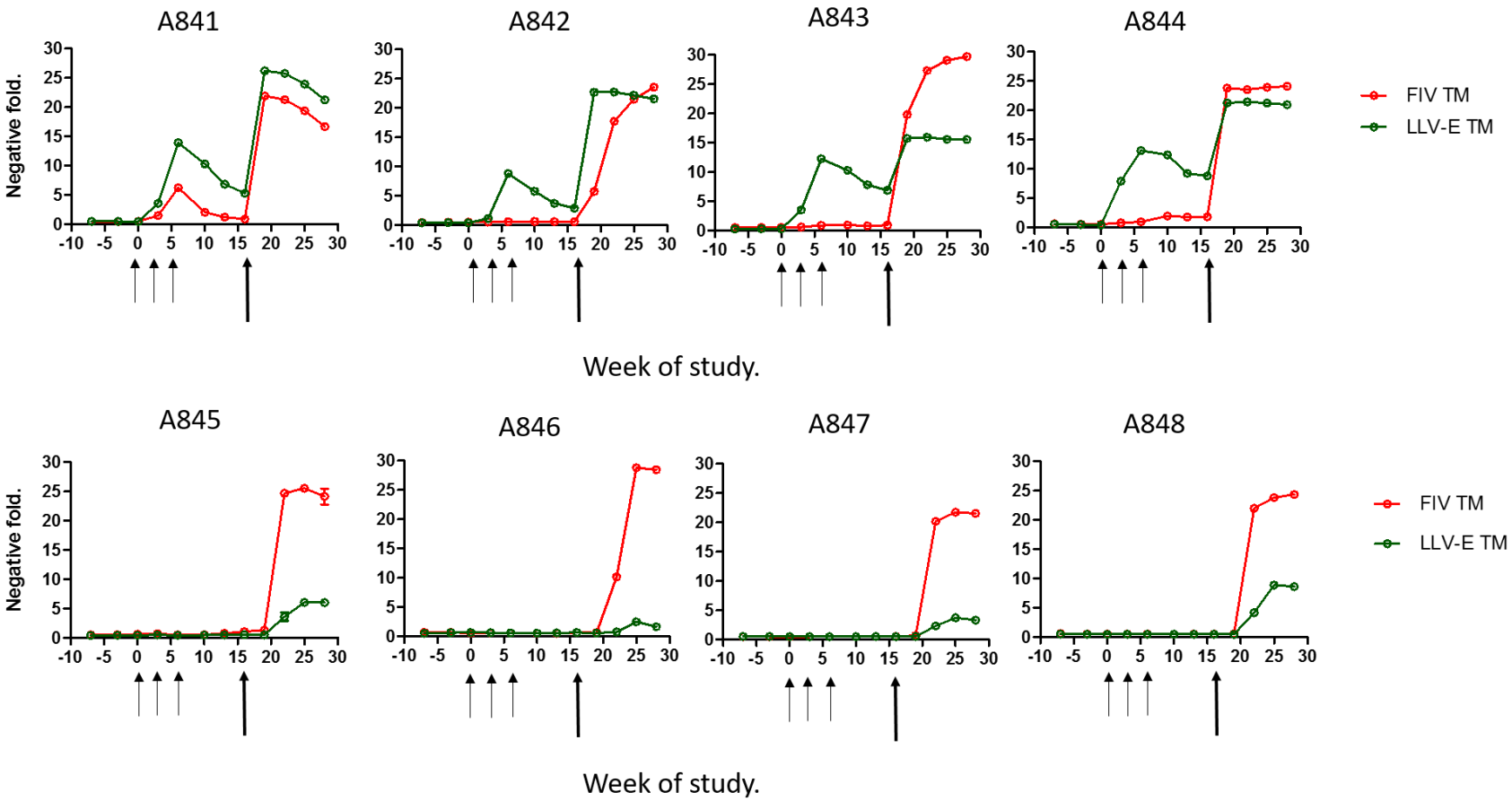


Figure 5-9. Serological differentiation of infected and vaccinated cats. Vaccinates (A841-4) and controls (A845-8) clearly displayed different reactivities against the two TM peptides tested. Until the day of challenge (large arrow) vaccinates demonstrated antibodies recognising the LLV-E TM, the antibody responses increasing with each immunisation (small arrows).

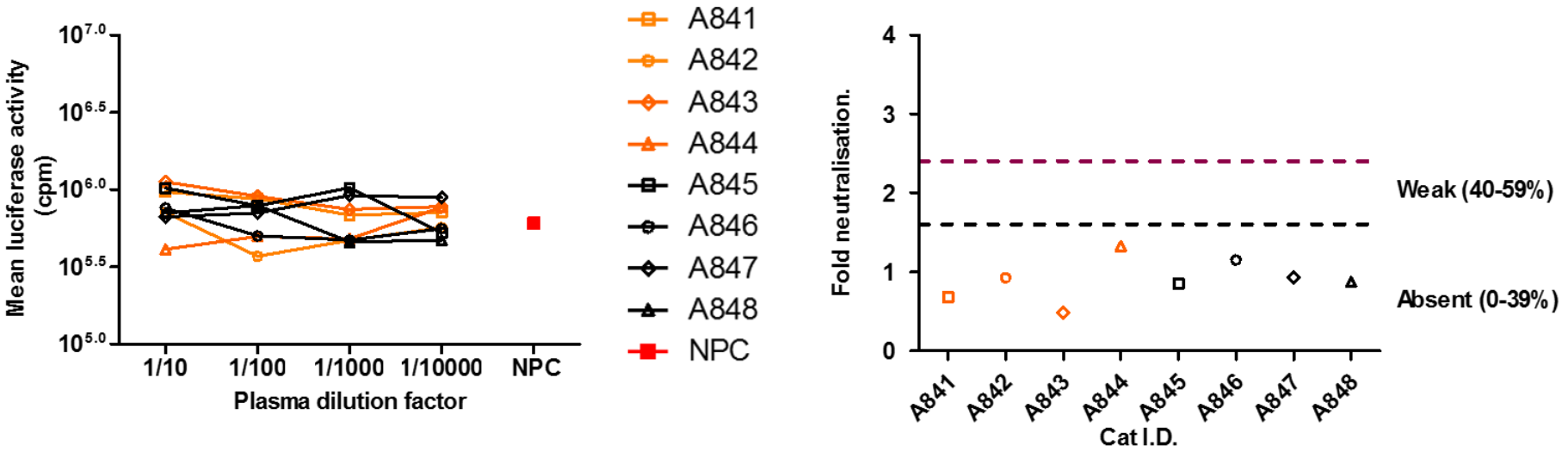


Figure 5-10. No neutralising antibodies were produced prior to challenge. The mean luciferase activity (Left) and fold neutralisation (Right) of vaccinates and controls, tested on the day of challenge, are shown. The mean luciferase activity observed for both vaccinates (Orange) and controls (Black) were both similar to that of the no plasma control (NPC), consistent with a lack of neutralisation. This was confirmed when the fold neutralisation was calculated (Right); no nAb were detected, responses were absent when fold neutralisation was related to percentage neutralisation (Right legend).

5.5.4 Virus isolation and proviral load.

Although the complete inactivation of the vaccine virus had previously been established *in vitro*, it was important to determine if any residual infectivity remained *in vivo*, since cats might be more sensitive to lower infecting doses than cells in culture. Since virus isolation is considered the reference standard for diagnosing FIV infection, PBMCs were purified from each cat on the day of challenge and cultured with Mya-1 cells for 21 days, as described in materials and methods. As shown in figure 5-11, no FIV p24 was detected in culture fluids after 21 days of co-cultivation. Next, DNA was extracted from the cultures and a nested PCR was performed that would amplify the *env* gene (data not shown). No *env* genes could be amplified from any sample, confirming that there had been no residual infectivity in the vaccine preparation. Additionally, this validates the inactivation protocol.

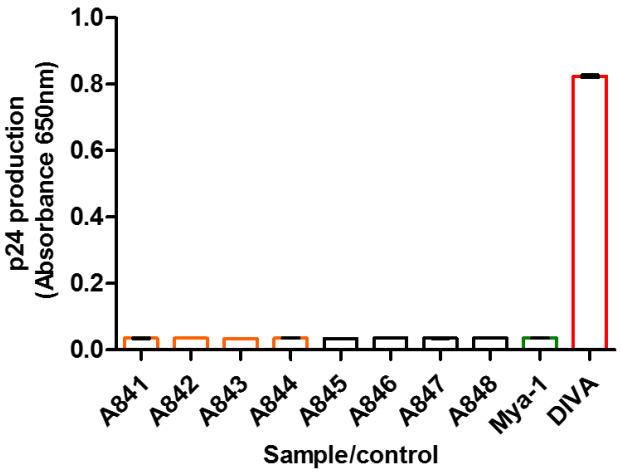


Figure 5-11. No FIV p24 was detected in the VI culture fluids derived from the day of challenge (as determined by p24 ELISA). Orange bars represent vaccinates and black bars controls. The negative control (Mya-1, green bar) was culture fluid collected from uninfected Mya-1 cells and the positive control (DIVA, red bar) was culture fluid derived from Mya-1 cells infected the DIVA virus.

Virus isolation was performed at study weeks +3 and +6 (Figure 5-12). At week +3 of the study, FIV p24 was detected in culture fluids from all but one cat (A846) after 21 days of co-culture. At study week +6, FIV p24 was detected in culture fluids of all co-cultures after 7 days. As the one cat that virus could not be isolated from at study week +3 was in the control group, it was possible that immunisation with the DIVA vaccine might have led to

enhanced infection, as virus was readily isolated from all the vaccinates before the controls. To assess whether or not enhancement had occurred, proviral load was determined using a qPCR method designed to amplify a conserved region of the *gag* gene.

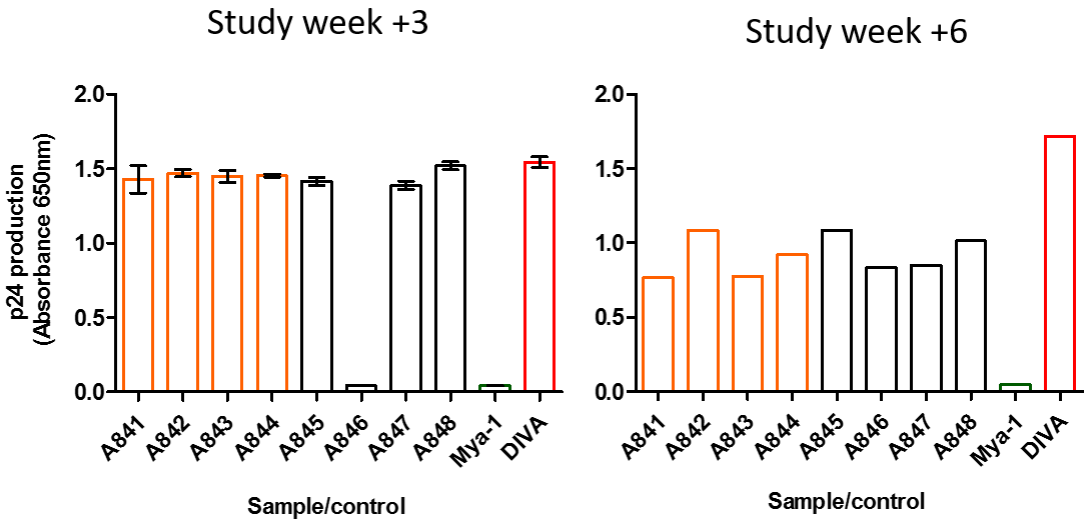


Figure 5-12. Levels of p24 detected in VI culture fluid at study weeks +3 and +6. At Study week +3 the individual co-cultures were maintained for 21 days and then the culture fluid tested in triplicate by ELISA. At study week +6, all samples tested positive by day 7; these samples were tested singularly. Virus isolation is used as a binary measure of FIV infection whereby samples are either positive or negative. As all individual co-cultivations had tested positive by day 7 on study week +6, it was deemed unnecessary to continue the experiment, resulting in enough material from each individual co-cultivation to be screen singularly for the presence of p24 antigen. Orange bars represent vaccinates and black bars controls. The negative control (Mya-1, green bar) is culture fluid from uninfected Mya-1 cells and the positive control (DIVA, red bar) is culture fluid derived from Mya-1 cells infected with the DIVA virus.

As shown in Figure 5-13, all cats displayed increasing proviral loads after challenge, which then decreased to the set point. However, it was noted that cat A842 displayed a markedly higher initial proviral load compared to the other cats. The proviral load of cat A842 then decreased to a level more similar to that of the other cats, however by study week +9 the viral load had increased again and continued to increase until the end of the study. When comparing the mean proviral loads of the 2 groups, it was clear that the vaccinates had a higher mean proviral load from study week +3 until the end of the trial. This difference in

proviral load was statistically insignificant between the 2 groups (p= 0.15, Kruskal-Wallis test).

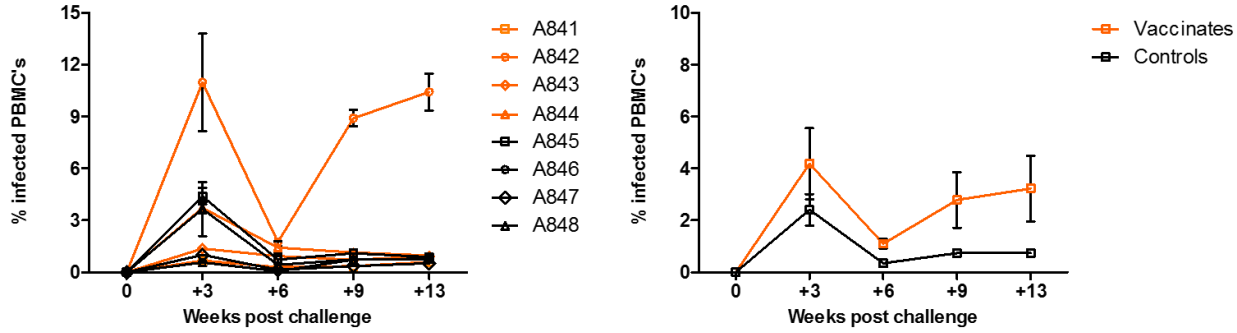


Figure 5-13. Proviral load of vaccinates and controls from the day of challenge until the end of the study. When the proviral loads of each cat at each time point was compared (Left) it was difficult to identify a trend between the vaccinates (Orange) and controls (Black). However, the proviral loads were comparable with other studies where FIV GL8 414 had been used as the challenge virus. When the mean proviral loads of both groups were compared at each time point (Right), an insignificant difference was observed, with the lower mean proviral load in the control group (p= 0.15, Kruskal-Wallis test).

5.5. Conclusion.

In this vaccine trial, it has been demonstrated that vaccination with DIVA WIV does not protect cats against low dose FIV-Glasgow8 challenge. Refectory to protection, vaccination with DIVA WIV appeared to enhance infection as demonstrated by the higher proviral load and the ability to isolate virus from PBMCs in all vaccinates sooner than the controls. The aim of this trial was to attempt and elicit protection against FIV-Glasgow8 by the production of VNA. Previous research on the ancestral envelope glycoprotein of the DIVA virus, designated 206394 (Samman, 2010. PhD thesis), characterised a unique mutation at the apex of hypervariable loop 2 (V2). Incorporation of the V2 PNGS mutation into the FIV-Glasgow8 *env* rendered the pseudotyped virus approximately 5 times more sensitive to neutralisation by plasmas containing BnAb. The evaluation of the chimeric virus, FIV ΔV2, demonstrated that the V2 PNGS mutation elicits strong homologous VNA but failed to elicit heterologous VNA (Chapter 4). In this study neither homologous or heterologous VNA were elicited. The prototype Fel-O-Vax FIV vaccine inactivates the incorporated virus in 1.25% paraformaldehyde compared to our inactivation method of 0.5%. This concentration of inactivate had been used previously by our lab (Hosie *et al*, 2000) and was chosen as it completely inactivated the virus and was believed to preserve the immunogenicity of the envelope glycoprotein, preserving the envelopes neutralisation sensitive phenotype. Other FIV vaccination trials utilising paraformaldehyde as the inactivating agent have reported it be a mild inactivant, however no studies have assessed the degree of cross-linkage induced by paraformaldehyde inactivation nor the preservation of immunogenic determinants. Studies by Rosso *et al* (1998) demonstrated that inactivation of HIV-1_{MN}/HIV-1_{LAI} and SIV_{mne} by formalin treatment to be as harsh as heat inactivating the virus at 56^{o}C for 2 hours with a resulting loss of around 75% of the antigenicity, when measured by whole virus precipitation using the HIV-1 monoclonal antibody gp120 d48. This protein modification of antigenicity was also shown for cellular derived proteins demonstrating that the loss of antigenicity to a function of the inactivating method and not the antigen. Preservation of antigenicity by inactivating HIV-1_{MN} and SIV_{mne} using 1,5 iodonaphthylazide (INA) was also demonstrated as determined by an ELISA utilising the BnAb as capture antibodies (Raviv *et al*, 2005). Extensive cross linkage of the viral envelope may explain the lack of detectable homologous and heterologous VNA. It has previously been demonstrated that alterations to the net charge of the V3 loop can facilitate alterations to the tropism and neutralisation

sensitivity of FIV (de Parseval *et al*, 2006. Lombardi *et al*, 1993). Should challenge with the FIV ΔV2 virus elicit V3 specific VNA, chemical inactivation of the DIVA virus may render it neutralisation resistant/inaccessible.

Although there was no statistically significant difference in proviral load between vaccinates and controls it is apparent that the vaccinates became infected before the controls. Regardless of whether one or all cats demonstrate enhancement, it is the third documentation of enhancement when using FIV-Glasgow8 as the challenge strain (Hosie *et al*, 1992. Dunham *et al*, 2006). Although in this study the causes of enhancement were not further examined, the use of FIV-Glasgow8 as a challenge strain offers the opportunity to study the mechanisms of lentivirus enhancement of infection. Since there appears to be no increase in the titre of pseudotyped virus when performing the neutralisation assay, this would indicate that the observed enhancement is not antibody mediated. As enhancement of infection has been demonstrated when vaccinating animals with FIV envelopes harbouring mutated PIDs (Richardson *et al*, 1997) it is not possible to claim the enhancement observed is mediated by complement as further studies would need to be carried out to delineate the cause of enhancement. Adding to this problem is the nature of the vaccine preparation administered.

Although the induction of immunological memory was not directly measured, the ELISA results would indicate that memory B-cells have been elicited. The large and rapid anamnestic antibody response observed immediately after challenge supports this hypothesis. Initially we wanted to assess the strength of the CTL response induced by vaccination with the DIVA virus, however as none of the cats were protected and the ELIspot assay is time consuming, we decided not to investigate this further. A positive aspect of the vaccination trial was the ability to differentiate infected from vaccinated animals using the DIVA virus. We observed an increase in the titre of antibodies recognising the LLV-E peptide with every boost immunisation. In two of the cats (A843 and A844) this titre remains at relatively high levels until the day of challenge, in cats A841 and A842 the titre drops to relatively low levels by the day of challenge, however it was still possible to DIVA at this point. The duration of the antibody response to the LLV-E peptide remains to be established and the usefulness of the DIVA ELISA would need to be evaluated for its ability to DIVA on plasma samples derived from cats that were last immunised further than

6 months ago. This is however the first documented case of DIVA of FIV, and unlike other FIV DIVA strategies, this allows for the differentiation of vaccinated from uninfected animals unlike previous attempts (Kusuhara *et al*, Levy *et al*, Westman *et al*, Litster *et al*). Due to the incorporation of the mutated PID in the vaccine it is also possible to identify vaccine break through cases as demonstrated in Figure 5-9, and differentiate infected from vaccinated animals. Although the vaccine did not protect, the plasma reactivities to either FIV or LLV-E peptide would be need to assessed in cats protected from FIV infection. For this reason, it would be of value to assess the utility of the DIVA epitope in the prototype Fel-O-Vax FIV vaccine (FIV-Petaluma WIV) to determine the ability of the mutated PID to DIVA cats upon challenge.

Chapter 6

Final discussion.

The aim of the studies described in this thesis was to investigate whether a DIVA FIV vaccine could be constructed, by exploiting the PID within TM as a DIVA epitope. We vaccinated cats with a WIV vaccine based on a chimaeric FIV that contained a mutated TM region and showed that it was possible to differentiate vaccinated from infected cats using a peptide ELISA test to measure antibodies recognising either the wild type FIV TM or the TM derived from LLV-E.

Although the WIV vaccine based on a chimaeric FIV showed promise as a DIVA vaccine, there was no evidence that the vaccine could protect cats against challenge with the virulent primary isolate FIV-GL8. Indeed, there was some evidence that vaccination enhanced infection, although this enhancement was largely attributed to one of the vaccinated cats. Clearly it will be important to identify the mechanism(s) of enhancement, as this is the first case of enhancement following vaccination with a WIV immunogen. It is not clear whether enhancement was antibody or complement mediated, or if the inclusion of the DIVA epitope played a role in the observed enhancement. In vaccine studies performed by Richardson *et al* (1997), randomly mutated PIDs were included in a DNA vaccine that induced little or no anti-Env humoral response and significant enhancement of infection was reported. The vaccine tested in this study did elicit a significant humoral immune response against FIV Env (as detected by immunostain and ELISA) and therefore antibody-mediated enhancement cannot be ruled out, although no enhancement of pseudotype entry was observed when plasma samples collected from vaccinated cats on the day of challenge were tested for neutralisation. To define the mechanism(s) of enhancement would inform future vaccine design and determine the utility of including mutated PIDs in the next generation of FIV vaccines.

It remains to be determined why none of the vaccinated cats developed homologous nAb. Since cats infected with the FIV ΔV2 chimaera demonstrated a rapid and robust homologous nAb response, it might have been predicted that immunisation with the WIV (which was based on this chimaera and differed from the FIV ΔV2 virus only in the PID amino acid sequence) might also induce similar, or higher, titres of nAb. It is unlikely that the lack of homologous nAb induced by the DIVA vaccine reflected low levels of Env in the

virus as, during experiments to optimise the growth of the DIVA virus in CLL-OX40 cells, high levels of Env were detected by immunostaining (Figure 5-4, chapter 4, section 5.3.3). One hypothesis for the lack of homologous nAb response is that nAb epitopes on Env were altered following paraformaldehyde inactivation, during the preparation of the WIV. Indeed, studies comparing the inactivation of HIV-1 and SIV showed that formalin inactivation is as severe as heat inactivation of viruses, with significant loss of protein antigenicity (Rosso *et al*, 1998).

Support for this hypothesis is derived from immunostain data generated from Fel-O-Vax FIV vaccinated samples. Serum samples from vaccinated cats failed to recognise homologous Env (FIV-Petaluma derived from FL4 cells) by immunostaining (Appendix 3iii-Immunostains of samples from Australian and US vaccinates). The commercial vaccine is produced using paraformaldehyde at 1.25% w/v for inactivation, inducing significant cross-linkage of the viral Env and potentially altering the antigenicity (as determined by immunostain). The DIVA vaccine virus tested in this study inactivated using 0.5% paraformaldehyde w/v. This appeared to retain the antigenicity of the Env as it was recognised by homologous (FIV ΔV2) and heterologous plasma (FIV-Glasgow8 and FIV-Petaluma). The degree of cross-linkage, however, could still be too great to permit the induction of nAb, by invoking mild conformational changes in Env.

