THE UNIVERSITY OF SYDNEY

Epidemiology and diagnosis of feline retroviruses (FIV and FeLV) in Australia and a trial of FIV vaccine effectiveness in the field

© Mark Edward Westman

BVSc (Hons I) MANZCVS (Animal Welfare)

A thesis submitted in fulfilment of the requirements for the degree of **Doctor of Philosophy**

School of Life and Environmental Sciences

Faculty of Veterinary Science

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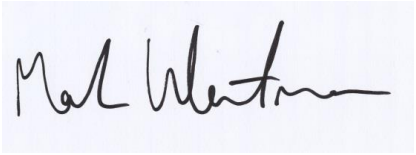
DECLARATION

This thesis is submitted to The University of Sydney in fulfilment of the requirements for the Degree of Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, in either full or in part, for a degree at this or any other institution.

Parts of this thesis have been published in the candidate's name (see List of Peer Reviewed Publications below).



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LIST OF PEER REVIEWED PUBLICATIONS

1. **Westman ME**, Malik R, Hall E, Harris M, Hosie MJ, Norris JM **(2016).** Duration of antibody response following vaccination against feline immunodeficiency virus (FIV). Accepted for publication in *Journal of Feline Medicine and Surgery*, 9th September, 2016. doi.org/10.1177/1098612X16673292

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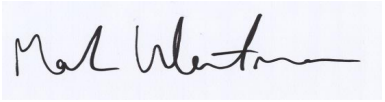
DISCLOSURE AND AUTHOR CONTRIBUTIONS

The essence of Chapters 2, 3, 4, 5 and 6 are published in peer-reviewed journals entitled:

- Seroprevalence of feline immunodeficiency virus and feline leukaemia virus in Australia: risk factors for infection and geographical distribution (2011-2013). *Journal of Feline Medicine and Surgery Open Reports 2016*. doi.org/10.1177/2055116916646388 **(Chapter 2)**
- Determining the feline immunodeficiency virus (FIV) status of FIV-vaccinated cats: using rapid point-of-care kits to solve this diagnostic dilemma *Journal of Comparative Immunology Microbiology and Infectious Diseases* 2015 4; *43-52*. doi.org/10.1016/j.cimid.2015.07.004 **(Chapter 3)**
- Diagnosing feline immunodeficiency virus (FIV) infection in FIV-vaccinated and FIV-unvaccinated cats using saliva. *Journal of Comparative Immunology Microbiology and Infectious Diseases 2016* 46; 66-72. doi.org/10.1016/j.cimid.2016.03.006 **(Chapter 4)**
- Duration of antibody response following vaccination against feline immunodeficiency virus (FIV). *Journal of Feline Medicine and Surgery* (in press), accepted for publication 9th September, 2016. doi.org/10.1177/1098612X16673292 **(Chapter 5)**
- The protective rate of the feline immunodeficiency virus vaccine: an Australian field study. *Vaccine 2016* 34; *4752-4758*. doi.10.1016/j.vaccine.2016.06.060 (**Chapter 6)**

I, *Mark Westman*, was primarily responsible for study design, conducting the laboratory work, statistical analysis of the data, interpretation of the findings and writing the manuscripts. I am very grateful to: Associate Professor Jacqui Norris, Professor Richard Malik, Associate Professor Paul Sheehy, Dr. Evelyn Hall, Dr. Phillip McDonagh, Dr. Amanda Paul, Professor Michael Ward, Professor Margaret Hosie, Matthew Harris, Dr. Andrea Harvey, Dr. Martine Perkins and Dr. Donna Schofield for their assistance with study designs, laboratory work, data analyses and editing of the manuscripts.



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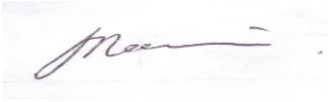

Jacqueline Norris, Primary Supervisor

5th July, 2016

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This thesis includes seven original papers, of which five been published in peer-reviewed journals, and two have been submitted to peer-reviewed journals and are awaiting review.

The ideas, development and writing up of all the papers in this thesis were the principal responsibility of the candidate, working within the Faculty of Veterinary Science, The University of Sydney, under the supervision of Jacqueline Norris and associate supervisors Richard Malik and Paul Sheehy. Peter Bracken (Boehringer Ingelheim) assisted with initial study design of the FIV vaccine effectiveness trial (Chapter 6). Evelyn Hall assisted with statistical assessments and finalising manuscripts. Karen Mathews provided guidance with some laboratory work including amplicon sequencing and plasmid cloning (Chapter 7). Christine Cole, Martine Perkins and Donna Schofield were incredibly helpful with recruitment and sampling of cats for the duration of antibody response following FIV vaccination study (Chapter 5). Yamamoto Laboratories (Janet Yamamoto, Florida, USA) and Veterinary Diagnostic Services (Margaret Hosie, Glasgow, UK) generously assisted with laboratory work that was unable to be performed in Australia. Portions of this thesis have been proof read by Alan Marcus, Phillip McDonagh, Andrea Harvey and Anna Dean.

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extra special, and going forward it will be my friends and colleagues who I will miss the most.

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ABBREVIATIONS

5UTR	Five prime untranslated region
ACT	Australian Capital Territory
BI	Boehringer Ingelheim
bp	Base pair
BVH	Bankstown Veterinary Hospital (NSW)
CAH	Campbelltown Animal Hospital (NSW)
cDNA	Complementary DNA
CID_{50}	Cat infectious dose (equivalent to the amount of virus required to cause infection in half of susceptible subjects)
CIs	Confidence intervals
CMI	Cell-mediated immunity
C_T	Cycle threshold
CV	Coefficient of variation
CVH	Casula Veterinary Hospital (NSW)
DOI	Duration of immunity
EDAH	Elizabeth Drive Animal Hospital (NSW)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FcaGHV1	*Felis catus* gammaherpesvirus 1
FCoV	Feline coronavirus
FDAH	Fort Dodge Animal Health
FeLV	Feline leukaemia virus
FeSV	Feline sarcoma virus
FGVS	Fulham Gardens Veterinary Surgery (SA)
FIV	Feline immunodeficiency virus
FIV_{Ao2} (B) (Aomori)	FIV isolated from Aomori, Japan

FIV_{Bang} (A/B)	FIV isolated from Massachusetts, USA
FIV_{FC1} (B)	FIV isolated from Florida, USA
$FIV_{FD/DutA}$ (A)	FIV isolated from the Netherlands
$FIV_{FD/US}$ (A)	FIV isolated from California, USA
FIV_{NZ1} (F'/C)	FIV isolated from Auckland, New Zealand (prime sign represents that a full sequence of subtype F has yet to be identified)
FIV_{Pet} (A)	FIV isolated from California, USA
FIV_{Shi} (D)	FIV isolated from Shizuoka, Japan
FIV_{UK8} (A)	FIV isolated from Glasgow, UK
GWAH	Great Western Animal Hospital (NSW)
HIV	Human immunodeficiency virus
IFA	Immunofluorescent antibody (FIV) / immunofluorescent antigen (FeLV)
IC	Immunochromatography
Ig	Immunoglobulin
IQR	Interquartile range
ISVH	Inner South Veterinary Hospital (ACT)
IV	Intravenous
IWC	Inactivated whole cell
IWV	Inactivated whole virus
LB	Lysogeny broth
LTR	Long terminal repeat
MAVH	Mt Annan Veterinary Hospital (NSW)
MUVH	Murdoch University Veterinary Hospital (WA)
NPV	Negative predictive value
NSW	New South Wales
NT	Northern Territory
OD	Optical density

OR	Odds ratio
Orfs	Open reading frames
PCR	Polymerase chain reaction
PBMCs	Peripheral blood mononuclear cells
PPV	Positive predictive value
PR	Protective rate
QLD	Queensland
qPCR	Real-time PCR
qRT-PCR	Real-time reverse-transcription PCR
REML	Restricted maximum likelihood
RT	Reverse transcription
SA	South Australia
SBA	Sheep blood agar
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
TAS	Tasmania
V	Volts
VI	Virus isolation
VIC	Victoria
vRNA	Viral RNA
WA	Western Australia

ABSTRACT

Feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) remain important infections of domestic cats in Australia and overseas. This thesis begins by providing up-to-date Australian prevalence data in three cohorts of cats, comprising approximately 4,300 cats. Interestingly, the prevalence of both retroviral infections is higher in Perth, Western Australia compared to the rest of the country (Chapter 2). The diagnosis of FIV infection in FIV-vaccinated cats using fast, readily accessible, cheap point-of-care antibody kits, previously thought impossible, is reported in Chapter 3, which includes also the results of FIV testing using PCR methodology. This finding will expedite the diagnosis of FIV by veterinarians in most clinical scenarios encountered in the field, and has already contributed to changed recommendations concerning the use of the FIV vaccine (WSAVA Vaccination Guidelines, changed from 'Not Recommended' in 2010 to 'Non-Core' in 2015). Using the same cohort of cats and same antibody test kits, it was discovered that FIV infection can also be reliably diagnosed in FIV-vaccinated and FIV-unvaccinated cats using saliva by two of the kits tested (Chapter 4). The duration of antibody response in FIV-vaccinated cats using four commercially available FIV antibody test kits and a prospective study of client-owned kittens and cats, and the effect on point-of-care testing for FIV infection, is reported in Chapter 5. The first ever study into the effectiveness of the only currently commercially available FIV vaccine in the field is reported in Chapter 6. The effectiveness (protective rate) of the vaccine was determined to be 56%, and five confirmed vaccine 'breakthroughs' are discussed, these being the first vaccine breakthroughs observed in the field. This finding has stimulated renewed discussion about the effectiveness of the FIV vaccine (e.g. meeting of Key Opinion Leaders organised by Boehringer Ingelheim, The Shangri-La Hotel, Sydney, 24th June, 2016) and will likely lead

to further studies in other countries using similar methodology. Diagnosis of FeLV infection was investigated using both blood and saliva as diagnostic specimens with the same three commercially available kits tested in Chapters 3 and 4 (kits were FIV/FeLV combination kits) and also an in-house real-time PCR assay, with one kit found to produce substantially more false-positive results using blood than the other two kits (Chapter 7). The combined outcomes of Chapters 3, 4 and 7 will provide guidance for Australian veterinarians on the most accurate FIV/FeLV test kits to use in practice, as well as introduce the concept to shelters and rescue organisations who currently routinely screen for FIV/FeLV that testing using saliva is a viable and less stressful alternative to testing using blood. Finally, an in-depth study of possible outcomes following FeLV exposure for the client-owned cats recruited for Chapter 6 and two cohorts of group-housed cats sampled from two different rescue facilities experiencing recent FeLV outbreaks is reported, leading to recommendations regarding FeLV testing and vaccination (Chapter 8).

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

The domestic cat (*Felis silvestris catus*) is host to three known exogenous retroviruses, all with worldwide distribution: feline immunodeficiency virus (FIV; subfamily Orthoretrovirinae, Genus *Lentivirus*), feline leukaemia virus (FeLV; subfamily Orthoretrovirinae, Genus *Gammaretrovirus*) and feline foamy virus (FFV; subfamily Spumaretrovirinae, Genus *Spumavirus*). While FFV is thought to be of minimal clinical significance, both FIV and FeLV result in a variety of immunologic perturbations that impact on morbidity and mortality.

1.1. Discovery and prevalence of FIV

FIV was first isolated in Petaluma, California, USA in 1986 following the investigation of an immunodeficiency syndrome in a cat colony. FIV was isolated by Pedersen and colleagues after inoculating two SPF kittens with whole blood and plasma obtained from three affected colony cats (Pedersen et al., 1987). IFA testing, using infected feline lymphocytes as the substrate, led to the diagnosis of 11/43 cats with FIV infection (then called feline T-lymphotropic lentivirus [FTLV]), of which one cat was clinically healthy and ten were sick. Signs of illness observed (to a varying extent in different individual cats) included gingivitis, dermatitis, otitis externa, rhinitis, conjunctivitis, cystitis, anaemia, thin body condition and diarrhoea. Over a period of four years, all ten 'sick' FIV-infected cats died. A short time later, an epidemiological survey found FIV to be endemic in the North American cat pet population (14% among 'high risk' cats) (Yamamoto et al., 1989).

FIV was subsequently shown to have worldwide distribution. An estimated 14.5 million pet cats are infected with FIV worldwide, and 33.5 million if feral cats are included

(Yamamoto et al., 2007). Healthy client-owned (pet) cat populations have been reported with infection rates of approximately 3% in Germany (Gleich et al., 2009b) and the USA (Levy et al., 2006), 6% in Canada (Ravi et al., 2010) and the United Kingdom (Hosie et al., 1989), 8% in Australia (Norris et al., 2007), 10% in New Zealand (Jenkins et al., 2013), 20% in Thailand (Sukhumavasi et al., 2012), 23% in Japan (Nakamura et al., 2010) and 31% in Malaysia (Bande et al., 2012). Serological surveys investigating the prevalence of FIV in stray and feral cat populations have found variable prevalence rates compared to the pet cat population. Feral and stray cats with full-time outdoor access and subjected to the dangers of fights with other potentially infected cats goes a long way to explaining the higher prevalence in these cats compared with pet cats, given the mode of transmission for FIV. For example, a small Australian survey of two feral cat colonies reported FIV prevalence of 20-25%, contrasting the 8% prevalence found in the pet cat sample population (Norris et al., 2007). Other studies have reported comparable FIV prevalence between shelter cats and pet cats, with differences likely relating to recruitment criteria (for example more sick cats are likely to be tested in veterinary clinics as part of an illness investigation, whereas shelters are more likely to test healthy cats as part of a standard screening process). For example, a North American study that recruited from 145 animal shelters located in the USA, Canada and Puerto Rico reported FIV prevalence of 2% in shelter cats (*versus* 3% in pet cats) (Levy et al., 2006), and a Canadian survey that recruited from 13 animal shelters representing 10 Canadian provinces reported FIV prevalence of 6% in shelter cats (*versus* 4% in pet cats) (Little et al., 2009).

An updated serosurvey of FIV prevalence rates in both client-owned and shelter cats in Australia forms the basis of the work presented in Chapter 2.

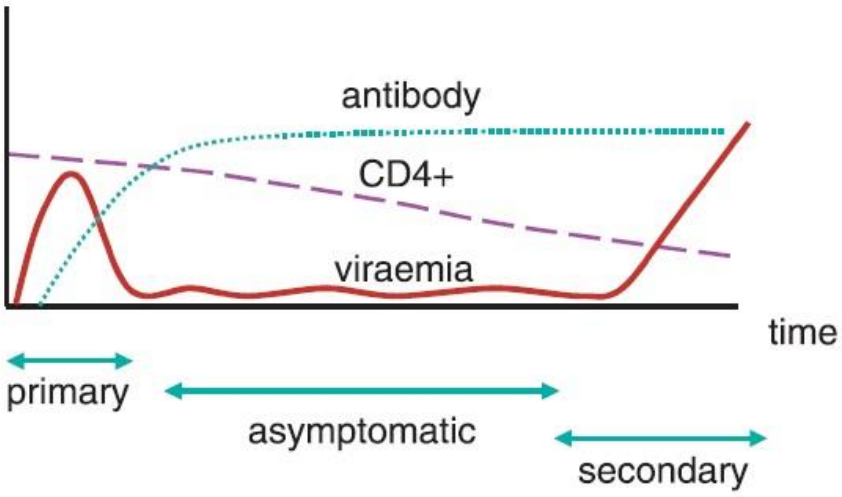
1.2. FIV transmission and disease associations

FIV has broad cellular tropism *in vivo*, targeting B lymphocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, macrophages and dendritic cells (Lecollinet and Richardson, 2008; Hosie et al., 2009). Cell entry is achieved by the FIV surface glycoprotein gp120 binding to a primary receptor on the cell surface, the CD134 molecule (Shimojima et al., 2004), with assistance from the ubiquitous chemokine receptor CXCR4 as co-receptor following conformational changes to gp120 (Willett et al., 1997; Lecollinet and Richardson, 2008). Within one week of experimental infection, FIV can be isolated from PBMCs and tissues including the thymus, spleen, lymph nodes, salivary glands and bone marrow of infected cats, and within two weeks it can be isolated from plasma and saliva (Yamamoto et al., 1989; Matteucci et al., 1993; Hosie et al., 2009). FIV transmission is usually via the inoculation of virus-laden saliva subcutaneously during a cat fight, thus entire male cats, castrated male cats and feral cats (all more likely to engage in displays of territorial aggression) are considered most at risk for infection (Yamamoto et al., 1989; Levy et al., 2006; Norris et al., 2007; Ravi et al., 2010). Vertical transmission of FIV has been demonstrated experimentally, although in the field this mechanism appears uncommon (Callanan et al., 1991; O'Neil et al., 1995; Allison and Hoover, 2003a, b).

Three main phases are recognised in cats experimentally infected with FIV. After initial infection, rapid viral replication leaks to peak viraemia 8-12 weeks later, which manifests clinically as generalised lymphadenomegaly, anorexia, depression and fever; this is called the *acute phase* of infection. Once viraemia declines, the infected cat enters a long period where few or no clinical signs are usually seen; this is called the *asymptomatic phase* of infection, and can last months but typically many years. By the time the infected cat reaches the final phase of infection, the *secondary phase*, the CD4:CD8 T lymphocyte ratio

has decreased due to a progressive CD4+ T lymphocytopenia and CD8+ T lymphocyte expansion, viral replication increases and a range of diseases classified as feline acquired immunodeficiency syndrome (FAIDS) may be observed (Torten et al., 1991; Willett et al., 1993; Hosie et al., 2009). Figure 1.1 illustrates schematically the three phases of infection described (Hosie and Beatty, 2007). Interestingly, although FIV causes progressive immune dysfunction due to a gradual depletion of CD4+ T lymphocytes, paradoxically a concurrent hyperimmunoactive state occurs which manifests as increased serum IgG levels reflective of FIV-induced virus-specific B-cell lymphocyte hyperactivity (Uhl et al., 2002). It is this B-cell hyperactivity that results in FIV-infected cats being at increased risk of developing high grade B-cell lymphomas compared with FIV-uninfected cats (Torten et al., 1991; Matsumura et al., 1993; Callanan et al., 1996).

Figure 1.1 Stages of FIV infection. (Reproduced from Hosie and Beatty, 2007).



In the field, many studies have reported some of the disease manifestations classified collectively as FAIDS. Often these disease manifestations are the result of opportunistic infections secondary to the animal's immunosuppressed state, rather than being a direct result of the virus; consequently, clinical signs associated with FIV-infection are widely

varied and generally non-specific (Hosie et al., 2009). The seminal FIV publication reporting the deaths of 10 FIV-infected cats listed a range of clinical signs including sudden death, enteritis, rhinitis, conjunctivitis, oral cavity disease, weight loss, neurological disease, anaemia, dermatitis, and 'terminal hysteria and rage' (Pedersen et al., 1987). A short time later, the same group studied 2,765 client-owned cats in USA and Canada and reported the most common clinical findings as oral cavity disease (56% of infected cats), anaemia (42%), upper respiratory tract infections (33%), leukopenia (30%), gastrointestinal disease (20%), neoplasia (3%) and central nervous system disease (< 1%) (Yamamoto et al., 1989). Around the same time, Ishida and colleagues reported a similar pattern of disease in 3,323 client-owned cats in Japan including oral cavity disease (52%), upper respiratory tract infections (29%), anaemia (18%), weight loss (15%), opportunistic bacterial infections (14%), skin disease (13%), enteritis (12%), renal disease (9%), lymphadenopathy (8%) and leukopenia (5%) (Ishida et al., 1989). Evidence for FIV-induced lymphomagenesis in pet cats, like experimentally infected cats, is strong yet largely circumstantial; Shelton and colleagues in America found FIV-infected cats were 5.6 times more likely to develop high-grade B-cell lymphoma than control cats negative for both FIV and FeLV (Shelton et al., 1990), while a study of client-owned cats in Australia diagnosed with lymphoma found 50% were FIV-positive using Western blot analysis, suggesting a causal relationship (Gabor et al., 2001b). Haematological and biochemical changes reported in FIV-infected cats include neutropenia, increased serum total protein concentration and increased ɣ–globulin concentration, compared to similarly aged control cats (Gleich and Hartmann, 2009a). In an Australian study of 911 cats, the prevalence of FIV among 'sick' cats was approximately three times that of 'healthy' cats (21% *versus* 8%) (Malik et al., 1997). An association between FIV infection and chronic

kidney disease (CKD) has been suggested from Australian and Italian field studies. Thomas and collaborators, investigating pet cats that had blood submitted to a Western Australian (WA) veterinary pathology laboratory, found FIV-infected cats were 3.1 times more likely to be azotaemic than FIV-uninfected cats (Thomas et al., 1993a). A case-control study using pet cats recruited from two veterinary hospitals in Sydney, NSW, found an increased incidence of CKD in FIV-infected cats < 11 years of age (compared to FIV-uninfected cats) (White et al., 2010). Researchers in Italy investigated and compared the renal tissue of naturally and experimentally infected cats and reported the presence of renal disease in both groups, consequently proposing a lentivirus-associated nephropathy (Poli et al., 2012). Recently, immunohistochemistry performed on the myocardium of five cats diagnosed with myocarditis or hypertrophic cardiomyopathy found an intense infiltration of FIV in lymphocytes and macrophages, leading to speculation about a possible disease association (Machado Rolim et al., 2016).

Other studies have not shown a clear relationship between FIV infection and the development of associated disease. Norris and colleagues did not demonstrate an unequivocal or immediate impact of FIV infection on feline health, finding equal FIV prevalence amongst ‘sick’ and ‘healthy’ Australian cats (8% *versus* 8%) (Norris et al., 2007). An Australian retrospective case-control study, recruiting client-owned cats from a university teaching hospital that were tested for FIV infection as part of a general work up for a variety of clinical signs, did not find a significant difference in survival age or survival time between FIV-infected and FIV-uninfected cats (Liem et al., 2013). A Canadian case-control study, which compared 58 FIV-infected and 58 FIV-uninfected cats, identified lethargy and oral cavity disease as being significantly associated with FIV-positivity, but did not find a significant difference in survival time between the two groups

(Ravi et al., 2010). A long-term study of a closed household in the UK consisting of 26 cats infected with a variety of viruses (FIV, FeLV and FCoV) actually found the survival time for FIV-infected cats was longer than the survival time for FIV-uninfected cats (median 51 *versus* 18 months), although this was not statistically significant (Addie et al., 2000). A recent study of naturally FIV-infected cats in America found a dramatic difference in outcomes between two cohorts, suggesting that management and housing conditions have a major impact on disease progression and survival times of FIV-infected cats. In this study, only 1/17 cats in the first group (FIV-infected cats kept in households with one or two cats) died during the 22-month observation period (from hypertrophic cardiomyopathy), while 17/27 cats in the second group (FIV-infected cats kept in a multi-cat household with more than 60 cats) died during the same period, including at least 11 cats diagnosed with lymphoma (Bęczkowski et al., 2015a).

An investigation of the impact of FIV-infection on the general health of Australian cats formed part of the epidemiological survey of retroviral infections presented in Chapter 2.

1.3. Structure of FIV

The FIV genome is composed of approximately 9,500 nucleotides, comprising three main open reading frames (orfs; *gag*, *pol* and *env)* encoding major capsid proteins (MA, matrix, p15; CA, capsid, p24; NC, nucleocapsid, p7), viral enzymes (PR, protease; RT, reverse transcriptase; IN, integrase) and envelope glycoproteins (TM, transmembrane, gp40; SU, surface, gp120), respectively (Olmsted et al., 1989).

FIV is subdivided into seven clades (subtypes) based on genetic diversity in the variable V3-5 region of the *env* gene (A, B, C, D, E, F and U-NZenv) (Duarte and Tavares, 2006; Hayward et al., 2007; Yamamoto et al., 2007; Hayward and Rodrigo, 2010). Subtypes A, B

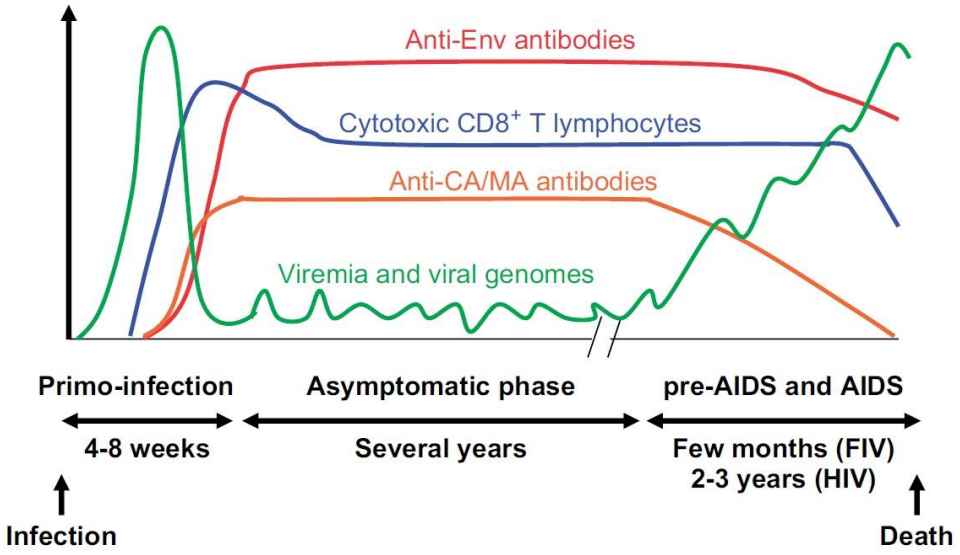
and C are most commonly encountered worldwide (Yamamoto et al., 2007), with subtype A predominant in Australia (Kann et al., 2006; Iwata and Holloway, 2008). Nucleotide sequence may vary up to 15% within a subtype and up to 38% between subtypes (Sodora et al., 1994; Duarte et al., 2002). Subtyping of FIV infections in each geographic area is important as the only commercially available FIV vaccine[1] contains only subtypes A and D and heterologous challenge may lower vaccine effectiveness (Kusuhara et al., 2005; Huang et al., 2010), although subtyping alone appears insufficient to predict vaccine performance (Hosie and Beatty, 2007).

1.4. Antibody production following FIV infection

FIV infection is life-long and results in persistently high antibody titres that are useful diagnostically for identifying infected patients (Jarrett et al., 1991; Tonelli, 1991; Hartmann et al., 2001; Hartmann et al., 2007), except in the terminal (FAIDS) stage of infection when antibody levels, particularly to p15 and p24, may wane (Figure 1.2; Lecollinet and Richardson, 2008). Areas of the FIV genome capable of evoking host antibody response (B-cell epitopes) have been identified in the p7, p15, p24, gp40 and gp120 domains, with immunodominant epitopes located in the highly variable V3 region of gp120 (Lecollinet and Richardson, 2008; Yamamoto et al., 2010). Antibodies to p24 and gp40 are usually detectable within three weeks of infection, while antibodies to p15 become detectable within four weeks of infection using Western blot (Yamamoto et al., 1988; O'Connor et al., 1989), although occasionally antibodies may take up to a year to develop following infection (Yamamoto et al., 1988). Antibodies to gp120 are usually not measurable by Western blot owing to shearing of the viral envelope during purification, unless cell lysate is used for antigen preparation, rather than concentrated virus (Yamamoto et al., 1988; Pedersen et al., 1989; Hosie and Jarrett, 1990). Variable

definitions of FIV positivity based on results from Western blotting have been proposed, including (i) presence of antibodies to gp120, (ii) antibodies to gp120 and at least one core protein (p7, p15, or p24); (iii) antibodies to at least two core proteins; or (iv) antibodies to three core proteins (O'Connor et al., 1989; Hosie and Jarrett, 1990).

Figure 1.2 Disease course of FIV and HIV infections, included to illustrate the variable antibody response to FIV infection depending on the B epitope studied. Using Western blot analysis, antibodies to p24 and gp40 appear slightly earlier than antibodies to p15. Antibodies to the envelope proteins (gp40 and gp120) persist throughout the course of FIV infection, while antibodies to matrix protein (p15) and capsid protein (p24) may wane in the terminal stages of FIV infection. (Reproduced from Lecollinet and Richardson, 2008).



1.5. Serological testing for FIV infection

Serologic testing for FIV infection is commonly undertaken by veterinarians when confronted with feline patients with severe stomatitis, sequential or persistent opportunistic infections, lymphoma and other malignancies, or signs of non-specific illness when a cause is not apparent after preliminary investigations. Veterinarians in shelters also typically perform serological testing for FIV infection prior to admission into a shelter, or prior to re-homing (Levy et al., 2008a; Hosie et al., 2009).

Point-of-care test kits to detect antibodies directed against FIV are inexpensive, easy to use and reliably diagnose FIV infection in FIV-unvaccinated cats (Hartmann et al., 2007). There is variation between commercially available antibody test kits in the methodology and target viral antigen for antibody detection. SNAP FIV/FeLV Combo[2] is a lateral flow ELISA kit that detects antibodies to p15 (matrix protein) and p24 (capsid protein) in kits sold in North America, Australia and New Zealand, with gp 40 (transmembrane glycoprotein) added to kits sold in Europe. Witness FeLV/FIV[3] is a lateral flow immunochromatography (IC) kit that detects antibodies to gp40, while Anigen Rapid FIV/FeLV[4] is a lateral flow IC kit that detects antibodies to p24 and gp40 (Figure 3.1 and Table 3.1). SNAP FIV/FeLV Combo Plus, available only in Europe, is a lateral flow ELISA kit that detects antibodies to p15, p24 and gp40. Published sensitivity and specificity of each test kit in FIV-unvaccinated cats are 94% and 100% for SNAP FIV/FeLV Combo (www.idexx.com/files/small-animal-health/products-and-services/snap-products/snap-fiv-felv-combo/snap-combo-test-accuracy.pdf), 100% and 100% for SNAP FIV/FeLV Combo Plus, 95% and 99% for Witness FeLV/FIV (Hartmann et al., 2007), and 89% and 100% for Anigen Rapid FIV/FeLV (Sand et al., 2010).

1.6. Antibody production following FIV vaccination

The only commercial FIV vaccine (Fel-O-Vax FIV)[1] currently available consists of formalin-inactivated whole cells (IWC) and whole virus (IWV) suspended together in an adjuvant (Yamamoto et al., 2007). It was presumed, on the basis of this composition, that the antibody response of FIV-vaccinated cats would be indistinguishable from those of FIV-infected cats (Uhl et al., 2002). Using laboratory-based ELISA testing, Huang and collaborators showed antibody against p24 and gp40 peaked 1–3 weeks after the third primary FIV vaccination (vaccines given three weeks apart) in kittens and decreased over

the following three months, with antibody levels maintained for 12 months in most cats (Huang et al., 2004; Huang et al., 2010). The same result was found by another group using ELISA testing for antibodies to whole FIV antigen and recombinant p24 (r-gag) in cats aged 7–12 months (Kusuhara et al., 2005), as well as from p24 ELISA determinations in experimental kittens vaccinated three times 2-3 weeks apart (unpublished data, Boehringer Ingelheim). Western blot analysis of four FIV-vaccinated cats confirmed antibody production to p15, p24 and gp40 three weeks after the second primary FIV vaccination, which persisted for at least 12 months following the third primary FIV vaccination (Uhl et al., 2002). Work by Levy and colleagues shortly after the release of the FIV vaccine reported that all FIV-vaccinated cats tested FIV-positive by three weeks after the first vaccine dose using a lateral flow ELISA kit (SNAP FIV/FeLV Combo),[2] and by 14 weeks used a microwell plate ELISA (PetChek FIV)[2] (Levy et al., 2004). Consequently, in FIV-vaccinated cats and cats of unknown FIV vaccination status, diagnosis of FIV shifted to more expensive molecular methods such as nucleic acid amplification, with variable results in terms of accuracy and reliability (Bienzle et al., 2004; Crawford et al., 2005; Crawford and Levy, 2007; Litster et al., 2012; Morton et al., 2012; Ammersbach et al., 2013).

Chapter 3 revisits the assertion that point-of-care antibody kits are unable to differentiate FIV-infected and FIV-vaccinated cats by testing FIV-vaccinated cats, with variable FIV infection status, using three different antibody kits available in Australia.

Chapter 5 expands on these results by prospectively recruiting a group of cats to administer the primary vaccination course against FIV and systematically and regularly test them over time to monitor their antibody response, as measured by four point-of-care antibody kits.

1.7. Diagnosis of FIV infection using saliva

Antibody testing using saliva accurately detects HIV infection in people; a meta-analysis of the OraQuick Advance Rapid HIV-1/2 In-Home HIV Test[5] identified similar specificity, and only a 2% reduction in sensitivity, when saliva was used instead of whole blood (Pai et al., 2012). As a result, this test kit has been approved by the USA Food and Drug Administration (FDA) for self-testing using saliva[6] (Delaney et al., 2006). Surprisingly, despite similarities between serological testing for HIV-1 and FIV infection, and the potential welfare benefits of collecting saliva instead of blood from a cat, few studies have been performed to investigate the potential for diagnosing FIV infection by detecting FIV antibodies in saliva. The first study to investigate this possibility reported a high frequency of false-positive and false-negative results (Poli et al., 1992), while a more recent study . found a good correlation between salivary antibody and salivary PCR results (Chang-Fung-Martel et al., 2013).

Chapter 4 reports the testing of the same cats recruited for Chapter 3 using the same three FIV antibody kits currently available in Australia, with saliva utilised instead of blood as the diagnostic specimen.

1.8. The FIV vaccine

The commercial release of a FIV vaccine (Fel-O-Vax FIV)[1] for use in domestic cats (USA in 2002; Australia in 2004) was the first time a vaccine had been registered for preventing infection by a *Lentivirus* in either human or veterinary medicine. More than 5,000 laboratory cats were used over 14 years to develop a dual-subtype (A and D) IWC and IWV vaccine. A further 689 client-owned cats were used for safety testing in the field before the vaccine was released commercially. The result was a vaccine registered with a

'preventable fraction' (efficacy) of 68%, based on combined results from two laboratory-based efficacy studies involving 105 cats (52 FIV-vaccinated, 53 FIV-unvaccinated) challenged one year after receiving three FIV vaccinations administered three weeks apart (Yamamoto et al., 2007). However, the vaccine wasn't registered in many jurisdictions (e.g. Europe), owing in part to concerns related to the production of antibodies in FIV-vaccinated cats indistinguishable from those produced in response to natural FIV infection.

To date, a total of 262 cats have been tested using the current commercial FIV vaccine in laboratory-based efficacy studies (including the 105 cats from the pre-registration studies), with an overall preventable fraction of 66% (Table 6.1) (Huang et al., 2004; Kusuhara et al., 2005; Pu et al., 2005; Dunham et al., 2006b; Yamamoto et al., 2007; Huang et al., 2010; Yamamoto et al., 2010; Coleman et al., 2014). Extremely high challenge doses, intravenous (IV) challenge (which avoids innate immunity barriers), and the use of highly pathogenic strains for challenge (e.g. FIV_{UK8}), have been proffered as possible explanations for the variation in reported protection rates (Hosie and Beatty, 2007; Yamamoto et al., 2007). It has therefore been suggested that Fel-O-Vax FIV efficacy may have been underestimated and there has been speculation that field trials involving natural challenge might report a preventable fraction in excess of 66-68% (Hosie and Beatty, 2007; Yamamoto et al., 2010).

The global estimate of cats infected with FIV (35 million) is similar to the estimated number of humans infected with HIV-1 (www.who.int/gho/hiv/en/). The FIV-cat model is often advocated as a 'test-bed' for HIV infection, and the FIV vaccine used as a 'proof of concept' for the development of sterilizing immunity against lentiviruses such as HIV-1 (Yamamoto et al., 2007; Lecollinet and Richardson, 2008; Bienzle, 2014). The successful development of a FIV vaccine would certainly provide encouragement for researchers

attempting to develop a HIV-1 vaccine. As some investigators have pointed out, however, the potential 'road map' provided by the development of the FIV vaccine has not been heeded by investigators in the HIV field. Indeed, the lack of translation of knowledge between fields has been described as being akin to 'flying without a compass' (Dunham, 2006a; Lecollinet and Richardson, 2008).

Chapter 6 reports the first ever field study investigating the effectiveness ('protective rate') of the FIV vaccine and discusses the significance of the observed findings in relation to both veterinary medicine and the development of a HIV vaccine.

1.9. Discovery and prevalence of FeLV

FeLV was first reported in 1964 following the recognition of a temporo-spatial cluster of lymphoma cases in a cattery (Jarrett et al., 1964). In this seminal study, electron microscopy enabled veterinary pathologist Bill Jarrett to identify a virus-like particle, similar in structure to murine leukaemia virus, in the mesenteric lymph node of a cat with lymphosarcoma (lymphoma). Jarrett's work later inspired an American biomedical researcher to search for a viral agent associated with human T-cell leukaemias, ultimately leading to the discovery of human T-cell leukaemia virus type 1 and HIV-1 (Willett and Hosie, 2013).

In many countries, feline leukemia virus (FeLV) infection is considered less prevalent, although no less pathogenic, than it was in the 1970s and 1980s (Levy et al., 2008; Lutz et al., 2009). This is the result of veterinary interventions, including rigorous testing and isolation of infected animals (Lutz et al., 2009; Willett and Hosie, 2013), pushing FeLV 'back into nature' (www.sockfip.info/about-fip/all-articles/67-about-fip). Yet recent prevalence serosurveys have demonstrated FeLV infection is still common in certain

regions, such as Malaysia (12%) (Bande et al., 2012), Singapore (16%) (Chan et al., 2013) and Thailand (25%) (Sukhumavasi et al., 2012), remaining an important agent of disease in domestic cats. Of client-owned cats presented to university veterinary hospitals and tested for FeLV antigenaemia (p27) using various test kits and for an assortment of reasons, FeLV-positivity based on antigenaemia ('progressive-infections') was 6% in east Austria (249/4,465; 1996–2011) (Firth and Möstl, 2015) and 4% in Canada (50/1,205; 1996–2006) (Ravi et al., 2010). FeLV-positivity in large mixed populations of client-owned and shelter cats was 2% in North America (409/18,038; 2004) (Levy et al., 2006) and 3% in Canada (383/11,144; 2007) (Little et al., 2009). Indeed, the risk of FeLV infection remains sufficiently high to result in shelter guidelines recommending FeLV testing of all incoming cats prior to re-homing (Möstl et al., 2013). FeLV has not disappeared completely, and should definitely not be forgotten.

In Australia, FeLV infection is considerably less prevalent than FIV infection in healthy client-owned cats (0-2% *versus* 8%) (Malik et al., 1997; Norris et al., 2007). Similarly, New Zealand and Singapore have reported lower seroprevalences of FeLV compared to FIV (6% *versus* 10% in a convenience sample of sick cats in New Zealand [Jenkins et al., 2013]; 9% *versus* 16% in healthy cats tested at a Singaporean veterinary clinic [Chan et al., 2013]). The difference between FeLV and FIV seroprevalences in New Zealand cats is likely to be greater than first reported; it was later discovered using PCR testing that many FeLV-positive results were actually false-positives (Severine Tasker, personal communication). The disparity between FeLV and FIV infection rates in domestic cats in Australia, New Zealand and Singapore seems out of step with other developed nations in Europe and North America, where the infection rates of FeLV and FIV are comparable (2% and 3% in North America (Levy et al., 2006), 3% and 4% in Canada (Little et al.,

2009), 5% and 6% in the United Kingdom (Hosie et al., 1989) and 4% and 3% in Germany (Gleich et al., 2009b) respectively).

An updated serosurvey of FeLV prevalence rates in both client-owned and shelter cats in Australia forms the basis of the work presented in Chapter 2.

1.10. FeLV transmission and disease associations

The main cellular target of FeLV is circulating lymphocytes, with additional cell tropism dependent on the FeLV subgroup present. FeLV-subtype A, which is involved in every infection and binds to target cells through an interaction with the thiamine transporter THTR1, has broad cellular tropism since THTR1 is expressed widely in feline tissues. FeLV-subtype B, which arises following recombination of FeLV-A with endogenous FeLV, attaches to cells via receptors Pit-1 and Pit-2 found on T-lymphocytes. FeLV-subtype C, which results from a mutation in the *env* gene, utilises the haem transporters FLVCR1 and FLVCR2 expressed widely in haematopoietic tissues for viral entry. As a consequence of these viral receptors having different transport functions and being expressed by different cells, infection with different FeLV subtypes results in distinct clinical manifestations (Lutz et al., 2009; Willett and Hosie, 2013)

FeLV is known as the ‘friendly cat disease’ since transmission only requires close contact in the form of allogrooming or shared food and watering stations. Bite wounds, however, are an increasingly recognised source of FeLV transmission (Goldkamp et al., 2008; Gleich et al., 2009b; Lutz et al., 2009). Vertical transmission of FeLV in the field is common and associated with high kitten mortality (Lutz et al., 2009).

FeLV-infected cats with persistent (progressive) infections have a 62-fold increased risk of developing lymphoma or leukaemia compared with cats not infected with FeLV, with

FeLV-B infections responsible for the majority of FeLV-induced lymphoma (Hartmann, 2012; Willett and Hosie, 2013). FeLV infection can also cause severe non-regenerative macrocytic anaemia (FeLV-A) and aplastic anaemia (FeLV-C), the latter often being fatal within weeks (Willett and Hosie, 2013). FeLV-infected cats are more to likely develop disorders of haematopoiesis (including aplastic anaemia, thrombocytopenia, neutropenia and lymphocytosis) compared to FeLV-uninfected cats (Gleich and Hartmann, 2009a). The survival rate for progressively FeLV-infected cats is estimated at 50% by two years and 20% by three years after infection (Lutz et al., 2009; Hartmann, 2012). Co-infection with FIV is common in certain regions (e.g. Malaysia, Singapore, Thailand) and generally results in more serious clinical outcomes (Shelton et al., 1990; Courchamp et al., 1997).

The impact of transient (regressive) FeLV infections on the health of cats is largely unknown. Some authors have suggested regressively FeLV-infected cats have a similar life-expectancy to cats never exposed to FeLV and do not seem to develop FeLV-associated disease (Lutz et al., 2009; Willett and Hosie, 2013). However, despite apparently clearing the viraemia and 'recovering', reactivation of regressive FeLV infection contained in the bone marrow has been demonstrated experimentally with and without the administration of corticosteroids (Rojko et al., 1982; Hofmann-Lehmann et al., 2007; Helfer-Hungerbuehler et al., 2015), and care is suggested when treating regressively FeLV-infected cats with immunosuppressive medication (e.g. chemotherapy, chlorambucil, ciclosporin) (Torres et al., 2005; Hartmann, 2012). Long-term follow up of five regressively-infected cats (following experimental inoculation) found all cats had detectable provirus and viral RNA (vRNA) loads in almost all of 27 tested tissues, even up to 12 years after virus exposure (Helfer-Hungerbuehler et al., 2015). Research in Australia and Canada has suggested a correlation between regressive FeLV infections and the

occurrence of lymphoma, with two studies reporting 80% (56/70) and 26% (25/97) of lymphomas were PCR-positive for FeLV provirus, respectively (Jackson et al., 1993; Gabor et al., 2001a). Furthermore, regressively-infected cats are capable of transmitting FeLV infection to recipient cats by blood transfusion, an important reminder that potential blood donors need to be tested for FeLV provirus, regardless of p27 antigen status using point-of-care test kits (Nesina et al., 2015).

1.11. Categories of FeLV infection

The host-pathogen interaction for FeLV in cats is complex and best represented as a spectrum of outcomes following exposure. The development of molecular methods for FeLV diagnosis, specifically qPCR testing to detect low levels of proviral DNA and qRT (reverse-transcription)-PCR to detect low levels of viral RNA in blood, has enriched our understanding of the cat-FeLV relationship, adding nuances to the diagnosis, pathogenesis and categorization of FeLV infection.

Currently, four putative outcomes are defined for cats following FeLV challenge: (i) At one end of the spectrum, some cats mount a timely and appropriate immune response and eliminate virus before it progresses beyond local replication in oropharyngeal tissue – so-called abortive-infection (20-30% of cases under laboratory conditions using specific pathogen free [SPF] cats); (ii) at the other end of the spectrum, some cats become persistently viraemic (progressive-infection; 30-40% of experimental infections); (iii) between these extremes, some cats are transiently viraemic before mounting a partial immune response to eliminate the viraemia after 2-16 weeks, but not before a latent infection is established as DNA provirus, predominantly in lymphoid cells in the bone marrow (regressive-infection; 30-40% of experimentally inoculated cats); and (iv) a small

number of cats follow an atypical course involving a latent state with variable antigenaemia and viraemia due to local foci of infection, e.g. in the eyes, bladder or mammary tissue (atypical/focal infection; 5%) (Lutz et al., 2009; Englert et al., 2012; Hartmann, 2012). From the lymphoma studies cited earlier, where results from blood ELISA testing and tissue FeLV PCR testing were both available in the same cat, latent infections (regressive/focal) were identified in 27% (7/26) and 22% (19/86) of cases, respectively (i.e. ELISA negative, PCR positive) (Jackson et al., 1993; Gabor et al., 2001a). Such a range of possible outcomes following FeLV exposure makes defining the expectations of diagnostic test kits important, before attempting to evaluate the performance of a given test (Chapter 7).

Chapter 8 investigates the prevalence of both progressive and regressive FeLV-infections in three groups of cats in Australia, including one group representative of the general cat population and two groups of group-housed rescue cats where index cases of FeLV infection had been identified.

1.12. Diagnosis of progressive FeLV infection using blood

While antibody detection has been the mainstay of FIV testing historically, antigen detection has been the most common method of FeLV diagnosis. This can be done via screening with a point-of-care ELISA/IC test kit (to detect viral capsid protein p27), direct IFA testing or VI, although confirmatory IFA testing is now rarely performed owing to the ready availability and superior sensitivity of real-time PCR (qPCR) testing to detect FeLV provirus, while VI is confined mainly to the research setting (Lutz et al., 2009; Adam and Dandrieux, 2011).

Prior to the commercial availability of qPCR assays, studies of four commercially available point-of-care FeLV antigen test kits (1989–1991) reported almost 100% sensitivity and 100% specificity for all kits compared to IFA testing (Lopez et al., 1989b; Hawks et al., 1991) but reduced sensitivity compared to VI (Hawks et al., 1991). Furthermore, discordant results were observed between test results for whole blood and serum due to a combination of false-positive (whole blood) and false-negative (serum) results (Hawks et al., 1991). Subsequent research (1998–2001) using a later generation of test kits reported 5/6 point-of-care FeLV antigen kits tested using serum (including SNAP FIV/FeLV Combo[2] and Witness FeLV/FIV[3]) to have good sensitivity and specificity using VI as the 'gold standard' (Hartmann et al., 2001), and SNAP FIV/FeLV Combo and Witness FeLV/FIV both performed well when tested side-by-side (Robinson et al., 1998). In one of the studies, SNAP FIV/FeLV Combo testing using whole blood instead of serum increased the number of equivocal results and was therefore advised against by the authors, despite the manufacturer's recommendations that whole blood could be used (Hartmann et al., 2001). More recently (2007–2010), testing of eight of the latest generation point-of-care FeLV antigen test kits (including SNAP FIV/FeLV Combo and Witness FeLV/FIV) with serum found all but one kit had similarly high sensitivity and specificity when compared to VI (Hartmann et al., 2007), while a separate study reported Anigen Rapid FIV/FeLV[4] to have comparable diagnostic accuracy to SNAP FIV/FeLV Combo using provirus PCR testing as the gold standard (Sand et al., 2010). Witness FeLV/FIV was found to have 'a high number of tests that were difficult to interpret' (14%) (Hartmann et al., 2007), although exactly what is meant by that is difficult to interpret.

Chapter 7 investigates the performance of three FeLV antigen test kits available in Australia using whole blood as the diagnostic specimen, and proviral PCR testing as the

gold standard. Of particular interest, considering the low prevalence of FeLV in Australia in the general cat population, was the PPV of each kit and the frequency of false-positive results encountered consequently (Hawkins, 1991; Beatty et al., 2011; Möstl et al., 2013).

1.13. Diagnosis of progressive FeLV infection using saliva

Diagnosis of FeLV infection using samples other than blood has been investigated using both laboratory-based ELISA testing and commercial point-of-care FeLV test kits. Saliva is the obvious alternative diagnostic specimen, as it is often easier to collect than blood, especially from fractious cats, and saliva contains on average five-times more FeLV per mL than plasma (Francis et al., 1977). Results from saliva testing have, however, been contradictory. One early study of experimentally infected and sick client-owned cats using laboratory-based ELISA testing found false-positive FeLV results occurred rarely (5/1,117; 0.4%) but false-negative results occurred in 23% (39/167) of FeLV-infected sick cats (Lutz and Jarrett, 1987). A later study from Switzerland, using the same sandwich ELISA, reported false-positive FeLV results in 5% (19/367) of FeLV-uninfected cats (Gomes-Keller et al., 2006). Other studies have investigated FeLV diagnosis using point-of-care ELISA saliva testing, finding a concordance of 98% (552/564) between saliva and serum tested concurrently using ViraChek FeLV[7] (Lewis et al., 1987), and 92% (94/102) between saliva and plasma using ClinEase-Virastat[7] (Lopez et al., 1990). Both studies concluded saliva testing was a useful rapid screening procedure using these kits, but recommended confirmatory testing (Lewis et al., 1987; Lopez et al., 1990). Despite these promising results, the American Association of Feline Practitioners (AAFP) declared that ‘antigen tests should not be performed on tears or saliva because these tests are prone to more errors’ (Levy et al., 2008a).

Chapter 7 also investigates the performance of three FeLV antigen test kits available in Australia using saliva as the diagnostic specimen, and proviral PCR testing as the gold standard.