Another potential explanation is that the strong homologous nAb response observed in the cats infected with FIV ΔV2 were induced by a fully replicating virus so that Env would be processed fully and presented to the immune system such that the appropriate immune response was obtained. The use of WIV circumvents the antigen processing and presentation that are likely required for the induction of nAb, for the lack of homologous nAb induced following vaccination.

A successful outcome of the vaccination trial was the incorporation of the DIVA epitope that allowed the differentiation of infected from vaccinated animals. Unlike previous attempts, the approach to constructing a DIVA vaccine described in this thesis allowed the differentiation of infected and uninfected cats and also vaccinated and uninfected cats. This differentiation has not been possible using either formalin inactivated virus and TM (Kusuhara *et al*, 2006. Levy *et al*, 2008), combinations of point of care kits (Westman *et al*, 2015), PCR (Wang *et al*, 2010. Ammersbach *et al*, 2013) or florescence activated cell sorting (Litster *et al*, 2014).

When developing a potential DIVA vaccine, it is necessary also to develop an appropriate DIVA assay. Other DIVA viral vaccines have utilised deletion mutants as the DIVA immunogen and rely on competition ELISAs to serve as the discriminatory test (Vu *et al*, 2013. van Oirschot *et al*, 1990). Competition ELISAs generally display lower sensitivities than conventional direct, indirect or sandwich ELISAs and are appropriate screening tests to assess the exposure of large numbers of animals to an infectious agent. However, such tests are not appropriate for screening companion animals for infection with infectious agents. By targeting the PID of FIV as the DIVA epitope, and exploiting the serological diagnosis of FIV using the DIVA ELISA, it should be possible to differentiate infected from vaccinated cats irrespective of FIV infection or vaccination status. As the PIDs of lentiviruses are highly conserved, the mutation of the PID could theoretically allow DIVA for any lentiviral infections should the vaccine contain the TM domain of Env.

Prospects for future work.

While the studies described in this thesis have demonstrated that it is possible to construct a DIVA vaccine for FIV, the duration of the humoral response to the mutated PID has yet to be determined. The antibody response against the LLV-E peptide decreased to low levels by the day of challenge. It will therefore be necessary to test cats that have been vaccinated with the DIVA virus, to assess the duration of the humoral antibody response recognising the DIVA epitope and to evaluate whether it will still be possible to differentiate vaccinated from infected cats 6-12 months after the final vaccination.

As the DIVA WIV used in this study did not protect cats from challenge with the virulent primary isolate GL-8, it is not known what the serological profile of DIVA vaccinated cats might have been had the vaccinated cats been protected. Therefore, it would be valuable to incorporate the DIVA mutation into the prototype FIV vaccine (WIV of FIV-Petaluma). As a WIV based on FIV-Petaluma has been shown to protect against low dose homologous and heterologous challenge (Yamamoto *et al*, 1991 and 1993), it would be possible to assess the serological profile of DIVA-vaccinated and protected cats.

The identification of potential immunogens that are capable of eliciting BnAb should be pursued further. Utilising complex gene screening assays and computational modelling, researchers are assessing HIV-1 Envs capable of eliciting BnAb in a prime boost regime by germline targeting (Jardine *et al*, 2015). Another possible approach is to utilise the FIV

model of lentivirus infection. Should FIV positive cats that have mounted a BnAb response be bred, the selected uninfected offspring should, theoretically, contain the optimal alleles required for the formation of BnAb, as well as the optimal combinations of VDJ genes required for BnAb production. Should the offspring be infected with a low dose of FIV (5 CID_{50}), it is possible that a BnAb response could be induced. By determining the structure of the Env of the circulating FIV, and comparing that with the development of BnAb, it might be possible to identify certain characteristic of the virus associated with the development of BnAb. Stocks of virus could then be expanded from selected time points (preceding the development of BnAb) and, used in a prime boost regime to test whether it is possible to elicit BnAb by vaccination.

Appendices

Appendix 2. Names, orientations and sequences of primers and probes used in this study.

PCR and sequencing primers

Primer	Forward (F) /reverse (R)	Sequence
1F4	F	5' -TGT AAT CAA CGY TTT GTK TCT CCT TAC AG- 3'
XR2	R	5' - CCT CAA AGG GAA GAA ATC AGC TCA-3'
GL8-Sal	F	5'- GGG **TCG A**CA CCA TGG CAG AAG GGT TTG CAG CA -3'
GL8-Not1	R	5'- GGG C**GG CC**G CCA TCA TTC CTC CTC TTT TT-3'
550F	F	5'-TTT TTT GGG ATT GTT GGG C-3'
7223F	F	5'- GTA CAG ACC CAT TAC AAA TCC-3'
8461R	R	5'- CCC CCA AAG TTA TAT TTT C-3'
FIV-env_890	R	5'- TCC CTT GTA ACC AAG TAT CTA CTC - 3'
BNC5 Nde1	R	5'- AAT GGA TTC A**TA TG**A CAC ATC TTC CTC-3'
BNC5 PID/Spe1	F	5'- GAG CAT CAA GTA **CTA G**TA ATA GGA TTA AAA GTA GAA GCT ATG GAA AAA TTC TTA TAT ACT GCT TTC GCT ATG CAA GAA TTG GGA TGT **AGA GAA CAA** CAA **TTT** TTC TGT AAA GTC CCT TTT-3'

Sequences highlighted in red denote restriction sites. Sequences underlined in bold highlight mutagenesis sequences.

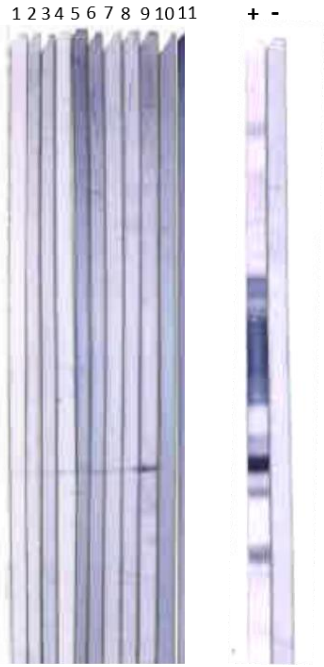
QPCR/PERT primer and probes

Primer/probe	Forward (F) /reverse (R)	Sequence
FIV-1360F	F	5'- GCA GAA GAA AGA TTT GCA CCA-3'
FIV-1437R	R	5'- TAT GGC GGC CAA TTT TCC T-3'
FIV Gag Probe	F	5' FAM- TGC CTC AAG ATA CCA TGC TCT ACA CTG CA-BHQ1-3'
rDNA 343F	F	5'- CCA TTC GAA CGT CTG CCC TA- 3'
rDNA 409R	R	5'- TCA CCC GTG GTC ACC ATG-3'
rDNA 370 Probe	F	5' FAM- CGA TGG TAG TCG CCG TGC CTA-BHQ1-3'
MS2 RNA F	F	5'-GCC TTT CTC ATT CGT TGT CG-3'
MS2 RNA R	R	5'-GCT TAT GAT GGA CTC ACC CG-3'
MS2 probe	F	5'-FAM-CCG TGG GAT GCT CCT ACA TGT CAG GA-TAM-3'

Appendix 3i. Serology of samples of a predetermined FIV sero-status.

Immunostain 1.

Sero-negative



1. 133
2. 132
3. 131
4. 127
5. 124
6. 123
7. 120
8. 116
9. 129
10. 122
11. 134

Sero-positive

1. 119

TM ELISA result

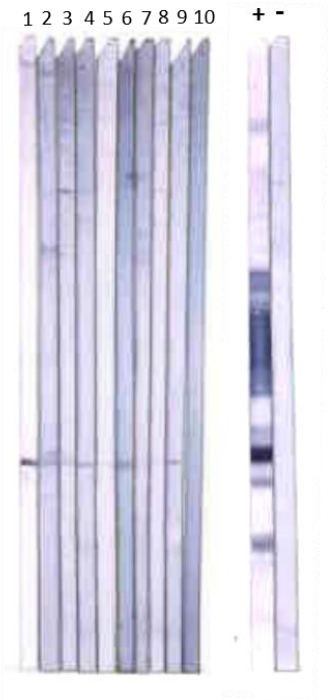
Cat I.D.	Mean O.D.	Negative fold
133	0.044	0.564
132	0.046	0.585
131	0.066	0.850
127	0.050	0.641
124	0.051	0.654
123	0.052	0.671
120	0.041	0.526
116	0.041	0.526
129	0.083	1.068
122	0.079	1.017
134	0.138	1.765
119	0.429	5.496
Positive	1.334	17.103
Negative	0.039	0.500

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

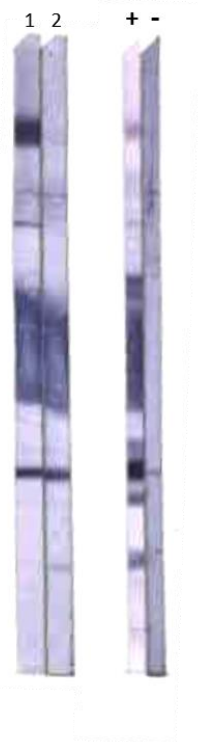
Immunostain 2.

Sero-negative



1. 114
2. 111
3. 104
4. 103
5. 101
6. 96
7. 95
8. 94
9. 93
10. 84

Sero-positive



1. 115
2. 108

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

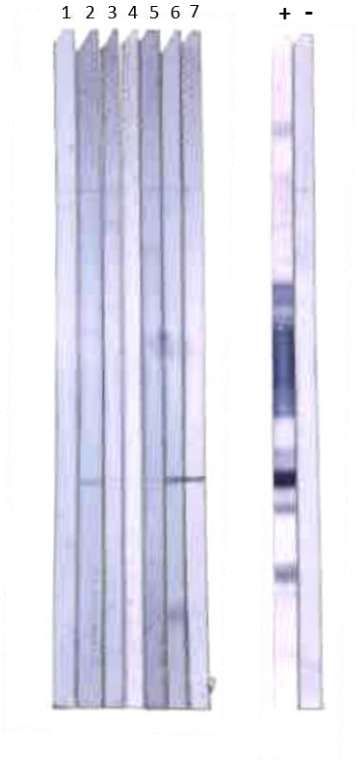
TM ELISA result

Cat I.D.	Mean O.D.	Negative fold
114	0.033	0.419
111	0.041	0.530
104	0.064	0.821
103	0.058	0.748
101	0.035	0.453
96	0.050	0.641
95	0.054	0.688
94	0.042	0.534
93	0.039	0.500
84	0.041	0.526
115	1.301	16.684
108	1.277	16.368
Positive	1.425	18.274
Negative	0.039	0.504

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 3.

Sero-negative



1. 83
2. 80
3. 78
4. 62
5. 61
6. 77
7. 64

Sero-positive

1. 79
2. 72
3. 71
4. 57

TM ELISA result

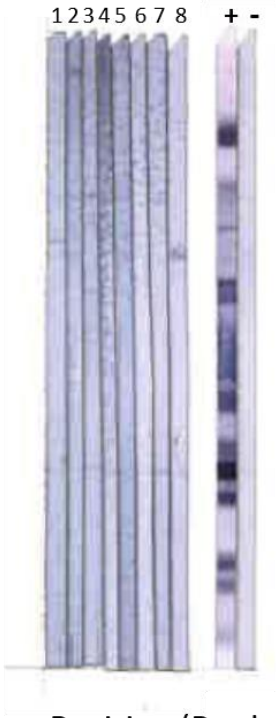
Cat I.D.	Mean O.D.	Negative fold
83	0.034	0.425
80	0.041	0.517
78	0.043	0.538
62	0.045	0.563
61	0.036	0.446
77	0.106	1.329
64	0.083	1.033
79	1.412	17.646
72	1.513	18.913
71	1.515	18.938
57	1.392	17.400
Positive	1.359	16.992
Negative	0.040	0.504

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 4.

Sero-negative



1. 55
2. 54
3. 50
4. 49
5. 47
6. 46
7. 30
8. 74

Sero-positive

1. 35
2. 14

TM ELISA result

Cat I.D.	Mean O.D.	Negative fold
55	0.050	0.641
54	0.058	0.739
52*	0.056	0.714
50	0.038	0.487
49	0.050	0.637
47	0.053	0.679
46	0.067	0.855
30	0.043	0.551
74	0.042	0.534
51**	1.359	17.427
35	1.266	16.231
14	1.408	18.056
Positive	1.288	16.509
Negative	0.039	0.496

*Blotted but not shown

** Not blotted. Insufficient material

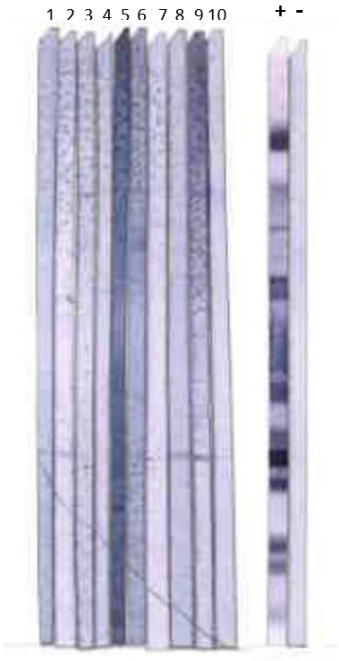
+ Positive (Pooled anti-GL8 plasma)

- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 5.

Sero-negative



1. 21
2. 26
3. 33
4. 11
5. 12
6. 16
7. 17
8. 367607
9. 367626
10. 367595

Sero-positive

1. 25
2. 367517

TM ELISA result

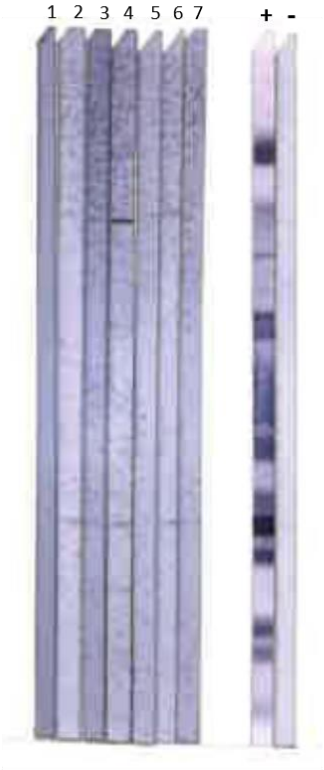
Cat I.D.	Mean O.D.	Negative fold
21	0.042	0.423
26	0.049	0.487
33	0.075	0.747
11	0.044	0.437
12	0.083	0.833
16	0.082	0.820
17	0.060	0.597
367607	0.039	0.387
367626	0.067	0.670
367595	0.034	0.340
25	1.564	15.643
367517	1.190	11.897
Positive	1.497	14.973
Negative	0.050	0.500

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

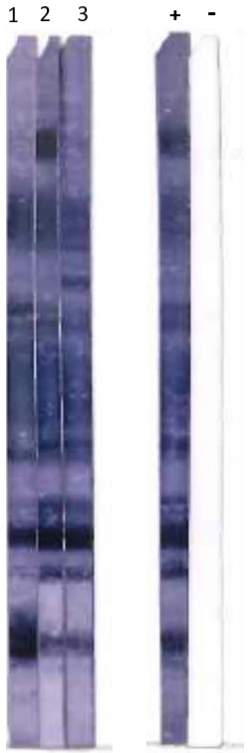
Immunostain 6.

Sero-negative



1. 66
2. 142
3. 143
4. 153
5. 155
6. 156
7. 157

Sero-positive



1. 42
2. 141
3. 146

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

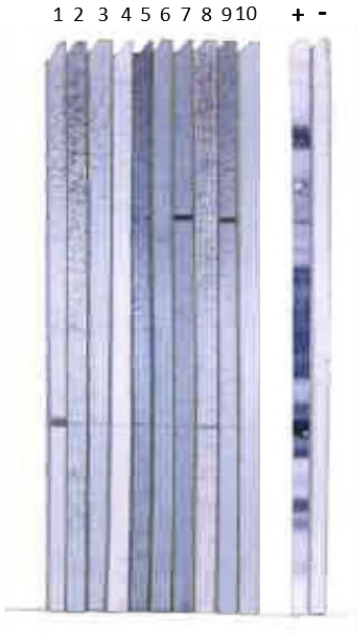
TM ELISA result

Cat I.D.	Mean O.D.	Negative fold
66	0.036	0.383
142	0.034	0.365
149	0.050	0.528
153	0.045	0.475
155	0.052	0.550
156	0.047	0.504
157	0.054	0.574
42	0.475	5.050
141	0.558	5.936
146	1.401	14.904
Positive	2.007	21.348
Negative	0.047	0.496

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 7.

Sero-negative



1. 185
2. 154
3. 180
4. 178
5. 176
6. 173
7. 172
8. 171
9. 168
10. 179

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Sero-positive

1. 183
2. 175

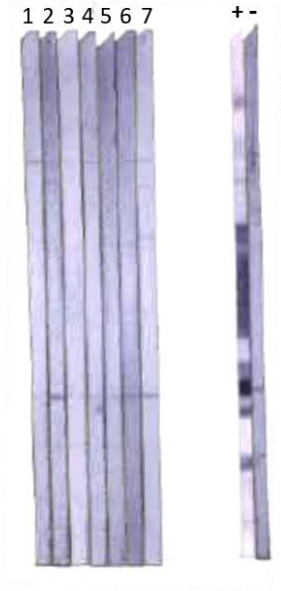
TM ELISA result

Cat I.D.	Mean O.D.	Negative fold
185	0.047	0.569
154	0.054	0.663
180	0.078	0.955
178	0.032	0.390
176	0.049	0.602
173	0.039	0.476
172	0.040	0.488
171	0.038	0.467
168	0.053	0.650
179	0.109	1.329
183	0.552	6.728
175	1.604	19.557
Positive	1.660	20.248
Negative	0.041	0.500

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive.

Immunostain 8.

Sero-negative



1. 147
2. 166
3. 159
4. 158
5. 320544
6. 230560
7. 320571

Sero-positive

1. 160
2. 182942
3. 182455
4. 182052
5. 182461

TM ELISA result

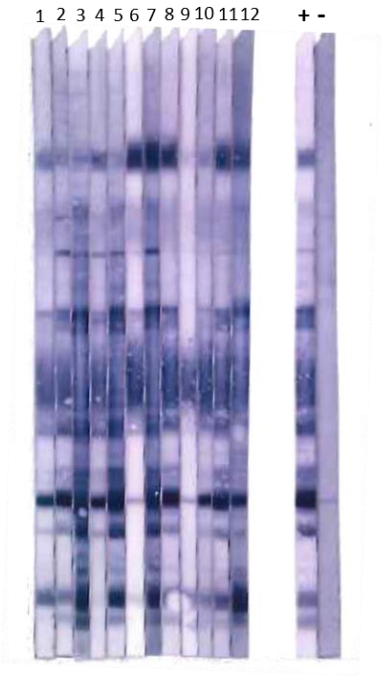
Cat I.D.	Mean O.D	Negative fold
147	0.044	0.516
166	0.034	0.399
159	0.041	0.477
158	0.039	0.453
320544	0.057	0.659
230560	0.044	0.516
320571	0.040	0.469
160	1.749	20.333
182942	1.660	19.298
182455	1.449	16.845
183053	1.757	20.434
182461	1.707	19.853
Positive	1.682	19.558
Negative	0.043	0.504

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 9.

Sero-positive



1. 182246
2. 182312
3. 180914
4. 182304
5. 180949
6. 182189
7. 181143
8. 182961
9. 184439
10. 181294
11. 181259
12. 367516

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

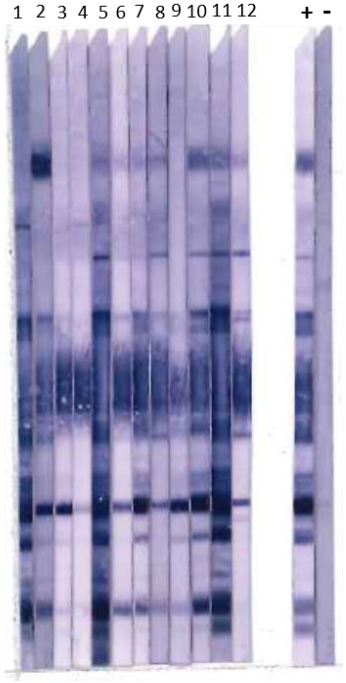
TM ELISA result

Cat I.D.	Mean O.D.	Negative fold
182246	1.533	15.029
182312	1.569	15.379
180914	1.565	15.340
182304	1.452	14.239
180949	1.579	15.484
182189	1.566	15.350
181143	1.611	15.791
180961	1.446	14.180
184439	0.789	7.739
181294	1.164	11.412
181259	1.440	14.118
367516	1.548	15.173
Positive	1.516	14.863
Negative	0.051	0.500

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 10.

Sero-positive



1. 180911
2. 186966
3. 182249
4. 182285
5. 181218
6. 181948
7. 181256
8. 181215
9. 181091
10. 181146
11. 182193
12. 186496

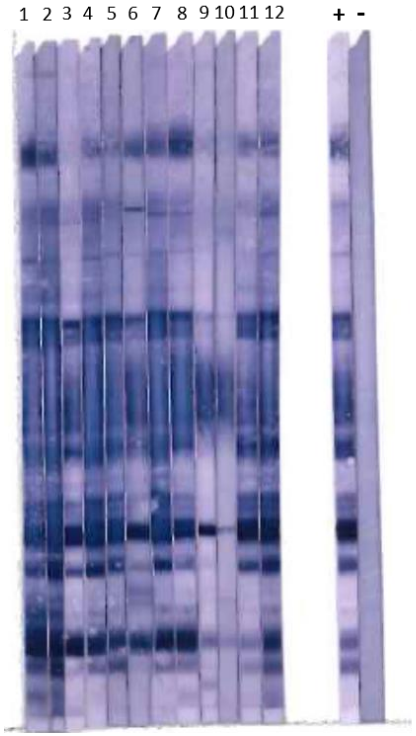
+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

TM ELISA result

Cat I.D.	Mean O.D.	Negative fold
180911	1.625	13.102
186966	1.428	11.519
182249	1.594	12.855
182285	1.213	9.785
181218	1.555	12.543
181948	0.945	7.621
181256	1.523	12.282
181215	1.632	13.164
181091	0.699	8.520
181146	1.553	12.524
182193	1.297	10.460
186496	1.596	12.868
Positive	1.734	13.984
Negative	0.062	0.497

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 11.