1.14. Impetus for this thesis: an explanation

The aforementioned literature review covers the two clinically important retroviral diseases of cats in a classic historical perspective. The background and impetus to the studies included within this thesis are outlined below, to provide some insights into the chronology.

Two of my supervisors (Norris and Malik) had been approached by Boehringer Ingelheim, the distributors of the FIV vaccine (originally developed by Fort Dodge), to conduct a field study to assess the effectiveness of this vaccine in an Australian setting. There had been, to date, no confirmed reports of 'vaccine breakthroughs', and it was anticipated that the data generated would provide further support for vaccination of at risk cats in an Australian setting. Although Norris and Malik argued persuasively for a prospective study of vaccine effectiveness, the commercial realities dictated to start the investigation using a retrospective study design to provide data in a timelier manner, and the samples required for this investigation form the major study cohort for this PhD dissertation. The requirement for stringent FIV testing of cats in this study mandated a highly structured study design, and the testing of cats provided an opportunity for head-to-head testing of the latest generation of test kits for FIV against the commercial qPCR offered by IDEXX Laboratories as the gold standard, although in some discordant cases it was necessary to utilise virus isolation in specialist laboratories as the final arbiter of FIV status. As most of the kits also tested for FeLV antigen, it was logical to examine the prevalence of FeLV in

the same study cohort, to get an appreciation of the occurrence of so-called progressive *versus* regressive infections in the field. Further, additional minimally-invasive sample collection facilitated an examination of the accuracy of testing using saliva *versus* whole blood as the diagnostic specimen for these test kits.

Early results suggested that some of the test kits for FIV could provide a reliable indication of true FIV status even in cats who had been given the complete course of FIV vaccinations, and the reasons for this unexpected observation provided a fertile ground for the more detailed prospective experiments in Chapter 5.

Finally, the serendipitous discovery of two 'hot spots' for FeLV disease, based on clinical investigation of FeLV related disease by colleagues in practice, provided us with the impetus to compare and contrast the epidemiology of FIV and FeLV in these small rescue facilities, against the overall wider client-owned feline population.

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[1] Fel-O-Vax® FIV, Boehringer Ingelheim, Fort Dodge, IA, USA.
[2] IDEXX Laboratories, Westbrook, ME, USA.
[3] Zoetis Animal Health, Lyon, France.
[4] BioNote, Gyeonggi-do, Korea.
[5] OraSure Technologies Inc, PA, USA.
[6] www.fda.gov/ForConsumers/ConsumerUpdates/ucm310545.htm
[7] Synbiotics Corporation, San Diego, CA, USA

CHAPTER 2. THE PREVALENCE OF FIV and FeLV IN DOMESTIC CATS IN AUSTRALIA

2.1 ABSTRACT

The aims were to (i) determine the current seroprevalence of FIV and FeLV in three large cohorts of cats from Australia and (ii) investigate potential risk factors for retroviral infection. Cohort 1 (*n* = 2,151 for FIV, *n* = 2,241 for FeLV) consisted of cats surrendered to a shelter on the west coast of Australia (Perth, WA). Cohort 2 (*n* = 2,083 for FIV, *n* = 2,032 for FeLV) consisted of client-owned cats with outdoor access recruited from around Australia through participating veterinary clinics. Cohort 3 (*n* = 169 for FIV, *n* = 166 for FeLV) consisted of cats presenting to MUVH for a variety of reasons. Fresh whole blood was collected and tested using a commercially available point-of-care lateral flow ELISA kit that detects p27 FeLV antigen and antibodies to FIV antigens (p15 and p24) (Cohorts 1 and 2), or one of two lateral flow IC kits that detect p27 antigen and antibodies to FIV antigen (p24 and/or gp40) (Cohort 3). Data recorded for cats in Cohort 2 included signalment, presenting complaint and postcode, allowing investigation of risk factors for FIV or FeLV infection as well as potential geographic 'hot spots' for infection. The seroprevalence of FIV was 6% (Cohort 1), 15% (Cohort 2) and 14% (Cohort 3) while the seroprevalence of FeLV was 1%, 2% and 4% in the same respective cohorts. Risk factors for FIV infection among cats in Cohort 2 included age (> 3 years), sex (male), neutering status (entire males) and location (WA had a significantly higher FIV seroprevalence compared with the ACT, NSW and VIC). Risk factors for FeLV infection among cats in Cohort 2 included health status ('sick') and location (WA cats were approximately three times more likely to be FeLV-infected compared with the rest of Australia). No geographic

hot spots of FIV infection were identified. Both FIV and FeLV remain important infections among Australian cats. WA has a higher seroprevalence of both feline retroviruses compared to the rest of Australia, which has been noted in previous studies. A lower de-sexing rate for client-owned male cats is likely responsible for the higher seroprevalence of FIV infection in WA cats, while the reason for the higher seroprevalence of FeLV in WA cats is currently unknown.

2.2 INTRODUCTION

To date, there has been one reported investigation of retroviral infection in Australian shelter cats. This was an underpowered study of only 20 cats from Melbourne, Victoria, which found six cats, with a median age of 3 years, to be FIV-positive (30%) (Friend et al., 1990). FeLV status was not investigated in that study (Friend et al., 1990). Consequently, a larger study into the retroviral status of Australian shelter cats has been long overdue.

While there has been a paucity of studies investigating the retroviral status of Australian shelter cats, there have been several investigations into the retroviral status of client-owned cats. These studies vary considerably in relation to location, design and recruitment, resulting in considerable variation in the reported seroprevalences of FIV and FeLV infection (Sabine et al., 1988; Belford et al., 1989; Friend et al., 1990; Robertson et al., 1990b; Thomas et al., 1993b; Malik et al., 1997; Winkler et al., 1999; Norris et al., 2007; Chang-Fung-Martel et al., 2013). The seroprevalence of FIV infection in these prior studies varies between 0-29% (for 'healthy' cats) and 4-32% (for 'sick' cats). The seroprevalence of FeLV infection in these prior studies varies between 0-7% (for 'healthy' cats) and 0-11% (for 'sick' cats) (Tables 2.1 and 2.2). Two of these studies investigated FIV and FeLV infection in WA cats: a 1990 study found seroprevalence rates of 29% (FIV) and 7% (FeLV) in 'healthy' cats, and 28% (FIV) and 11% (FeLV) in 'sick' cats (Robertson et al., 1990b); while a 1993 study found seroprevalence rates of 24% (FIV) and 6% (FeLV) in 'sick' cats (Thomas et al., 1993b).

The aim of this study was to determine the seroprevalence of FIV and FeLV in three different Australian feline cohorts: cats surrendered to a rescue facility (shelter) in WA (Cohort 1), client-owned cats recruited from around Australia through participating

veterinary clinics (Cohort 2) and cats presenting to MUVH (Perth, WA) (Cohort 3) for a variety of reasons (mostly illness-related). This third cohort was recruited to provide further insights into the high seroprevalence of FIV and FeLV in WA detected in preliminary data analysis of cats from Cohort 2, and reported in previous studies (Robertson et al., 1990b; Thomas et al., 1993b). Detailed information was recorded for cats in the second cohort, which permitted investigation of risk factors for retroviral infection as well as the use of spatial statistical methods to identify potential geographic 'hot spots' of infection in Australia.

Table 2.1 Summary of previous Australian studies investigating FIV and progressive FeLV infection amongst 'healthy' cats.

Reference	Location	Study design	Age	FIV prevalence	FeLV prevalence
Sabine *et al.* (1988)	Sydney, NSW	'Healthy' cats (*n* = 30), serum/plasma supplied by Webster's Vaccine Company	NP	2/30; 7%	2/30; 7%
Robertson *et al.* (1990b)	Perth, WA	'Healthy' client-owned cats (*n* = 72), recruited by random selection of households from the Perth electoral rolls	NP	21/72; 29%	5/72; 7%
Malik *et al.* (1997)	Sydney, NSW	'Healthy' client-owned cats (*n* = 200), prospective sampling from four veterinary clinics	Median age 4 years	15/200; 8%	4/200; 2%
Norris *et al.* (2007)	Sydney, NSW	'Healthy' client-owned cats (*n* = 170), prospective sampling from 3 veterinary clinics stringently designed to reflect a typical hospital population	Median age 7 years	13/170; 8%	4/170; 2% (unpublished data)
Beatty *et al.* (2011)	Sydney, NSW	'Healthy' client-owned cats (*n* = 169), most acquired from rescue societies), prospective sampling from 3 veterinary clinics	Mean age 3 months (all < 1 year)	0/169; 0%	0/169; 0%
Chang-Fung-Martel *et al.* (2013)	Townsville, QLD	'Healthy' cats (*n* = 96), door-to-door survey using a random sampling approach, saliva collected	Median age 5 years	10/96; 10%	NP

Table 2.2 Summary of previous Australian studies investigating FIV and progressive FeLV infection amongst 'sick' cats.

Reference	Location	Study design	Age	FIV prevalence	FeLV prevalence
Sabine *et al*. (1988)	Sydney, NSW	'Sick' cats (*n* = 23), convenience sample using serum/plasma sent to VPDS, The University of Sydney with many specimens dating back to the 1970s	NP	1/23; 4%	2/23; 9%
Belford *et al*. (1989)	QLD and northern NSW	'Sick' cats and in-contact cats (*n* = 65), convenience sampling using serum/plasma sent to VPS from cats suspected to be FIV infected based on clinical or laboratory findings (break up of sick *versus* in-contact cats not specified)	NP	21/65; 32%	NP
Robertson *et al*. (1990b)	Perth, WA	'Sick' client-owned cats (*n* = 211), convenience sample using serum sent to MUVH Clinical Pathology Laboratory for diagnostic work up of clinical disease (not specifically suggestive of FIV)	NP	59/211; 28%	23/211; 11%
Friend *et al*. (1990)	Melbourne, VIC	'Sick' cats (*n* = 467, consisting of 447 client-owned and 20 shelter cats), convenience sample using serum sent to CVDL or SVS, most cats displaying clinical disease compatible with immunodeficiency	NP	120/467; 26%	16/467; 3%
Thomas *et al*. (1993b)	WA	'Sick' client-owned cats (*n* = 326), convenience sample using blood sent to a private laboratory for diagnostic work up of clinical disease	NP	78/326; 24%	21/326; 6%

Malik *et al*. (1997)	NSW	'Sick' client-owned cats (*n* = 894), convenience sample using serum sent to a private clinical pathology laboratory for diagnostic work up of suspected immunodeficiency (not all samples tested for both FIV and FeLV)	NP	148/711; 21%	11/761; 1%
Winkler *et al*. (1999)	Adelaide, SA	Client-owned cats of unknown health status (*n* = 389), convenience sample using serum sent to VPS (presumably cats 'sick' and sampled for diagnostic work up of their illness)	NP	39/389; 10%	NP
Norris *et al*. (2007)	Sydney, NSW	'Sick' client-owned cats (*n* = 170), prospective sampling from three veterinary clinics stringently designed to reflect a typical hospital population, cats were 'systemically unwell' and sampled for diagnostic work up of their illness	Median age 7 years	14/170; 8%	4/170; 2% (unpublished data)
Beatty *et al.* (2011)	Sydney, NSW	'Sick' client-owned cats (*n* = 75), convenience sample using cats presented to VCCC for further work up of anaemia, cytopenia, lymphoma and other illnesses	Mean age 11.5 years	8/75; 11%	0/75; 0%

NP = not provided; VPDS = Veterinary Pathology Diagnostic Services; VPS = Veterinary Pathology Services; MUVH = Murdoch University Veterinary Hospital; CVDL = Central Veterinary Diagnostic Laboratory; SVS = School of Veterinary Science, University of Melbourne; VCCC = Valentine Charlton Cat Centre, University of Sydney.

2.3 MATERIALS AND METHODS

2.3.1 Sample population

Cohort 1 consisted of cats surrendered to a shelter on the west coast of Australia in Perth, WA between January 2011 and March 2013. Entire male cats older than 7 months of age were tested routinely, while entire female cats older than 7 months of age were tested at the discretion of the attending veterinarian (personal communication). Age was determined either by paperwork completed by the surrendering owner or estimated by the veterinarian, based on dentition.

Cohort 2 consisted of client-owned cats recruited through participating veterinary clinics in Australia between January 2012 and December 2012. Clinics from five Australian states and one territory participated (New South Wales [NSW], Victoria [VIC], Queensland [QLD]. South Australia [SA], West Australia [WA] and Australian Capital Territory [ACT]); only one poorly populated island state (Tasmania [TAS]) and one sparsely populated territory (Northern Territory [NT]) were not included in the study design (Figure 2.2). BI technical representatives offered to supply selected clinics with up to 30 free point-of-care FIV/FeLV test kits, on the condition that veterinary staff recorded in a spreadsheet test results and basic information, including signalment, postcode, reason for presentation and a subjective assessment of 'sick' *versus* 'healthy' on all cats sampled. Veterinarians were instructed to test cats only if individuals were 2 years of age or older (although this criterion was not strictly adhered to), had some level of outdoor access and had not been vaccinated against FIV. This scheme was part of a BI marketing program to raise the profile of a FIV vaccine[8] in Australia by demonstrating presence of FIV infection to clinicians in their local area, and thus to cat owners. Clinics selected included both those

which already recommended FIV vaccination and others that didn't routinely recommend FIV vaccination owing to a perceived low prevalence of FIV in their vicinity. A free map of the local area displaying the location of FIV-positive cats was offered as an inducement to participating clinics at the conclusion of the study with the intent of encouraging owners to vaccinate against FIV (Figure 2.1).

Cohort 3 consisted of cats presenting to MUVH between January 2011 and December 2013. The majority were cats presenting to the emergency or feline medicine units for signs of non-specific illness; other reasons for FIV/FeLV testing included health assessments of stray animals or prior to blood donation or commencement of immunosuppressive therapy.

2.3.2 Serological testing

Whole blood was collected by cephalic or jugular venipuncture for immediate in-clinic testing. All cats in Cohorts 1 and 2 were tested using SNAP FIV/FeLV Combo[9] according to the manufacturer's instructions. This kit is a lateral flow ELISA that detects antibodies to FIV matrix protein (p15) and FIV capsid protein (p24), and FeLV antigen (specifically core viral capsid protein p27). Cats in Cohort 3 were tested using either Witness FeLV/FIV[10] or SensPERT FeLV/FIV.[11] Both of these kits use lateral flow IC to either detect antibodies to FIV glycoprotein (gp40) and FeLV antigen (p27) (Witness), or antibodies to FIV capsid protein (p24) and FIV glycoprotein (gp40), and FeLV antigen (p27) (SensPERT).

2.3.3 Data collection

Results from cats in Cohort 1 were entered into a database at the time of testing by veterinary staff. A summary of results was retrieved in May 2013 using a summary search

function.[12] Pertinent data such as signalment, medical history, vaccination history and information on previous outdoor access was unavailable for these cats.

Results from cats in Cohort 2 were entered into a spreadsheet at the time of testing by veterinary staff, collated at the end of the testing period by a BI employee and then supplied to the first author for analysis. Signalment information (excluding breed), clinic postcode, primary presenting complaint and a subjective assessment of 'healthy' *versus* 'sick' made by the attending veterinarian were recorded alongside the cat's FIV and FeLV results. Based on the reason for presentation, cats in Cohort 2 were reclassified as 'healthy' or 'sick' according to previously published definitions.(Malik et al., 1997; Norris et al., 2007) 'Healthy' cats were those for whom the purpose of blood collection was not disease investigation; rather, it was as part of a routine health check, for routine testing prior to the dispensing of behaviour modifying medication, for routine pre-anaesthetic testing prior to sedation or general anaesthesia for de-sexing, grooming, dental disease or cat fight abscess treatment, or investigation and treatment of traumatic injuries. Dental disease was not graded by the attending veterinarian and so this category may have included minor teeth scaling and polishing to remove tartar, as well as extensive extractions attributable to periodontal disease. 'Sick' cats were those for whom the reason for presentation was suggestive of systemic illness, such as vomiting, diarrhoea, weight loss, respiratory signs, neoplasia, and severe illness warranting euthanasia. Cats were classified as 'unknown' if the reason for presentation did not easily fit either the 'healthy' or 'sick' definitions.

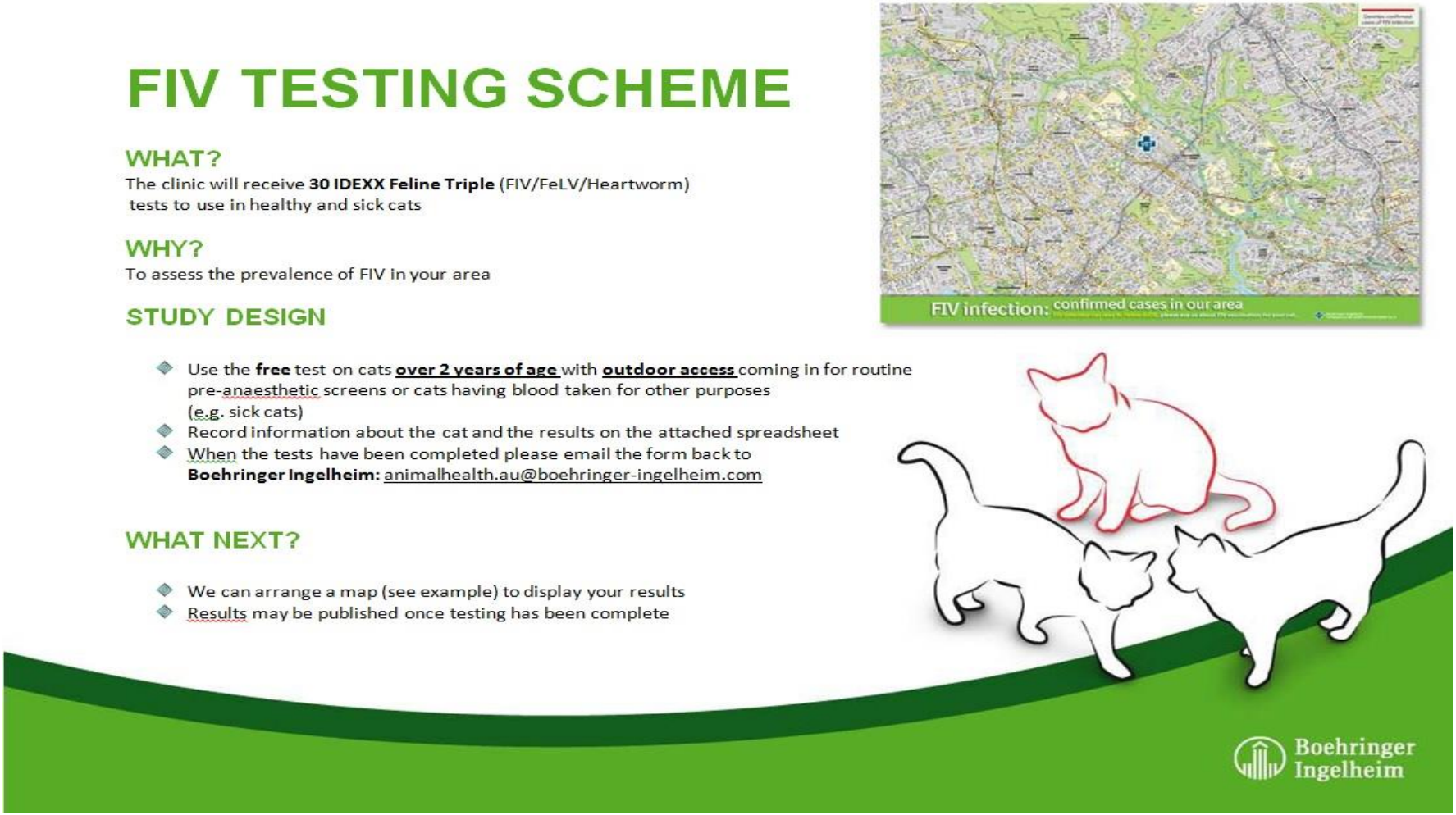
Results from cats in Cohort 3 were entered into the patient's medical records at the time of testing by the attending veterinarian and a summary of results retrieved in May 2015 by searching for invoiced FIV and FeLV point-of-care test kits.[13]

2.3.4 Statistical analysis

Numerical analyses were performed using a commercial statistical software package[14] with *P* values < 0.05 considered significant, and 95% CIs were calculated based on a normal approximation and the Wald method.[15] Probability of infection was used, where possible, since the measured outcome was binomial. Univariate and multivariate logistic regression modelling was performed to determine the effect of age, sex, neutering status, health assessment ('healthy' *versus* 'sick') and location (state/territory) on the retroviral status of cats in cohort 2. A two-tailed Fisher's exact test was used to investigate whether entire male cats were over-represented in WA in Cohort 2. The 2-sample *z*-test was used to compare FIV seroprevalence between 'healthy' and 'sick' cats in cohort 2 using an online calculator.[16] Potential geographic hot spots of FIV infection based on postcode were investigated using the scan statistic (SaTScan version 7). A scanning window of 5% of the study area size and a Bernoulli model (case-control) was used.

[8] Fel-O-Vax FIV, BI, Fort Dodge, IA, USA.
[9] IDEXX Laboratories, Maine, USA.
[10] Zoetis Animal Health, Lyon, France.
[11] VetAll Laboratories, Gyeonggi-do, Korea.
[12] Animal Shelter Manager Version 2.8.12.
[13] RxWorks Version 4.7.3200.
[14] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.
[15] Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.
[16] www.epitools.ausvet.com.au/content.php?page=z-test-2

Figure 2.1 Marketing material used by BI to recruit veterinary clinics for the study.



2.4 RESULTS

2.4.1 Cohort 1 (Shelter cats, WA)

Of 2,151 cats tested, 124 were FIV-positive (6%; 95% CI 4.8-6.7). Of 2,241 cats tested, 22 cats were FeLV-positive (1%; 95% CI 0.6-1.4). We were unable to determine the FIV/FeLV co-infection rate for this cohort, owing to limitations with the summary search function.

2.4.2 Cohort 2 (Client-owned cats, Australia)

2.4.2.1 Sample population

A total of 2,222 cats were recruited from 130 veterinary clinics in five states and one territory of Australia (Figure 2.2). Cats with incomplete details recorded were excluded from the final analyses, as were kittens 6 months of age or younger, owing to the possibility of maternal antibodies giving false-positive results with FIV testing (Hosie et al., 2009). Some cats had a FIV result recorded but no FeLV result. Ultimately, 2,083 cats remained available for analysis, of which 2,032 also had a recorded FeLV result.

The age of cats recruited ranged from 7 months to 22 years (median age 6 years; interquartile range [IQR] 3-11 years). Castrated male cats were the most common category (974/2,083; 47%), followed by spayed female cats (671/2,083; 32%), entire male cats (245/2,083; 12%) and entire female cats (193/2,083; 9%). Overall, there was a gender bias resulting in more males (1,219/2,083; 59%) than females (864/2,083; 41%) being tested (Figure 2.3). Entire male cats were significantly over-represented in WA compared to the rest of the country (54/239; 23% *versus* 191/1,844; 11%, $P = 0.001$).

2.4.2.2 Serological testing

Of 2,083 cats tested, 305 were FIV-positive (15%; 95% CI 13.1-16.2). Of 2,032 cats tested, 32 were FeLV-positive (2%; 95% CI 1.0-2.1) (Figure 2.2, Table 2.3). Of the 32 FeLV-positive cats, 11 also tested FIV-positive (34%), giving a FIV/FeLV co-infection rate of 11/2,032 (0.5%; 95% CI 0.2-0.9). The median age of FIV-infected cats was 7 years (IQR 4-11 years). The median age of FeLV-infected cats was 6 years (IQR 3-10 years). FIV and FeLV seroprevalence rates by location (state/territory), sex and neutering status are provided in Tables 2.4.1 and 2.4.2.

2.4.2.3 Risk factors for FIV seropositivity

The seroprevalence of FIV infection was significantly higher in cats older than 3 years of age compared to cats younger than 3 years of age ($P < 0.001$). Male cats were significantly more likely than female cats to be FIV-infected ($P < 0.001$), while entire male cats were significantly more likely than castrated male cats to be FIV-infected ($P = 0.001$).

When FIV seroprevalence was assessed using a multivariate model to account for the significant effects of age, sex and neutering status, a significant difference between sampling locations was found ($P = 0.03$). Specifically, the ACT, NSW and VIC had a significantly lower FIV seroprevalence compared with WA, while ACT and NSW had a significantly lower FIV seroprevalence compared with QLD. When WA was compared with the rest of the country, cats domiciled in that state were significantly more likely to be FIV-infected (OR 1.7) (Figure 2.4). Although SA had the lowest recorded FIV seroprevalence (3/38; 8%), the low sample number and resulting large SE precluded this difference from reaching statistical significance.

The seroprevalence of FIV infection was not significantly different between 'healthy' and 'sick' cats using the aforementioned definitions (14% *versus* 16%; $P = 0.17$), although when cats classified with dental disease were excluded from analysis there was a trend towards significance ($P = 0.06$). When the attending veterinarian's assessment of health status was considered, however, the prevalence of FIV infection among 'sick' cats was almost twice that of 'healthy' cats (11% *versus* 20%, $P < 0.0001$).

One potential geographical hot spot of FIV infection was identified in WA (postcodes 6024, 6060, 6090, $P = 0.06$). When investigated further, this cluster of infections was found to be the result of biased sampling, with a higher proportion of entire cats sampled compared with the rest of cohort 2 ($P < 0.001$). Socioeconomic data from the Australian Bureau of Statistics (ABS)[17] for these postcodes was examined and compared to ABS data for other postcodes in cohort 2; no significant differences were found between the cluster postcodes and other postcodes for socioeconomic disadvantage ($P = 0.74$), resources ($P = 0.74$) or education ($P = 0.94$).

2.4.2.4 Risk factors for FeLV seropositivity

Age, sex and neutering status were not found to be risk factors for FeLV infection ($P = 0.87$, 0.50 and 0.63, respectively), and sampling location only just failed to reach significance ($P = 0.06$). When results for WA were compared with the rest of the country, cats from that state were approximately three times as likely to be FeLV-infected (OR 3.0) (Figure 2.4). ACT did not record any FeLV-infected cats (0/45), while WA recorded the highest seroprevalence (9/239; 4%). The seroprevalence of FeLV infection was approximately three times higher among 'sick' cats than 'healthy' cats, using both the

aforementioned definitions and the attending veterinarian's assessment of health status (1% *versus* 3%, $P = 0.02$; 1% *versus* 3%, $P < 0.001$, respectively).

A summary of significant risk factors for FIV and FeLV infection of cats in cohort 2 is provided in Table 2.5.

2.4.3 Cohort 3 (Cats presenting to MUVH, WA)

2.4.3.1 Sample population

A total of 170 cats were tested for FIV and/or FeLV, ranging in age from 2 months to 19 years (median age 6 years; IQR 2-10 years). These cats comprised 80 castrated males (47%), 66 spayed females (39%), 17 entire males (10%) and seven entire females (4%). Most were domestic crossbred cats (127/170; 75%); the remainder comprising a range of pedigree breeds. The majority of cats were tested as part of a medical work up for non-specific illness (114/170; 67%), followed by testing prior to commencement of immunosuppressive therapy (31/170; 18%), testing prior to blood donation (12/170; 7%) health assessment of stray animals (11/170; 7%) and routine testing prior to FIV/FeLV vaccination (2/170; 1%).

2.4.3.2 Serological testing

Of 169 cats tested for FIV, 24 were positive (14%; 95% CI 8.9-19.5). FIV positive cats ranged from 2 months to 13 years (median age 4 years; IQR 2-8 years), comprised mostly of domestic crossbred cats (19/24; 79%) with a preponderance of male cats (14 castrated males, seven entire males, two spayed females and one entire female).

Of 166 cats tested for FeLV, 7 cats were positive (4%; 95% CI 1.2-7.3). FeLV positive cats ranged from 10 months to 8 years (median age 4 years; IQR 2-8 years), comprised mostly

of domestic crossbred cats (6/7; 86%) and entirely male cats (four castrated males and three entire males). Of the seven FeLV-positive cats, four also tested FIV-positive (57%), giving a FIV/FeLV co-infection rate of 4/165 (2%; 95% CI 0.1-4.7).[18]

[17] www.abs.gov.au. 2011 census data used.
[18] 165/170 cats had both FIV and FeLV results recorded.

Figure 2.2 Map of Australia showing the distribution of client-owned cats recruited for testing (Cohort 2). A larger circle indicates a higher number of cats sampled in that area. Only one poorly-populated island state (TAS) and one sparsely populated territory (NT) were not included in the study. FIV (top line) and FeLV (bottom line) seroprevalence rates are shown for each state/territory.

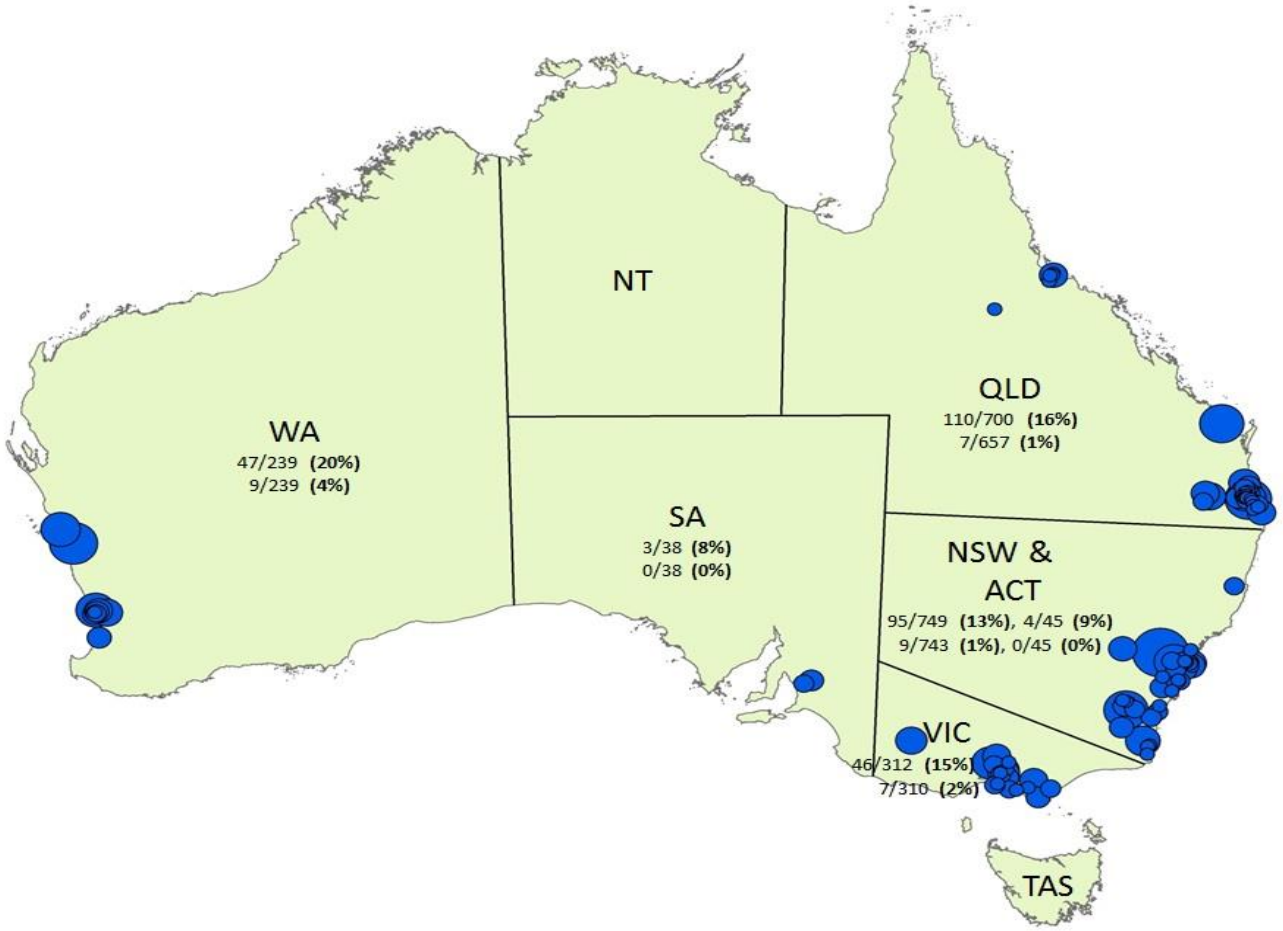
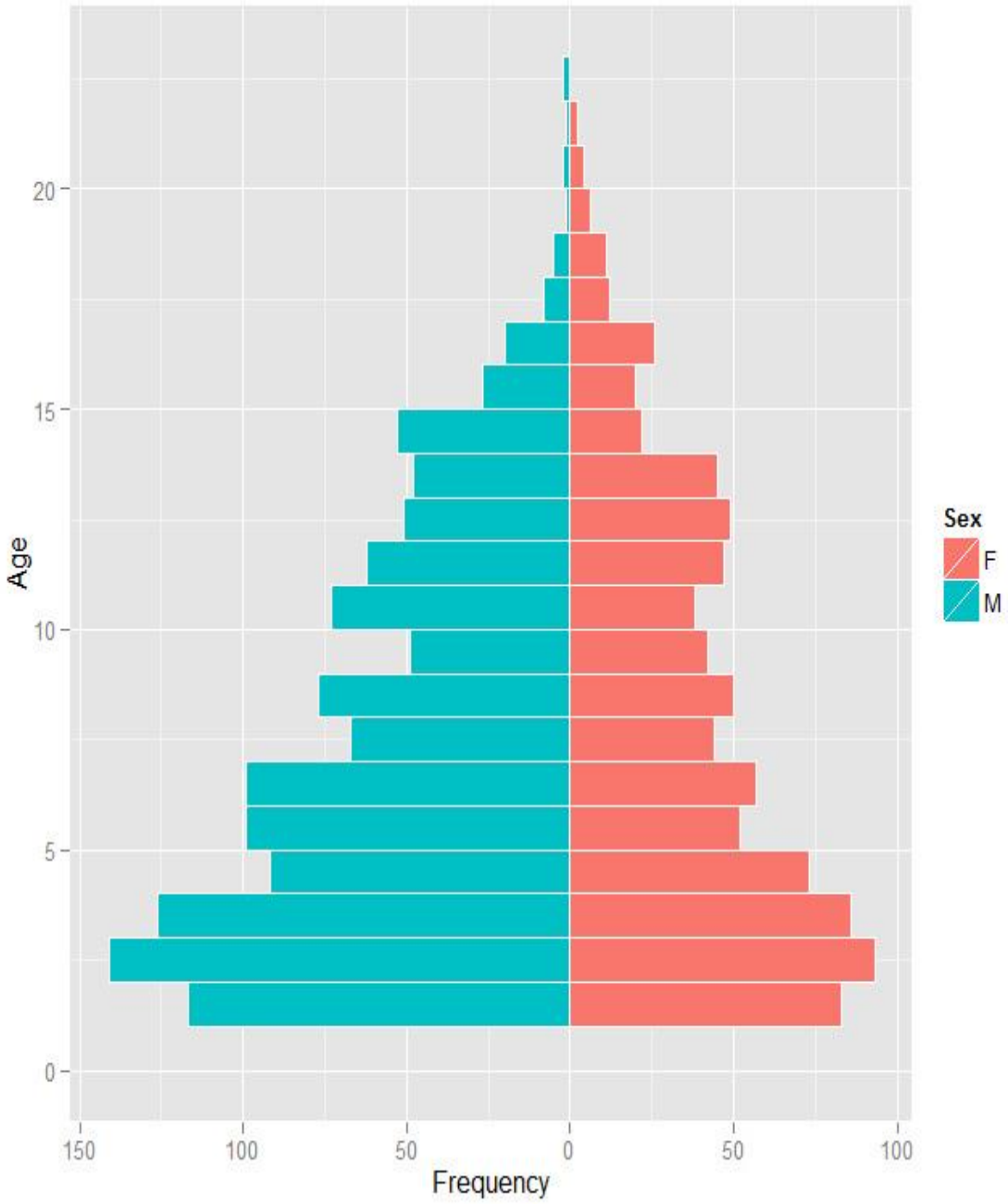


Figure 2.3 Age and sex pyramid for client-owned cats recruited for testing (Cohort 2), highlighting the skew towards male cats. The age of cats is displayed on the *y*-axis, while the number of cats of each sex is displayed on either side of the *x*-axis.



F = female, M = male.

Table 2.3 FIV and FeLV seroprevalence amongst client-owned cats (Cohort 2) by location (raw data).

Location	FIV seroprevalence	FeLV seroprevalence
ACT	4/45 **(9%)**	0/45 **(0%)**
NSW	95/749 **(13%)**	9/743 **(1%)**
VIC	46/312 **(15%)**	7/310 **(2%)**
QLD	110/700 **(16%)**	7/657 **(1%)**
SA	3/38 **(8%)**	0/38 **(0%)**
WA	47/239 **(20%)**	9/239 **(4%)**
Total	**305/2,083 (15%)**	**32/2,032 (2%)**

Table 2.4.1 FIV seroprevalence rates amongst client-owned cats (Cohort 2) by location, sex and neutering status (raw data).

	FIV seroprevalence				
Location	**Entire male**	**Castrated male**	**Entire female**	**Spayed female**	**TOTAL**
ACT	2/8 (25%)	2/20 (10%)	0/4 (0%)	0/13 (0%)	**4/45 (9%)**
NSW	16/94 (17%)	57/327 (17%)	5/81 (6%)	17/247 (7%)	**95/749 (13%)**
VIC	8/34 (24%)	30/160 (19%)	3/24 (13%)	5/94 (5%)	**46/312 (15%)**
QLD	16/49 (33%)	80/369 (22%)	0/39 (0%)	14/243 (6%)	**110/700 (16%)**
SA	1/6 (17%)	1/17 (6%)	0/1 (0%)	1/14 (7%)	**3/38 (8%)**
WA	13/54 (24%)	27/82 (33%)	5/44 (11%)	2/59 (3%)	**47/239 (20%)**
TOTAL	**56/245 (23%)**	**197/975 (20%)**	**13/193 (7%)**	**39/670 (6%)**	**305/2,083 (15%)**

Table 2.4.2 FeLV seroprevalence rates amongst client-owned cats (Cohort 2) by location, sex and neutering status (raw data).

	FeLV seroprevalence				
Location	**Entire male**	**Castrated male**	**Entire female**	**Spayed female**	**TOTAL**
ACT	0/8 (0%)	0/20 (0%)	0/4 (0%)	0/13 (0%)	**0/45 (0%)**
NSW	1/93 (1%)	2/325 (1%)	1/81 (1%)	5/244 (2%)	**9/743 (1%)**
VIC	1/34 (3%)	2/159 (1%)	0/24 (0%)	4/93 (4%)	**7/310 (2%)**
QLD	0/48 (0%)	5/343 (2%)	0/39 (0%)	2/227 (1%)	**7/657 (1%)**
SA	0/6 (0%)	0/17 (0%)	0/1 (0%)	0/14 (0%)	**0/38 (0%)**
WA	4/54 (7%)	3/82 (4%)	1/44 (2%)	1/59 (2%)	**9/239 (4%)**
TOTAL	**6/243 (3%)**	**12/946 (1%)**	**2/193 (1%)**	**12/650 (2%)**	**32/2,032 (2%)**

Figure 2.4 FIV and FeLV seroprevalence amongst client-owned cats (Cohort 2) for WA compared with the rest of Australia (model adjusted data). The *y*-axis shows the probability of FIV infection at a fixed point in time. SE bars are shown.

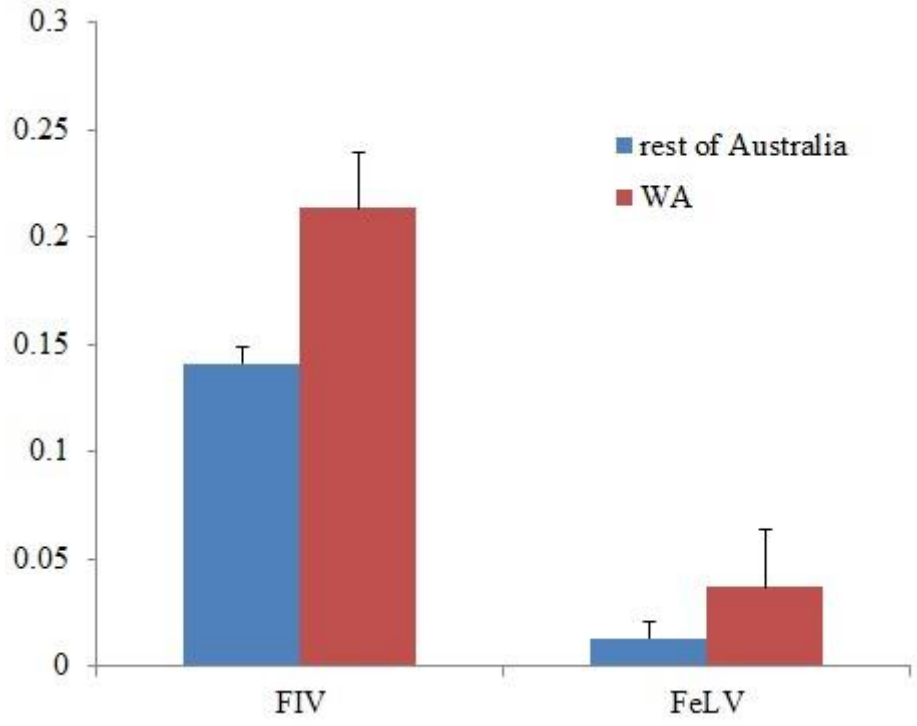


Table 2.5 Summary of statistically significant risk factors ($P < 0.05$) for retroviral infection amongst client-owned cats (Cohort 2).

FIV infection	FeLV infection
Age (> 3 years)	Health status ('sick')
Sex (male)	Location (WA)
Neutering status (entire males)	
Location (WA, QLD)	

2.5 DISCUSSION

This is the largest study to date of FIV and FeLV infection amongst Australian cats and provides important epidemiological information regarding feline retroviral infections in Australia. Results from feline Cohort 1 in the current study confirmed the notion that FIV infection is more common in Australia (6% in the WA shelter) than North America, with a large US-based study finding the seroprevalence of FIV infection amongst relinquished shelter cats to be just 1% (Levy et al., 2006). The most novel finding from this investigation was the higher seroprevalences of both FIV and FeLV in WA cats in Cohort 2 compared with the rest of Australia.

Interestingly, like the last Australian FIV serosurvey (Norris et al., 2007), the current study did not find a difference in FIV seroprevalence between 'healthy' and 'sick' client-owned cats in Cohort 2 using a similar classification system. This finding does not equate to FIV infection being apathogenic in Australian cats, but rather that a different study design targeting specific disease associations (e.g. B-cell lymphoma), older cats (due to the long asymptomatic phase of FIV infection) and different wild strains (due to variability in pathogenicity) is required to investigate the impact of FIV infection on mortality and morbidity. When the attending veterinarian's assessment of 'healthy' or 'sick' was considered, however, a significant difference in FIV seroprevalence was found, suggesting that elements of health assessment are subjective, possibly intuitive and without doubt informative, although not easily captured by strict objective criteria. The current study found a significant difference in FeLV seroprevalence between 'healthy' and 'sick' client-owned cats in Cohort 2, regardless of the classification system utilized, reflecting the well-known impact of FeLV infection on feline health.

The comparatively higher FIV and FeLV infection rates (20% and 4%, respectively) reported in the current study for WA cats in Cohort 2, compared to the rest of the country, appear consistent with earlier studies (Robertson et al., 1990b; Thomas et al., 1993b). In the current study, cats in Cohort 2 domiciled in WA were 1.7 times more likely to be FIV-infected, and 3.0 times more likely to be FeLV-infected compared with the rest of the country. The difference in FIV seroprevalence between shelter and client-owned cats in the current study (i.e. Cohort 1 *versus* Cohort 2), and particularly the lower FIV seroprevalence in WA shelter cats compared to WA client-owned cats, likely reflects a difference in demographics between the two cohorts. Although signalment details for shelter cats were unavailable, and Australian data on characteristics of cats entering shelters is lacking, one US study found 639/1,200 (53%) adult cats (6 months of age or older) entering the shelter were younger than 3 years of age (New et al., 2000). If this trend is also true for Australian shelters, which we think likely, and as the cumulative risk for acquiring FIV infection increases with age, it is reasonable to assume the lower seroprevalence of FIV in shelter cats compared with client-owned cats was due to a lower median sampling age in Cohort 1 compared to Cohort 2. Furthermore, based on previous Australian studies, it is likely that around a quarter of shelter cats sampled were previously housed exclusively indoors and therefore had very low, if any, exposure to FIV, compared with the client-owned cats, which because of the study design, all had some level of outdoor access (Toribio et al., 2009; Chang-Fung-Martel et al., 2013). Although the skew towards male cats in Cohort 2 (59% *versus* 41%) would have resulted in a slight overestimation of FIV seroprevalence, the same likely was likely true for cats in Cohort 1, owing to the described sampling bias towards males.

This is the first FIV seroprevalence study conducted since the introduction of the FIV vaccine in Australia in October 2004 (Norris and collaborators tested blood specimens collected prior to the release of the vaccine). FIV vaccination results in the production of FIV antibodies indistinguishable from those used for the diagnosis of FIV infection when using SNAP FIV/FeLV Combo (Uhl et al., 2002; Chapter 3), so that in the absence of additional testing an overestimation of FIV seroprevalence is possible. However, cats surrendered to shelters have typically received a lower level of care compared to non-surrendered cats (Salman et al., 1998), and thus are less likely to have been vaccinated against FIV. Furthermore, although not specifically stated in the study instructions provided to veterinary clinics by BI, it is our presumption that cats vaccinated against FIV would not have been selected by veterinarians for testing. It therefore seems unlikely that previous FIV vaccination would have caused sufficient false-positive antibody test results to substantially impact the reported FIV seroprevalence for either shelter or client-owned cats.

Seroprevalence studies for FeLV in Australia are impacted by the low FeLV infection rate in the general cat population and the resulting low positive predictive value of point-of-care antigen test kits, despite excellent sensitivity and specificity of the current generation of kits (www.idexx.com/files/small-animal-health/products-and-services/snap-products/snap-fiv-felv-combo/snap-combo-test-accuracy.pdf). The European ABCD Guidelines recommend confirmatory testing for suspected cases of FeLV infection using proviral PCR, particularly in healthy cats without clinical signs of disease (Lutz et al., 2009). If a true FeLV seroprevalence of 0.5-1.0% in Australia is postulated, then the occurrence of false-positive FeLV results with point-of-care antigen testing is similar to the prevalence of FeLV antigenemia (Hartmann et al., 2007; Adam and Dandrieux, 2011;

Beatty et al., 2011). Therefore, it is likely that the true FeLV infection rate for both cohorts of cats in the current study was actually lower than reported.

Age, sex and neutering status in males were important risk factors for FIV infection in client-owned cats (Cohort 2), reinforcing findings from other studies and confirming that fighting between cats continues to be the main mechanism for FIV transmission, particularly in Australia where the climate permits outdoor access for most months of the year (Norris et al., 2007; Hosie et al., 2009). Conversely, age, sex and neutering status were not found to be risk factors for FeLV infection in client-owned cats. The susceptibility of cats to infection with FeLV has traditionally thought to be age-dependent, with young cats more likely to be FeLV-infected (and older cats more likely to be FIV-infected) (Norris et al., 2007; Lutz et al., 2009). The current study was not consistent with this 'age effect' on retroviral status and instead found a similar median age for FIV and FeLV infection in client-owned cats (7 years and 6 years, respectively). This finding is consistent with recent research from Germany (Gleich et al., 2009b), suggesting a changing landscape for FeLV infection, where older cats are as likely to be FeLV-infected as younger cats. The resulting impact of FeLV infection on morbidity and mortality in older cats needs confirmation and further investigation.

2.6 CONCLUSION

This study reports the largest number of client-owned cats recruited over a large proportion of the populated continent to investigate the epidemiology of retroviral infections. FIV continues to be a common infection of client-owned cats with outdoor access, making Australia an ideal location for testing the effectiveness of the FIV vaccine in the field. FIV and FeLV are significantly more common in client-owned cats in Perth, WA, compared with the rest of the country. The reasons for this might be related to lower rates of neutering, and require further investigation and intervention. FeLV infection, although uncommon, should not be forgotten, even in older cats, as a potential cause of feline morbidity and mortality, including late 'downstream' effects such as lymphomagenesis. Owing to the low prevalence of FeLV antigenaemia in Australia, confirmatory testing by real-time PCR should always be pursued for any cat testing positive for FeLV antigen using point-of-care test kits. In cats infected with FeLV, co-infection with FIV is common.

CHAPTER 3. DIAGNOSIS OF FIV INFECTION IN THE DOMESTIC CAT USING BLOOD

3.1 ABSTRACT

This study challenges the commonly held view that the FIV infection status of FIV-vaccinated cats cannot be determined using point-of-care antibody test kits due to indistinguishable antibody production in FIV-vaccinated and naturally FIV-infected cats. The performance of three commercially available point-of-care antibody test kits was compared in a mixed population of FIV-vaccinated (n = 119) and FIV-unvaccinated (n = 239) cats in Australia. FIV infection status was assigned by considering the results of all antibody kits in concert with results from a commercially available PCR assay (FIV RealPCR). Two lateral flow IC test kits (Witness FeLV/FIV; Anigen Rapid FIV/FeLV) had excellent overall sensitivity (100%; 100%) and specificity (98%; 100%) and could discern the true FIV infection status of cats, irrespective of FIV vaccination history. The lateral flow ELISA test kit (SNAP FIV/FeLV Combo) could not determine if antibodies detected were due to previous FIV vaccination, natural FIV infection, or both. The sensitivity and specificity of FIV RealPCR for detection of viral and proviral nucleic acid was 92% and 99%, respectively. These results will potentially change the way veterinary practitioners screen for FIV in jurisdictions where FIV vaccination is practiced, especially in shelter scenarios where the feasibility of mass screening is impacted by the cost of testing.