1. 179114
2. 179105
3. 179181
4. 179176
5. 179120
6. 179118
7. 179117
8. 179297
9. 179288
10. 179200
11. 179323
12. 179369

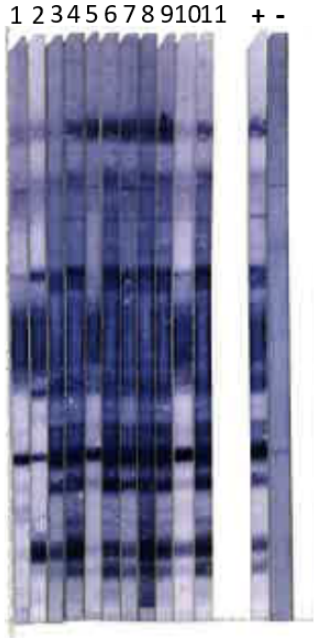
Cat I.D.	Mean O.D.	Negative fold
179114	1.048	17.467
179105	0.982	16.361
179181	0.961	16.022
179176	1.061	17.689
179120	0.749	12.489
179118	0.450	7.506
179117	0.687	11.450
179297	1.076	17.939
179288	0.731	12.183
179200	0.487	8.117
179323	0.737	12.278
179369	0.867	14.444
Positive	0.943	15.717
Negative	0.030	0.500

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 12.

Sero-positive



1. 179898
2. 179876
3. 179195
4. 179837
5. 179845
6. 179850
7. 179582
8. 179638
9. 179550
10. 179466
11. 179481

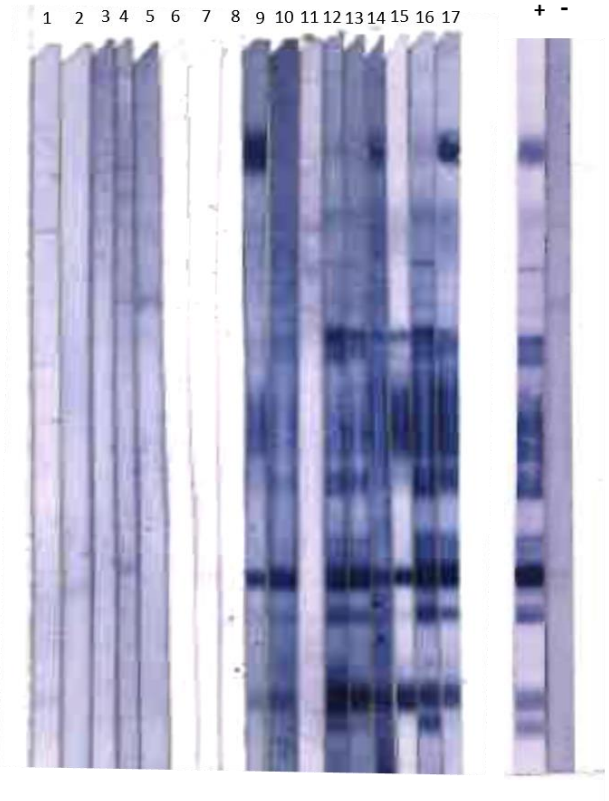
+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

TM ELISA result

Cat I.D.	Mean O.D	Negative fold
179898	0.507	7.687
179876	0.995	15.081
179195	0.967	14.657
179837	0.726	10.995
179845	0.868	13.157
179850	0.679	10.288
179582	0.979	14.828
179638	0.822	12.455
179550	0.856	12.970
179466	0.798	12.091
179481	0.805	12.197
179562	0.031	0.470
Cat +	0.954	14.455
Cat -	0.033	0.495

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 13.



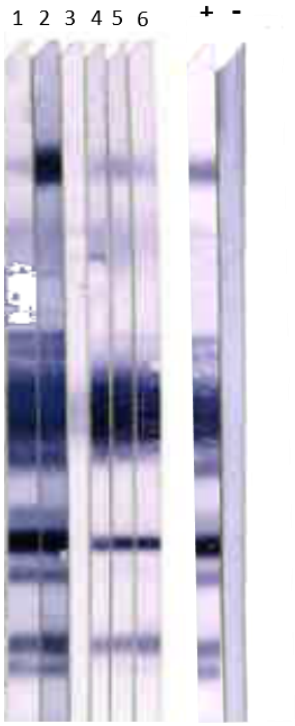
1. 378633
2. 377299
3. 378305
4. 378352
5. 378358
6. 378644
7. 367645
8. 378188
9. 378253
10. 378262
11. 378263
12. 378264
13. 378282
14. 378291
15. 378385
16. 378477
17. 378479

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Cat I.D.	Mean O.D.	Negative fold
378633	0.039	0.552
377299	0.048	0.686
378305	0.045	0.648
378352	0.050	0.719
378358	0.075	1.067
367644	0.028	0.519
367645	0.032	0.586
378188	0.035	0.642
378253	0.208	3.846
378262	0.496	5.764
378263	0.050	0.920
378264	0.442	8.185
378282	0.248	4.586
378291	0.665	9.505
378385	1.004	14.338
378477	1.439	20.562
378479	1.326	18.938
Positive	1.178	16.829
Negative	0.035	0.500

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 14.



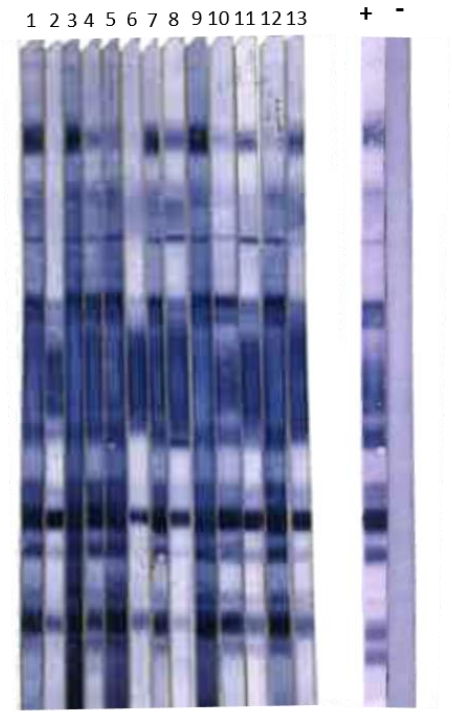
1. 183039
2. 182421
3. 182631
4. 182337
5. 182313
6. 182387

Cat I.D.	Mean O.D.	Negative fold
183039	0.647	7.519
182421	0.491	5.705
182631	0.041	0.481
182337	0.452	5.256
182313	0.690	8.027
182387	0.561	6.519
Positive	0.604	7.023
Negative	0.043	0.504

\+ Positive (Pooled anti-GL8 plasma)
\- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 15.



+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

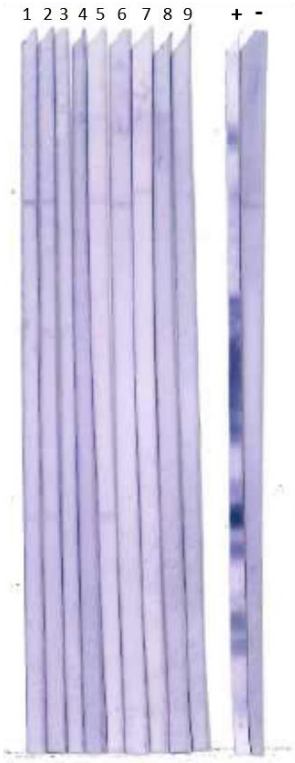
1. 180724
2. 180652
3. 180795
4. 180801
5. 180800
6. 180659
7. 180647
8. 180644
9. 180638
10. 180389
11. 180536
12. CPG-41 (experimental plasma)
13. 180498

Cat I.D.	Mean O.D.	Negative fold
180724	1.675	18.203
180652	1.686	18.330
180795	1.642	17.844
180801	1.745	18.964
180800	1.564	16.996
180659	1.552	16.866
180647	1.647	17.906
180644	1.682	18.286
180638	1.586	17.243
180389	1.400	15.214
180536	1.552	16.866
CPG41	1.698	18.460
180498	1.673	18.181
Positive	1.599	17.377
Negative	0.046	0.500

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Appendix 3ii. Serology of samples with an inconclusive FIV sero-status.

Immunostain 1.



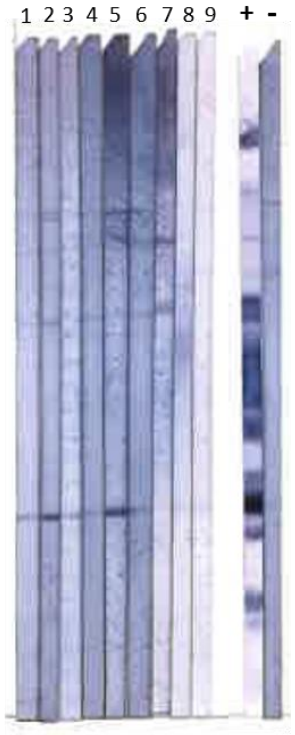
1. 363027
2. 347317
3. 347445
4. 347226
5. 347132
6. 346532
7. 347140
8. 346816
9. 346793

Cat I.D.	Mean O.D.	Negative fold
363027	0.055	0.565
347317	0.060	0.616
347445	0.052	0.527
347226	0.056	0.568
347132	0.057	0.585
346532	0.060	0.616
347140	0.041	0.415
346816	0.104	1.061
346793	0.056	0.575
Positive	1.978	20.180
Negative	0.049	0.500

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 2.



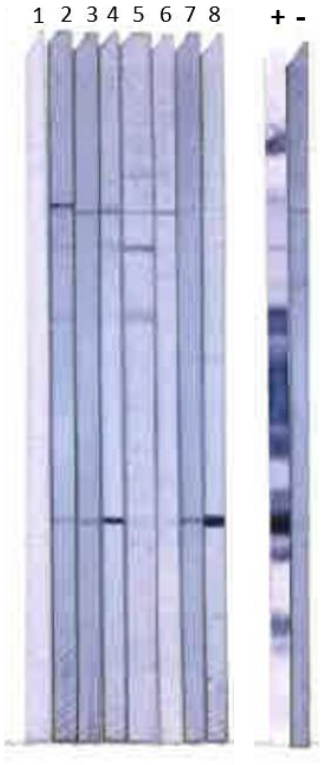
1. 345089
2. 346405
3. 346097
4. 363779
5. 364503
6. 364144
7. 364376
8. 346495
9. 346444

Cat I.D.	Mean O.D.	Negative fold
345089	0.096	1.067
346405	0.074	0.819
346097	0.056	0.619
363779	0.092	1.022
364503	0.092	1.022
364144	0.072	0.796
364376	0.058	0.648
346495	0.034	0.374
346444	0.043	0.478
Positive	2.009	22.322
Negative	0.045	0.504

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive.

Immunostain 3.



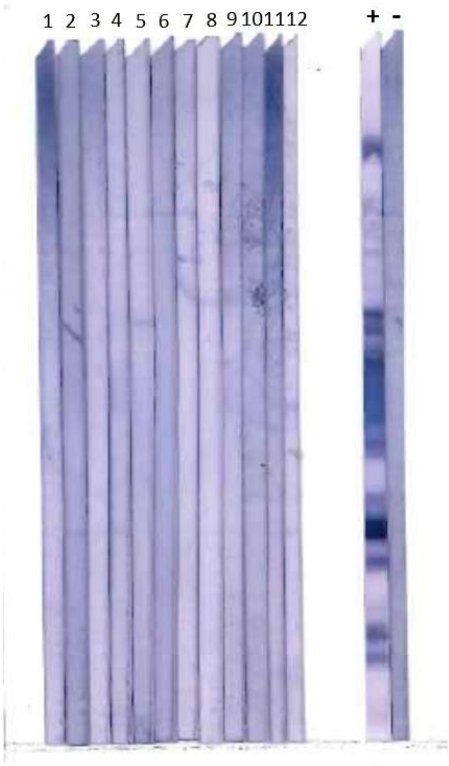
1. 346440
2. 345547
3. 345855
4. 347307
5. 364094
6. 346533
7. 346515
8. 346170

Cat I.D.	Mean O.D.	Negative fold
346440	0.052	0.634
345547	0.085	1.033
345855	0.059	0.715
347307	0.052	0.634
364094	0.062	0.752
346533	0.055	0.667
346515	0.093	1.134
346170	0.074	0.907
Positive	2.032	24.776
Negative	0.041	0.504

\+ Positive (Pooled anti-GL8 plasma)
\- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 4.



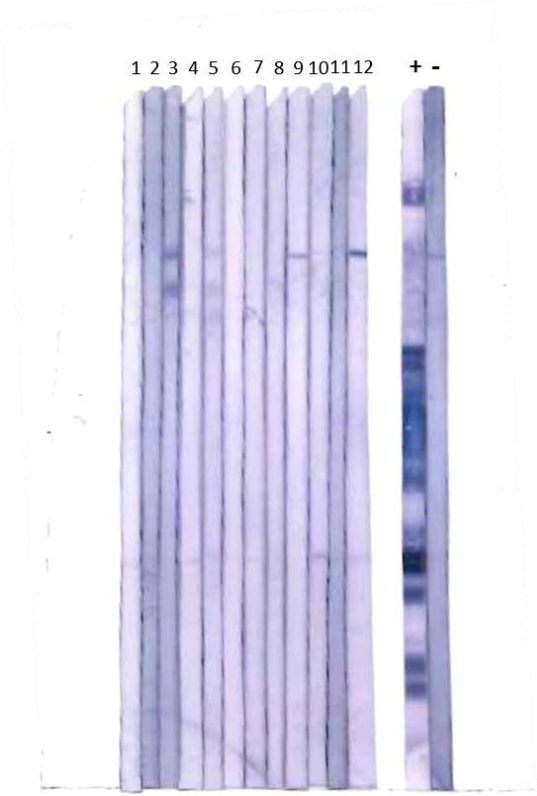
1. 346228
2. 346594
3. 363764
4. 346639
5. 346596
6. 346595
7. 363127
8. 363038
9. 346577
10. 363703
11. 346368
12. 346572

Cat I.D.	Mean O.D.	Negative fold
346228	0.063	0.712
346594	0.063	0.712
363764	0.047	0.538
346639	0.065	0.735
346596	0.084	0.955
346595	0.089	1.008
363127	0.055	0.629
363038	0.043	0.485
346577	0.056	0.636
363703	0.086	0.981
346368	0.056	0.636
346572	0.051	0.583
Positive	1.995	22.667
Negative	0.044	0.500

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 5.



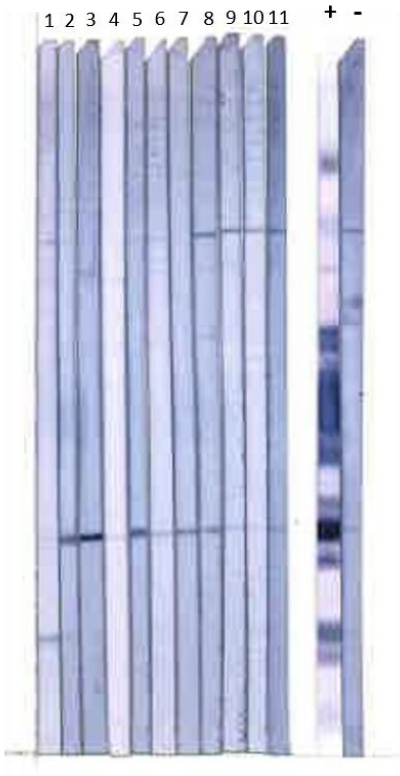
+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

1. 346573
2. 346933
3. 346909
4. 346823
5. 346736
6. 347257
7. 347467
8. 347644
9. 347721
10. 347177
11. 347398
12. 347573

Cat I.D.	Mean O.D.	Negative fold
346573	0.048	0.456
346933	0.096	0.909
346909	0.049	0.459
346823	0.037	0.349
346736	0.059	0.560
347257	0.050	0.469
347467	0.046	0.437
347644	0.073	0.692
347721	0.087	0.821
347177	0.114	1.079
347398	0.131	1.239
347573	0.127	1.195
Positive	1.752	16.528
Negative	0.053	0.500

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 6.



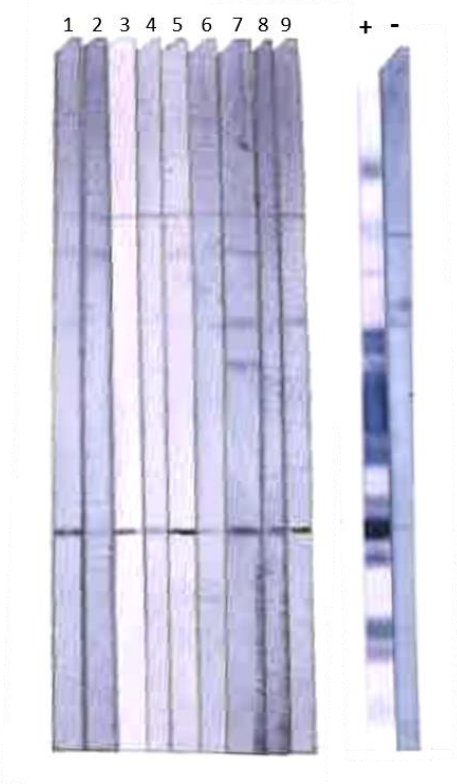
1. 347447
2. 347621
3. 347761
4. 347929
5. 347309
6. 347464
7. 346700
8. 345182
9. 346538
10. 346262
11. 347731

Cat I.D.	Mean O.D.	Negative fold
347447	0.061	0.545
347621	0.080	0.717
347761	0.086	0.771
347929	0.043	0.384
347309	0.044	0.396
347464	0.069	0.613
346700	0.047	0.420
345182	0.059	0.527
346538	0.081	0.720
346262	0.064	0.574
347731	0.181	1.619
Positive	1.856	16.571
Negative	0.056	0.500

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 7.



+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

1. 363782
2. 346132
3. 364790
4. 345879
5. 347760
6. 347074
7. 364888
8. 364869
9. 364867

Cat I.D.	Mean O.D.	Negative fold
363782	0.090	0.941
346132	0.071	0.736
364790	0.044	0.462
345879	0.069	0.722
347760	0.070	0.733
347074	0.107	1.115
364888	0.159	1.660
364869	0.146	1.517
364867	0.151	1.569
Positive	1.843	19.201
Negative	0.048	0.503

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 8.

1. 346639
2. 364988
3. 365011
4. 365081
5. 364945
6. 365083
7. 365080

Cat I.D.	Mean O.D.	Negative fold
346639	0.065	0.577
364988	0.085	0.756
365011	0.046	0.408
365081	0.060	0.539
364945	0.061	0.548
365083	0.080	0.714
365080	0.051	0.455
Positive	1.689	15.083
Negative	0.056	0.500

+ Positive (Pooled anti-GL8 plasma)
- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 9.

1. 378601
2. 378747
3. 378475

Cat I.D.	Mean O.D.	Negative fold
378601	0.031	0.448
378474	0.050	0.714
378475	0.050	0.710
Positive	1.178	16.829
Negative	0.035	0.500

\+ Positive (Pooled anti-GL8 plasma)
\- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive

Immunostain 10.

1. 367445
2. 367547
3. 378531

Cat I.D.	Mean O.D.	Negative fold
367445	0.033	0.611
367547	0.028	0.525
378531	0.028	0.525
Positive	1.178	21.815
Negative	0.027	0.500

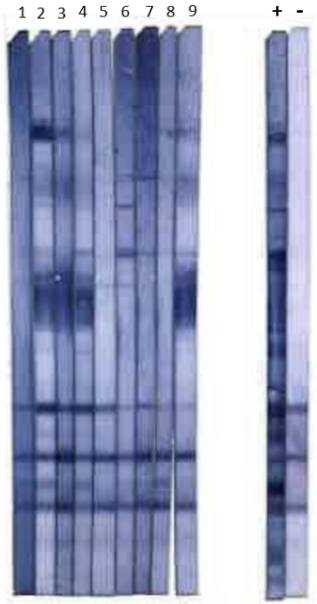
\+ Positive (Pooled anti-GL8 plasma)
\- Negative (SPF plasma)

Green = Sero-negative, Yellow= inconclusive, Red = Sero-positive.

Immunostains 11-13

A PDVF membrane containing reduced and disrupted FIV virus (FIV ΔV2). The membrane was blocked in 2% MPBST and probed with antibody diluted 1/200 in 2% MPBST.

A PDVF membrane containing reduced and disrupted FIV virus (FIV ΔV2). The membrane was blocked in 25% Foetal bovine serum (diluted in deionised water) and probed with antibody diluted 1/200 in 25% foetal bovine serum (diluted in deionised water).



A PDVF membrane containing reduced and disrupted FIV virus (FIV ΔV2). The membrane was blocked in 25% Foetal bovine serum (diluted in deionised water) and probed with antibody diluted 1/200 in X1 casein (diluted in deionised water).

1. 346430
2. 345592
3. 364637
4. 346406
5. 364982
6. 364989
7. 347930
8. 346657
9. 345387

Immunostains 14-16.

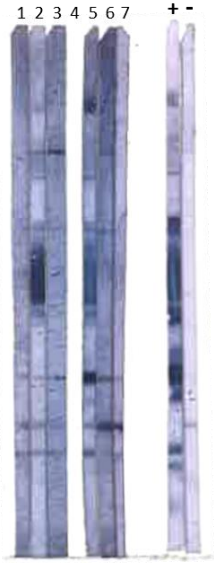
A.

1. 346430
2. 345592
3. 346657
4. 363353
5. 346406
6. 364982
7. 364989
8. 347930
9. 364637

B.

1. 364637
2. 363353
3. 364982
4. 347930
5. 346657
6. 345387

C.



1. 364637
2. 363353
3. 364982
4. 347930
5. 346657
6. 345387
7. 364503*

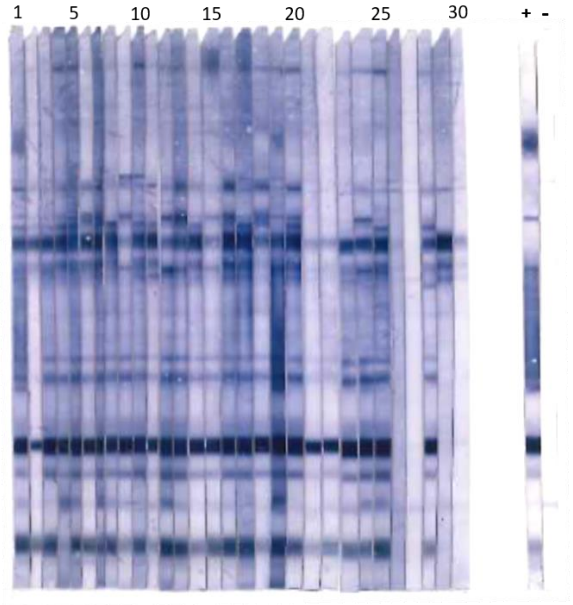
All membranes contain reduced and disrupted FIV virus (FIV ΔV2). All membranes were blocked with X 1 casein solution and probed with antibody diluted to 1/200 in X 1 casein solution unless other wise stated. A, the membrane strip was process as described in materials and methods. **B**, Samples not giving a definitive immunoblot result were re-examined with plasma/sera diluted to 1/600 in X1 casein solution and processed as described in materials and methods. **C**, Membrane strips were incubated with sample plasma/sera over night at 4^{o}C overnight whilst being agitated at 40rpm. Samples were then processed as described in materials and methods. *364503 and 364637 are sequential samples from the same cat respectively.