3.2 INTRODUCTION

The introduction of the FIV vaccine in 2002 complicated FIV diagnosis because vaccination was reported to result in the production of antibodies to FIV indistinguishable from those produced in response to natural infection (Uhl et al., 2002). Consequently, for FIV-vaccinated cats and cats of unknown vaccination status, FIV diagnostics shifted towards molecular methods such as nucleic acid amplification (Crawford and Levy, 2007; Morton et al., 2012). Some studies have also explored alternative methods for FIV diagnosis with excellent results, such as a discriminatory ELISA based on antibody response to two different FIV antigens (Kusuhara et al., 2007; Levy et al., 2008b), and by calculating the CD4:$CD8^{low}$ T-lymphocyte ratio (Litster et al., 2014).

In this study, we reappraised the assertion that point-of-care kits are unable to distinguish antibodies produced following FIV vaccination from antibodies produced in response to natural FIV infection, and therefore are unable to determine the true FIV infection status of FIV-vaccinated cats, using three commercially available test kits (SNAP FIV/FeLV Combo, Witness FeLV/FIV and Anigen Rapid FIV/FeLV) with each antibody kit using a different panel of viral epitopes (Figure 3.1 and Table 3.1).

Figure 3.1 Schematic of FIV emphasising the different target antigens for antibody testing.

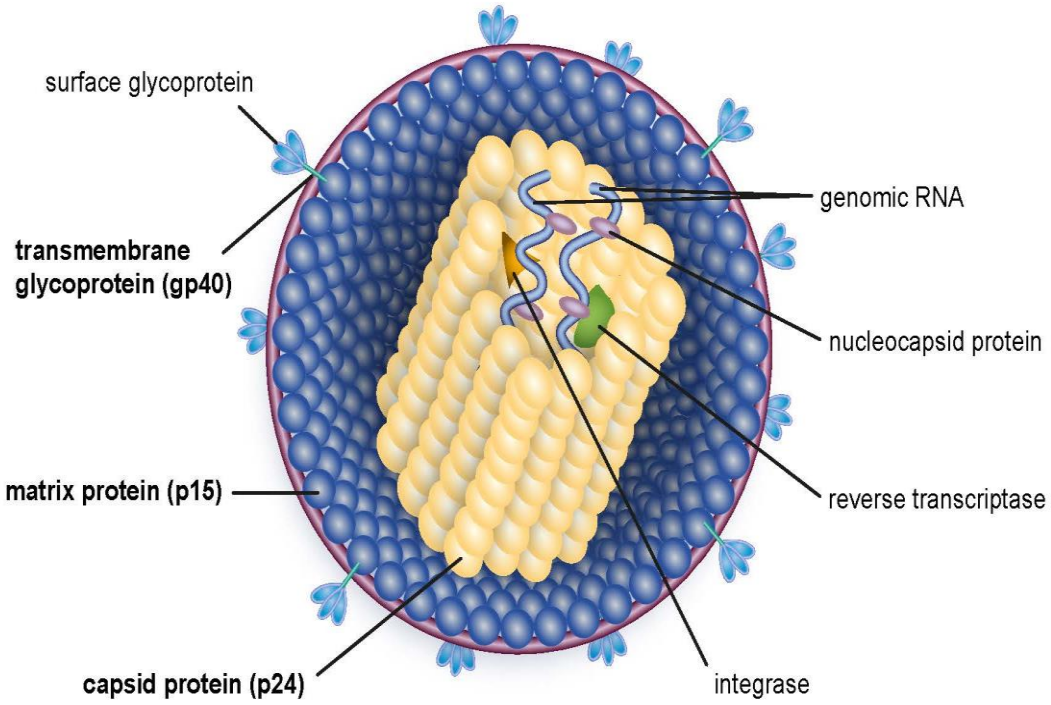


Table 3.1 Summary of the antibodies detected using four different point-of-care FIV antibody test kits.

FIV Antibody Detection Kit	FIV Target Antigen		
	p15	p24	gp40
SNAP FIV/FeLV Combo (Australia, NZ, North America)	●	●	
SNAP FIV/FeLV Combo Plus (Europe)[a]	●	●	●
Witness FeLV/FIV			●
Anigen Rapid FIV/FeLV		●	●

[a]Not used in this study, but used in Hartmann et al., 2007.

3.3 MATERIALS AND METHODS

3.3.1 Sample population

Cats with known FIV vaccination history were recruited through veterinary clinics in Australia during 2013-2014, most commonly at the same time as an annual health check or some routine procedure (e.g. dental scaling and polishing). Very occasionally, cats were sampled during hospitalisation for further work up of systemic illness; however no FIV-infected cats would have been classified as being in the feline-AIDS (FAIDS) phase of infection. Cats or kittens were excluded from the study if they were less than six months of age (due to the possibility of maternal antibodies being present), had an unclear FIV vaccination history or had a known FIV infection status (due to prior testing). Cats were included in the 'FIV-vaccinated' group if they had received one or more FIV vaccines at any time in their life, regardless of whether or not the administration of vaccine had been in accordance with the manufacturer's guidelines[1]. Cats were included in the 'FIV-unvaccinated' group if they had never been vaccinated against FIV. Clinical records of all patients from both groups were carefully interrogated to enforce this inclusion criterion. Cases were recruited from veterinary practices servicing areas where the prevalence of FIV infection was perceived to be high (Chapter 2).

Animal ethics approval was granted by the University of Sydney (Approval number 5920).

3.3.2 Serological and molecular detection of FIV infection

Blood was collected by the primary author using jugular venipuncture and immediately aliquoted into three EDTA tubes and stored at 4^{o}C. Testing for FIV antibodies was performed within 24 hours of blood collection[19] with three commercially available point-of-care kits tested concurrently, using whole blood from the same EDTA tube, according

to the manufacturer's instructions. The antibody kits tested were SNAP FIV/FeLV Combo[2], Witness FeLV/FIV[3], and Anigen Rapid FIV/FeLV[4] (Table 3.1). The antibody results panel for each cat was digitally photographed at the time of testing. Blood from this tube was also used for routine haematologic examination.[20] The second EDTA tube was centrifuged within 6 h of collection for 3 min at 12,000 *x g* and the plasma transferred to a plain tube using a sterile pipette. Plasma specimens and EDTA cell pellets were stored subsequently at -80^{o}C for confirmatory testing of discrepant samples at a later time. The third EDTA tube was sent for FIV nucleic acid amplification (FIV RealPCR),[21] a commercially available PCR assay targeting a conserved *gag* region in both viral RNA (using cDNA following a reverse transcription step) and proviral DNA. FIV subtype was determined in infected cats using subtype specific primer pairs for subtypes A, B, D and F (www.idexx.com.au/pdf/en_au/smallanimal/education/realpcr-test-for-fiv.pdf).

3.3.3 Defining FIV infection status (Table 3.2)

At the beginning of the study FIV RealPCR testing was used as the 'gold standard' for FIV diagnosis, with a published sensitivity and specificity of 94% and 94% (Litster et al., 2012). As the study progressed, however, it became clear that two of the antibody detection kits were able to determine the true FIV infection status of cats, irrespective of FIV vaccination history. In light of this finding, revised definitions for 'FIV-infected' and 'FIV-uninfected' were employed, which considered results from all three antibody kits in concert with the FIV RealPCR result. Where there was complete agreement between the three antibody kits and the FIV RealPCR result (either all negative or all positive), assigning a given cat's FIV infection status was straightforward. Where two of the three antibody kits matched the FIV RealPCR^{T}result (i.e. three out of four results were in agreement), FIV infection status was assigned and it was assumed the conflicting antibody

kit was a false-positive or false-negative result. Where all three antibody kits tested negative and the FIV RealPCR result was positive, FIV RealPCR testing was repeated using either stored sample or a fresh blood specimen (collected at a second venipuncture), and the second FIV RealPCR result was taken as being definitive. Where all three antibody kits tested positive and the FIV RealPCR result was negative, PCR testing was repeated at a second commercial laboratory using stored sample and a methodologically distinct assay (www.gribblesvets.com.au/index.php/download_file/view/90/142/)[22] and FIV RealPCR testing was repeated using fresh blood collected at several time points over 12-18 months. For such cats, additional fresh blood was also collected into a heparinised tube and sent refrigerated to a third laboratory for VI.[23] The VI result was considered definitive. The same additional testing was also undertaken to confirm FIV infection in FIV-vaccinated cats that tested positive with all three antibody kits as well as FIV RealPCR, i.e. vaccination 'failures'. Where there were two positive and two negative results, regardless of which tests were positive, additional blood was collected into a heparanised tube and sent refrigerated to a fourth laboratory for VI,[24] with the VI result taken as being definitive.

When determining the results of the antibody test kits, even a faint band or spot was subjectively recorded as a 'faint positive' result. Although the manufacturer's instructions for Witness FeLV/FIV and Anigen Rapid FIV/FeLV contain no guidelines for interpreting faint results, instructions for SNAP FIV/FeLV Combo advise that any colour development in the FIV sample spot should be considered significant (www.idexx.com/resource-library/smallanimal/snap-combo-package-insert-en.pdf). Antibody testing was repeated using stored plasma thawed from -80^{o}C where there was disagreement between all three antibody kits and the FIV RealPCR result, where there were two positive and two negative results, and where a 'faint positive' result was recorded using any of the antibody kits.

For the purpose of this study, SNAP FIV/FeLV Combo, Witness FeLV/FIV and Anigen Rapid FIV/FeLV are antibody test kits sold for the sole purpose of diagnosing FIV infection. Therefore, by definition, a positive antibody test result in a FIV-uninfected cat, regardless of FIV vaccination history, was considered a false-positive result. Conversely, a negative antibody test result in a FIV-uninfected cat, regardless of FIV vaccination history, was considered a true-negative result.

Table 3.2 Determination of FIV infection status of cats in the study. Classification of FIV status was based on the overall combination of results from (a) three commercially available FIV antibody test kits (SNAP FIV/FeLV Combo, Witness FeLV/FIV and Anigen Rapid FIV/FeLV) and FIV RealPCR testing. Additional testing (repeat FIV RealPCR testing, PCR testing using different primers and methodology and/or virus isolation) was pursued when the results panel was equally divided (two positive results, two negative results), when there was complete agreement between antibody results but disagreement with the FIV RealPCR result, and to confirm FIV infection in FIV-vaccinated cats.

Antibody test kit[a]	FIV RealPCR	Additional tests		FIV status
		Repeat PCR	Virus isolation	
+++	+	+ (vaccinated cats only)	+ (vaccinated cats only)	+
+++	-	+	+	+
++-	+	NP	NP	+
++-	-	-	-	-
+--	+	-	NP	-
+--	-	NP	NP	-
---	-	NP	NP	-
---	+	-	NP	-

+ = positive, - = negative, NP = not performed. Red = FIV-infected, yellow = FIV-uninfected.

3.3.4 Statistical analysis

Numerical analyses were performed using a commercial programme (Genstat 16th Edition).[25] Statistical significance was considered at $P < 0.05$ and 95% CIs were calculated using Microsoft Excel.[26] Fisher's exact test was used to investigate whether SNAP FIV/FeLV Combo and Witness FeLV/FIV false-positive results were more common in FIV-vaccinated cats than FIV-unvaccinated cats by comparing false-positive and true-negative results. Fisher's exact tests were also used to examine whether 'faint positive' results with any of the three antibody test kits was associated with FIV infection status. A two-sample *t*-test was used to investigate whether there was a correlation between time since last FIV vaccination and false-positive antibody results recorded with Witness FeLV/FIV in the FIV-vaccinated group.

[19] Faculty of Veterinary Science, The University of Sydney, Sydney, NSW, Australia.
[20] Veterinary Pathology Diagnostic Services, The University of Sydney, Sydney, NSW, Australia.
[21] IDEXX Laboratories, East Brisbane, Queensland, Australia.
[22] Gribbles Veterinary Pathology, Melbourne, Victoria, Australia.
[23] Yamamoto Laboratory, The University of Florida, Gainesville, FL, USA.
[24] Veterinary Diagnostic Services, The University of Glasgow, Scotland, UK.
[25] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.
[26] Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.

3.4 RESULTS

3.4.1 Sample population

Blood samples were obtained from 358 client-owned cats recruited from 12 veterinary clinics distributed over four states of Australia (NSW, VIC, QLD and SA) (Table 3.3).

A total of 119 FIV-vaccinated cats were recruited, ranging from six months to 18 years (median 7 years; IQR 5-10 years). These cats comprised 66 castrated males and 53 spayed females. Most were domestic crossbred cats (103/119; 87%); the remainder comprising a range of pedigree breeds. Most cats in this cohort (109/119; 92%) had received three primary FIV vaccinations, two to four weeks apart (i.e. the protocol recommended by the vaccine manufacturer), and three or more annual FIV vaccinations before being sampled. For these 109 cats sampling took place between 2 and 462 days following their last FIV vaccination (median 237 days; IQR 152-317 days), with 10/109 (9%) cats sampled within eight weeks of their last annual FIV vaccination. Seven cats (out of 119) were considered overdue for their annual FIV vaccination (more than 15 months since last vaccination; median 5.4 years, range 3-7 years), and three cats were overdue for their second or third primary FIV vaccination (by 46 days, 74 days and 3 years).

A total of 239 FIV-unvaccinated cats were recruited, ranging from 2 to 20 years (median 7 years; IQR 6 to 10 years). These cats comprised 112 castrated males, 123 spayed females, and 4 entire males. Most were domestic crossbred cats (207/239; 87%); the remainder comprising a range of pedigree breeds.

Table 3.3 List of twelve recruited veterinary clinics including clinic address and number of cats tested.

Name of clinic	Clinic details	No. of cats
Great Western Animal Hospital	469 Great Western Hwy, Pendle Hill NSW 2145, (02) 9631 9322	114
Newtown Veterinary Clinic	121 West Fyans St, Newtown VIC 3220, (03) 5221 5333	47
Elizabeth Drive Animal Hospital	Cnr Elizabeth Drive and Woodlands Rd, Liverpool NSW 2170, (02) 9602 7018	46
Campbelltown Animal Hospital	15 Chamberlain St, Campbelltown NSW 2560, (02) 4626 4222	33
Bankstown Veterinary Hospital	14 Marshall St, Bankstown NSW 2200, (02) 9790 1101	32
Fulham Gardens Veterinary Surgery	441 Tapleys Hill Rd, Fulham Gardens SA 5024, (07) 8355 5475	24
Mt Annan Veterinary Hospital	17/2-4 Main St, Mt Annan NSW 2567, (02) 4647 7722	21
Casula Veterinary Hospital	674 Hume Hwy, Casula NSW 2170, (02) 9602 9863	18
The Cat Clinic	189 Creek Road, Mt Gravatt, QLD 4122, (07) 3349 0811	13
Kardinia Veterinary Clinic	355 Moorabool St, Geelong VIC 3220, (03) 5221 5122	7
RSPCA Veterinary Hospital	201 Rookwood Rd, Yagoona NSW 2199, (02) 9770 7555	2
Sylvania Veterinary Hospital	335 Princes Hwy, Sylvania NSW 2224, (02) 9522 7088	1

3.4.2 Serological and molecular detection of FIV infection

3.4.2.1 FIV-vaccinated cohort (n = 119, Tables 3.4 and 4.5)

All FIV-vaccinated cats (119/119) tested FIV positive using SNAP FIV/FeLV Combo. In contrast, only a small number of the 119 FIV-vaccinated cats tested FIV positive using Witness FeLV/FIV (11 cats) and Anigen Rapid FIV/FeLV (5 cats) (Figures 3.2 and 3.3).

All five cats that tested FIV positive using Anigen Rapid FIV/FeLV also tested FIV positive with the other two antibody kits. Initial FIV RealPCR testing confirmed 3/5 cats to be FIV-infected, which was further confirmed by VI in two of these cats (one cat was unavailable for further sampling). The remaining two cats were initially negative with both FIV RealPCR and PCR testing at the second laboratory. Subsequent resampling and re-testing, however, found both cats positive with FIV RealPCR and VI. Thus, all five cats testing FIV positive using Anigen Rapid FIV/FeLV were truly FIV-infected. Of the five FIV-infected cats, three were castrated males and two were spayed females.

The six cats that tested FIV positive with SNAP FIV/FeLV Combo and Witness FeLV/FIV, but FIV negative with Anigen Rapid FIV/FeLV and FIV RealPCR, were considered to be FIV-uninfected based on VI results (i.e. the positive antibody kit results were false-positives).[27] Another FIV-vaccinated cat had FIV RealPCR testing repeated due to possible contamination in the PCR facility; this cat, which initially was FIV RealPCR positive, subsequently tested FIV RealPCR negative and was ultimately considered to be FIV-uninfected.

No false-negative FIV results were recorded with any of the antibody kits.

[27] Only 5/6 cats had VI performed due to the unfortunate death (unrelated to this study) of one of the cats.

Figure 3.2 Photograph of test kit results from a FIV-vaccinated/FIV-infected cat, showing three positive FIV antibody test kit results: (a) Anigen Rapid FIV/FeLV, (b) Witness FeLV/FIV, and (c) SNAP FIV/FeLV Combo. For both IC kits the top strip is for FIV antibody testing and the bottom strip is for FeLV antigen testing. One band in the FIV or FeLV strip indicates a negative result, while two bands in the FIV or FeLV strip indicates a positive result. For SNAP FIV/FeLV Combo, two spots in the displayed formation indicates a positive FIV result. This result was obtained in 5/119 cats.

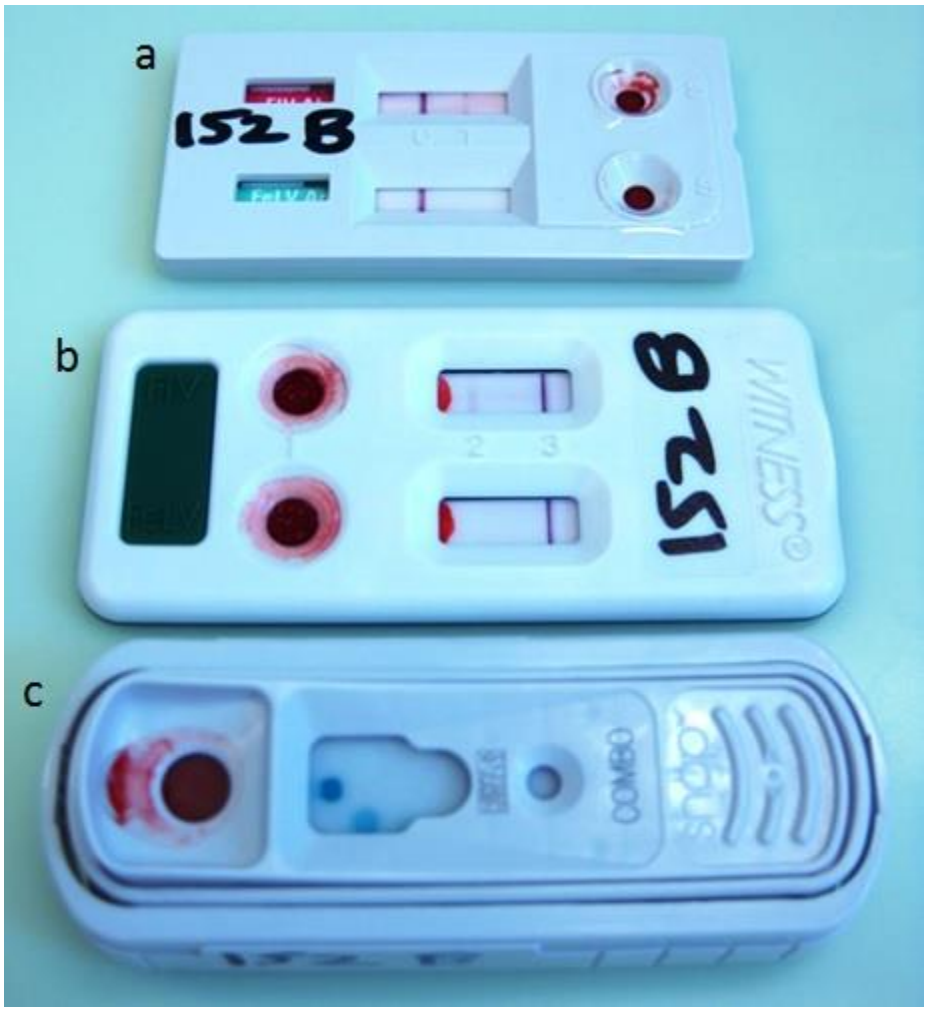


Figure 3.3 Photograph of test kit results from a FIV-vaccinated/FIV-uninfected cat, showing two negative FIV antibody test kit results: (a) Anigen Rapid FIV/FeLV, and (b) Witness FeLV/FIV; and one positive FIV antibody test kit result, (c) SNAP FIV/FeLV Combo. For both IC kits the top strip is for FIV antibody testing and the bottom strip is for FeLV antigen testing. One band in the FIV or FeLV strip indicates a negative result, while two bands in the FIV or FeLV strip indicates a positive result. For SNAP FIV/FeLV Combo, two spots in the displayed formation indicates a positive FIV result. This result was obtained in 108/119 cats.

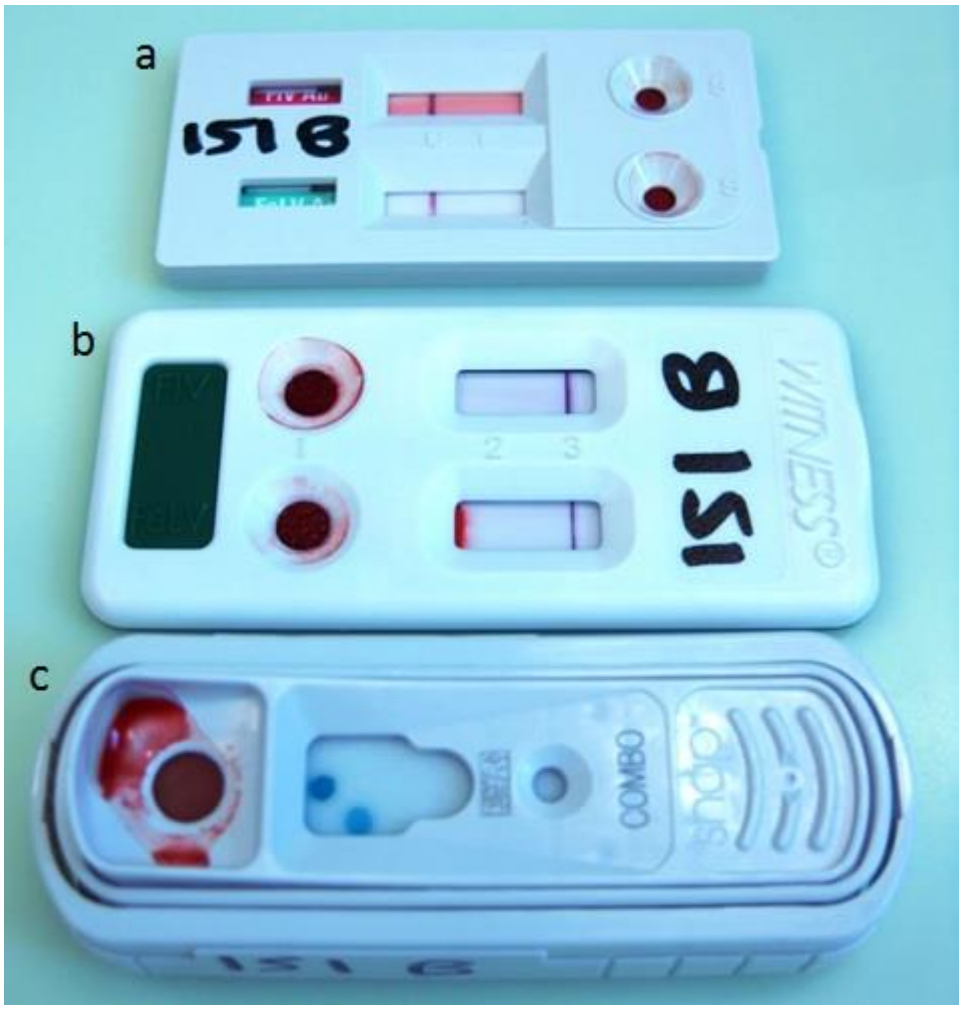


Table 3.4 Summary of results from FIV-vaccinated cats (*n* = 119), highlighting general trends as well as discrepant results. Only 2/3 FIV-vaccinated/FIV-infected, initially FIV RealPCR positive, cats had virus isolation performed. Both FIV-vaccinated/FIV-infected, initially FIV RealPCR negative, cats had blood sent to a second commercial laboratory for confirmatory PCR testing. Cat #102 was negative with repeat FIV RealPCR testing as well as PCR testing at the second commercial laboratory. Discordant cats were re-tested at a later date using thawed plasma stored at -80^{o}C.