		VDS immunoblot				RRL immunoblot[#]							
Identification number	**IFA titre**	**p24**	**p17**	**p24 Ag ELISA**	**RT activity (PERT)**	**SU**	**p55**	**TM**	**p24**	**p17**	**p10**	**Negative fold (TM ELISA)**	**FIV status Positive/Negative**
364503[1]	0	+/-	-	-	>40	-	-	-	+	-	+/-	1.022	N
364637[2]	0	+/-	-	-	>40	-	-	+	+	+	+/-	3.625	P
345387	10^3	+	-	+	25.094	+	+	+	+	-	+	17.826	P
345592	10^4	+	-	+	36.259	+	+	+	+	+	+	22.337	P
346430	10^4	+	-	+	36.942	+	+	+	+	-	-	20.799	P
346406	10^4	+	-	+	26.541	+	+	+	+	+	+	11.782	P
346657	10^3	+	-	+	19.476	+	+	+	+	+	+	4.306	P
347930	0	+	-	-	38.468*	-	+	+/-	+	-	+	6.544	P
364982	10^2	+/-	+/-	+	25.612	-	+	+	+	-	+	6.423	P
364989	10^4	+/-	-	+	27.733	+	+	+	+	-	+	15.923	P
363353	0	+	-	n/a	n/a	+	-	+	+	-	+	2.997	P

Tabulation of all IFA inconclusive, TM ELISA positive samples. Sample 364503 is included in the tabulation as this the first or 2 sequential samples taken from this animal. The cat was re-sampled just 6 days later (364637) and clearly shows a different anti-FIV serology profile. Out the 10 cats represented by the 11 samples, IFA was unable to detect antibodies in 3/10 of these samples. Although 2 of these cats gave an inconclusive result by TM ELISA, immunostain analysis clearly shows specific FIV antibodies are present in the sera of these cats. Although the remaining 7 IFA positive cats gave high titres of FIV antibodies, these results could not be reproduced by VDS immunostain and thus were classed as inconclusive. **Green= negative result, Yellow = inconclusive result, Pink= positive result. + = present, - = not present, +/- = very weak presence/not specific, *= RT activity detected in 1 well of the triplicate, # = Tabulated immunostain data from several screenings.**

Appendix 3iii. Serology of the Australian and American samples.

Immunostain 1.

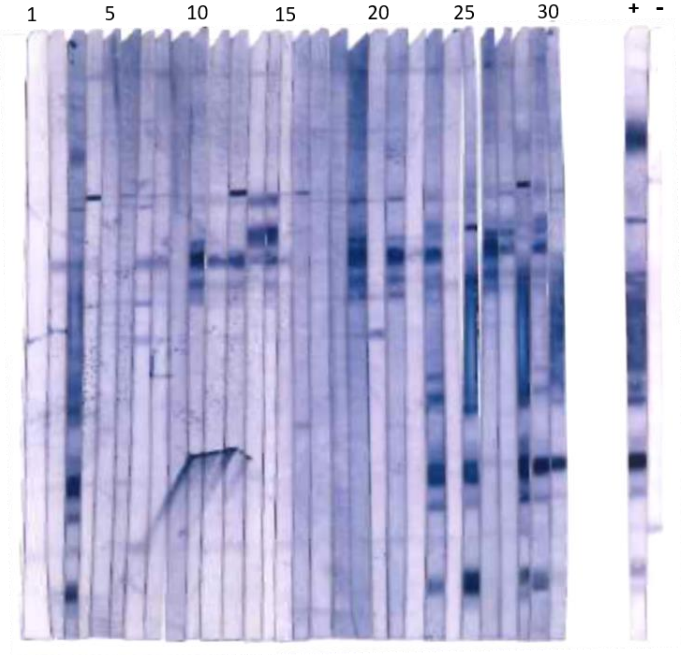


+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-1	0.920	11.500
2	FIV-2	0.044	0.554
3	FIV-3	0.419	5.242
4	FIV-4	0.129	1.617
5	FIV-5	0.334	4.179
6	FIV-6	0.087	1.083
7	FIV-7	0.553	6.908
8	FIV-8	0.128	1.596
9	FIV-9	0.133	1.663
10	FIV-10	0.059	0.738
11	FIV-11	0.099	1.242
12	FIV-12	0.154	1.925
13	FIV-13	0.159	1.988
14	FIV-14	0.128	1.604
15	FIV-15	0.268	3.346
16	FIV-16	0.162	2.021
17	FIV-17	0.429	5.367
18	FIV-18	0.075	0.942
19	FIV-19	1.007	12.592
20	FIV-20	0.363	4.542
21	FIV-21	0.068	0.850
22	FIV-22	0.157	1.963
23	FIV-23	0.329	4.113
24	FIV-24	0.179	2.238
25	FIV-25	0.126	1.571
26	FIV-26	0.072	0.896
27	FIV-27	0.039	0.488
28	FIV-28	0.109	1.358
29	FIV-29	0.048	0.604
30	FIV-30	0.045	0.563
+	POSITIVE	1.084	13.550
-	NEGATIVE	0.040	0.500

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Immunostain 2.

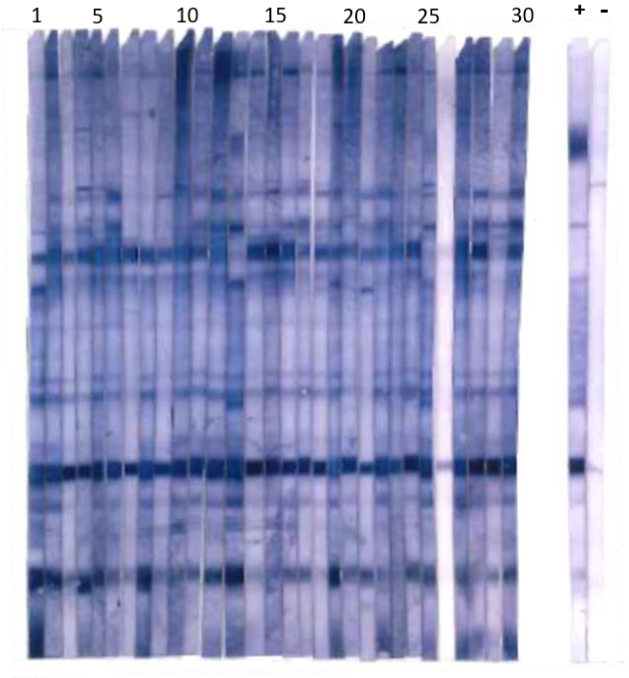


+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-31	0.106	1.078
2	FIV-32	0.067	0.687
3	FIV-33	1.250	12.759
4	FIV-34	0.102	1.044
5	FIV-35	0.087	0.888
6	FIV-36	0.099	1.014
7	FIV-37	0.077	0.789
8	FIV-38	0.146	1.486
9	FIV-39	0.084	0.857
10	FIV-40	0.070	0.714
11	FIV-41	0.069	0.701
12	FIV-42	0.061	0.626
13	FIV-43	0.041	0.418
14	FIV-44	0.056	0.575
15	FIV-45	0.055	0.561
16	FIV-46	0.073	0.741
17	FIV-47	0.078	0.799
18	FIV-48	0.068	0.690
19	FIV-53	0.120	1.224
20	FIV-54	0.052	0.531
21	FIV-56	0.078	0.799
22	FIV-57	0.053	0.537
23	FIV-59	0.345	3.520
24	FIV-61	0.064	0.656
25	FIV-62	0.870	12.088
26	FIV-65	0.090	0.915
27	FIV-71	0.064	0.656
28	FIV-80	0.988	13.727
29	FIV-84	0.113	1.156
30	FIV-85	0.654	9.088
+	POSITIVE	1.158	11.813
-	NEGATIVE	0.049	0.497

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Immunostain 3.

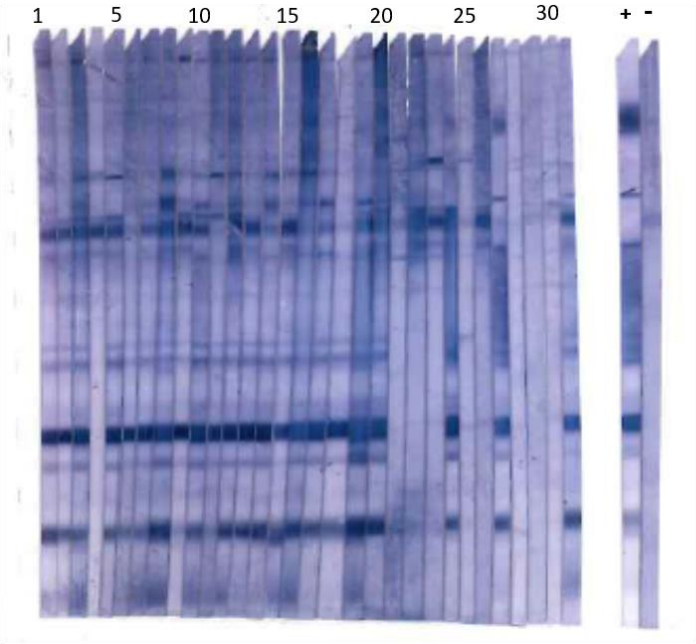


+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-92	0.945	13.120
2	FIV-93	0.240	2.923
3	FIV-95	0.076	0.931
4	FIV-97	0.526	6.415
5	FIV-98	0.243	2.959
6	FIV-99	0.202	2.467
7	FIV-100	0.101	1.232
8	FIV-101	0.465	5.675
9	FIV-102	0.065	0.789
10	FIV-103	0.237	2.890
11	FIV-104	0.069	0.837
12	FIV-105	0.345	4.211
13	FIV-106	1.112	13.565
14	FIV-107	0.282	3.443
15	FIV-108	0.115	1.407
16	FIV-109	0.411	5.016
17	FIV-110	0.222	2.707
18	FIV-116	0.095	1.154
19	FIV-117	0.393	4.789
20	FIV-118	0.148	1.809
21	FIV-119	0.060	0.728
22	FIV-120	0.195	2.382
23	FIV-121	0.361	4.398
24	FIV-122	0.467	5.695
25	FIV-123	0.080	0.972
26	FIV-127	0.846	11.750
27	FIV-131	0.128	1.565
28	FIV-132	0.093	1.138
29	FIV-133	0.106	1.293
30	FIV-96	0.186	2.268
+	POSITIVE	1.193	14.553
-	NEGATIVE	0.041	0.496

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Immunostain 4.

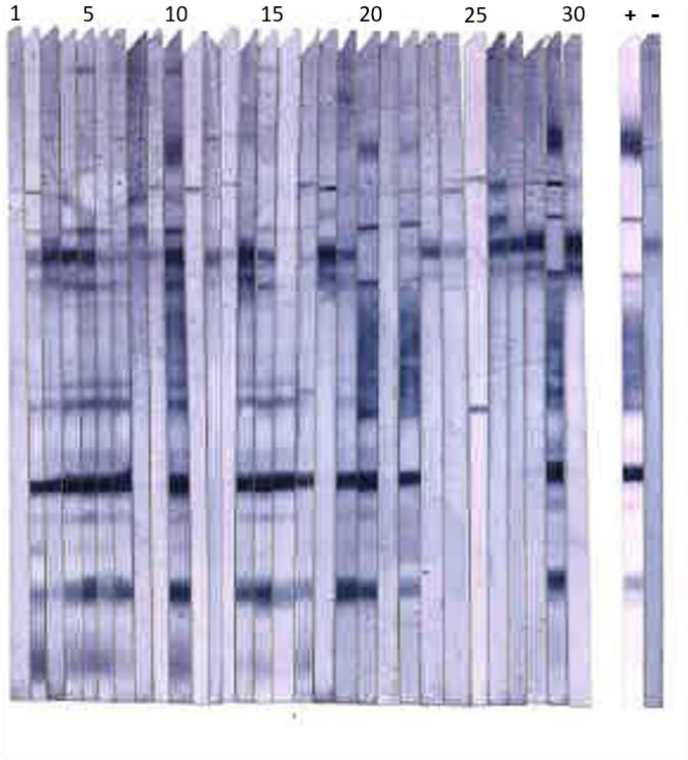


+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-134	0.121	1.640
2	FIV-135	0.106	1.437
3	FIV-136	0.090	1.216
4	FIV-137	N/A	N/A
5	FIV-138	0.075	1.009
6	FIV-139	0.107	1.441
7	FIV-140	0.125	1.685
8	FIV-141	0.252	3.410
9	FIV-142	0.081	1.095
10	FIV-143	0.143	1.937
11	FIV-144	0.091	1.225
12	FIV-145	0.280	3.788
13	FIV-146	0.053	0.716
14	FIV-147	0.295	3.982
15	FIV-148	0.126	1.698
16	FIV-149	0.208	2.811
17	FIV-150	0.064	0.860
18	FIV-151	0.083	1.122
19	FIV-152	1.021	13.797
20	FIV-153	0.074	1.000
21	FIV-154	0.055	0.748
22	FIV-155	0.051	0.694
23	FIV-156	0.042	0.568
24	FIV-157	0.999	13.875
25	FIV-159	0.074	1.000
26	FIV-160	0.078	1.054
27	FIV-163	0.991	13.764
28	FIV-165	0.040	0.545
29	FIV-166	0.051	0.685
30	FIV-167	0.047	0.631
31	FIV-168	0.086	1.158
+	POSITIVE	1.094	14.779
-	NEGATIVE	0.037	0.505

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Immunostain 5.



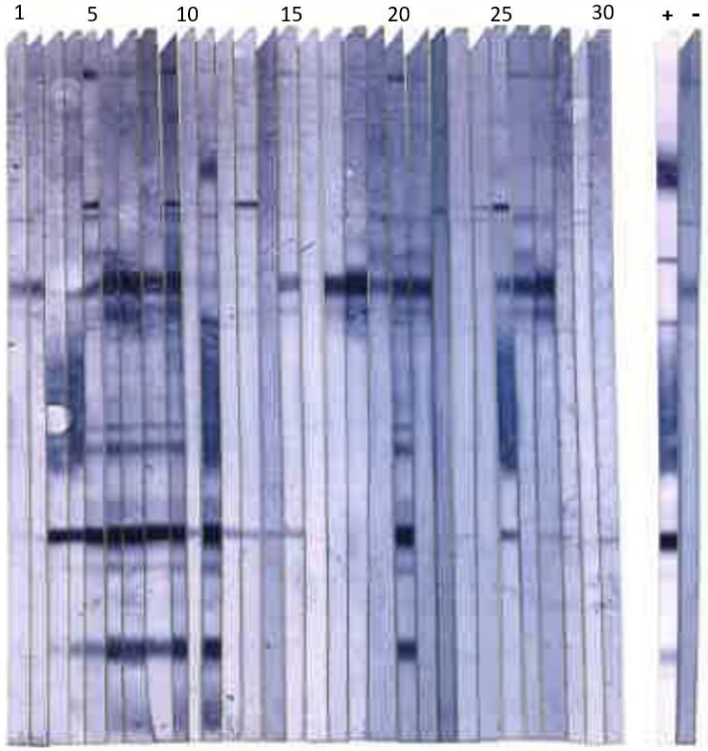
+ . Pooled anti-FL4 plasma

- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-169	0.107	1.185
2	FIV-170	0.138	1.533
3	FIV-171	0.093	1.030
4	FIV-172	0.310	3.441
5	FIV-173	0.343	3.811
6	FIV-174	0.175	1.941
7	FIV-175	0.155	1.726
8	FIV-177	0.095	1.059
9	FIV-178	0.091	1.011
10	FIV-179	0.253	2.807
11	FIV-180	0.045	0.504
12	FIV-181	0.061	0.678
13	FIV-182	0.040	0.441
14	FIV-183	0.066	0.733
15	FIV-184	0.234	2.600
16	FIV-185	0.066	0.730
17	FIV-186	0.059	0.652
18	FIV-188	0.045	0.500
19	FIV-189	0.059	0.652
20	FIV-190	1.012	14.056
21	FIV-191	0.057	0.637
22	FIV-192	1.014	14.079
23	FIV-193	0.050	0.556
24	FIV-196	0.053	0.589
25	FIV-198	0.049	0.548
26	FIV-199	0.101	1.126
27	FIV-200	0.073	0.811
28	FIV-201	0.065	0.719
29	FIV-203	1.105	15.352
30	FIV-204	0.053	0.589
+	POSITIVE	1.148	12.752
-	NEGATIVE	0.045	0.496

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Immunostain 6.



+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-205	0.061	0.740
2	FIV-206	0.073	0.894
3	FIV-208	0.832	10.671
4	FIV-209	0.938	12.030
5	FIV-212	0.072	0.882
6	FIV-213	0.273	3.333
7	FIV-214	0.122	1.492
8	FIV-215	0.159	1.935
9	FIV-216	0.224	2.728
10	FIV-217	0.049	0.602
11	FIV-218	1.009	12.932
12	FIV-219	0.065	0.793
13	FIV-220	0.062	0.752
14	FIV-225	0.078	0.951
15	FIV-230	0.049	0.602
16	FIV-231	0.050	0.610
17	FIV-232	0.052	0.634
18	FIV-233	0.055	0.667
19	FIV-235	0.082	0.996
20	FIV-236	0.115	1.407
21	FIV-242	0.057	0.699
22	FIV-243	0.053	0.650
23	FIV-244	0.059	0.715
24	FIV-245	0.166	2.028
25	FIV-250	0.964	12.359
26	FIV-259	0.045	0.553
27	FIV-260	0.050	0.614
28	FIV-261	0.061	0.748
29	FIV-262	0.035	0.427
30	FIV-263	0.054	0.663
+	POSITIVE	1.153	14.057
-	NEGATIVE	0.041	0.496

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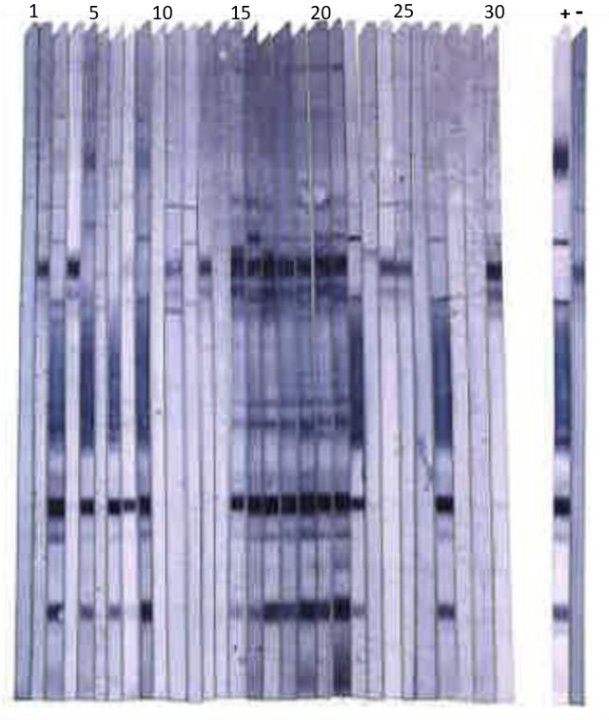
Immunostain 7.

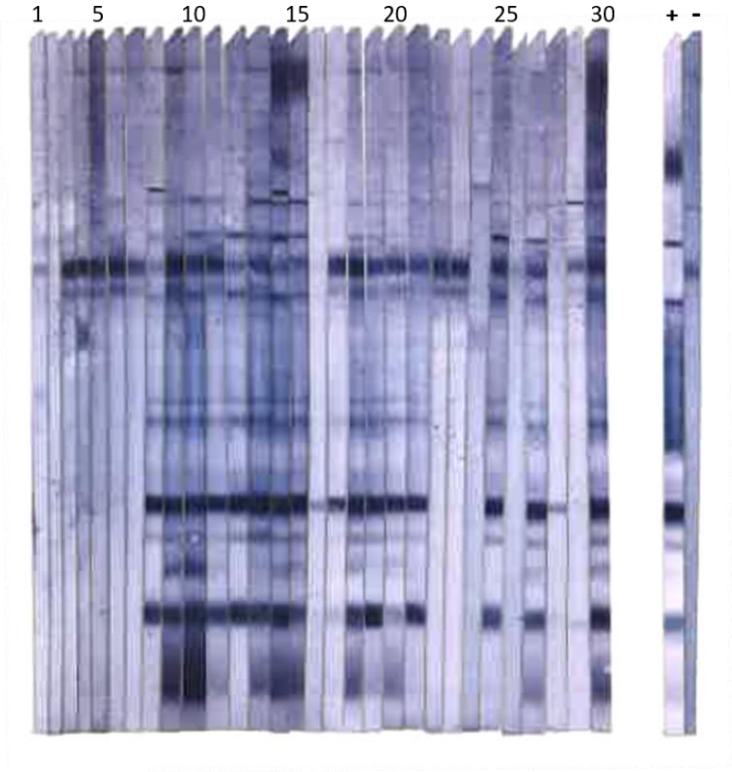


+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-265	0.054	0.543
2	FIV-271	0.039	0.393
3	FIV-272	0.191	2.444
4	FIV-273	0.034	0.340
5	FIV-274	0.588	7.534
6	FIV-277	0.038	0.380
7	FIV-279	0.827	10.598
8	FIV-280	0.038	0.383
9	FIV-281	0.978	12.543
10	FIV-283	0.038	0.377
11	FIV-284	0.041	0.410
12	FIV-285	0.045	0.453
13	FIV-291	0.043	0.427
14	FIV-295	0.038	0.377
15	FIV-296	0.054	0.537
16	FIV-297	0.228	2.277
17	FIV-298	0.061	0.613
18	FIV-299	0.152	1.517
19	FIV-300	0.112	1.123
20	FIV-301	0.074	0.737
21	FIV-302	0.130	1.297
22	FIV-303	0.898	11.513
23	FIV-304	0.052	0.517
24	FIV-309	0.041	0.407
25	FIV-314	0.062	0.623
26	FIV-316	0.036	0.363
27	FIV-319	0.888	11.389
28	FIV-320	0.053	0.530
29	FIV-321	0.049	0.493
30	FIV-322	0.038	0.377
+	POSITIVE	0.924	9.243
-	NEGATIVE	0.050	0.500

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Immunostain 8.



+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-323	0.044	0.437
2	FIV-324	0.050	0.500
3	FIV-325	0.042	0.417
4	FIV-326	0.075	0.747
5	FIV-327	0.047	0.473
6	FIV-333	0.042	0.423
7	FIV-337	0.038	0.377
8	FIV-339	0.054	0.543
9	FIV-340	0.242	2.420
10	FIV-341	0.232	2.320
11	FIV-342	0.081	0.810
12	FIV-343	0.080	0.800
13	FIV-344	0.235	2.350
14	FIV-345	0.356	3.563
15	FIV-346	0.162	1.623
16	FIV-347	0.042	0.420
17	FIV-348	0.050	0.503
18	FIV-349	0.093	0.930
19	FIV-350	0.253	2.533
20	FIV-351	0.058	0.583
21	FIV-352	0.093	0.930
22	FIV-353	0.042	0.423
23	FIV-354	0.043	0.430
24	FIV-355	0.051	0.510
25	FIV-357	0.151	1.510
26	FIV-358	0.042	0.420
27	FIV-359	0.060	0.597
28	FIV-360	0.039	0.393
29	FIV-361	0.042	0.417
30	FIV-362	0.112	1.120
+	POSITIVE	0.942	9.417
-	NEGATIVE	0.050	0.500

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Immunostain 9.

+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	FIV-369	0.070	0.676
2	FIV-370	0.062	0.593
3	FIV-371	1.063	13.632
4	FIV-372	0.049	0.468
5	FIV-374	1.032	13.226
6	FIV-375	0.051	0.490
7	FIV-377	0.047	0.452
8	FIV-378	0.049	0.474
9	FIV-380	0.057	0.548
10	FIV-381	0.037	0.356
11	FIV-386	0.058	0.558
12	FIV-388	0.103	0.987
13	FIV-389	0.041	0.397
14	FIV-390	0.113	1.090
+	POSITIVE	0.934	8.984
-	NEGATIVE	0.052	0.497

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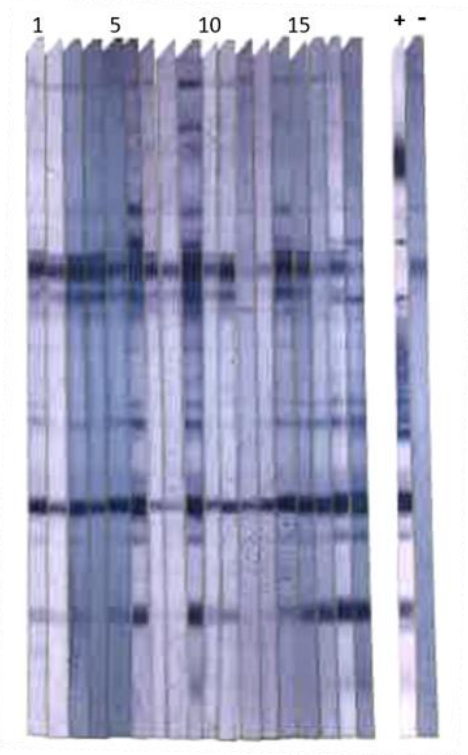
Immunostain 10.



+ . Pooled anti-FL4 plasma
- . SPF plasma.

Blot number	Cat I.D.[a]	Mean O.D.	Negative fold[b]
1	US-1	0.048	0.833
2	US-2	0.042	0.724
3	US-3	0.050	0.856
4	US-4	0.035	0.603
5	US-5	0.079	1.356
6	US-6	0.064	1.109
7	US-7	0.040	0.684
8	US-8	0.047	0.810
9	US-9	0.115	1.977
10	US-10	0.055	0.948
11	US-11	0.041	0.713
12	US-12	0.070	1.201
13	US-13	0.037	0.638
14	US-14	0.081	1.391
15	US-15	0.059	1.011
16	US-16	0.064	1.103
17	US-17	0.120	2.069
18	US-18	0.218	3.753
+	POSITIVE	1.081	18.644
-	NEGATIVE	0.029	0.506

[a]Red = infected, Yellow = vaccinated, Grey = None infected. [b]Pink = Positive result, Yellow= inconclusive, Green = Negative result

Signalment table 1a. Samples of a known FIV sero-status (Sero-negative)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
133	0.5	MN	DSH	0	0.564		Severe gingivitis
132	12.5	F	NG	0	0.585		Inflammation conjunctiva, oedema
131	6.9	MN	DSH	0	0.850	FCV positive	Severe gingivitis and weight loss
127	5.5	FN	DSH	0	0.641	FCoV 320	Gastroenteritis and very large mesenteric lymph nodes
124	5.0	MN	DSH	0	0.654	FCV positive	Unknown
123	4.9	F	DSH	0	0.671	FCV positive	Moderate/severe dental disease
120	0.6	F	DSH	0	0.526	FeLV/FIV neg	Routine screen
116	9.8	FN	BSH	0	0.526		Disorientated
129	9.8	FN	BSH	0	1.068	FeLV/FIV neg	Weight loss
122	0.6	F	DSH	0	1.017	FeLV/FIV neg	Routine screen
114	NG	F	DSH	0	0.419		Confirmatory screen
111	4.2	MN	DSH	0	0.530	FCoV 0	Pyrexic and enlarged kidneys
104	7.0	M	DSH	0	0.821		Confirmatory screen
103	2.1	MN	Siamese	0	0.748	FCoV 80, α1-AGP 2000	Abdominal discomfort
101	2.0	FN	DSH	0	0.453	FCoV 80, α1-AGP 1260 FeLV/FIV neg	Unknown

*****F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given

#DSH= Domestic short hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

† FCV= Feline Calcivirus, FCoV= Feline Coronavirus (Feline intraperitoneal virus), FeLV= Feline leukaemia virus, FIV= Feline immunodeficiency virus, FHV= Feline herpes virus, α1-AGP= Alpha-1 acid glycoprotein.

‡ URT= Upper respiratory tract disease, UTI= Urinary tract infection.

Table 1a continued. Samples of a known FIV sero-status (Sero-negative)

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Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
96	8.7	MN	BSH	0	0.641	FCoV>1280, α1-AGP 2460	Lethargy and weight loss
95	3.5	MN	BSH	0	0.688	FeLV/FIV/FCoV neg	Neutropenia, gingivitis and UTI
94	NG	NG	NG	0	0.534	FeLV/FIV/FCV/FHV neg	Gingivitis.
93	3.2	M	NF	0	0.500	FCV positive. FIV/FeLV neg	Gingivo-stomatitis
84	3.0	MN	DSH	0	0.526	FeLV/FIV/FCoV/Toxoplasma neg	Unknown
83	0.5	F	DLH	0	0.425	FeLV Ag Pos. FeLV/FIV/FCoV ab neg	Acute onset dysponea, ataxia and pyrexia
80	0.5	F	DLH	0	0.517	Positive in house	Unknown
78	8.6	MN	NG	0	0.538		Panleukapaenia
62	8.0	MN	DSH	0	0.563		Confirmatory screen
61	1.6	FN	Bengal	0	0.446	FCV positive	Gingivitis.
77	7.0	MN	DSH	0	1.329	FCoV 80	Weight loss
64	NG	M	DSH	0	1.033	FCV positive	History of severe stomatitis
55	13.2	FN	DR	0	0.641	FCoV >1280	Unknown
54	7.6	MN	BSH	0	0.739	FeLV/FIV/FCoV neg	Weight loss.
52	3.0	MN	DSH	0	0.714	FHV positive	Anorexia.

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‡ URT= Upper respiratory tract disease, UTI= Urinary tract infection.

Table 1a continued. Samples of a known FIV sero-status (Sero-negative)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
50	2.3	F	DSH	0	0.487	FCV positive	Severe gingivitis.
49	2.3	FN	DSH	0	0.637		Seizures
47	10.0	MN	AB	0	0.679	FCV positive	Stomatitis
46	Adult	F	DLH	0	0.855		Unknown
30	1.4	F	DSH	0	0.551	1 Snap +, 1 Snap -.	Confirmatory screen
74	0.4	F	Siamese	0	0.534	FCoV 160	Extended abdomen
21	1.1	MN	MC	0	0.423	FCV positive	History of URT disease
26	0.6	M	Siamese	0	0.487		Chronic gingivitis
33	NG	NG	NG	0	0.747	α1-AGP 500	Progressive ataxia
11	1.4	FN	DLH	0	0.437		Severe gingivitis.
12	9.7	MN	DSH	0	0.833		Routine screen
16	4.0	MN	BI	0	0.820	FCoV >1280	Weight loss. Mass in abdomen. Anaemia.
17	19.7	F	DSH	0	0.597		Disorientated
367607	0.9	M	DSH	0	0.387	FeLV/FIV/FCV/FHV neg	URT signs
367626	3.0	MN	DSH	0	0.670	Toxoplasma Ab >50. FeLV/FIV/FCoV neg	Ataxic fore limbs

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Table 1a continued. Samples of a known FIV sero-status (Sero-negative)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
367595	5.0	FN	DSH	0	0.340	FCV positive. FIV/FeLV neg	Gingivitis
66	4.0	FN	DLH	0	0.383	α1-AGP 2740. FeLV/FIV/FCoV neg	Rapid weight loss
142	NG	NG	NG	0	0.365	FeLV/FIV neg	Weight loss
149	5.0	F	DSH	0	0.528	FCoV 40. FeLV/FIV neg	Unknown
153	1.8	MN	DSH	0	0.475	FCV positive FeLV/FIV neg	Gingivo-stomatitis
155	8.0	MN	DSH	0	0.550	FeLV/FIV/FCoV neg	Lethargy and neutropenia
156	6.0	F	DSH	0	0.504	FeLV/FIV neg	Gingivitis
157	NG	FN	DSH	0	0.574	FIV neg	Unknown
185	NG	F	DSH	0	0.569	Faint in house positive	weight loss
154	1.8	FN	DSH	0	0.663	FeLV Ag neg/FIV/FHV/FCV neg	Gingivo-stomatitis
180	0.6	M	DSH	0	0.955	FCoV 40 (None specific) FIV/FeLV neg	Chronic diarrhoea
178	0.8	F	DSH	0	0.390	FeLV/FIV/FCoV/Toxoplasma neg	History and ongoing pyrexia
176	2.5	MN	DLH	0	0.602	FCV positive FeLV/FIV neg	Gingivitis.
173	1d	FN	DSH	0	0.476	FeLV/FIV/FCoV neg	Seizures
172	0.8	F	MC	0	0.488	FCoV >1280 FeLV/FIV/Toxoplasma neg	Poor development

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Table 1a continued. Samples of a known FIV sero-status (Sero-negative)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
171	7.8	MN	DSH	0	0.467	FeLV/FIV neg	Sick
168	3.0	F	DSH	0	0.650	FCoV 80, α1-AGP 1420 FIV/FeLV neg	Dsyponea
179	0.2	M	BSH	0	1.329	FCoV >1280, α1-AGP 1520. FeLV/FIV neg	Lethargy. Inappetence. Pyrexia
147	2.0	M	DSH	0	0.516	FeLV/FIV neg	Mandibular symphysis
166	0.6	MN	DSH	0	0.399	FeLV Ag neg/FIV/FHV/FCV neg	Unknown
159	1.0	F	NG	0	0.477	FeLV/FIV neg	Routine screen
158	0.8	M	DSH	0	0.453	FeLV/FIV neg	Routine screen
320544	4.0	MN	DSH	0	0.659	Feral. FeLV/FIV neg	Plasma cell pododermatitis
320560	1.7	F	Birman	0	0.516	FCoV positive	Unknown
320571	1.9	MN	DSH	0	0.469	FeLV/FIV/FCoV neg	Unexplained weight loss
179562	1.0	M	Bengal	0	0.470	FCoV >1280. α1-AGP 2240. FeLV Ag/Ab neg. FIV neg	Unknown
134	Adult	MN	DLH	0	1.765	FeLV/FIV neg	Plasma cell pododermatitis
378633	Adult	F	DSH	0	0.552	FeLV/FIV neg	Sanctuary cat
377299	6.0	FN	DSH	0	0.686	FeLV/FIV/FCoV neg	Weight loss, pale mms, chronic vomiting.
378305	13.0	NG	Bengal	0	0.648	VDS FIV neg	Confirmatory screen

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Table 1a continued. Samples of a known FIV sero-status (Sero-negative)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
378352	5.1	FN	DSH	0	0.719	FeLV/FIV/FCV/FHV neg	Gingivostomatitis
378358	13.0	MN	DLH	0	1.067	FeLV/FIV/FCV/FHV neg	Gingivitis
367644	15.0	FN	PLH	0	0.519	FeLV/FIV neg	Confirmatory screen
367645	4.2	FN	DSH	0	0.586	FeLV/FIV neg. FCV positive	Gingivitis, moderate dental disease
378188	11.6	FN	DSH	0	0.642	VDS FIV neg	Confirmatory screen
378263	3.0	FN	DSH	0	0.920	FeLV/FIV neg	Lethargy, inappetence, weight loss ?FIP
182631	9.0	MN	DSH	0	0.481	FeLV/FIV neg	Unknown

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Signalment table 1b. Samples of a known FIV sero-status (Sero-positive)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
119	5.0	MN	DSH	1K	5.496	Previous FIV inconclusive. FCoV= 640	Recurrent pyrexia. Treated for abscess and neutering
115	NG	M	DSH	1K	16.684		Well but abscess.
108	12.0	MN	DSH	10K	16.368	FeLV Ag neg/ FCV positive	Gingivitis. Confirmatory screen.
79	21.9	M	DSH	10K	17.646		Conjunctivitis left eye. Sneezing
72	4.0	M	DSH	10K	18.913		Confirmatory screen
71	NG	M	DSH	10K	18.938		Unknown
57	6.9	MN	DSH	10K	17.400	FHV/Mycoplasma felis/Chlamydophila felis negative	Unilateral conjunctivitis
51	NG	MN	PL	10K	17.427		Confirmatory screen
35	6.5	MN	DSH	10K	16.231		Anaemic/not eating. Confirmatory screen.
14	15.6	MN	DSH	10K	18.056	FCV/FeLV neg	Severe gingivitis. All teeth removed
25	2.0	MN	DSH	10K	15.643	FeLV neg	Confirmatory screen
367517	0.6	F	DSH	10K	11.897	FeLV Ag/FIV positive. FCV positive	Enlarged submandibular Lymph node. Pyrexia, gingivitis
42	NG	MN	DSH	10K	5.050		Unknown
141	NG	MN	DSH	10K	5.936		Confirmatory screen
146	6.0	M	DSH	10K	14.904		Confirmatory screen

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† FCV= Feline Calcivirus, FCoV= Feline Coronavirus (Feline intraperitoneal virus), FeLV= Feline leukaemia virus, FIV= Feline immunodeficiency virus, FHV= Feline herpes virus, α1-AGP= Alpha-1 acid glycoprotein. **‡** URT= Upper respiratory tract disease, UTI= Urinary tract infection.

Table 1b continued. Samples of a known FIV sero-status (Sero-positive)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
183	5.0	MN	DSH	1K	6.728		Confirmatory screen
175	4.0	M	DSH	10K	19.557		Confirmatory screen
160	2.9	M	DLH	10K	20.333		Confirmatory screen
182942	Adult	M	NG	1K	19.298	FeLV Ag neg/ FCoV 0	Stray
182455	NG	F	DSH	10K	16.845		NG
183053	Adult	F	DSH	10K	20.434		Healthy, previous positive
182461	12.0	M	DSH	10K	19.853		Sick
182246	9.0	MN	DSH	10K	15.029		Sick, severe gingivitis
182312	7.8	MN	DSH	10K	15.379	FeLV Ag neg/FCoV 0	Sick, severe depression. Normal TPR
180914	5.0	MN	DSH	10K	15.340	FeLV Ag neg	Sick, lethargy, mild dysponea, history of fights
182304	1.8	M	DSH	10K	14.239	FeLV Ag neg	Off food, mild dehydration, lameness
180949	Adult	M	DSH	10K	15.484	NG	NG
182189	10.9	MN	DSH	10K	15.350	FeLV Ag neg	lethargy, weight loss
181143	Adult	FN	DSH	10K	15.791	NG	NG
180961	5.0	MN	DSH	10K	14.180	FeLV Ag neg	

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Table 1b continued. Samples of a known FIV sero-status (Sero-positive)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
181294	5.7	M	DSH	10K	11.412		History of fights
181259	2.0	MN	DSH	10K	14.118		Stray- fighter
367516	6.0	M	DSH	10K	15.173		Confirmatory screen
180911	2.0	F	DSH	10K	13.102	FeLV Ag neg	
182249	NG	NG	NG	10K	12.855		NG
182285	7.0	FN	DSH	10K	9.785	FeLV Ag neg/ FCoV 0	NG
181218	0.3	NG	DSH	10K	12.543		NG
181948	5.0	M	DSH	10K	7.621		Chronic weight loss
181256	0.9	F	DSH	10K	12.282		General Malaise. Lingual ulcers, halitosis, marginal anaemia
181215	14.0	M	DSH	10K	13.164	FeLV Ag neg	Weight loss. Tongue ulceration.
181146	0.4	M	DSH	10K	12.524	NG	NG
182193	NG	M	DSH	10K	10.460	FeLV Ag neg	Stray
186496	11.1	MN	DSH	10K	12.868	FCoV 40, α1-AGP 620, FeLV Ag neg	NG
179114	10.1	MN	DSH	10K	17.467	FeLV Ag neg	NG
179105	3.0	M	DSH	10K	16.361	FeLV Ag neg	NG

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Table 1b continued. Samples of a known FIV sero-status (Sero-positive)

Cat I.D.	Age	Sex *	Breed #	IFA	Negative fold.	Other†	Clinical signs‡
179181	2.9	MN	DSH	10K	16.022	FeLV Ag neg	NG
179176	Adult	MN	DSH	10K	17.689		Healthy
179120	NG	MN	DSH	10K	12.489	FeLV ag neg/FCoV >1280	Healthy
179118	8.0	M	DSH	10K	7.506	FeLV Ag neg	Sick, weight loss.
179117	0.9	MN	DLH	10K	11.450		Healthy
179297	7.4	MN	DSH	10K	17.939		Healthy
179288	3.0	MN	DSH	1K	12.183	FeLV Ag neg/FCV neg	Gingivitis. Neck lesion
179200	10.0	MN	DSH	10K	8.117		Weight loss, lethargy, pyrexia
179323	10.9	M	DSH	10K	12.278	FeLV Ag neg/FCoV 320	Raised liver enzymes. Abdominal fluid
179369	Adult	M	DSH	10K	14.444	FeLV Ag neg	NG
179898	NG	FN	DSH	1K	7.687	FCoV >1280	NG
179876	10.0	FN	DSH	10K	15.081	FeLV Ag neg	General malaise- neutropaenia
179195	NG	NG	NG	1K/10K	14.657	FeLV Ag neg/ FCoV 0	Intermittent vomiting, weight loss. Pale mucous membrane
179837	1.4	NG	PL	10K	10.995		NG
179845	11.8	MN	DLH	10K	13.157	FeLV Ag neg. FCoV 0	Gingivitis, pancytopaenia, hypoalbuminaenia, hyperglobulinaemia

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Table 1b continued. Samples of a known FIV sero-status (Sero-positive)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
179850	13.0	FN	DSH	10K	10.288		NG
179582	2.0	M	DSH	10K	14.828		NG
179638	8.0	MN	DSH	10K	12.455	FeLV Ag neg	Healthy
179550	1.4	FN	DSH	10K	12.970		Healthy
179466	5.0	M	NG	10K	12.091		NG
179481	14.0	MN	DSH	10K	12.197	FeLV Ag neg	Confirmatory screen
181091	2.0	F	DSH	10K	8.733	FeLV Ag neg	Healthy
378291	2.1	M	DSH	10K	9.505	FeLV Ag neg/ FCoV 0	Lethargy. Abscess on neck
378385	11.0	F	DSH	10K	14.338		Confirmatory screen
378477	2.9	M	DSH	10K	20.562		Castrated
378479	4.0	FN	DSH	10K	18.938	FeLV Ag neg/FCV positive	Gingivostomatitis
378264	0.7	FN	DSH	10K	8.185		Confirmatory screen
378282	NG	MN	DSH	10K	4.586		Confirmatory screen
378253	2.9	M	DSH	10K	3.846		Confirmatory screen
378262	8.2	FN	PLH	10K	5.764		Confirmatory screen

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Table 1b continued. Samples of a known FIV sero-status (Sero-positive)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
183039	5.6	MN	DSH	10K	7.519	FeLV Ag neg	Keratitis. Anterior uveitis
182421	5.0	M	DSH	10K	5.705	FeLV Ag neg	Severe back trauma (very pruritic). Mild gingivitis
182337	4.0	MN	DSH	10K	5.256	FeLV Ag neg	Stray. Uncontrollable bleeding upon castration
182313	NG	M	DSH	10K	8.027		Inappetence, lethargy, ocular discharge
182387	2.9	M	DSH	10K	6.519		Healthy
180724	10.0	M	DSH	10K	18.203	FeLV Ag neg/FCV positive	9-month history of gingivitis/dental problems
180652	4.0	M	DSH	10K	18.330	Previous FIV IFA inconclusive.	NG
180795	Adult	MN	DSH	10K	17.844	FeLV Ag neg/FCV positive	Tongue ulcerations- purulent discharge
180801	2.5	M	PL	10K	18.964		Healthy
180800	NG	M	DSH	10K	16.996		NG
180659	13.6	FN	Siames	10K	16.866		NG
180647	NG	M	DSH	10K	17.906		NG
180644	9.4	MN	DSH	10K	18.286		NG
180638	2.0	M	DSH	10K	17.243	FeLV Ag neg. FCV positive.	Severe periodontitis, all teeth lost. Swollen and lysed jaw bones. Thickened bowel loop.
180389	8.0	MN	DSH	1K	15.214		Diabetes. Influenza like symptoms

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Table 1b continued. Samples of a known FIV sero-status (Sero-positive)

Cat I.D.	Age	Sex*	Breed#	IFA	Negative fold.	Other†	Clinical signs‡
180536	10.8	M	BSH	10K	16.866	FeLV Ag neg. FCV positive	Unceraltive glossitis
CPG41	N/A	N/A	N/A	10k	18.460		
180498	8.0	F	DSH	10K	18.181	FCoV 1280	Chronic gingivitis

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Signalment table 2a. IFA inconclusive samples – individual samples.

Cat I.D.	Age	Sex*	Breed#	IFAΘ	Negative FoldΦ	Other†	Clinical symptoms/history‡
347731	4.0	M	DSH	100 NS	1.619	Giardia positive. Nematode/protozoan free	Pyrexic for 2 weeks with occasional incontinence.
346577	2.0	M	DSH	0	0.636	FeLV Ag neg	Confirmatory screen
378475	4.0	M	DSH	0	0.710	FeLV Ag neg	Confirmatory screen
364988	NG	M	DSH	0	0.756		Confirmatory screen
364869	2.0	M	NG	0	1.517	FeLV Ag neg	Stray. Routine screen
364888	NG	M	NG	0	1.660		None given
364376	NG	M	DSH	0	0.648		Confirmatory screen
346495	0.3	M	NG	0	0.374		Confirmatory screen
346515	10.3	M	DSH	0	1.134	FeLV Ag neg	Severe infected Stomatitis
346538	4.0	M	DSH	0	0.720		Confirmatory screen
346639	2.3	M	DSH	0	0.735		Confirmatory screen
346816	NG	M	DSH	0	1.061		Confirmatory screen.
364790	8.1	MN	DSH	0	0.462		Confirmatory screen
378601	5.0	MN	DSH	10/100	0.448		Confirmatory screen
367547	14.0	MN	DSH	0	0.525		Confirmatory screen. No History

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Θ NS= None specific fluorescence, Toxic= Sample adversely reacts with fixed culture.

Φ Green = TM ELISA negative, Yellow= TM ELISA inconclusive, Pink= TM ELISA positive

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Table 2a continued. IFA inconclusive samples – individual samples.