Category	SNAP	Witness	Anigen	PCR	VI
FIV-vaccinated/FIV-uninfected (n = 107)	+	-	-	-	NP
FIV-vaccinated/FIV-infected (n = 3)	+	+	+	+	+ (2/3)
FIV-vaccinated/FIV infected (n = 2)	+	+	+	-	+ (2/2)
FIV-vaccinated/FIV-uninfected discordant cats (n = 7)					
(i) Cat #97	+	Faint +	-	-	-
(ii) Cat #173	+	Faint +	-	-	NP[27]
(iii) Cat #340	+	Faint +	-	-	-
(iv) Cat #341	+	Faint +	-	-	-
(v) Cat #345	+	Faint +	-	-	-
(vi) Cat #350	+	Faint +	-	-	-
(vii) Cat #102	+	-	-	+	NP

+ = positive, - = negative, NP = not performed.

Table 3.5 Results of three point-of-care FIV antibody test kits in FIV-vaccinated cats (*n* = 119). Confidence intervals (95%) are given in brackets.

Test kit	SNAP Combo	Witness	Anigen Rapid
True positive	5	5	5
False negative	0	0	0
True negative	0	108	114
False positive	114	6	0
Sensitivity (%)	5/5 = **100**	5/5 = **100**	5/5 = **100**
Specificity (%)	0/114 = **0**	108/114 = **95** (91-99)	114/114 = **100**
PPV (%)	5/119 = **4** (0-8)	5/11 = **45** (16-75)	5/5 = **100**
NPV (%)	0/0 = **0**	108/108 = **100**	114/114 = **100**

3.4.2.2 FIV-unvaccinated cohort (n = 239, Tables 3.6 and 3.7)

In this group of FIV-unvaccinated cats, 21 cats tested FIV positive with all three antibody kits and were confirmed to be FIV-infected with FIV RealPCR testing. Of 21 FIV-infected cats, 15 were male (14 castrated, one entire) and 6 were spayed females.

Of the remaining 218 FIV-uninfected cats in this group, most (212/218) tested FIV negative with all three antibody kits. Six false-positive FIV results were recorded using SNAP FIV/FeLV Combo, one false-positive was recorded using Witness FeLV/FIV, while no false-positive results were recorded using Anigen Rapid FIV/FeLV.

One FIV-uninfected cat in this group (cat #305) tested FIV positive with both SNAP FIV/FeLV Combo and Witness FeLV/FIV, but FIV negative with Anigen Rapid

FIV/FeLV and FIV RealPCR; VI was subsequently performed and confirmed the negative FIV status.

Three cats tested negative with all three antibody kits but initially positive with FIV RealPCR with varying C_T values: cat #126 tested positive for subtype A (C_T 32), cat #259 subtype F (C_T 39) and cat #277 subtype D (C_T 32). Repeat FIV RealPCR testing found all three cats to be FIV-uninfected and presumably there was contamination at the PCR facility.

No false-negative FIV results were recorded with any of the antibody kits.

Table 3.6 Summary of results from FIV-unvaccinated cats (*n* = 239), highlighting general trends as well as discrepant results. Cat #126, cat #259 and cat #277 were negative with repeat FIV RealPCR testing. Discordant cats were re-tested at a later date using thawed plasma stored at -80^{o}C.

Category	SNAP Combo	Witness	Anigen	PCR	VI
FIV-unvaccinated/FIV-uninfected (n = 209)	-	-	-	-	NP
FIV-unvaccinated/FIV-infected (n = 21)	+	+	+	+	NP
FIV-unvaccinated/FIV-uninfected discordant cats (n = 9)					
(i) Cat #60	Faint +	-	-	-	NP
(ii) Cat #263	Faint +	-	-	-	NP
(iii) Cat #280	Faint +	-	-	-	NP
(iv) Cat #326	Faint +	-	-	-	NP
(v) Cat #335	+	-	-	-	NP
(vi) Cat #305	+	Faint +	-	-	-
(vii) Cat #126	-	-	-	+	NP
(viii) Cat #259	-	-	-	+	NP
(ix) Cat #277	-	-	-	+	NP

+ = positive, - = negative, NP = not performed.

Table 3.7 Results of three point-of-care FIV antibody test kits in FIV-unvaccinated cats (*n* = 239). Confidence intervals (95%) are given in brackets.

Test kit	SNAP Combo	Witness	Anigen Rapid
True positive	21	21	21
False negative	0	0	0
True negative	212	217	218
False positive	6	1	0
Sensitivity (%)	21/21 = **100**	21/21 = **100**	21/21 = **100**
Specificity (%)	212/218 = **97** (95-99)	217/218 = **100** (99-100)	218/218 = **100**
PPV (%)	21/27 = **78** (62-93)	21/22 = **95** (87-100)	21/21 = **100**
NPV (%)	212/212 = **100**	217/217 = **100**	218/218 = **100**

3.4.2.3 Combined FIV-vaccinated and FIV-unvaccinated cohorts (n = 358, Table 3.8)

In total there were 120 false-positive FIV results recorded with SNAP FIV/FeLV Combo, seven false-positive FIV results recorded with Witness FeLV/FIV, and no false-positive FIV results recorded with Anigen Rapid FIV/FeLV. False-positive FIV results were significantly more common in FIV-vaccinated cats than FIV-unvaccinated cats for both SNAP FIV/FeLV Combo (114/120; 95%; $P < 0.001$) and Witness FeLV/FIV (6/7; 86%; $P = 0.007$). For Witness FeLV/FIV 10/33 (30%) positive results were recorded as 'faint positives', while 7/146 (5%) positive SNAP FIV/FeLV Combo and 7/26 (27%) positive Anigen Rapid FIV/FeLV results were recorded as 'faint positives'. 'Faint positive' results with Witness FeLV/FIV were strongly associated with absence of FIV infection and thus likely to be false-positive results ($P < 0.001$); only 3/26 FIV-infected cats recorded a 'faint positive' result with Witness FeLV/FIV, and 7/7 (100%) of false-positive Witness

FeLV/FIV results were recorded as 'faint positives'. There was no association between 'faint positive' results and absence of FIV infection (i.e. false-positive results) for either the SNAP FIV/FeLV Combo or Anigen Rapid FIV/FeLV kit (P = 1.000 for both). Time between last FIV vaccination and sampling was not a risk factor for false-positive FIV results with Witness FeLV/FIV (P = 0.82 with outliers [more than 15 months since last vaccination] removed); only 1/11 recently vaccinated cats (8 weeks or less since last FIV vaccination, cat #173) tested false-positive with Witness FIV/FeLV. The other five false-positive FIV results with Witness FeLV/FIV in vaccinated cats were recorded 139, 196, 259, 337 and 354 days after last FIV vaccination. All 6 FIV-vaccinated cats that had a false-positive FIV result with Witness FeLV/FIV had a 'faint positive' result recorded.

Discrepant samples that underwent repeat antibody testing with stored plasma thawed from -80^{o}C recorded almost identical antibody results as fresh whole blood tested initially (27 samples re-tested using 81 antibody test kits with 80/81 [99%] agreement).

Based on this study's definition for FIV positivity (Table 3.2), and considering only the initial FIV RealPCR result, molecular detection of FIV using RealPCR testing produced four false-positive and two false-negative results, giving a sensitivity of 92% (95% CI 82.1 to 100) and specificity of 99% (95% CI 97.7 to 99.9). One false-positive result and the two false-negative results were from the FIV-vaccinated group, while three false-positive results were from the FIV-unvaccinated group. Repeat FIV RealPCR testing, either using the original sample or a subsequent sample, was able to correctly assign FIV status in all six cats. Subtyping results for the 26 FIV-infected cats are given in Table 3.9, and C_T values are given in Table 3.10. Two subtypes were identified in almost half of FIV-infected cats (11/26 cats; 42%). Infection with FIV subtype A was identified most

commonly (22/26 cats; 85%), followed by subtype F (12/26 cats; 46%) and subtype D (3/26 cats; 12%). Subtype B was not identified in any FIV-infected cats.

Table 3.8 Combined results of three point-of-care FIV antibody test kits in FIV-vaccinated and FIV-unvaccinated cats (*n* = 358). Note that this composite population was biased by FIV-vaccinated cats (119/358; 33%). In practice, the percentage of vaccinated cats in an area will be heavily dependent on the vaccination protocols of local veterinary clinics and may differ considerably from this value. Confidence intervals (95%) are given in brackets.

Test kit	SNAP Combo	Witness	Anigen Rapid
True positive	26	26	26
False negative	0	0	0
True negative	212	325	332
False positive	120	7	0
Sensitivity (%)	26/26 = **100**	26/26 = **100**	26/26 = **100**
Specificity (%)	212/332 = **64** (59-69)	325/332 = **98** (96-99)	332/332 = **100**
PPV (%)	26/146 = **18** (12-24)	26/33 = **79** (65-93)	26/26 = **100**
NPV (%)	212/212 = **100**	325/325 = **100**	332/332 = **100**
Overall accuracy (%)	238/358 = **66** (62-71)	351/358 = **98** (97-99)	358/358 = **100**

Table 3.9 Subtyping results from FIV RealPCR testing (*n* = 26). Primers pairs for FIV subtypes A, B, D and F were included in the PCR reaction.

FIV subtype	Frequency
FIV A only	14/26 = 54%
FIV B only	0
FIV D only	0
FIV F only	1/26 = 4%
FIV A/F	8/26 = 31%
FIV D/F	3/26 = 12%

Table 3.10 C_T values by FIV subtype from qPCR testing of FIV-infected cats using blood (*n* = 26).

Cat	Group	qPCR CT by FIV subtype[a]
Cat #1[b,c]	FIV-vaccinated	FIV A 31.7
Cat #19[c]	FIV-vaccinated	FIV A 32.23
Cat #106[b,c]	FIV-vaccinated	FIV A 32.01, FIV5UTR 31.15
Cat #152[c]	FIV-vaccinated	FIV A 29.73, FIV5UTR 29.24
Cat #297	FIV-vaccinated	FIVA 31.5, FIVF 36.99, FIV5UTR 29.92
Cat #33	FIV-unvaccinated	FIV A 30.8, FIV F 38.34, FIV5UTR 29.38
Cat #62	FIV-unvaccinated	FIV A 28.02, FIV5UTR 26.84
Cat #80	FIV-unvaccinated	FIV D 30.0, FIV F 29.12, FIV5UTR 28.31
Cat #85	FIV-unvaccinated	FIV A 28.6, FIV5UTR 28.63
Cat #92	FIV-unvaccinated	FIV D 29.0, FIV F 29.23 FIV5UTR 29.75
Cat #127	FIV-unvaccinated	FIV A 32.08, FIV5UTR 31.03
Cat #157	FIV-unvaccinated	FIV A 30.53, FIV5UTR 30.27
Cat #163	FIV-unvaccinated	FIV A 32.50, FIV5UTR 31.62
Cat #190	FIV-unvaccinated	FIV A 32.50, FIV5UTR 33.1
Cat #192	FIV-unvaccinated	FIV F 32.31, FIV5UTR 32.35

Cat #203	FIV-unvaccinated	FIV A 29.26, FIV F 36.14, FIV5UTR 30.06
Cat #208	FIV-unvaccinated	FIV A 32.87, FIV5UTR 32.86
Cat #209	FIV-unvaccinated	FIV A 30.57, FIV F 36.14, FIV5UTR 31.11
Cat #218	FIV-unvaccinated	FIV A 31.15, FIV F 35.93, FIV5UTR 30.13
Cat #250	FIV-unvaccinated	FIV A 31.04, FIV5UTR 31.64
Cat #272	FIV-unvaccinated	FIV A 31.48, FIV5UTR 30.84
Cat #274	FIV-unvaccinated	FIV D 35.00, FIV F 34.31, FIV5UTR 31.17
Cat #279	FIV-unvaccinated	FIV A 30.88, FIV F 36.00, FIV5UTR 30.21
Cat #281	FIV-unvaccinated	FIV A 32.00, FIV5UTR 31.37
Cat #303	FIV-unvaccinated	FIV A 28.39, FIV F 35.53, FIV5UTR 28.46
Cat #319	FIV-unvaccinated	FIV A 30.44, FIV F 35, FIV5UTR 30.09

[a]FIV subtyping was determined using subtype specific primers. Primers pairs for FIV subtypes A, B, D and F were included in the qPCR reaction.
5UTR = five prime untranslated region (also known as the 'Leader Sequence').
[b]Cats were sampled and tested three times before positive PCR results were produced.
[c]Confirmed with VI performed by Yamamoto Laboratory.

3.5 DISCUSSION

FIV infection was reliably diagnosed in FIV-vaccinated and FIV-unvaccinated cats using two inexpensive, fast, simple to use antibody detection kits made by different manufacturers (Witness FeLV/FIV and Anigen Rapid FIV/FeLV). If results of the two IC kits were considered together there was agreement in 351/358 (98%) of cats, with FIV RealPCR testing and VI required to clarify the FIV status of only seven cats. Consequently, a potential algorithm for FIV screening in a group of cats of known or unknown FIV vaccination history is (i) start with Anigen Rapid FIV/FeLV or Witness FeLV/FIV testing; (ii) repeat testing with the other antibody kit if a positive FIV test result is encountered; (iii) pursue further confirmatory testing such as PCR (or VI) for cats only when there is disagreement between the two test kits or a high index of suspicion for FIV remains due to the clinical presentation, such as sequential opportunistic infections or wasting syndromes (Figure 3.4).

This finding will facilitate veterinary practices and shelters to quickly and confidently determine the FIV infection status of cats, regardless of FIV vaccination history, thereby providing a less expensive option than testing using serology and confirmatory testing with a PCR assay.

Immediately following the release of the FIV vaccine in the USA, one study demonstrated that FIV-vaccinated cats tested FIV positive using SNAP FIV/FeLV Combo as early as three weeks after the second primary FIV vaccine, and that vaccinated cats would remain seropositive for at least 12 months post vaccination (Uhl et al., 2002). This finding was recapitulated when another group found that 26 FIV-vaccinated cats all tested FIV positive using SNAP FIV/FeLV Combo within three weeks of the third primary FIV vaccine. In the

same study, all 26 FIV-vaccinated cats also tested positive using a microwell plate ELISA (Petchek FIV, IDEXX Laboratories) at 14 weeks after the third primary FIV vaccine (Levy et al., 2004). At this time, registration restrictions limited the availability of other FIV antibody test kits for use in these studies in North America. Later research found it was possible to accurately distinguish FIV-vaccinated from FIV-infected cats using a discriminatory ELISA that considered antibody response to both formalin-treated whole FIV and a synthetic transmembrane (TM) peptide (Kusuhara et al., 2007; Levy et al., 2008b). Recently, investigation using the CD4:$CD8^{low}$ T-lymphocyte ratio to differentiate between FIV-vaccinated and FIV-infected cats showed promise (Litster et al., 2014). However, both of these methods are currently unavailable to veterinarians in practice. To our knowledge, the current study is the first to extensively investigate the performance of Witness FeLV/FIV and Anigen Rapid FIV/FeLV antibody test kits in FIV-vaccinated cats.

The Witness FeLV/FIV and Anigen Rapid FIV/FeLV antibody kits demonstrated excellent sensitivity and specificity using our definition for FIV positivity, even in a study population where 33% of cats (119/358) were FIV-vaccinated. The cause of the seven false-positive FIV results with the Witness FeLV/FIV kit may be a lower threshold for antibody detection compared to the Anigen Rapid FIV/FeLV kit, as most (6/7) were in FIV-vaccinated cats. Presumably there is a low titre of antibody to gp40 following FIV vaccination that is detectable in a small subset of cats using Witness FeLV/FIV, manifesting as a ‘faint positive’ test result. In another diagnostic study, faint results were classified as equivocal if the colour change for the sample spot was less than 50% of the positive control, as determined by a plate reader (Pinches et al., 2007a).

It was not possible to attribute false-positive FIV results with the Witness FeLV/FIV kit as a result of recent FIV vaccination; false-positive results actually occurred most frequently

in cats not recently vaccinated (5/6 cats had not been vaccinated for at least four months). In contrast, a recent abstract reported a high proportion of false-positive FIV results using the Witness FeLV/FIV kit in experimentally vaccinated kittens in a research colony. For example, five weeks after FIV vaccination 14/19 (74%) tested FIV positive, while by 34 weeks after vaccination all kittens tested FIV negative (Lappin, 2015). Clearly, further research needs to be conducted to better understand humoral immune response following FIV vaccination, in particular the time course of antibody production directed against gp40.

All FIV-infected and FIV-vaccinated cats tested FIV positive using SNAP FIV/FeLV Combo. Critically, this antibody kit was not able to distinguish the 114 FIV-vaccinated/FIV-uninfected cats from the five FIV-vaccinated/FIV-infected cats, in agreement with previous reports. The FIV-vaccinated group included cats that had not been vaccinated for up to seven years, demonstrating that vaccination induces production of antibodies which are detectable using this kit for an extended period of time. A previous study found 100% of cats tested (n = 5) still had detectable FIV antibodies over two years after initial vaccination (Levy et al., 2004). In adult cats of unknown FIV-vaccination status, a positive FIV test result with SNAP FIV/FeLV Combo could therefore indicate FIV vaccination, FIV infection, or both. A major consequence of this uncertainty is in a shelter setting where incorrect diagnosis of FIV infection can result in euthanasia (Crawford and Levy, 2007; Levy et al., 2008a). It should be noted that the reported specificity of SNAP FIV/FeLV Combo for the entire study population (64%; 95% CI 59-69%; Table 3.7) was directly affected by the inclusion of 119 FIV-vaccinated cats to create a composite population of FIV-vaccinated (119/358; 33%) and FIV-unvaccinated (239/358; 67%) cats. As the percentage of FIV-vaccinated cats in a population decreases

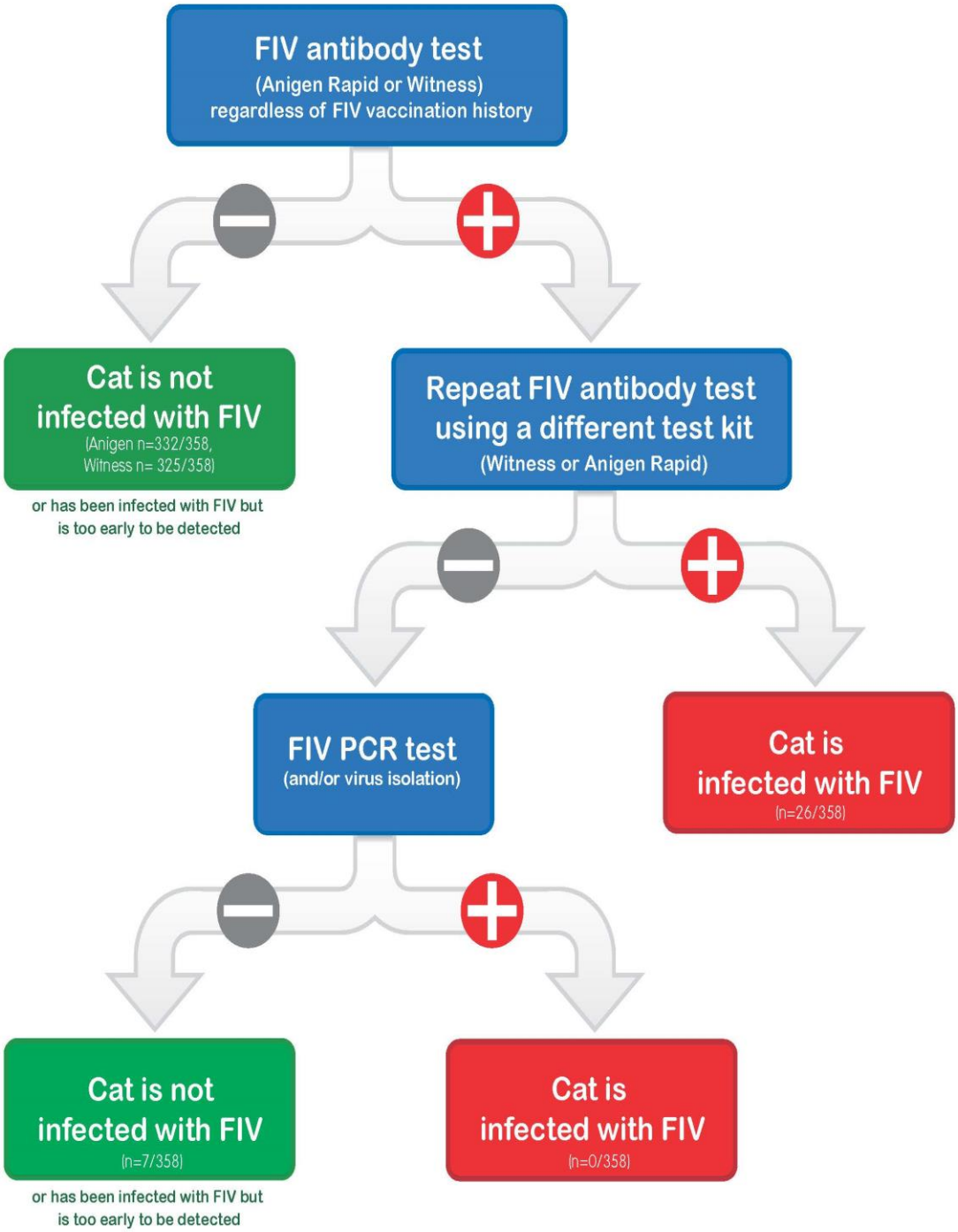
from 33%, the specificity of SNAP FIV/FeLV Combo will progressively increase (and vice versa). In practice, the percentage of vaccinated cats in an area will be heavily dependent on the vaccination protocols of local veterinary clinics and may differ considerably from the 33% of this study cohort. The performance of SNAP FIV/FeLV Combo was comparable to the performance of the two IC test kits in FIV-unvaccinated cats.

Fel-O-Vax® FIV vaccine contains formalin-inactivated whole virus and infected Fet-J cells (Coleman et al., 2014). One possible explanation for the variation in results between the ELISA-based kit (SNAP FIV/FeLV Combo) and IC kits (Witness FeLV/FIV and Anigen Rapid FIV/FeLV) in FIV-vaccinated cats is that relevant antigenic determinants of p15 may be well preserved in the formalin-fixed vaccine, whereas vaccine production may render p24 and gp40 less persistently immunogenic. This would result in the production of a high titre of host antibodies to p15 following FIV vaccination, indistinguishable from antibodies produced against p15 in response to natural FIV infection, but lower titres, less persistent titres, or even no production of antibodies to p24 or gp40. A similar hypothesis was suggested by the researchers who developed a discriminatory ELISA measuring antibody response to two different FIV antigens, formalin-treated whole FIV and a synthetic TM peptide, the latter which may closely resemble the gp40 capture antigen used in the Witness FeLV/FIV antibody test kit. This group, however, was unable to distinguish FIV-vaccinated from FIV-infected cats using only one type of antigen. On further review, this inability to distinguish FIV vaccination from FIV infection may have been a consequence of including results from 16 recently infected cats that when first tested, only had low levels of detectable antibodies to gp40, but when sampled 3-4 weeks later had much higher antibody responses to gp40 (Kusuhara et al., 2007). There are major

methodological differences between western blot, ELISA and IC which presumably lead to threshold differences in the level of detection of antibodies for each method; these differences may help explain variation in results between the current study and earlier work conducted into antibody production in FIV-vaccinated cats (Uhl et al., 2002).

The accuracy of FIV RealPCR testing in the current study was comparable to results for a group of FIV-unvaccinated cats (Litster et al., 2012). Initially, false-negative PCR results were obtained from two FIV-vaccinated cats that tested positive with all antibody kits and later were proven to be FIV-infected by VI. However, serial sampling of these two cats eventually resulted in positive results with FIV RealPCR, suggesting that the initial viraemia was below the limit of detection for the assay. Interestingly, both of these cats also initially tested negative using a methodologically distinct PCR assay at a second commercial laboratory. False-positive results with RealPCR were produced in one FIV-vaccinated cat and three FIV-unvaccinated cats; retesting of these samples produced negative results. False-positive PCR results are usually thought to occur as a result of contamination during testing.

Figure 3.4 Suggested algorithm for diagnosis of FIV infection. If there is the possibility of recent FIV infection, re-testing is recommended due to delays in seroconversion and potentially low viral loads early in infection. Repeat testing on negative cats should be performed at least 8 weeks later for antibody testing and 4 weeks later for PCR testing following last potential exposure. Currently, virus isolation is not commercially available for diagnostic samples in Australia.



3.6 CONCLUSION

Two point-of-care FIV antibody test kits (Witness FeLV/FIV and Anigen Rapid FIV/FeLV) could reliably identify natural FIV infection in client-owned cats in Australia, irrespective of their FIV vaccination history. Where FIV vaccination is practiced, there is an advantage to using these kits for initial screening of FIV infection, particularly in shelters where large numbers of cats need to be assessed quickly and affordably and where vaccination history is often unknown. A third point-of-care FIV antibody test kit (SNAP FIV/FeLV Combo) was useful for confirming a humoral response to FIV vaccination, but could not distinguish FIV-vaccinated from FIV-infected cats. All three antibody detection kits gave comparable and highly accurate results in determining FIV infection status in FIV-unvaccinated cats.