Cat I.D.	Age	Sex*	Breed#	IFAΘ	Negative Fold Φ	Other†	Clinical symptoms/history‡
347644	8.0	MN	DSH	0	0.692		Confirmatory screen
346405	12.5	MN	DSH	0	0.819		Weight loss, Jaundice, constipation, anaemic.
346594	2.0	MN	DSH	0	0.712	From multi-cat house (57 total)	Confirmatory screen
347621	0.1	MN	DSH	0 (toxic)	0.717		Confirmatory screen
347721	4.1	MN	DSH	0	0.821		Confirmatory screen
347140	0.6	MN	DSH	0 NS (toxic)	0.415	FeLV Ag neg	Unwell, off food, vomiting blood.
347309	8.0	MN	DSH	0	0.396		Confirmatory screen. Weight loss
363127	7.0	MN	DSH	0 (toxic)	0.629	FeLV/FCV/FHV neg	Chronic facial dermatitis and ocular discharge.
364094	2.1	MN	DSH	0	0.752		Confirmatory screen
363764	NG	F	DSH	0	0.538		Confirmatory screen
346532	11.0	F	Siamese	0	0.616	FeLV Ag neg/ FCoV 0	No history
365011	0.4	F	DSH	0	0.408	FeLV Ag neg	Confirmatory screen
365080	1.0	F	DSH	0 NS	0.455	FCoV 1280. FeLV Ag neg	Confirmatory screen, from same house hold
365081	1.0	F	DSH	0 NS	0.539	FCoV 1280. FeLV Ag neg	
365083	4.0	M	DSH	0	0.714	FCoV 320. FeLV Ag neg	

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Table 2a continued. IFA inconclusive samples – individual samples.

Cat I.D.	Age	Sex*	Breed#	IFAΘ	Negative Fold Φ	Other†	Clinical symptoms/history‡
346700	Adult	F	NG	0	0.420		Confirmatory screen
345182	NG	F	DSH	0	0.527		Thin, renal disease, anaemic
346533	3.1	F	DR	0	0.667	FeLV Ag neg/toxoplasma 50	Head and Neck puritis
347177	NG	F	DSH	0	1.079		Confirmatory screen
378474	3.0	F	DLH	0	0.714		Confirmatory screen
346444	0.3	F	DLH	0.1/1K	0.478	FeLV Ag neg	Routine screen. Littermate FIV positive
346595	NG	FN	DSH	0	1.008		Confirmatory screen
346596	NG	FN	NG	0	0.955		Chronic gingivitis. Confirmatory screen
346793	1.5	FN	DSH	100? NS?	0.575	FeLV Ag neg/FCoV 0	Peripheral Lymphadenopathy, persistent PUO 3mths
346823	0.2	FN	DSH	0	0.349	Different results from different test	Confirmatory screen
346909	0.2	FN	DSH	0	0.459	FeLV neg.	No History
346933	12.0	FN	DSH	0 (toxic)	0.909	FeLV Ag neg/FCoV 0	Uveitis. Intra ocular haemorrhage
347467	2.0	FN	DSH	0	0.437		Confirmatory screen
347398	2.3	FN	NG	0 (toxic)	1.239	FeLV/FCoV neg	Weight loss
364945	5.0	FN	MC	0 (toxic)	0.548	FeLV Ag neg. FCV positive	Gingivo-stomatitis

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Table 2a continued. IFA inconclusive samples – individual samples.

Cat I.D.	Age	Sex*	Breed#	IFAΘ	Negative Fold Φ	Other†	Clinical symptoms/history‡
346639	2.0	NG	DSH	0/10	0.577		Confirmatory screen. Sick cat
346262	11.0	NG	NG	0	0.574		None given
347447	5.2	NG	DSH	0	0.545		Gingivitis and stomatitis
347464	Adult	NG	PLH	0	0.613		Confirmatory screen
378531	NG	M	DSH	10/100NS	0.525	FCoV 1280. FeLV Ag neg Prev:366983	Pyrexic.
367445	13.5	NG	DSH	0	0.611	366983 re-test	None healing ulcerated wound to rear flank.

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Table 2a continued. IFA inconclusive samples – individual samples.

Cat I.D.	Age	Sex*	Breed#	IFAϴ	Negative Fold Φ	Other†	Clinical symptoms/history‡
345387	NG	M	DSH	1k	17.826		Gingivitis
364982	NG	M	DSH	100	6.423		Stray trapped for neutering. Many fight abscesses
345592	2.0	MN	DSH	10k	22.337	FeLV Ag neg	Sanctuary cat. No clinical signs
346406	8.0	MN	DSH	10K	11.782	FeLV neg/FCV positive	No history
346657	2.0	MN	DSH	1K?	4.306		Stray fighter. Abscess. Confirmatory screen
346430	7.5	F	DSH	10K	20.799	Blood Glucose 8.8mmol/l. Taking Noroclair	Acute weight loss, history of gingivitis, mass in mouth.
347930	4.0	F	DSH	0	6.544		Confirmatory screen
364989	8.5	FN	DSH	10K	15.923	FeLV Ag neg. FCV/FHV neg	Chronic suppurative Cheilitis
363353	NG	M	DSH	0	2.997		Confirmatory screen

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Φ Green = TM ELISA negative, Yellow= TM ELISA inconclusive, Pink= TM ELISA positive
† FCV= Feline Calcivirus, FCoV= Feline Coronavirus (Feline intraperitoneal virus), FeLV= Feline leukaemia virus, FIV= Feline immunodeficiency virus, FHV= Feline herpes virus, α1-AGP= Alpha-1 acid glycoprotein.
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Signalment table 2b. IFA inconclusive samples – sequential samples.

Cat I.D.	Re-sample date	Age	Sex*	Breed#	IFAΘ	Negative Fold Φ	Other†	Clinical symptoms/history‡
347073[1]	04/09/2014	2.0	F	DSH	0	1.115		Confirmatory screen
347573[2]	30/09/2014				0	1.195		
345089 [1]	08/05/2014	3.0	M	DSH	0	1.067		Confirmatory screen/healthy
345547 [2]	09/06/2014				0 NS	1.033		
346097 [1]	11/07/2014	5.0	M	DSH	0 NS	0.619		Confirmatory screen
346440 [2]	01/08/2014				0	0.634		
346132[1]	14/07/2014	5.0	M	PLH	0 (toxic)	0.736	FeLV Ag neg/ FCoV 1280/ Toxoplasma 40	Seizures
346736[2]	19/08/2014				0	0.560	FCoV 320/ α1-AGP = 1300	
346228 [1]	18/07/2014	3.0	M	DSH	0	0.712		Confirmatory screen
346368 [2]	28/07/2014				0	0.636		
363779[1]	01/12/2014	12.0	M	DSH	0	1.022		Stray. Non-healing wound, broken teeth, plaque, oral infected stomatitis.
364144[2]	22/12/2014				0	0.796		

*****F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given

#DSH= Domestic short hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

Θ NS= None specific fluorescence, Toxic= Sample adversely reacts with fixed culture.

Φ Green = TM ELISA negative, Yellow= TM ELISA inconclusive, Pink= TM ELISA positive

† FCV= Feline Calcivirus, FCoV= Feline Coronavirus (Feline intraperitoneal virus), FeLV= Feline leukaemia virus, FIV= Feline immunodeficiency virus, FHV= Feline herpes virus, α1-AGP= Alpha-1 acid glycoprotein.

‡ URT= Upper respiratory tract disease, UTI= Urinary tract infection, Confirmatory screen = Has tested positive in house

Table 2b. IFA inconclusive samples – sequential samples.

Cat I.D.	Re-sample date	Age	Sex*	Breed#	IFAΘ	Negative Fold Φ	Other†	Clinical symptoms/history‡
363703[1]	26/11/2014	Adult	M	DSH	0 NS	0.981		Confirmatory screen. Semi-feral - neglect case
364867[2]	30/01/2015				0	1.569		
346170 [1]	16/07/2014	3.4	MN	DSH	0	0.907	FeLV Ag neg	Fighter.
347929 [2]	20/10/2014				0	0.384		
347132 [1]	10/09/2014	4.0	MN	DSH	0	0.585		Confirmatory screen
347317 [2]	17/09/2014				0	0.616		
363027 [3]	22/10/2014					0.565		
347761[1]	13/10/2014	10.0	MN	DSH	100 NS	0.771		Persistent pyrex. Weight loss, confirmatory screen. Rescue case.
363782[2]	01/12/2014				0	0.941		
347257[1]	16/09/2014	3.0	FN	DLH	0(toxic)	0.469	FCoV 160/FeLV neg	Dehydrated and anorexic. Pyrexic
363038[2]	22/10/2014				0 (toxic)	0.485		

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Θ NS= None specific fluorescence, Toxic= Sample adversely reacts with fixed culture.

Φ Green = TM ELISA negative, Yellow= TM ELISA inconclusive, Pink= TM ELISA positive

† FCV= Feline Calcivirus, FCoV= Feline Coronavirus (Feline intraperitoneal virus), FeLV= Feline leukaemia virus, FIV= Feline immunodeficiency virus, FHV= Feline herpes virus, α1-AGP= Alpha-1 acid glycoprotein.

‡ URT= Upper respiratory tract disease, UTI= Urinary tract infection, Confirmatory screen = Has tested positive in house

Table 2b continued. IFA inconclusive samples – sequential samples.

Cat I.D.	Re-sample date	Age	Sex*	Breed#	IFA$^{\Theta}$	Negative Fold $^{\Phi}$	Other†	Clinical symptoms/history‡
347226 [1]	15/09/2014	13.0	FN	DSH	0	0.568		Poor coat, lethargic
347445 [2]	25/09/2014				0	0.527		
347307[1]	17/09/2014	9.0	FN	DSH	0	0.634	FeLV Ag neg	Precaution screen.
347760[2]	13/10/2014				100 NS	0.733		
345855 [1]	27/06/2014	Adult	NG	NG	0 NS	0.715		Confirmatory screen
346573 [2]	12/08/2014				0NS	0.456		
345879 [1]	27/06/2014	Adult	NG	NG	0 NS	0.722		Confirmatory screen
346572 [2]	08/08/2014				0NS	0.583		
364503[1]	14/01/2015	8.0	MN	DSH	0	1.022		Confirmatory screen
364637[2]	20/01/2015				0	3.625		

***F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given
#DSH= Domestic short hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.
Θ NS= None specific fluorescence, Toxic= Sample adversely reacts with fixed culture.
Φ Green = TM ELISA negative, Yellow= TM ELISA inconclusive, Pink= TM ELISA positive
† FCV= Feline Calcivirus, FCoV= Feline Coronavirus (Feline intraperitoneal virus), FeLV= Feline leukaemia virus, FIV= Feline immunodeficiency virus, FHV= Feline herpes virus, α1-AGP= Alpha-1 acid glycoprotein.
‡ URT= Upper respiratory tract disease, UTI= Urinary tract infection, Confirmatory screen = Has tested positive in house

Signalment table 3a. Australian vaccinate samples.

Cat I.D.	Age	Sex*	Breed#
FIV-2	2.4	MN	Bengal
FIV-3	16.3	FN	DSH
FIV-4	11.5	FN	DSH
FIV-5	11	FN	DSH
FIV-6	5	FN	DSH
FIV-7	13	MN	DSH
FIV-8	13	MN	DSH
FIV-9	3	FN	DSH
FIV-10	8.8	MN	DSH
FIV-11	5.3	FN	Himalayan
FIV-12	4.3	MN	DSH
FIV-13	5.3	FN	DSH
FIV-14	5.3	MN	DSH
FIV-15	6	FN	DSH
FIV-16	7.3	FN	DSH
FIV-17	9.2	FN	DSH
FIV-18	5	MN	DSH
FIV-20	8.3	FN	DSH
FIV-21	4	MN	DSH
FIV-22	13.2	MN	DSH
FIV-23	13.2	FN	DSH

Cat I.D.	Age	Sex*	Breed#
FIV-24	4	FN	DSH
FIV-25	7.5	MN	DSH
FIV-28	5.4	MN	DSH
FIV-59	5.3	FN	DMH
FIV-84	6.8	FN	DSH
FIV-93	7	FN	Russian Blue X
FIV-95	8.8	FN	DMH
FIV-96	7.8	FN	DSH
FIV-97	8.4	FN	DSH
FIV-98	9.6	MN	DMH
FIV-99	5.3	MN	DSH
FIV-100	9.7	MN	DSH
FIV-101	6.9	FN	DSH
FIV-102	13.7	MN	DSH
FIV-103	5.5	MN	DSH
FIV-104	6.3	MN	DSH
FIV-105	7.3	FN	DMH
FIV-107	5	FN	DSH
FIV-108	8.8	FN	DSH
FIV-109	12	FN	Himalayan
FIV-110	5.3	MN	DSH

Cat I.D.	Age	Sex*	Breed#
FIV-116	5.3	FN	DSH
FIV-117	10.8	FN	DSH
FIV-118	10	MN	DSH
FIV-120	7.7	FN	DSH
FIV-121	7.7	FN	DSH
FIV-122	4.8	MN	Russian Blue X
FIV-123	10.8	MN	DMH
FIV-131	4.3	FN	DSH
FIV-132	4.5	FN	DSH
FIV-133	4.8	FN	DSH
FIV-134	5.8	MN	DSH
FIV-135	4.7	FN	DSH
FIV-136	4.7	MN	DSH
FIV-138	8.7	MN	DSH
FIV-139	7	MN	DLH
FIV-140	4.6	FN	DSH
FIV-141	15.8	MN	Tonkinese
FIV-142	9.7	MN	DSH
FIV-143	10	MN	DLH
FIV-144	10	FN	DSH
FIV-145	11.1	MN	DSH

***F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given, #DSH= Domestic short hair, DMH= Domestic medium hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

Table 3a continued. Australian vaccinate samples.

Cat I.D.	Age	Sex*	Breed#
FIV-146	12.3	FN	DSH
FIV-147	10.7	MN	DSH
FIV-148	10.7	MN	DSH
FIV-149	4.8	MN	DMH
FIV-150	4	FN	DSH
FIV-151	7.2	FN	Ragdoll X
FIV-153	4.8	FN	DSH
FIV-168	6.3	MN	DSH
FIV-170	13.8	MN	DSH
FIV-171	6.3	FN	DSH
FIV-172	5.4	FN	Abyssinian
FIV-173	3.5	MN	Abyssinian
FIV-174	5.1	MN	DSH
FIV-175	10.3	MN	DSH
FIV-179	4	FN	DSH
FIV-183	7.8	FN	Tonkinese
FIV-184	7.8	MN	Tonkinese
FIV-185	6.7	MN	DLH
FIV-186	6.5	MN	Burmese
FIV-189	6.8	MN	DSH
FIV-212	13.5	FN	DSH

Cat I.D.	Age	Sex*	Breed#
FIV-213	7.2	FN	Burmese
FIV-214	7.2	MN	Burmese
FIV-215	6.4	MN	DMH
FIV-216	6.2	FN	DSH
FIV-236	3.9	MN	DMH
FIV-296	13.3	MN	DSH
FIV-297	6.8	MN	DSH
FIV-298	6.8	MN	Burmese X
FIV-299	6.3	MN	DSH
FIV-300	4.7	FN	DMH
FIV-301	4.7	MN	Burmese
FIV-302	3.7	FN	DSH
FIV-339	4.2	MN	DSH
FIV-340	11.8	MN	Siamese
FIV-341	4.9	MN	DLH
FIV-342	18.1	MN	Burmese
FIV-343	4.9	MN	DSH
FIV-344	10.3	FN	DSH
FIV-345	9.2	FN	DSH
FIV-346	9.2	MN	DSH
FIV-349	10.2	MN	DSH

Cat I.D.	Age	Sex*	Breed#
FIV-350	6.9	FN	DSH
FIV-351	8.4	MN	DSH
FIV-352	8.4	MN	DSH
FIV-357	6.6	FN	DSH
FIV-359	5.8	FN	DSH
FIV-362	9.2	MN	DMH
FIV-388	3.3	MN	DSH
FIV-390	4.5	MN	DSH

*****F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given

#DSH= Domestic short hair, DMH= Domestic medium hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

Signalment table 3b. American vaccinate samples

Cat I.D.	Age	Sex*	Breed#
US-1	5.3	MN	DLH
US-2	5.1	MN	DLH
US-3	6.3	FN	DSH
US-4	13.4	MN	Manx/Siamese
US-5	12.6	MN	DSH
US-6	2.5	FN	DSH
US-7	4.6	FN	DSH
US-8	4.6	MN	DSH
US-9	4.1	MN	DSH
US-10	17.7	MN	DSH
US-11	4.9	FN	DLH
US-12	11.3	MN	DLH
US-13	3.0	FN	DSH
US-14	13.7	FN	DSH
US-15	6.3	MN	DSH
US-16	9.2	FN	DSH
US-17	4.7	FN	DSH
US-18	3.1	FN	DMH

*****F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given**, #**DSH= Domestic short hair, DMH= Domestic medium hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

Signalment table 3c. Australian uninfected samples.

Cat I.D.	Age	Sex*	Breed#
FIV-26	6.5	FN	DSH
FIV-27	20.2	FN	DSH
FIV-29	12	FN	Siamese
FIV-30	12	MN	Siamese
FIV-31	13	MN	DLH
FIV-32	4	FN	DSH
FIV-34	8.3	MN	DSH
FIV-35	7	FN	DSH
FIV-36	7	MN	DSH
FIV-37	7	MN	DSH
FIV-38	8	FN	DSH
FIV-39	7	MN	DSH
FIV-40	6.9	MN	DSH
FIV-41	15	MN	DSH
FIV-42	12.5	FN	DMH
FIV-43	6.5	FN	DMH
FIV-44	2.3	MN	DSH
FIV-45	16	FN	DLH
FIV-46	8.5	FN	DSH
FIV-47	3	FN	DSH
FIV-48	12	MN	DSH

Cat I.D.	Age	Sex*	Breed#
FIV-53	5	MN	DSH
FIV-54	7	MN	DSH
FIV-56	14.2	MN	DSH
FIV-57	2.5	FN	DSH
FIV-61	4.4	FN	Abyssinian X
FIV-65	5.6	FN	DMH
FIV-71	6	FN	DSH
FIV-154	8.2	MN	DSH
FIV-155	12.4	MN	DSH
FIV-156	5.2	MN	DSH
FIV-159	7	MN	DSH
FIV-160	6.8	MN	DSH
FIV-165	5.6	MN	DSH
FIV-166	7.2	MN	DSH
FIV-167	4.1	FN	DSH
FIV-169	5.1	MN	DSH
FIV-177	3.4	FN	Persian
FIV-178	3.6	FN	DSH
FIV-180	5.1	MN	DSH
FIV-181	6.8	MN	Burmese
FIV-182	5.2	MN	Burmese

Cat I.D.	Age	Sex*	Breed#
FIV-188	4	MN	DSH
FIV-191	11.3	MN	DSH
FIV-193	7.1	FN	Spotted Mist
FIV-196	12.2	MN	DLH
FIV-198	7.5	FN	DSH
FIV-199	6.3	FN	DSH
FIV-200	7.9	FN	DMH
FIV-201	7	FN	DSH
FIV-204	8.3	MN	DSH
FIV-205	12.5	FN	DSH
FIV-206	12.5	MN	DSH
FIV-217	5.3	MN	Ragdoll
FIV-219	7.4	MN	DSH
FIV-220	7.1	FN	DSH
FIV-225	5.1	FN	DSH
FIV-230	6.7	FN	DSH
FIV-231	7.6	FN	DSH
FIV-232	7.5	MN	DMH
FIV-233	7.6	MN	DSH
FIV-235	5.3	MN	DSH
FIV-242	6.3	FN	DSH

*****F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given**, #**DSH= Domestic short hair, DMH= Domestic medium hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

Signalment table 3c. Australian uninfected samples.

Cat I.D.	Age	Sex*	Breed#
FIV-243	12	MN	DSH
FIV-244	13	MN	DSH
FIV-245	10.9	FN	DSH
FIV-259	10.7	FN	Birman
FIV-260	6.4	FN	DSH
FIV-261	6.1	MN	DSH
FIV-262	11.6	FN	DSH
FIV-263	6	FN	DSH
FIV-265	7	FN	DSH
FIV-271	6.3	MN	DSH
FIV-273	8.3	FN	DSH
FIV-277	7.4	FN	DSH
FIV-280	6.5	MN	DSH
FIV-283	5.6	MN	DLH
FIV-284	5.8	FN	DSH
FIV-285	6	MN	DSH
FIV-291	9.2	MN	DSH
FIV-295	4	FN	DSH
FIV-304	6y	FN	DSH
FIV-309	10.5	MN	DMH
FIV-314	12	MN	Burmese

Cat I.D.	Age	Sex*	Breed#
FIV-316	5.3	FN	DSH
FIV-320	6.5	MN	DLH
FIV-321	13	FN	DSH
FIV-322	9.7	FN	DLH
FIV-323	9	MN	DLH
FIV-324	12.1	MN	Ragdoll
FIV-325	8.7	MN	Ragdoll
FIV-326	7.8	FN	DSH
FIV-327	6.7	FN	Siamese X
FIV-333	13.2	MN	DSH
FIV-337	4.8	MN	DSH
FIV-347	14.3	FN	DSH
FIV-348	11.8	MN	Ragdoll
FIV-353	6.2	FN	DSH
FIV-354	8.2	FN	DMH
FIV-355	6.1	MN	DMH
FIV-358	4.2	FN	DSH
FIV-360	5.8	MN	DSH
FIV-361	5.8	FN	DSH
FIV-369	4.1	MN	DSH
FIV-370	9.5	FN	DLH

Cat I.D.	Age	Sex*	Breed#
FIV-372	6.3	MN	DSH
FIV-375	7.7	MN	DMH
FIV-377	6.3	MN	DMH
FIV-378	4.8	MN	Burmese
FIV-380	6.5	MN	DSH
FIV-381	7.6	MN	DSH
FIV-386	11.4	MN	DLH
FIV-389	3.5	FN	DLH

***F** = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given**, #**DSH= Domestic short hair, DMH= Domestic medium hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

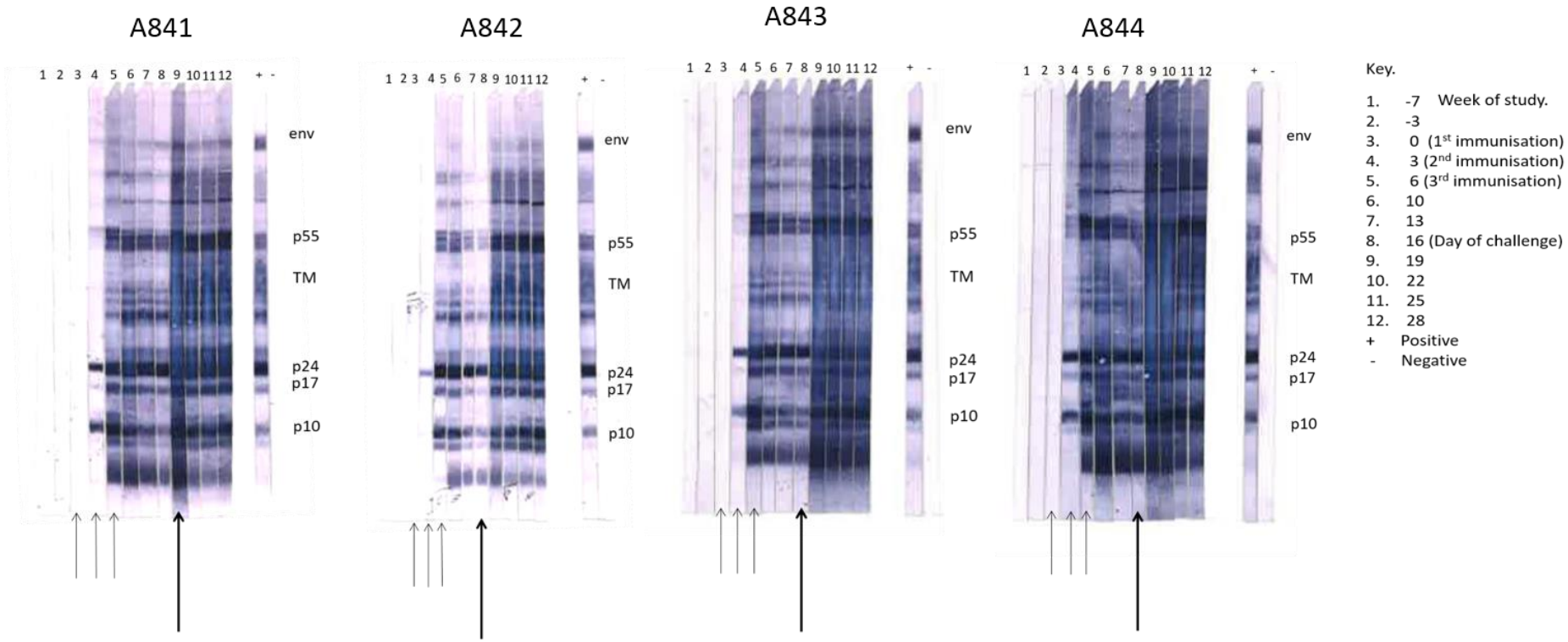
Signalment table 3d. Australian infected samples.

Cat I.D.	Age	Sex*	Breed#
FIV-1	7.3	MN	DSH
FIV-19	8.8	FN	DSH
FIV-106	5.5	MN	DLH
FIV-152	8.1	FN	DSH
FIV-33	13.4	MN	DSH
FIV-62	3.4	MN	DSH
FIV-80	4	FN	DSH
FIV-85	9.5	FN	Persian X
FIV-92	5	MN	DSH
FIV-127	7.1	FN	DLH
FIV-157	4.1	FN	DLH
FIV-163	13.6	MN	DSH
FIV-190	9.4	MN	DSH
FIV-192	9.3	MN	DSH
FIV-203	11.3	MN	DSH
FIV-208	4.6	MN	DSH
FIV-209	6.1	MN	DSH
FIV-218	5.7	FN	Himalayan
FIV-250	5.5	MN	DSH
FIV-272	4.3	M	DSH
FIV-274	6.4	MN	DSH

Cat I.D.	Age	Sex*	Breed#
FIV-279	10.1	MN	DSH
FIV-281	9.3	MN	DSH
FIV-303	16.1	MN	DSH
FIV-319	6.4	FN	DSH
FIV-371	10.9	MN	Bengal
FIV-374	4.9	MN	DSH

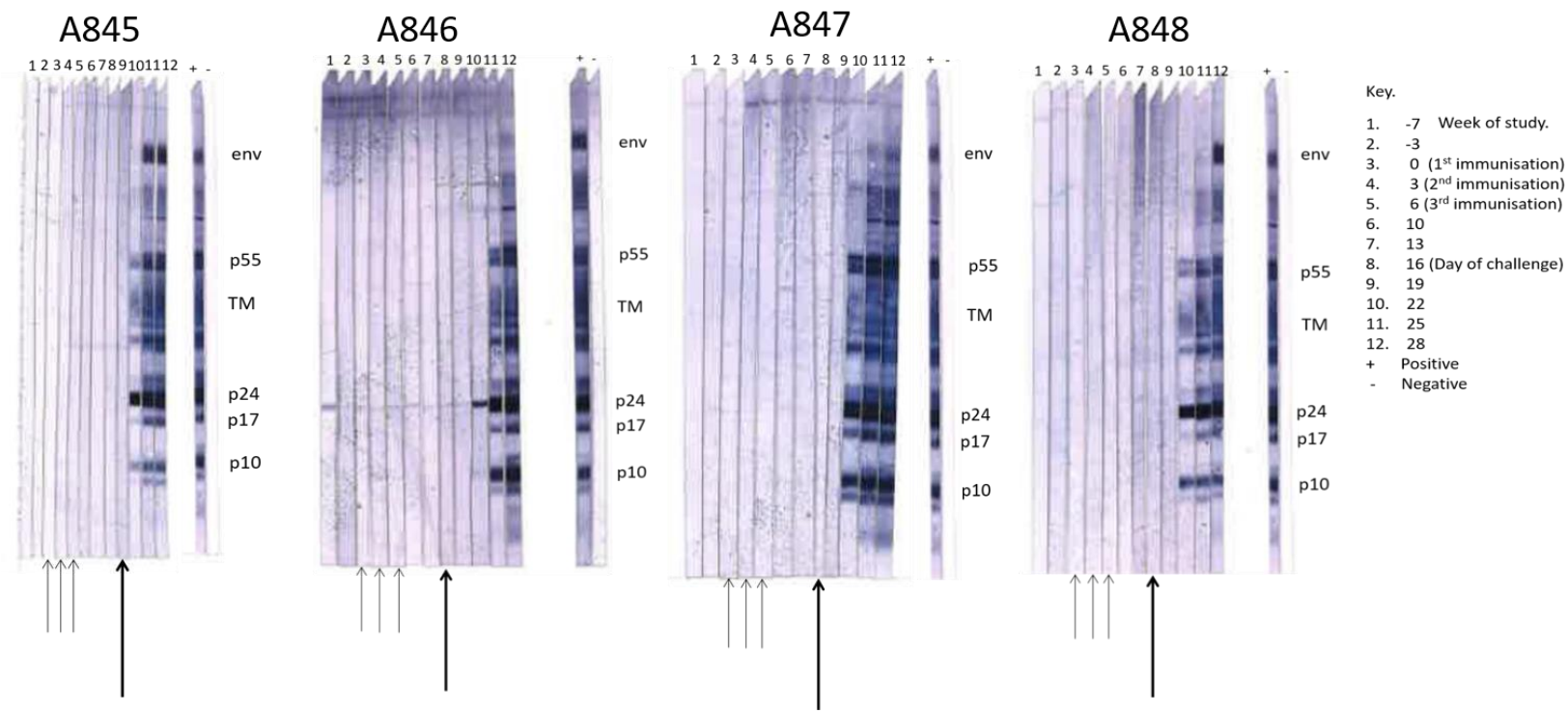
***F = Female, FN= Female neutered, M=Male, MN=Male neutered, NG=Not given, #DSH= Domestic short hair, DMH= Domestic medium hair, DLH=Domestic long hair, PLH=Persian long hair, BSH=British short hair, DR=Devon rex, MC=Main coon, AB=Abbysian, BI=Birman, NF=Norwegian forest.

Appendix 5. Immunostain analysis; vaccinates.



Seroconversion of vaccinated animals following immunisation with the DIVA virus and challenge with FIVGL8-414. Small arrows indicate the timings of FIV vaccine immunisations, whilst the large arrow denotes the week of challenge.

Appendix 5. Immunostain analysis; controls.



Seroconversion of control cats following immunisation with the control vaccine and challenge with FIVGL8-414. Small arrows indicate the timings of control vaccine immunisations; large arrows indicate the week of challenge.

Bibliography.

Addie D.D., Toth S., Reid S., Jarret O., Dennis J.M., Callanan J.J., 2000. Long-term impact on a closed household of pet cats of natural infection with feline coronavirus, feline leukaemia virus and feline immunodeficiency virus. *Veterinary Record,* 146: 419-424.

Ammersbach M., Little S., Bienzle D., 2013. Preliminary evaluation of a quantitative polymerase chain reaction assay for diagnosis of feline immunodeficiency virus infection. *Journal of Feline Medicine and Surgery*, 15(8): 725-729.

Andersen P.R., Tyrrell P., 2004. Feline immunodeficiency virus diagnosis after vaccination. *Animal Health Research Reviews*, 5 (2):327-330.

Arnold B.A., Helper R.W., Keller P.M., 1998. One-step fluorescent probe product-enhanced reverse transcriptase assay. *BioTechniques*. 25: 98-106.

Avrameas A., Strosberg A.D., Moraillon A., Songio P., Pancino G., 1993. Serological diagnosis of feline immunodeficiency virus infection based on synthetic peptides from Env glycoproteins. *Research in Virology*, 144: 209-218.

Avrameas A., Strosberg A.D., Moraillon A., Sonigo P., Pancino G., 1993. Serology diagnosis of feline immunodeficiency virus infection based on synthetic peptides from Env glycoproteins. *Research in Virology* 144: 209-218.

Baldinotti F., Matteucci D., Mazzetti P., Giannelli C., Bandecchi P., Tozzini F., Bendinelli M., (1994). Serum Neutralization of Feline Immunodeficiency Virus Is Markedly Dependant on Passage History of the Virus and Host System. *Journal of Virology*, 68 (7): 4572-4579.

Beczkowski P., (2012). Virus evolution in the progression of natural feline immunodeficiency virus infection. PhD Thesis. Institute of infection, immunity and inflammation.
https://glasgow.summon.serialssolutions.com/?s.q=phd+thesis&s.cmd=addFacetValueFilt

ers%28ContentType%2CNewspaper+Article%2Ct%7CContentType%2CBook+Review%2Ct%29#!/search?ho=t&fvf=ContentType,Newspaper%20Article,t%7CContentType,Book%20Review,t%7CContentType,Dissertation,f&l=en-UK&q=phd%20thesis%20beczkowski

Beczkowski P., Harris M., Techakriengkrai N., Beatty J. A., Willett B. J., Hosie M.J., 2015. Neutralising antibody response in domestic cats immunised with a commercial feline immunodeficiency (FIV) vaccine. *Vaccine* 33 (8): 977-984.

Bienzle D., Reggeti F., Wen X., Little S., Hobson J., Kruth S., 2004. The variability of serological and molecular diagnosis of feline immunodeficiency virus infection. *Canadian Veterinary Journal*, 45: 753-757.

Bishop S.A., Stokes C.R., Gruffydd-Jones T.J., Whiting C.V., Humphries J.E., Osborne R., Papanastasopoulou M., Harbour D.A., 1996. Vaccination with fixed feline immunodeficiency virus (FIV) infected cells: protection, breakthrough and specificity of response. *Vaccine*, 14 (13): 1243-1250.

Broche-Pierre S., Richardson J., Moraillon A., Songio P., 2005. Evaluation of line feline immunodeficiency virus vaccines with modified antigenic properties. *Journal of General Virology*, 86: 2495-2506.

Carlson J.R., Yee J., Hinrichs S.H., Bryant M.L., Gardner M.B., Pedersen N.C., 1987. Comparison of Indirect Immunofluorescence and Immunostain for Detection of Anti-Human Immunodeficiency Virus Antibodies. *Journal of Clinical Microbiology*, 25 (3): 494-497.

Coleman J.K., Pu R., Martin M.M., Noon-Song E.N., Zwijnenberg R., Yamamoto J.K., 2014. Feline immunodeficiency virus (FIV) vaccine efficacy and FIV neutralising antibodies. *Vaccine*, 32:746-754.

Crawford P.C., Levy J.K., 2007. New Challenge for the Diagnosis of Feline Immunodeficiency Virus Infection. *Veterinary Clinics Small Animal Practice*, 37: 335-350.

Crawford P.C., Slater M.R., Levy J.K., 2005. Accuracy of polymerase chain reaction assays for diagnosis of feline immunodeficiency virus infection in cats. *Journal of the American Veterinary Medical Association,* 226 (9): 1503-1507.

de Parseval A., Grant C.K., Sastry J., Elder J.H., (2006). Sequential CD134-CXCR4 interactions in Feline Immunodeficiency Virus (FIV): Solusble CD134 Activates FIV Env for CXCR4-Dependant Entry and Reveals a Cryptic Neutralisation Epitope. *Journal of Virology*, 80 (6): 3088-3091.

de Ronde A., Stam J.G., Boers P., LAngedijk H., Meloen R., Hesselink W., Keldermans L.C.E.J.M., van Vliet A., Verschoor E.J., Horzinek M.C., Egberink H.F., 1994. Antibody Response in Cats to the Envelope Proteins of Feline Immunodeficiency Virus: Identification of an Immunodominant Neutralization Domain. *Virology*, 198: 257-264.

de Rozieres S., Mathiason C.K., Rolston M.R., Chatterji U., Hoover E.A, Elder J.H., 2004. Characterization of a Highly Pathogenic Molecular clone of Feline Immunodeficiency Virus Clade C. *Journal of Virology*, 78(17): 8971-8982.

Diehl L.J., Mathiason-Dubard C.K., O'Neil L.L., Obert L.A., Hoover E.A., 1995. Induction of Accelerated Feline Immunodeficiency Virus Disease by Acute-Phase Virus Passage. *Journal of Virology*, 69(10): 6149-6157.

Dunham S.P., Bruce J., Klein D., Flynn J.N., Golder M.C., MacDonald S., Jarrett O., Neil J.C., 2006. Prime-boost vaccination using a DNA and whole inactivated virus vaccines provides limited protection against virulent feline immunodeficiency virus. *Vaccine*, 24:7095-7108.

Dunham S.P., Bruce J., Mackay S., Golder M., Jarrett O., Neil J.C., 2006. Limited efficacy of an inactivated feline immunodeficiency virus vaccine. *The Veterinary Record*, 158: 561-562.

Elder J.H., Schnolzer M., Hasselkus-Light C.S., Henson M., Lerner D.A., Phillips T.R.,

English R.V., Johnson C.M., Gebhard D.H., Tampkins M.B., (1993). In Vivo Lymphocyte Tropism of Feline Immunodeficiency Virus. *Journal of Virology*, 69 (9): 5175-5186.

Ferrantelli F., Ruprecht R.M., (2002). Neutralising antibodies against HIV- back in the major league? *Current Opinion in Immunology*, 14: 495-502.

Flynn J.N., Cannon C.A., Lawrence C.E., Jarrett O., (1994). Polyclonal B-cell activation in cats infected with feline immunodeficiency virus. *Immunology,* 81: 626-630.

Flynn J.N., Cannon C.A., Neil J.C., Jarrett O., 1997. Vaccination with a feline immunodeficiency virus multiepitopic peptide induces cell-mediated and humoral immune responses in cats but does not confer protection. *Journal of Virology*, 71 (10):7589-7592.

Fontenot J.D., Hoover E.A., Elder J.H., Montelaro R.C., 1992. Evaluation of Feline Immunodeficiency Virus and Feline Leukemia Virus Transmembrane Peptides for Serological Diagnosis. *Journal of Clinical Microbiology*, 30(7): 1885-1890.

Freed E.O., (1998). HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology*, 251: 1-15.

Furuya T., Hasegawa A., Miyazawa T., Miki K., Mikami T., 1992. Detection of anti-gag antibodies of feline immunodeficiency virus in cat sera by enzyme-linked immunosorbent assay. *Archives in Virology*, 124: 355-361.

Gleich S.E., Krieger S., Hartmann K., 2009. Prevalence of feline immunodeficiency virus and feline leukaemia virus among client-owned cats and risk factors for infection in Germany. *Journal of Feline Medicine and Surgery*, 11: 985-992.

Gnann J.W., Nelson J.A., Oldstone M.B.A., (1987). Fine Mapping of an Immunodominant Domain in the Transmembrane Glycoprotein of Human Immunodeficiency Virus. *Journal of Virology*, 61 (8): 2639-2641.

Graham F.L., Smiley J., Russell W.C., Nairn R., 1977. Characteristics of a Human cell line transformed by DNA from Human Adenovirus type 5. Journal of general Virology 36: 59-72.7

Hartmann K., 2012. Clinical Aspects of Feline Retroviruses: A Review. *Virology*, 4: 2684-2710.

Hartmann K., Vidyashankar A., Jarrett O., Egberink H., Greene C.E., Griessmayr P., 2007. Comparison of different new test for feline immunodeficiency virus and feline leukemia virus infection. *Journal of Feline Medicine and Surger*y 9: 439-445.

Haynes B.F., Kelsoe G., Harrison S.C., Kepler T.B., (2012). B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. *Nature Biotechnology*, 30 (5): 423-433.
Hayward J.J., Taylor J., Rodrigo A.G., (2007). Phylogenetic analysis of feline immunodeficiency virus in feral and companion domestic cats of New Zealand. *Journal of Virology,* 81 (6): 2999-3004.

Hofmann-Lehmann R., Holznagel E., Ossent P., Lutz H., (1997). Parameters of Disease Progression in Long-term Experimental Feline Retrovirus (Feline Immunodeficiency Virus and Feline Leukemia Virus) Infection: Hematology, Clinical Chemistry and Lymphocyte Subsets. *Clinical and Diagnostic Laboratory Immunology*, 4 (1): 33-42.

Hohdatsu T., Okada S., Motokawa K., Aizawa C., Yamamoto J.K., Koyama H., 1997. Effect of dual-subtype vaccine against feline immunodeficiency virus infection. *Veterinary Microbiology*, 58: 155-165.

Hosie M.J., Addie D., Belak S., Boucraut-Baralon C., Egberink H., Frymus T., Gruffydd-Jones T., Hartmann K., Lloret A., Lutz H., Marsilio F., Grazia Pennisi M., Radford A.D., Thiry E., Truyen U., Horzinek M.C., 2009. Feline Immunodeficiency ABCD guidelines on the prevention and management. *Journal of Feline Medicine and Surger*y, 11: 575-584.

Hosie M.J., Dunsford T., Klein D., Willett B.J., Cannon C., Osborne R., MacDonald J., Spibey N., Mackay N., Jarrett O., Neil J.C., 2000. Vaccination with inactivated Virus but not viral

DNA reduces virus load following challenge with a Heterologous and Virulent Isolate of Feline Immunodeficiency Virus. *Journal of Virology*, 74(20): 9403-9411.

Hosie M.J., Flynn J.N., Rigby M.A., Cannon C., Dunsford T., Mackay N.A., Argyle D., Willett B.J., Miyazawa T., Onions D.E., Jarrett O., Neil J.C., 1998. DNA vaccination affords significant protection against Feline Immunodeficiency Virus Infection without Inducing Detectable Antiviral Antibodies. *Journal of Virology*, September:7310-7319.

Hosie M.J., Jarrett O., 1990. Serological responses of cats to feline immunodeficiency virus. *AIDS,* 4: 215-220.

Hosie M.J., Klein D., Binley J.M., Dunsford T.M., Jarrett O., Neil J.C., Knapp E., Giannecchini S., Matteucci D., Bendinelli M., Hoxie J.A., Willett B.J., 2005. Vaccination with an Inactivated Virulent Feline Immunodeficiency Virus Engineered to Express High Levels of Env. *Journal of Virology*, 79(3):1954-1957.

Hosie M.J., Osborne R., Reid G., Neil J.C., Jarrett O., 1992. Enhancement after feline immunodeficiency virus vaccination. *Veterinary Immunology and Immunopathology*, 35: 191-197.

Hosie M.J., Osborne R., Yamamoto J.K., Neil J.C., Jarrett O., 1995. Protection against Homologous but not heterologous challenge induced by inactivated Feline Immunodeficiency Virus Vaccines. *Journal of Virology*, February: 1253-1255.

Hosie M.J., Pajek D., Samman A., Willet B.J., (2011). Feline immunodeficiency virus (FIV) Neutralisation: A Review. *Viruses*, 3: 1870-1890.

Hosie M.J., Robertson C., Jarret O., 1989. Prevalence of feline leukaemia and antibodies to feline immunodeficiency virus in cats in the United Kingdom. *Veterinary Record,* 128: 293-297.

Hosie M.J., Willett B.J., Klein D., Dunsford T.H., Cannon C., Shimojima M., Neil J.C., Jarrett O., 2002. Evolution of Replication Efficiency following Infection with a Molecularly Cloned Feline Immunodeficiency Virus of Low Virulence. *Journal of Virology*, 76(12):6062-6072.

Huang C., Conlee D., Gill M., Chu H.J., 2010. Dual-subtype feline immunodeficiency virus vaccine provides 12 months of protective immunity against heterologous challenge. Journal of *Feline Medicine and Surgery*, 12: 451-457.

Huang C., Conlee D., Loop J., Champ D., Gill M., Chu H.J., 2004. Efficacy and safety of a feline immunodeficiency virus vaccine. *Animal Health Research Reviews*, 5 (2): 295-300.

Huisman W., Karlas J.A., Siebelink K.H.J., Huisman R.C., de Ronde A., Francis M.J., Rimmelzwaan G.F., Osterhaus A.D.M.E., 1998. Feline immunodeficiency virus subunit vaccines that induce virus neutralising antibodies but no protection against challenge infection. *Vaccine*, 16 (2/3):181-187.

Ishida T., Washizu T., Toriyabe K., Motoyoshi S., Tomoda I., Pedersen N.C., 1989. Feline immunodeficiency virus infection in cats of Japan. *Journal of the American Veterinary Medical Association,* 194 (2): 221-225.

Jardine J.G., Ota T., Sok D., Pauthner M., Kulp D.W., Kalyuzhniy O., Skog P.D., Thinnes T.C., Bhullar D., Briney B., Menis S., Jones M., Kubitz M., Spencer S., Adachi Y., Burton D.R., Schief W.R., Nemazee D., (2015). Priming a broadly neutralising antibody response to HIV-1 using a germline-targeting immunogen. *Science*, 349(6244): 156-161.

Kakinuma S., Motokawa K., Hohdatsu T., Yamampto J.K., Koyama H., Hashimoto H., 1995. Nucleotide Sequence of Feline Immunodeficiency Virus: Classification of Japanese Isolates into Two Subtypes Which Are Distinct from Non-Japanese Subtypes. *Journal of Virology*, 69 (6): 3639-3646.

Kania S.A., Kennedy M.A., Potgieter L.N.D., (1997). Serologic reactivity using conserved envelope epitopes in feline lentivirus-infected felids. *Journal of Veterinary Diagnostic Investigation,* 9: 125-129.

Karlas J.A., Siebelink K.H.J., van Peer M.A., Huisman W., Cuisinier A.A., Rimmelzwaan G.F., Osterhaus A.D.M.E., 1999. Vaccination with experimental feline immunodeficiency virus vaccines, based on autologous infected cells, elicits enhancement of homologous challenge infection. *Journal of General Virology*, 80:761-765.

Kenyon J.C., Lever A.M.L., (2011). The Molecular Biology of Feline Immunodeficiency Virus (FIV). *Viruses,* 3: 2192-2213.