CHAPTER 4. DIAGNOSIS OF FIV IN THE CAT USING SALIVA

4.1 ABSTRACT

Chapter 3 showed that two IC point-of-care FIV antibody test kits (Witness FeLV/FIV and Anigen Rapid FIV/FeLV) were able to correctly assign FIV infection status, irrespective of FIV vaccination history, using whole blood as the diagnostic specimen. A third FIV antibody test kit, SNAP FIV/FeLV Combo (a lateral-flow ELISA), was unable to differentiate antibodies produced in response to FIV vaccination from those incited by FIV infection. The aim of this study was to determine if saliva is a suitable diagnostic specimen using the same well characterized feline cohort. FIV infection status of these cats had been determined previously using a combination of serology, PCR testing and VI. This final assignment was then compared to results obtained using saliva as the diagnostic specimen utilizing the same three point-of-care FIV antibody test kits and commercially available PCR assay (FIV RealPCR). In a population of cats where one third (117/356; 33%) were FIV-vaccinated, both IC test kits accurately diagnosed FIV infection using saliva via a centrifugation method, irrespective of FIV vaccination history. For FIV diagnosis using saliva, the specificity of Anigen Rapid FIV/FeLV and Witness FeLV/FIV was 100%, while the sensitivity of these kits was 96% and 92% respectively. SNAP FIV/FeLV Combo had a specificity of 98% and sensitivity of 44%, while FIV RealPCR testing had a specificity of 100% and sensitivity of 72% using saliva. A revised direct method of saliva testing was trialed on a subset of FIV-infected cats (n = 14), resulting in 14, 7 and 0 FIV positive results using Anigen Rapid FIV/FeLV, Witness FeLV/FIV and SNAP FIV/FeLV Combo, respectively. These results demonstrate that saliva can be used to diagnose FIV infection,

irrespective of FIV vaccination history, using either a centrifugation method (Anigen Rapid FIV/FeLV and Witness FeLV/FIV) or a direct method (Anigen Rapid FIV/FeLV). Collection of a saliva specimen therefore provides an acceptable alternative to venipuncture (i) in fractious cats where saliva may be easier to obtain than whole blood, (ii) in settings when a veterinarian or trained technician is unavailable to collect blood and (iii) in shelters where FIV testing is undertaken prior to adoption but additional blood testing is not required.

4.2 INTRODUCTION

Whole saliva is composed mainly of fluid produced by the salivary glands, which contains small amounts of locally produced Ig molecules (mainly IgA, but also IgM and IgG), and crevicular fluid (Brandtzaeg, 2007). Crevicular fluid is derived from the capillary bed beneath the buccal mucosa, has an antibody content similar to that of plasma, and is responsible for most of the IgM and IgG content of whole saliva (Chappel et al., 2009). Consequently, saliva may be regarded as a transudate of plasma (Parry et al., 1987). In people, the concentration of total IgG in whole saliva is approximately 1000 times less than in plasma (Connell et al., 1993). Investigation of total IgG in cat saliva using healthy subjects found whole saliva contained 190 times less IgG than serum using a radial immunodiffusion assay, and 340 times less total IgG than serum using an ELISA. There were no cats in which salivary IgG could not be detected using these methodologies (Harley et al., 1998). The same researchers found that both salivary and serum total IgG were higher in cats with chronic gingivostomatitis (Harley et al., 2003), a disease process FIV-infected cats are more likely to have and to have more severely than FIV-negative cats (Tenorio et al., 1991; Ravi et al., 2010).

Despite IgG being reliably detectable in cat saliva (Harley et al., 1998), only three studies have investigated using saliva to diagnose FIV infection in cats. Poli and colleagues reported that detection of FIV antibodies in saliva using ELISA was extremely unreliable, with a high frequency of false-positive and false-negative results, although the exact numbers and details of the commercial ELISA kits used were not provided (Poli et al., 1992). In contrast, an indirect immunofluorescence assay and Western blot testing (WB-IgG) detected FIV antibodies in the saliva of 15/16 (94%) FIV-seropositive cats and no

false-positive results were recorded amongst the 16 FIV-seronegative cats (Poli et al., 1992). Matteucci et al., (1993) attempted to isolate FIV from the saliva, plasma and peripheral blood mononuclear cells (PBMC) of naturally FIV-infected cats; the isolation rate of FIV from saliva was considerably lower than from PBMC (18% *versus* 81% of cats) (Matteucci et al., 1993). The third study investigating saliva testing to diagnose FIV infection was a prevalence survey of client-owned cats using a later generation of a commercially available ELISA kit (SNAP FIV/FeLV Combo) to detect FIV antibodies in addition to utilizing nucleic acid amplification (PCR testing) to detect proviral DNA (Chang-Fung-Martel et al., 2013). Although blood was not obtained in the main study, preliminary evaluation using three FIV-infected and two FIV-uninfected cats found results for FIV antibody testing using the ELISA kit to be identical when blood and saliva from the same cat were tested concurrently. There was also good correlation between the ELISA antibody test kit results and combined results from the three PCR assays using saliva (Kappa value 0.76; 95% confidence interval [CI] 0.64-0.87) (Chang-Fung-Martel et al., 2013).

The aim of the present study was to systematically investigate the use of saliva to diagnose FIV infection in FIV-vaccinated and FIV-unvaccinated cats, using three point-of-care FIV antibody (IgG) detection kits and a commercially available real-time qPCR assay, in a well characterized cohort.

4.3 MATERIALS AND METHODS

4.3.1 Sample population

Client-owned cats from Chapter 3 were recruited. Briefly, cats of known FIV vaccination history were recruited through veterinary clinics and classified as 'FIV-vaccinated' (had received at least one FIV vaccine at any time in their life) or 'FIV-unvaccinated' (had never received a FIV vaccine). Clinical records were interrogated to enforce this criterion. Practices where the prevalence of FIV infection was perceived to be high were targeted. Animal ethics approval was granted by The University of Sydney (Approval number N00/1-2013/3/5920).

4.3.2 Blood collection, blood testing and defining FIV infection status

The procedures for blood collection, FIV antibody testing of whole blood using three point-of-care test kits, nucleic acid amplification of blood (FIV RealPCR), use of VI in rare discrepant cases and final assignment of FIV status were described in Chapter 3.

All three FIV antibody kits tested in the current study, despite differing methodology, detect IgG. SNAP FIV/FeLV Combo is a lateral flow ELISA that detects antibodies to FIV matrix protein (p15) and capsid protein (p24), Witness FeLV/FIV uses IC to detect antibodies to FIV surface glycoprotein (gp40) and Anigen Rapid FIV/FeLV uses IC to detect antibodies to p24 and gp40.

Consideration of all four FIV test results (three antibody tests and PCR testing) led to FIV status being assigned when there was a majority, either of negative or positive FIV results (i.e. 3-1 or 4-0). In seven cases, where test results were equally split (i.e. 2-2), VI was

undertaken as the 'tie-breaker'. VI was also undertaken to confirm FIV-vaccinated/FIV-infected cats, even though in all cases there was a clear FIV-positive test majority.

Four FIV-vaccinated cats (4/117; 3%) were determined to be FIV-infected. All four cats tested FIV-positive using whole blood with SNAP FIV/FeLV Combo, Witness FeLV/FIV and Anigen Rapid FIV/FeLV. Two of the four cats tested FIV-negative with FIV RealPCR initially, although with repeat testing (three times over 18 months) these two cats eventually tested positive with FIV RealPCR. Serial re-testing was undertaken following positive VI results to investigate whether FIV RealPCR would be sensitive enough to detect FIV infection in these two cats.

Of the 113 FIV-vaccinated/FIV-uninfected cats, SNAP FIV/FeLV Combo recorded zero FIV-negative results (i.e. all 113 cats tested FIV-positive using this kit), Witness FeLV/FIV recorded 107 FIV-negative results and Anigen Rapid FIV/FeLV recorded 113 FIV-negative results. A total of 112/113 cats tested FIV-negative with FIV RealPCR.

Twenty-one of the 239 FIV-unvaccinated cats (9%) were determined to be FIV-infected. All 21 cats tested FIV-positive using whole blood with SNAP FIV/FeLV Combo, Witness FeLV/FIV, Anigen Rapid FIV/FeLV and FIV RealPCR. Of the 218 FIV-unvaccinated/FIV-uninfected cats, SNAP FIV/FeLV Combo recorded 212 FIV-negative results, Witness FeLV/FIV recorded 217 FIV-negative results and Anigen Rapid recorded 218 FIV-negative results. A total of 215/218 cats tested FIV-negative with FIV RealPCR.

4.3.3 Saliva collection and saliva testing

Saliva collection was performed immediately following blood collection. Two sterile, individually cased cotton swabs mounted on plastic rods[28] were used to obtain saliva. Each swab was rubbed one after the other against the buccal mucosa on each side of the mouth,

with the cheek pressed gently against the upper dental arcade while slowly twisting the swab, for approximately 10 s per side. Swabs with frank blood on the cotton tip due to gingivitis were noted. The average weight of a total of ten saliva swabs from five cats after sampling was determined using a Precision Plus electronic balance[29] and compared to the average weight of ten unused swabs. Both saliva swabs were then refrigerated at 4^{o}C. One of the saliva swabs (selected randomly) was used for FIV antibody testing[30] within 24 hours of collection by cutting the plastic rod approximately 2 cm from the cotton tip and transferring it to a sterile microcentrifuge tube.[28] After placing the cotton tip in the tube, with the plastic rod at the bottom of the tube, 450 µL of sterile phosphate buffered saline (PBS) was added and the tube shaken vigorously by hand for 10 s. The tube, still containing the cut cotton swab, was centrifuged for 30 s at 10,000 g.[31] The swab was then removed from the tube using forceps and the supernatant tested using three FIV antibody test kits designed for FIV antibody detection in whole blood, plasma or serum (SNAP FIV/FeLV Combo, Witness FeLV/FIV and Anigen Rapid FIV/FeLV). Testing was performed per manufacturers' instructions except that an equivalent volume of saliva-containing supernatant was substituted for blood in the test protocol. None of the manufacturers endorses using saliva as a diagnostic specimen for their test kits. The primary author performed FIV antibody testing using saliva immediately following FIV antibody testing using blood, meaning samples were not blinded for saliva antibody testing.

The manufacturer's instructions for SNAP FIV/FeLV Combo advise that any colour development in the FIV sample spot should be considered significant.[32] Although the manufacturers' instructions for Anigen Rapid FIV/FeLV and Witness FeLV/FIV contain no guidelines for interpreting faint results using whole blood, plasma or serum, a faint band

(IC) or spot (ELISA) was recorded as a positive result. This was the same criteria used for our previous study using blood (Chapter 3).

The second saliva sample was stored at -80^{o}C within 24 hours of collection. At the conclusion of the study, stored samples were shipped on dry ice for nucleic acid extraction and FIV testing using a commercially available qPCR assay (FIV RealPCR).[33] Primers for this assay target the conserved *gag* region in both viral RNA (using cDNA following a reverse transcription step) and proviral DNA. FIV subtype was determined using subtype specific primer pairs for subtypes A, B, D and F.[34] The laboratory does not endorse using saliva as a diagnostic specimen for FIV RealPCR testing. Laboratory technicians performing FIV RealPCR testing using saliva were blind to saliva FIV antibody test results.

At the conclusion of the study a small subset of FIV-infected cats was resampled using three new cotton swabs and the aforementioned collection technique. However, instead of using PBS and centrifugation to extract a supernatant sample, a single cotton swab was used exclusively for each antibody test kit (randomly ordered), using a revised and simpler methodology. For each test kit the saliva swab was directly applied to the sample well spot, soaking the cotton tip with twice the volume of buffer recommended in the manufacturers' instructions, and rolling the cotton tip on the sample spot for 10 s while the buffer was added. The result was read 10 min later. This revised 'direct application' technique for antibody testing using saliva was investigated to determine the accuracy of a more practical method for patient-side use.

4.3.4 Statistical analysis

Numerical analyses were performed using a commercial program (Genstat 16th Edition).[35] Statistical significance was considered at $P < 0.05$ and 95% CI were calculated based on a normal approximation and the Wald method using Microsoft Excel.[36] PPV and NPV were calculated using the standard formulas (PPV = 'number of true positives' / ('number of true positives' + 'number of false positives'); NPV = 'number of true negatives' / ('number of true negatives' + 'number of false negatives')). Overall test accuracy was determined by the formula ('number of true positives' + 'number of true negatives' / total number of cats sampled). Two-tailed Fisher's exact tests were used to investigate whether false-positive and false-negative FIV results were more common in FIV-vaccinated cats than FIV-unvaccinated cats, and whether false-positive FIV results were more common in cats returning a blood-tinged saliva swab than a non-bloody swab. Binomial logistic regression with a logit link function was conducted on the test results to determine if there was a significant difference in sensitivity between test methodologies using saliva, and also to compare sensitivity and specificity between blood and saliva for each test methodology. Cohen's Kappa Index Value (κ) for each test methodology was determined to calculate agreement between blood and saliva using the standard formula ($\kappa = 1 - (1 - P_o) / (1 - P_e)$), where P_o was the observed agreement and P_e was the expected agreement (0.5).

[28] Sarstadt, Mawson Lakes, South Australia, Australia (Plastic Stem Cotton Tip Catalogue No. 80.625; 1.5 mL Micro Tube Catalogue No. 72.706.400).
[29] Ohaus, Parsippany, NJ, USA.
[30] Faculty of Veterinary Science, The University of Sydney, Sydney, NSW, Australia.
[31] Eppendorf AG, Hamburg, Germany (Model 5424).
[32] www.idexx.com/resource-library/smallanimal/snap-combo-package-insert-en.pdf
[33] IDEXX Laboratories, East Brisbane, Queensland, Australia.
[34] www.idexx.com.au/pdf/en_au/smallanimal/education/realpcr-test-for-fiv.pdf
[35] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.
[36] Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.

4.4 RESULTS

4.4.1 Sample population

Blood and saliva samples were obtained from 356 client-owned cats recruited from eleven veterinary clinics distributed over four states of Australia (NSW, VIC, QLD and SA).

A total of 117 FIV-vaccinated cats were recruited, ranging from 2 to 18 years (median 7 years; IQR 5–10 years). This is two less cats than reported in our previous study, because saliva was not obtained from two cats at the same time as blood was collected. These cats comprised 64 castrated males and 53 spayed females. Most were domestic crossbred cats (101/117; 86%), the remainder comprising a range of pedigree breeds. Most cats in this cohort (109/117; 93%) had received three primary FIV vaccinations, two to four weeks apart, and three or more annual FIV vaccinations before being sampled. For these 109 cats, sampling took place between 2 and 462 days following their last FIV vaccination (median 237 days; IQR 152–317 days). Seven cats were considered overdue for their annual FIV vaccination (more than 15 months since last vaccination; median 5 years, range 3-7 years) and one cat was 3 years overdue for its second primary FIV vaccination.

A total of 239 FIV-unvaccinated cats were recruited, ranging from 2 to 20 years (median 7 years; IQR 6–10 years). These cats comprised 112 castrated males, 123 spayed females and 4 entire males. Most were domestic crossbred cats (207/239; 87%), the remainder comprising a range of pedigree breeds.

4.4.2 Saliva testing

The median weight of saliva collected per cotton swab was 0.07 g (IQR 0.03–0.11 g), based on the five cats where this was studied.

4.4.2.1 FIV-vaccinated cohort (n = 117)

Of the four FIV-vaccinated/FIV-infected cats, three tested FIV-positive with SNAP FIV/FeLV Combo (i.e. there was one false-negative result), two tested FIV-positive with Witness FeLV/FIV and all four tested FIV-positive with Anigen Rapid FIV/FeLV. Two of the four cats tested FIV-positive with FIV RealPCR; the same two cats that were initially FIV-negative with FIV RealPCR testing using blood were also FIV-negative using saliva.

Considering the 113 FIV-vaccinated/FIV-uninfected cats, SNAP FIV/FeLV Combo recorded 107 FIV-negative results (i.e. there were six false-positive results), while both Witness FeLV/FIV and Anigen Rapid FIV/FeLV recorded 113 FIV-negative results. Four of the six false-positive FIV results recorded with SNAP FIV/FeLV Combo were in cats that returned a blood-tinged saliva swab. All 113 cats tested negative with FIV RealPCR.

A summary of results for each test methodology in FIV-vaccinated cats, including sensitivity, specificity, PPV and NPV, is provided in Table 4.1.

4.4.2.2 FIV-unvaccinated cohort (n = 239)

Of the 21 FIV-unvaccinated/FIV-infected cats, eight tested FIV-positive with SNAP FIV/FeLV Combo (i.e. there were 13 false-negative results), 21 tested FIV-positive with Witness FeLV/FIV and 20 tested FIV-positive with Anigen Rapid FIV/FeLV. A photograph of one of the eight FIV-unvaccinated/FIV-infected cats that tested positive with all three antibody kits (cat #92) is shown in Figure 4.1. A total of 16/21 cats tested FIV-positive with FIV RealPCR.

Of the 218 FIV-unvaccinated/FIV-uninfected cats, SNAP FIV/FeLV Combo recorded 217 FIV-negative results (one false-positive result from a non-bloody swab) while Witness

FeLV/FIV, Anigen Rapid FIV/FeLV and FIV RealPCR all recorded 218 FIV-negative results.

A summary of results for each test methodology in FIV-unvaccinated cats, including sensitivity, specificity, PPV and NPV, is provided in Table 4.2.

4.4.2.3 Combined FIV-vaccinated and FIV-unvaccinated cohorts (n = 356)

Combined results for saliva testing of both cohorts, including overall accuracy of each test methodology, are summarized in Table 4.3.

False-positive results were significantly more common using SNAP FIV/FeLV Combo in FIV-vaccinated cats (6/113; 5%) than FIV-unvaccinated cats (1/218; 0.5%) (P = 0.007). There was no significant difference in the proportion of false-negative results using SNAP FIV/FeLV Combo between FIV-vaccinated (1/4; 25%) and FIV-unvaccinated cats (13/21; 62%) (P = 0.29). False-positive FIV results using SNAP FIV/FeLV Combo were significantly more common in FIV-vaccinated cats that returned a blood-tinged swab (4/20; 20%) compared to a non-bloody swab (2/93; 2%) (P = 0.009). Blood contamination of swabs did not lead to an increased number of false-positive results in FIV-unvaccinated cats using SNAP FIV/FeLV Combo (0/24 *versus* 1/194; P = 1.00).

The specificity of all four test methodologies (SNAP FIV/FeLV Combo, Witness FeLV/FIV, Anigen Rapid FIV/FeLV and FIV RealPCR) using saliva was comparable, while a significant effect of test methodology on sensitivity was found (P < 0.001). Witness FeLV/FIV and Anigen Rapid FIV/FeLV had comparable sensitivities (P = 0.55), Anigen Rapid FIV/FeLV was significantly more sensitive than FIV RealPCR (P = 0.038), and all three methodologies were significantly more sensitive than SNAP FIV/FeLV

Combo (Witness FeLV/FIV and Anigen Rapid FIV/FeLV [$P = 0.001$]; FIV RealPCR [$P = 0.048$]).

Subtyping results and C_T values from FIV RealPCR testing of both blood and saliva from the 25 FIV-infected cats are provided in Tables 4.4 and 4.5.

4.4.2.4 Repeat saliva testing using revised technique (n = 14)

More than half of the FIV-infected cats (14/25; 56%) were available for repeat testing with saliva using the revised 'direct application' technique. With initial centrifugation testing, 7/14 (50%) of these cats tested FIV positive with SNAP FIV/FeLV Combo, 12/14 (86%) tested FIV positive with Witness FeLV/FIV and 13/14 (93%) tested FIV positive with Anigen Rapid FIV/FeLV. Using the direct application technique, 0/14 (0%) of these cats tested FIV-positive with SNAP FIV/FeLV Combo, 7/14 (50%) tested FIV-positive with Witness FeLV/FIV and 14/14 (100%) tested FIV-positive with Anigen Rapid FIV/FeLV.

Figure 4.1 Photograph of data from a FIV-infected cat (cat #92) using saliva as the diagnostic specimen, showing three positive FIV antibody test kit results using: (a) Anigen Rapid FIV/FeLV, (b) Witness FeLV/FIV, and (c) SNAP FIV/FeLV Combo. For both IC kits, the top strip is for FIV antibody testing and the bottom strip is for FeLV antigen testing. One band in the FIV or FeLV strip indicates a negative result, two bands in the FIV or FeLV strip indicates a positive result. For the SNAP FIV/FeLV Combo kit, two spots in the illustrated conformation indicates a positive FIV result. This result was found in 11/25 FIV-infected cats.

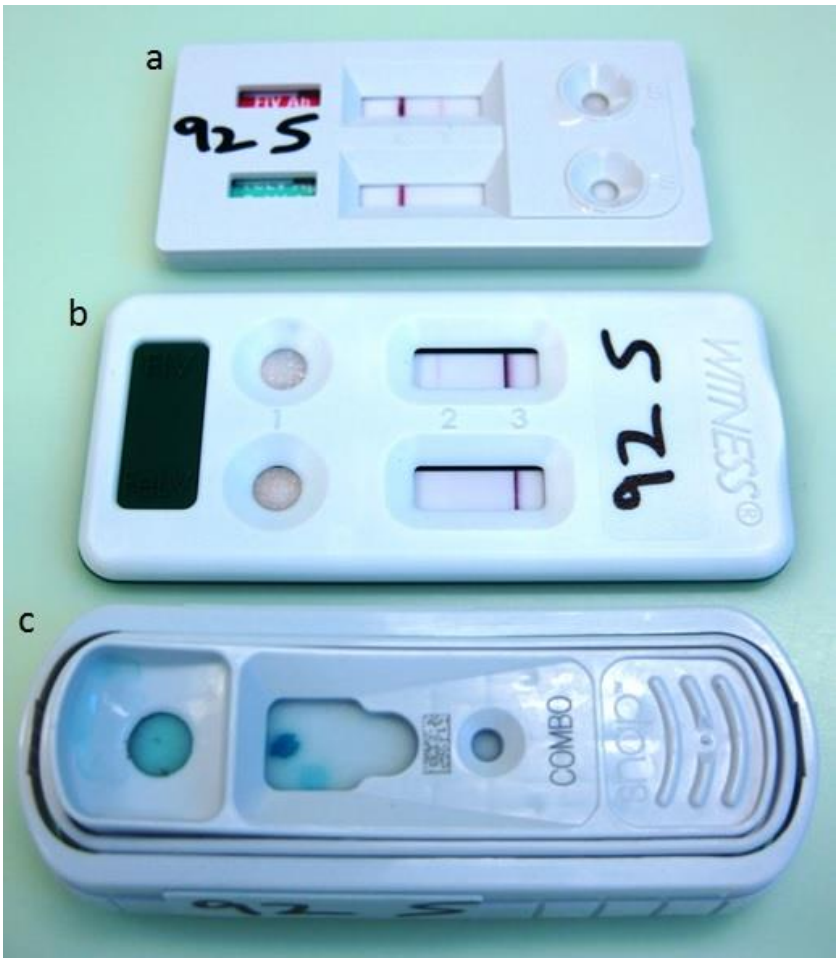


Table 4.1 (FIV-vaccinated cohort) Results of testing using three point-of-care FIV antibody kits and FIV RealPCR testing in FIV-vaccinated cats using saliva (*n* = 117; comprising 113 FIV-uninfected and 4 FIV-infected cats). PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

Test kit	SNAP Combo	Witness	Anigen Rapid	FIV Real PCR
True positive	3	2	4	2
False negative	1	2	0	2
True negative	107	113	113	113
False positive	6	0	0	0
Sensitivity (%)	3/4 = **75** **(33-100)**	2/4 = **50** **(1-99)**	4/4 = **100**	2/4 = **50** **(1-99)**
Specificity (%)	107/113 = **95** **(91-99)**	113/113 = **100**	113/113 = **100**	113/113 = **100**
PPV (%)	3/9 = **33** **(2-64)**	2/2 = **100**	4/4 = **100**	2/2 = **100**
NPV (%)	107/108 = **99** **(97-100)**	113/115 = **98** **(96-100)**	113/113 = **100**	113/115 = **98** **(96-100)**

Table 4.2 (FIV-unvaccinated cohort) Results of testing using three point-of-care FIV antibody kits and FIV RealPCR testing in FIV-unvaccinated cats using saliva (*n* = 239; comprising 218 FIV-uninfected and 21 FIV-infected cats). PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

Test kit	SNAP Combo	Witness	Anigen Rapid	FIV Real PCR
True positive	8	21	20	16
False negative	13	0	1	5
True negative	217	218	218	218
False positive	1	0	0	0
Sensitivity (%)	8/21 = **38** **(17-59)**	21/21 = **100**	20/21 = **95** **(86-100)**	16/21 = **76** **(58-94)**
Specificity (%)	217/218 = **99.5** **(99-100)**	218/218 = **100**	218/218 = **100**	218/218 = **100**
PPV (%)	8/9 = **89** (68-100)	21/21 = **100**	20/20 = **100**	16/16 = **100**
NPV (%)	217/230 = **94** **(91-97)**	218/218 = **100**	218/219 = **99.5** **(99-100)**	218/223 = **98** **(96-100)**

Table 4.3 Combined results of three point-of-care FIV antibody test kits in FIV-vaccinated and FIV-unvaccinated cats using saliva (*n* = 356). Note that this composite population was biased by the inclusion of many FIV-vaccinated cats (117/356; 33%). In practice, the percentage of FIV-vaccinated cats in an area will be heavily dependent on the vaccination protocols of the local veterinary clinics and may differ considerably from this value. PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

Test kit	SNAP	Witness	Anigen Rapid	FIV Real PCR
True positive	11	23	24	18
False negative	14	2	1	7
True negative	324	331	331	331
False positive	7	0	0	0
Sensitivity (%)	11/25 = **44** **(25-64)**	23/25 = **92** **(81-100)**	24/25 = **96** **(88-100)**	18/25 = **72** **(54-90)**
Specificity (%)	324/331 = **98** **(96-99)**	331/331 = **100**	331/331 = **100**	331/331 = **100**
PPV (%)	11/18 = **61** **(39-84)**	23/23 = **100**	24/24 = **100**	18/18 = **100**
NPV (%)	324/338 = **96** **(94-98)**	331/333 = **99** **(99-100)**	331/332 = **99.7** **(99-100)**	331/338 = **98** **(96-99)**
Overall accuracy (%)	**335/356 = 94**	**354/356 = 99**	**355/356 = 99.7**	**349/356 = 98**

Table 4.4 Subtyping results from FIV RealPCR testing of FIV-infected cats using saliva as the diagnostic specimen (*n* = 18). Primers pairs for FIV subtypes A, B, D and F were included in the qPCR reaction.