KOHL, N. E., EMINI, E. A., SCHLEIF, W. A., DAVIS, L. J., HEIMBACH, J. C., DIXON, R. A., SCOLNICK, E. M. & SIGAL, I. S., (1988). Active human immunodeficiency virus protease is required for viral infectivity. *Proceedings of the National Academy of Sciences of the United States of America,* 85**:** 4686-4690.

Kusuhara H., Hohdatsu T., Okumura M., Sato K., Suzuki Y., Motokawa K., Gemma T., Wantanabe R., Huang C., Arai S., Koyama, 2005. Dual-subtype vaccine (Fel-O-Vax FIV) protects against contact challenge with a heterologous subtype B FIV infected cats. *Veterinary Microbiology*, 108: 155-165.

Kusuhara H., Hohdatsu T., Seta T., Nemoto K., Motokawa K., Gemma T., Watanabe R., Huang C., Arai S., Koyama H., 2007. Serological differentiation of FIV-infected cats from dual-subtype feline immunodeficiency virus vaccine (Fel-O-Vax FIV) inoculated cats. *Veterinary Microbiology* 120: 217-225.

Leutenegger C.M., Klein D., Hofmann-Lehmann R., Mislin C., Hummel U., Boni J., Boretti F., Guenzburg., Lutz H., (1999). Rapid feline immunodeficiency provirus quantitation by polymerase chain reaction using the TaqMan® fluorogenic real-time detection system. *Journal of Virological Methods*, 78: 105-116.

Lever A.M., (2007). HIV-1 RNA packaging. *Advances in Pharmacology*, 55:1–32.

Levy J. K., Crawford P.C., Slater M. R., 2004. Effect of vaccination against feline immunodeficiency virus on the results of serological testing in cats. *Journal of American Veterinary Medical Association* 225 (10): 1558-1562.

Levy J.K., Crawford P.C., Kusuhara H., Motokawa K., Gemma T., Watanabe R., Arai S., Bienzle D., Hohdatsu T., 2008. Differentiation of Feline Immunodeficiency Virus Vaccinated, Infected, or Vaccination and Infection in Cats. *Journal of Veterinary International Medicine*, 22: 330-334.

Levy J.K., Crawford P.C., Slater M.R., 2004. Effect of vaccination against feline immunodeficiency virus on results of serological testing in cats. *Journal of the American Veterinary Medical Association*, 225(10): 1558-1561.

Levy J.K., Scott H. M., Lachtara J.L., Crawford P.C., 2006. Seroprevalence of feline leukemia virus and feline immunodeficiency virus infection among cats in North America and risk factors for seropositivity. *Journal of the American Veterinary Medical Association*, 228 (3): 371- 376.

Liao H.X., Lynch R., Zhou T., Gao F., Alam S.M., Boyd S.D., Fire A.Z., Roskin K.M., Schramm C.A., Zhang Z., Zhu J., Shapiro L., NISC Comparative Sequencing Program, Mullikin J.C., Gnanakaran S., Hraber P., Wiehe K., Kelsoe G., Yang G., Xia S.M., Montefiori D.C., Parks R., Lloyd K.E., Scearce R.M., Soderberg K.A., Cohen M., Kamanga G., Louder M.K., Tran L.M., Chen Y., Cai F., Chen S., Moquin S., Du X., Joyce M.G., Srivatsan S., Zhang B., Zheng A., Shaw G.M., Hahn B.H., Kepler T.B., Korber B.T.M., Kwong P.D., Mascola J.R., Haynes B.F., (2013). Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature,* 0: 1-11.

Litster A., Lin JM., Nichols J., Weng HY., 2014. Diagnostic utility of CD4%:CD8^{low}% T-lymphocyte ratio to differentiate feline immunodeficiency virus (FIV)- infected from FIV-vaccinated cats. *Veterinary Microbiology* 170(3-4): 197-205.

Lombardi S., Garzelli C., Pistello M., Massi C., Matteucci D., Baldinotti F., Cammarota G., Da Proto L., Bandecchi P., Tozzini F., Bendinelli M., (1994). A Neutralising Antibody

Inducing Peptide of the V3 Domain of Feline Immunodeficiency Virus Envelope Glycoprotein Does Not Induce Protective Immunity. *Journal of Virology*, 68 (12): 8374-8379.

Luttge, B. G. & Freed, E. O., (2010). FIV Gag: virus assembly and host-cell interactions. *Veterinary Immunology and Immunopathology,* 134**:** 3-13.

Matteucci D., Pistello M., Mazzatti P., Giannecchini S., Del Mauro D., Zaccaro L., Bandecchi P., Tozzini F., Bendinelli M., 1996. Vaccination protects against in vivo-grown feline immunodeficiency virus even in the absence of detectable neutralising antibodies. *Journal of Virology*, 70(1):617-622.

Matteucci D., Pistello M., Mazzetti P., Giannecchini S., Del Mauro D., Lonetti I., Zaccaro L., Pollera C., Specter S., Bendinelli M., 1997. Studies of AIDS vaccination using an ex vivo feline immunodeficiency virus model: protection conferred by a fixed-cell vaccine against cell-free and cell-associated challenge differs in duration and is not easily boosted. *Journal of Virology*, 71(11): 8368-8376.

Miyazawa T., Furuya T., Itagaki S., Tohya Y., Takahashi E., Mikami T., 1989. Establishment of a feline T-lymphoblastoid cell line highly sensitive for replication of feline immunodeficiency virus. *Archives in Virology* 108:131-135

Muirden A., 2002. Prevalence of feline leukaemia virus and antibodies to feline immunodeficiency virus and feline coronavirus in stray cats sent to a RSPCA hospital. *The Veterinary Record*, 150: 621-625.

Nakamura Y., Nakamura Y., Ura A., Hirata M., Sakuma M., Sakata Y., Nishigaki K., Tsujimoto H., Setoguchi A., Endo Y., 2010. An Updated Nation-Wide Epidemiological Survey of Feline Immunodeficiency Virus (FIV) Infection in Japan. *Journal of Veterinary Medical Science,* 78 (8): 1051-1056.

Nichols J., Weng H.Y., Litster A., Leutenegger C., Guptill L., 2016. Commercially available Enzyme-Linked Immunosorbent Assay and Polymerase Chain Reaction Tests for Detection of Feline Immunodeficiency virus infection. *Journal of Veterinary Internal Medicine*: 1-5.

Norris J.M., Bell E.T., Hales L., Toribio J.A.L.M.L., white J.D., Wigney D.I., Baral R.M., Malik R., (2007). Prevalence of feline immunodeficiency virus infection in domesticated and feral cats in eastern Australia. *Journal of Feline Medicine and Surgery,* 9: 300-308.

O'Connor T.P., Tanguay S., Steinman R., Smith R., Barr M.C., Yamamoto J.K., Pedersen N.C., Andersen P.R., Tonelli Q., 1989. Development and Evaluation of Immunoassay for Detection of Antibodies to the Feline T-Lymphotropic Lentivirus (Feline Immunodeficiency Virus). *Journal of Clinical Microbiology*, 27 (3), 474-479.

O'Rourke S.M., Schweighardt B., Phung P., Mesa K.A., Vollrath A.L., Tatsuno G.P., To B., Sinangil F., Limoli K., Wrin T., Berman P.W., (2012). Sequences in Glycoprotein gp41, the CD4 Binding Site, and the V2 Domain Regulate Sensitivity and Resistance of HIV-1 to Broadly Neutralising Antibodies. *Journal of Virology*, 86 (22): 12105-12114.

Pancino G., Camoin L., Songio P., 1995. Structural Analysis of the Principal Immunodominant Domain of the Feline Immunodeficiency Virus Transmembrane Glycoprotein. *Journal of Virology*, 69(4): 2110-2118.

Pancino G., Songio P., 1997. Retention of Viral Infectivity after Extensive Mutation of the Highly Conserved Immunodominant Domain of the Feline Immunodeficiency Virus Envelope. *Journal of Virology*, 71(6): 4339-4346.

Pecoraro M.R., Tomonaga K., Miyazawa T., Kawaguchi Y., Sugita S., Tohya Y., Kai C., Etcheverrigaray M.E., Mikami T., (1996). Genetic diversity of Argentine isolates of feline immunodeficiency virus. *Journal of General Virology*, 77: 2031-2035.

Pedersen N.C., Ho E.W., Brown M.L., Yamamoto J.K., 1987. Isolation of a T-Lymphotropic Virus from Domestic Cats with an Immunodeficiency-Like Syndrome. *Science,* 235:790-793.

Pedersen N.C., Yamamoto J.K., Ishida T., Hansen H., 1989. Feline Immunodeficiency Virus Infection. *Veterinary Immunology and Immunopathology,* 21: 111-129.

Petrov R.V., Khaitov R.M., Sidorovich I.G., Pavlikov S.P., Nikolaeva I.A., Ivachenko M.E., Andreev S.M., Sklyarov L.Y., (1990). The use of synthetic peptides in the diagnosis of HIV infections. *Biomedical Science*, 1: 239-244.

Pinches M.D.G., Diesel G., Helps C.R., Tasker S., Egan K., Gruffydd-Jones T.J., 2007. An update on FIV and FeLV test performance using a Bayesian statistical approach. *Veterinary Clinical Pathology*, 36(2): 141-147.

Pinter A., Honnen W.J., He Y., Gorny M.K., Zolla-Pazner S., Kayman S.C., (2004). The V1/V2 Domain of the gp120 Is a Global Regulator of the Sensitivity of Primary Human Immunodeficiency Virus Type 1 Isolates to Neutralization by Antibodies Commonly Induced upon Infection. *Journal of Virology*, 78 (10): 5205-5215.

Pistello M., Bonci F., Zabogli E., Conti F., Freer G., Maggi F., Stevenson M., Bendinelli M., 2010. Env-Expressing Autologous T Lymphocytes Induce Neutralising Antibodies and afford Marked Protection against Feline Immunodeficiency Virus. *Journal of Virology*, 84 (8):3845-3856.

Pu R., Coleman J., Coisman J., Sato E., Tanabe T., Arai M., Yamamoto J.K., 2005. Dual-subtype FIV vaccine (Fel-O-Vax® FIV) protection against a heterologous subtype B FIV isolate. *Journal of Feline Medicine and Surgery* 7:65-70.

Pu R., Coleman J., Omori M., Arai M., Hohdatsu T., Huang C., Tanabe T., Yamamoto J.K., 2001. Dual-subtype FIV vaccine protects cats against in vivo swarms of both homologous and heterologous subtype FIV isolates. *AIDS*, 15:1225-1237.

Pu R., Coleman J., Omori M., Arai M., Hohdatsu T., Huang C., Tanabe T., Yamamoto J.K., 2001. Dual-subtype FIV vaccine protects cats against in vivo swarms of both homologous and heterologous subtype FIV isolates. *AIDS* 15:1225-1237.

Ravi M., Wobeser G.A., Taylor S.M., Jackson M.L., 2010. Naturally acquired feline immunodeficiency virus (FIV) infection in cats from western Canada: Prevalence, disease association, and survival analysis. *Canadian Veterinary Journal,* 51: 271-276.

Raviv Y., Viard M., Bess J.W., Chertova E., Blumenthal R., (2005). Inactivation of retroviruses with Preservation of Structural Integrity by Targeting the Hydrophobic Domain of the Viral Envelope. *Journal of Virology,* 79 (19): 12394-12400.

Reid G., Rigby M.A., McDonald M., Hosie M.J., Neil J.C., Jarrett O., 1991. Immunodiagnosis of feline immunodeficiency virus infection using recombinant viral p17 and p24. *AIDS*, 5: 1477-1483.

Richardson J., Moraillon A., Baud S., Cuisinier A.M., Sonigo P., Pancino G., 1997. Enhancement of feline Immunodeficiency Virus (FIV) Infection after DNA Vaccination with the FIV Envelope. *Journal of Virology*, December: 9640-9649.

Rimmelzwaan G.F., Siebelink K.H.J., Broos H., Drost G.A., Weijer K., van Herwijnen R., Osterhaus A.D.M.E., 1994. *gag*- And *env*-specific serum antibodies in cats after natural and experimental infection with feline immunodeficiency virus. *Veterinary Microbiology*, 39: 153-165.

Rolland M., Edlefsen P.T., Larsen B.B., Tovanabutra S., Sanders-Buell E., Hertz T., decamp A.C., Carrico C., Menis S., Margaret C.A., Ahmed H., Juraska M., Chen L., Konopa P., Nariya S., Stoddard J.N., Wong K., Zhao H., Deng W., Maust B.S., Bose M., Howell S., Bates A., Lazzaro M., O'Sullivan A., Lei E., Bradfield A., Ibitamuno G., Assawadarachai V., O'Connell R. J., deSouza M.S., Nitayaphan S., Rerks-Ngarm S., Robb M.L., McLellan J.S., Georgiev I., Kwong P.D., Carlson J.M., Michael N.L., Schief W.R., Gilbert P.B., Mullins J.I., Kim J.H., (2012). Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. *Nature*, 490: 417-421.

Rossio J.L., Esser M.T., Suryanarayana K., Schneider D.K., Bess J.W., Vasquez G. M., Wiltrout T.A., Chertova E., Grimes M.K., Sattentau Q., Arthur L.O., Henderson L.E., Lifson J.D., (1998). Inactivation of Human Immunodeficiency Virus Type 1 Infectivity with Preservation of Conformational and Functional Integrity of Virion Surface Proteins. *Journal of Virology,* 72 (10): 7992-8001.

Samman A., (2010). The role of virus neutralisation in immunity to feline immunodeficiency virus infection. PhD Thesis. Institute of infection, immunity and inflammation. https://glasgow.summon.serialssolutions.com/?s.q=phd+thesis&s.cmd=addFacetValueFilters%28ContentType%2CNewspaper+Article%2Ct%7CContentType%2CBook+Review%2Ct%29#!/search?ho=t&fvf=ContentType,Newspaper%20Article,t%7CContentType,Book%20Review,t%7CContentType,Dissertation,f&l=en-UK&q=phd%20thesis%20samman

Sanchez-Merino J., Fabra-Garcia A., Gonzalez N., Nicolas D., Merino-Mansilla A., Manzardo C., Ambrosioni J., Schultz A., Meyerhans A., Mascola J.R., Gatell J.M., Alcami J., Miro J.M., Yuste E., (2016). Detection of Broadly Neutralizing Activity within the First Months of HIV-1 Infection. *Journal of Virology*, 90 (11): 5231-5245.

Schupbach J., Tomasik Z., Knuchel M., Opravil M., Gunthard H.F., Nadal D., Boni J., and The Swiss HIV cohort study (SHCS) and the Swiss HIV mother + child study (MoCHiV) 2006. Optimized virus disruption improves detection of HIV-1 p24 in particles and uncovers p24 reactivity in patients with undetectable HIV-1 RNA under long-term HAART. *Journal of Medical Virology* 78:1003-1010.

Shimojima M., Miyazawa T., Ikeda Y., McMonagle E. L., Haining H., Akashi Y., Takeuchi Y., Hosie M.J., Willett B.J., 2004. Use of CD134 as a primary receptor by the feline immunodeficiency virus. *Science* 303: 1192:1195.

Shin S.Y., Lee M.K., Kim S.Y., Jang S.Y., Hahm K.S., (1997). The use of multiple antigenic peptide (MAP) in the Immunodiagnosis of Human Immunodeficiency Virus infection. *Biochemistry and Molecular Biology International*, 43 (4): 713-721.

Siebelink K.H., Tijhaar E., Huisman R.C., Huisman W., de Ronde A., Darby I.H., Francis M.J., Rimmelzwaan G.F., Osterhaus A.D., 1995. Enhancement of feline immunodeficiency virus infection after immunization with envelope glycoprotein subunit vaccines. *Journal of Virology*, 69(6):3704-3711.

Siebelink K.H.K., Karlas J.A., Rimmelzsaan G.F., Osterhaus A.D.M.E., Bosch M.L., (1995). A determinant of feline immunodeficiency virus involved in Crandell Feline Kidney cell tropism. *Veterinary Immunology and Immunopathology*, 465: 61-69.

Sodora D.L., Shpaer E.G., Kitchell B.E., Dow S.E., Hoover E.A., Mullins J.I., (1994). Identification of three Feline Immunodeficiency Virus (FIV) *env* gene subtypes and comparision of the FIV and Human Immunodeficiency Virus type 1 evolutionary patterns. *Journal of Virology*, 68 (4): 2230-2238.

Sparger E.E., Luciw P.A., Elder J.H., Yamamoto J.K., Lowenstine L.J., Pedersen N.C., 1989. Feline immunodeficiency virus is a lentivirus associated with an AIDS-like disease in cats. *AIDS*, 3 (supplement 1): S43-S49.

Steinman R., Dombrowski J., O'Connor T., Montelaro R.C., Tonelli Q., Lawrence K., Seymour C., Goodness J., Pedersen N.C., (1990). Biochemical and immunological characterization of the major structural proteins of feline immunodeficiency virus. *Journal of General Virology*, 71: 701-706.

Stephens E.B., Monck E., Reppas K., Butfiloski E.J., (1991). Processing of the Glycoprotein of Feline Immunodeficiency Virus: Effects of Inhibitors of Glycosylation. *Journal of Virology*, 65 (3): 1114-1123.

Talbott R.L., Sparger E.E., Lovelave K.M., Fitch W.M., Pedersen N.C., Luciw P.A., Elder J.H., (1989). Nucleotide sequence and genomic organisation of feline immunodeficiency virus. *Proceeding of the National Academy of Sciences*, 86: 5743-5747.

Tilton G.K., O'Connor T., Seymour C.L., Lawrence K.L., Cohen N.D., Andersen P.R., Tonelli Q.J., 1990. Immunoassay for Detection of Feline Immunodeficiency Virus Core Antigen. *Journal of Clinical Microbiology*, 28 (5): 898-904.

Uhi E.W., Heaton-Jones T.G., Pu R., Yamamoto J.K., 2002. FIV vaccine development and its importance to veterinary and human medicine: a review FIV vaccine 2002 update and review. *Veterinary Immunology and Immunopathology*, 90: 113-132.

van Vuuren M., Stylianides E., Kania S.A., Zuckerman E.E., Hardy W.D., (2003). Evaluation of an indirect enzyme-linked immunosorbent assay for the detection of feline lentivirus-reactive antibodies in wild felids, employing a puma lentivirus-derived synthetic peptide antigen. *Onderstepoort Journal of Veterinary Research*, 70: 1-6.

Vereschoor E.J., Boven L.A., Blaak H., van Vliet A.L.W., Horzinek M.C., de Ronde A., (1995). A single mutation within the V3 envelope neutralisation domain of feline immunodeficiency virus determines it tropism for CRFK cells. *Journal of Virology*, 69 (8): 4752-4757.

Verschoor E.J., Hulskotte E.G., Ederveen J., Koolen M.J.M., Horzinek M.C., Rottier P.J.M., (1993). Post-translational Processing of the Feline Immunodeficiency Virus Envelope Precursor Protein. *Virology*, 193: 433-438.

Verschoor E.J., van Vliet A.L.W., Egberink H.F., Hesselink W., Horzinek M.C., de Ronde A., 1993. Expression of feline Immunodeficiency Virus *gag* and *env* Precursor Proteins in *Spodoptera frugiperda* Cells and Their Use in Immunodiagnosis, *Journal of Clinical Microbiology*, 31(9): 2350-2355.

Wagaman P.C., Kent S.B., (1993). Identification of proteolytic processing sites within the gag and pol polyproteins of feline immunodeficiency virus. *Journal of Virology*, 67: 1869–1876.

Wang C., Johnson C. M., Ahluwalia S.K., Chowdhury E., Li Y., Gao D., Poudel A., Rahman K.S., Kaltenboech B., 2010. Dual-Emission Fluorescence Resonance Energy Transfer (FRET)

Real-Time PCR Differentiates Feline Immunodeficiency Virus Subtypes and Discriminates Infected from Vaccinated Cats. *Journal of Clinical Microbiology* 48(5): 1667-1672.

Weaver E.A., Collisson E.W., Slater M., Zhu G., (2004). Phylogenetic analysis of Texas isolates indicates an evolving subtype of the clade B feline immunodeficiency virus. *Journal of Virology,* 78 (4): 2158-2163.

Weijer K., Langedijk H., van Herwijnen R., Pfauth A., Siebelink K., Rimmelzwaan G., Osterhaus A., Jarrett O., 1995. Location of B cell epitopes on the core protein p24 of feline immunodeficiency virus. Feline immunology and immunodeficiency, *Oxford science publication.* 279-305.

Westman M.E., Malik R., Hall E., Sheehy P.A., Norris J.M., 2015. Determining the feline immunodeficiency virus (FIV) status of FIV-Vaccinated cats using point-of-care antibody kits. *Comparative immunology, Microbiology and Infectious Disease* 42: 43-52.

Willett B.J., Hosie M.J., Callanan J.J., Neil J.C., Jarrett O., (1992). Infection with feline immunodeficiency virus is followed by the rapid expansion of a $CD8^{+}$ lymphocytes subset. *Immunology*, 78: 1-6.

Willett B.J., McMonagle E.L., Ridha S., Hosie M., 2006. Differential utilization of CD134 as a functional receptor by diverse strains of Feline immunodeficiency virus. *Journal of Virology* 80(7): 3386-3394.

Yamamoto J.K., Ackley C.D., Zochlinski H., Louie H., Pembroke E., Torten M., Hansen H., Munn R., Okuda T., 1991. Development of IL-2-Independent Feline Lymphoid Cell Lines Chronically Infected with Feline Immunodeficiency Virus: Importance for Diagnostic Reagents and Vaccines. *Intervirology*, 32:361-375.

Yamamoto J.K., Hansen H., Ho E.W., Morishita T.Y., Okuda T., Sawa T.R., Nakamura R.M., Pedersen N.C., 1989. Epidemiological and clinical aspects of feline immunodeficiency virus

infection in cats from the continental United States and Canada and possible mode of transmission. *Journal of the American Veterinary Medical Association*, 194 (2): 213-220.

Yamamoto J.K., Hohdatsu T., Olmsted R.A., Pu R., Louie H., Zochlinski H.A., Acevedo V., Johnson H.M., Soulds G.A., Gardner M.B., 1993. Experimental vaccine protection against homologous and heterologous strains of feline immunodeficiency virus. *Journal of Virology*, 67 (1): 601-605.

Yamamoto J.K., Okuda T., Ackley C.D., Louie H., Pembroke E., Zochlinski H., Munn R. J., Gardener M.B., 1991. Experimental Vaccine protection against feline immunodeficiency virus. *AIDS Research and Human Retroviruses*, 7 (11): 911-922.

Zemba M., Alke A., Bodem J., Winkler I.G., Flower R.L.P., Pfrepper K.l., Delius H., FluÈgel R.M., LoÈchelt M., 2000. Construction of Infectious Feline Foamy Virus Genomes: Cat Antisera Do Not Cross-Neutralize Feline Foamy Virus Chimera with Serotype-Specific Env Sequences. *Virology* 266: 150-156.