FIV subtype	Frequency
FIV A	11/18 = 61%
FIV B	0
FIV D	0
FIV F	0
FIV A/F	2/18 = 11%
FIV D/F	2/18 = 11%
5UTR only	3/18 = 17%

5UTR = five prime untranslated region (also known as the 'Leader Sequence').

Table 4.5 C_T values by FIV subtype from FIV RealPCR testing of FIV-infected cats using blood and saliva (*n* = 25).

Cat	Group	C_T value – BLOOD[a]	cDNA QC –	C_T value - SALIVA[a]	cDNA QC –
Cat #1[b,c]	FIV-vaccinated	FIV A 31.7	15.62	-	24.96
Cat #19[c]	FIV-vaccinated	FIV A 32.23	22.31	FIV A 32.0	25.02
Cat #106[b,c]	FIV-vaccinated	FIV A 32.01, FIV5UTR 31.15	14.98	-	22.44
Cat #152[c]	FIV-vaccinated	FIV A 29.73, FIV5UTR 29.24	21.8	FIV5UTR 33.57	24.06
Cat #33	FIV-unvaccinated	FIV A 30.8, FIV F 38.34, FIV5UTR 29.38	16.58	FIV A 33.29	25.2
Cat #62	FIV-unvaccinated	FIV A 28.02, FIV5UTR 26.84	17.18	FIV A 31.83, FIV5UTR 32.13	22.72
Cat #80	FIV-unvaccinated	FIV D 30.0, FIV F 29.12, FIV5UTR 28.31	16.94	FIV D 35.11, FIV F 36.65, FIV5UTR 32.97	22.91
Cat #85	FIV-unvaccinated	FIV A 28.6, FIV5UTR 28.63	14.37	FIV A 30.23, FIV5UTR 32.74	22.26
Cat #92	FIV-unvaccinated	FIV D 29.0, FIV F 29.23 FIV5UTR 29.75	14.6	FIV D 31.89, FIV F 36.6, FIV5UTR 33.94	24.06
Cat #127	FIV-unvaccinated	FIV A 32.08, FIV5UTR 31.03	17.35	-	23.44
Cat #157	FIV-unvaccinated	FIV A 30.53, FIV5UTR 30.27	19.39	FIV A 31.0, FIV5UTR 32.07	24.5

Cat #163	FIV-unvaccinated	FIV A 32.50, FIV5UTR 31.62	21.51	FIV5UTR 33.05	24.2
Cat #190	FIV-unvaccinated	FIV A 32.50, FIV5UTR 33.1	16.04	-	22.15
Cat #192	FIV-unvaccinated	FIV F 32.31, FIV5UTR 32.35	16.01	-	24.77
Cat #203	FIV-unvaccinated	FIV A 29.26, FIV F 36.14, FIV5UTR 30.06	15.01	-	23.09
Cat #208	FIV-unvaccinated	FIV A 32.87, FIV5UTR 32.86	15.6	-	21.35
Cat #209	FIV-unvaccinated	FIV A 30.57, FIV F 36.14, FIV5UTR 31.11	14.3	FIV A 32.0, FIV5UTR 31.69	21.28
Cat #218	FIV-unvaccinated	FIV A 31.15, FIV F 35.93, FIV5UTR 30.13	23.04	FIV A 30.82, FIV F 37.72, FIV5UTR 31.29	19.27
Cat #250	FIV-unvaccinated	FIV A 31.04, FIV5UTR 31.64	21.67	FIV A 32.00, FIV5UTR 31.72	19.66
Cat #272	FIV-unvaccinated	FIV A 31.48, FIV5UTR 30.84	15.78	FIV A 33.02, FIV5UTR 32.45	21.6
Cat #274	FIV-unvaccinated	FIV D 35.00, FIV F 34.31, FIV5UTR 31.17	15.99	FIV5UTR 33.32	22.59
Cat #279	FIV-unvaccinated	FIV A 30.88, FIV F 36.00,	17.34	FIV A 32.0, FIV5UTR	22.1

		FIV5UTR 30.21		32.75	
Cat #281	FIV-unvaccinated	FIV A 32.00, FIV5UTR 31.37	23.61	FIV A 32.5, FIV5UTR 32.78	22.25
Cat #303	FIV-unvaccinated	FIV A 28.39, FIV F 35.53, FIV5UTR 28.46	17.79	FIV A 32.66, FIV5UTR 32.2	24.02
Cat #319	FIV-unvaccinated	FIV A 30.44, FIV F 35, FIV5UTR 30.09	18.7	FIV A 32.84, FIV F 37.14, FIV5UTR 32.5	21.2

[a]FIV subtyping was determined using subtype specific primers. Primers pairs for FIV subtypes A, B, D and F were included in the qPCR reaction.
5UTR = five prime untranslated region (also known as the 'Leader Sequence').
[b]Cats were sampled and tested three times before positive PCR results were produced.
[c]Confirmed with VI performed by Yamamoto Laboratory.
- = negative result.

4.4.3 Comparing blood and saliva results (*n* = 356)

Considering the four FIV-vaccinated/FIV-infected cats, SNAP FIV/FeLV Combo did not identify one cat as FIV-positive (i.e. one false-negative result) using saliva as the diagnostic specimen instead of blood, Witness FeLV/FIV recorded two false-negative results using saliva and Anigen Rapid FIV/FeLV recorded zero false-negative results using saliva (i.e. saliva testing was equivalent to blood testing). FIV RealPCR did not identify two cats as FIV-positive using saliva; the same two cats that were FIV-negative with FIV RealPCR using saliva were also FIV-negative with initial FIV RealPCR testing using blood.

In the 113 FIV-vaccinated/FIV-uninfected cats, saliva testing was equivalent to blood testing using the Anigen Rapid FIV/FeLV kit. For SNAP FIV/FeLV Combo, Witness FeLV/FIV and FIV RealPCR testing, using saliva instead of blood reduced the number of false-positive results (107, 6 and 1 less false-positive results, respectively).

Considering the 21 FIV-unvaccinated/FIV-infected cats, saliva testing was equivalent to blood testing using the Witness FeLV/FIV kit (i.e. 21 true-positive results). However, 13 cats were not identified as FIV-positive (i.e. 13 false-negative results) with SNAP FIV/FeLV Combo using saliva, Anigen Rapid FIV/FeLV recorded one false-negative result using saliva and FIV RealPCR recorded five false-negative results using saliva.

In the 218 FIV-unvaccinated/FIV-uninfected cats, saliva testing was equivalent to blood testing using the Anigen Rapid FIV/FeLV kit. For SNAP FIV/FeLV Combo, Witness FeLV/FIV and FIV RealPCR testing, using saliva instead of blood resulted in a reduced number of false-positive results (5, 1 and 3 less false-positive results, respectively).

In total, considering both saliva and blood results for each test methodology at the same time, saliva testing and blood testing of cats correctly identified the FIV infection status in 223/356 (63%) using SNAP FIV/FeLV Combo ($\kappa = 0.29$), 347/356 (97%) using Witness FeLV/FIV ($\kappa = 0.95$), 355/356 (99.7%) using Anigen Rapid FIV/FeLV ($\kappa = 0.99$) and 345/356 (97%) using FIV RealPCR ($\kappa = 0.95$).

A full comparison of blood and saliva results is provided in Table 4.6, while a comparison of sensitivity and specificity for each antibody test kit and FIV RealPCR testing using blood and saliva is found in Table 4.7. Using saliva instead of blood for testing with SNAP FIV/FeLV Combo significantly increased specificity ($P < 0.001$) but concurrently reduced sensitivity ($P = 0.002$), while using saliva instead of blood for FIV RealPCR testing trended towards reduced sensitivity ($P = 0.081$). All other comparisons were statistically similar.

Table 4.6 Comparing blood (Chapter 3) and saliva results (*n* = 356). The non-highlighted number indicates how many cats tested FIV-positive.

FIV vaccination and FIV infection status	SNAP Combo		Witness		Anigen Rapid		FIV RealPCR	
	BLOOD	SALIVA	BLOOD	SALIVA	BLOOD	SALIVA	BLOOD	SALIVA
FIV-vaccinated/FIV-infected (*n* = 4)	**4**	**3 (1)**	**4**	**2 (2)**	**4**	**4**	**2 (2)**	**2 (2)**
FIV-vaccinated/FIV-uninfected (*n* = 113)	**113 (113)**	**6 (6)**	**6 (6)**	**0**	**0**	**0**	**1 (1)**	**0**
FIV-unvaccinated/FIV-infected (*n* = 21)	**21**	**8 (13)**	**21**	**21**	**21**	**20 (1)**	**21**	**16 (5)**
FIV-unvaccinated/FIV-uninfected (*n* = 218)	**6 (6)**	**1 (1)**	**1 (1)**	**0**	**0**	**0**	**3 (3)**	**0**

Yellow = number of false-positive results in FIV-uninfected cats. Witness FeLV/FIV, Anigen Rapid and FIV RealPCR did not record any false-positive results using saliva as the diagnostic specimen. SNAP Combo FIV/FeLV (as well as Witness FeLV/FIV and FIV RealPCR) recorded less false-positive results using saliva as the diagnostic specimen instead of blood.
Green = number of false-negative results in FIV-infected cats.

Table 4.7 Comparison of overall sensitivity and specificity for the three antibody test kits and FIV RealPCR testing for blood (Chapter 3) and saliva (*n* = 356). Note that this composite population was biased by the inclusion of many FIV-vaccinated cats (117/356; 33%). In practice, the percentage of FIV-vaccinated cats in an area will be heavily dependent on the vaccination protocols of the local veterinary clinics and may differ considerably from this value.

	SNAP Combo		Witness		Anigen Rapid		FIV RealPCR	
	BLOOD	SALIVA	BLOOD	SALIVA	BLOOD	SALIVA	BLOOD	SALIVA
Sensitivity	**100[a]**	**44[a]** (25-64)	**100**	**92** (81-100)	**100**	**96** (88-100)	**92[c]** (82-100)	**72[c]** (54-90)
Specificity	**64[b]** (59-69)	**98[b]** (96-99)	**98** (96-99)	**100**	**100**	**100**	**99** (98-100)	**100**

[a]*P* = 0.002.
[b]*P* = 0.001.
[c]*P* = 0.081.
CIs (95%) are given in brackets.

4.5 DISCUSSION

All three FIV antibody detection kits were able to detect IgG in whole saliva from FIV-infected cats, with varying accuracy. SNAP FIV/FeLV Combo had similar specificity to Witness FeLV/FIV and Anigen Rapid FIV/FeLV using saliva but significantly lower sensitivity. Despite the similar specificity, SNAP FIV/FeLV Combo produced seven false-positive FIV results (7/356; 2%), and these false-positive results were significantly more common in FIV-vaccinated cats than FIV-unvaccinated cats. Interestingly, 4/6 (67%) false-positive FIV results in the FIV-vaccinated cohort were recorded in cats that returned a blood-tinged saliva swab. False-positive FIV responses in FIV-vaccinated cats were described for SNAP FIV/FeLV Combo and Witness FeLV/FIV when testing whole blood (Chapter 3) and we theorized that p15 retains immunogenicity during FIV vaccine (Fel-O-Vax FIV) production to a greater extent and/or for a longer period of time compared to p24 or gp40. If this is the case, SNAP FIV/FeLV Combo would be unable to differentiate p15 antibodies produced in response to FIV vaccination from natural FIV infection. Interestingly, this phenomenon of false-positive results in FIV-vaccinated cats was much less obvious when saliva was used instead of blood (6 false-positive results compared to 113), presumably because the concentration of anti-FIV IgG in whole saliva in FIV-vaccinated/FIV-uninfected cats fell below the detection threshold of the SNAP FIV/FeLV Combo kit due to the dilution of crevicular fluid by saliva. However, this inability of the SNAP FIV/FeLV kit to detect low levels of anti-FIV IgG in saliva resulted in a significantly lower sensitivity than Witness FeLV/FIV and Anigen Rapid FIV/FeLV, with 14 false-negative FIV results recorded (out of 25 FIV-infected cats).

The current study provides a large, rigorous and systematic study design for the evaluation of saliva as a diagnostic specimen for determining FIV infection status. We found it possible to accurately determine the FIV status of both FIV-vaccinated and FIV-unvaccinated cats using IC methodology, without the need for further confirmatory testing such as nucleic acid amplification. The two IC antibody test kits did not produce any false-positive FIV results (0/356) and thus a positive result with either test kit represented a true-positive result. Witness FeLV/FIV produced two false-negative test results (2/356; 0.6%), while Anigen Rapid FIV/FeLV produced only one false-negative test result (1/356; 0.3%). These three false-negative results occurred in three different cats. This slight reduction in test kit sensitivity when using saliva instead of blood (8% reduction for Witness FeLV/FIV, 4% reduction for Anigen Rapid FIV/FeLV) is similar to the 2% reduction reported with the OraQuick Advance Rapid HIV-1/2 In-Home HIV Test which is commercially available over the counter for private use (Pai et al., 2012).

Two previous studies evaluating the detection of FIV antibodies in saliva as a means of diagnosing FIV infection had contradictory findings. The first study reported a high frequency of false-positive and false-negative results (Poli et al., 1992). Our belief is that the high number of erroneous results was attributable to limitations in the test kits used, as point-of-care test kits available in the early 1990s were far less refined than kits currently available. A subsequent study conducted more recently found a good correlation between salivary antibody and salivary PCR results (Chang-Fung-Martel et al., 2013), in accord with the present results. Chang-Fung-Martel and colleagues reported a higher proportion of false-positive FIV results in FIV-vaccinated cats using SNAP FIV/FeLV Combo (2/9; 22% *versus* 6/113; 5%) and a lower proportion of false-negative FIV results in FIV-infected cats (3/10; 30% *versus* 14/25; 56%) than the current study, although neither difference was

statistically significant (Fisher's exact test; $P = 0.11$ and 0.26, respectively) (Chang-Fung-Martel et al., 2013).

The main challenge to the routine use of salivary antibody testing is the requirement to purchase specific consumables, such as microcentrifuge tubes and a centrifuge. A cotton swab mounted on a plastic rod, rather than a cotton or wooden rod, is required to avoid absorption of saline by the rod in order to collect sufficient sample for testing. The centrifugation method for salivary antibody testing outlined in the present study was chosen as it had proved effective in a previous study (Chang-Fung-Martel et al., 2013). Unfortunately the centrifugation method is more complicated than the process required for antibody testing of whole blood and this may be off-putting for some veterinarians. Simplifying the testing process, as described in a subset of cats that were retested, potentially provides a method far more suitable for use in a busy practice or animal shelter.

4.6 CONCLUSION

Two point-of-care FIV antibody test kits (Witness FeLV/FIV and Anigen Rapid FIV/FeLV) could accurately identify natural FIV infection in client-owned Australian cats using saliva as the diagnostic specimen, irrespective of FIV vaccination history. In areas where FIV vaccination is practiced, and when venipuncture is not possible without skilled physical restraint or heavy sedation, collecting and testing saliva for the presence of FIV antibodies using either of these two kits is an accurate method for diagnosing FIV infection. This methodology may prove particularly helpful in shelters where large numbers of cats need to be screened for FIV infection quickly and affordably, additional haematologic tests are not indicated and vaccination history is often unknown.

CHAPTER 5. DURATION OF ANTIBODY RESPONSE FOLLOWING PRIMARY FIV VACCINATION

5.1 ABSTRACT

Chapter 3 reported that two point-of-care FIV antibody test kits (Anigen Rapid and Witness) were able to differentiate FIV-vaccinated from FIV-infected cats at a single time point, irrespective of the gap between testing and last vaccination (0–7 years). The aim of the current study was to systematically investigate anti-FIV antibody production over time in response to the recommended primary FIV vaccination series. First, residual plasma from Chapter 3 was tested using a laboratory-based ELISA to determine whether negative results with point-of-care testing were due to reduced as opposed to absent antibodies to gp40. Second, a prospective study was performed using immunologically naïve client-owned kittens and cats given a primary FIV vaccination series using a commercially available inactivated whole cell/inactivated whole virus vaccine (Fel-O-Vax FIV, three subcutaneous injections at four week intervals) and tested systematically (up to 11 times) over six months, using four commercially available point-of-care FIV antibody kits (SNAP FIV/FeLV Combo [detects antibodies to p15/p24], Anigen Rapid FIV/FeLV [p24/gp40], Witness FeLV/FIV [gp40], and VetScan FeLV/FIV Rapid [p24]). ELISA testing showed cats from the original study vaccinated within the previous 0–15 months had detectable levels of antibodies to gp40, despite testing negative with two kits that use gp40 as a capture antigen (Anigen Rapid and Witness kits). The prospective study showed that antibody-testing with SNAP Combo and VetScan Rapid was positive in all cats two weeks after the second primary FIV vaccination, and remained positive for the duration of the study (12/12 and 10/12 cats positive, respectively). Antibody-testing with Anigen Rapid

and Witness was also positive in a high proportion of cats two weeks after the second primary FIV vaccination (7/12 and 8/12, respectively), but antibody levels declined below the level of detection in most cats (10/12) by one month after the third (final) primary FIV vaccination. All cats tested negative using Anigen Rapid and Witness six months after the third primary FIV vaccination. This study has shown that a primary course of FIV vaccination does not interfere with FIV antibody-testing in cats using Anigen Rapid or Witness, provided primary vaccination has not occurred within the previous six months, but the findings following annual booster FIV vaccination need to be determined. The mechanism for the variation in FIV antibody test kit performance remains unclear.

5.2 INTRODUCTION

Areas of the FIV genome capable of evoking host antibody response (B-cell epitopes) have been identified in the p15, p24, p7, gp40 and gp120 domains, with immunodominant epitopes located in the highly variable region (V3) of gp120 (Lecollinet and Richardson, 2008; Yamamoto et al., 2010). A cascade of antibody responses occurs following natural FIV infection, with antibodies to p24 and gp40 detectable within three weeks of infection and antibodies to p15 detectable within four weeks of infection using Western blot analysis (Yamamoto et al., 1988; O'Connor et al., 1989).

Results from Chapter 3 led to speculation that p15 retains immunogenicity during FIV vaccine production to a greater extent than p24 or gp40. This finding was questioned by another researcher, who used only Witness kits and a small cohort of kittens (n = 19) given a primary FIV vaccination series not in accordance with the manufacturer's recommendations (two injections administered instead of three), with a high FIV false-positive rate reported using Witness (Lappin, 2015). Another larger study (n = 104), however, confirmed the ability of the Anigen Rapid and Witness kits to differentiate FIV-vaccinated and FIV-infected cats, but poor results from a fourth test kit that only detects antibodies to p24 (VetScan FeLV/FIV Rapid) challenged the notion that the ability of test kits to differentiate is solely linked to the choice of FIV antigen for antibody capture (Crawford, 2016). Further work is therefore required to precisely and prospectively determine the antibody response following FIV vaccination in relation to point-of-care test kit methodology.

The aims of the current study were (i) to determine if FIV-vaccinated cats produce antibodies to gp40 at concentrations below the detection threshold of Anigen Rapid

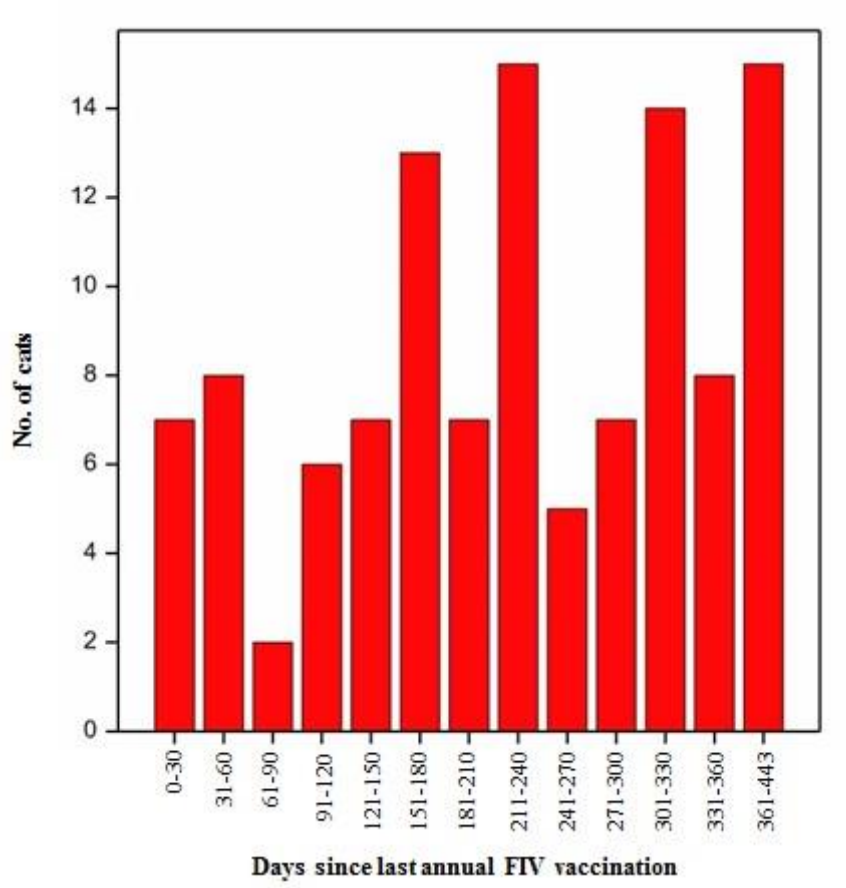
FIV/FeLV and Witness FeLV/FIV FIV/FeLV kits, using a laboratory well-based ELISA; and (ii) to investigate semi-quantitatively the duration of antibody response to p15, p24 and gp40 in cats following a primary course of FIV vaccination using four point-of-care FIV antibody test kits (SNAP FIV/FeLV Combo, Anigen Rapid FIV/FeLV, Witness FeLV/FIV and VetScan FeLV/FIV Rapid).

5.3 MATERIALS AND METHODS

5.3.1 Sample population (Study 1)

Residual blood from Chapter 3 was utilized for the first arm of this study. A total of 118 FIV-vaccinated cats had been recruited, comprising 4 FIV-infected and 114 FIV-uninfected cats. The median age of these cats was seven years (range 2–18 years, IQR 5–10 years) and the procedure for final assignment of FIV status was described in Chapter 3. All recruited cats had received a primary course of FIV vaccination consisting of three vaccines 2–4 weeks apart, in accordance with the manufacturer's recommendations, and a minimum of two annual booster vaccines, with no more than 15 months gap between annual vaccinations. 110/118 had received three or more annual boosters. Most cats (105/118) had been vaccinated within one year of sampling, and all cats had been vaccinated within 15 months of sampling (range 2–443 days, median 215 days, IQR 126–308 days) (Figure 5.1). Seven cats from the original study overdue for their annual FIV vaccination by 3–7 years were not tested. Of the 114 FIV-uninfected cats, 114 had tested FIV-positive with SNAP Combo, six with Witness and zero with Anigen Rapid. Each cat was only available for sampling at a single time point, although occasionally a discordant cat had subsequent follow-up testing. A total of 23 FIV-unvaccinated/FIV-infected cats from the original study were also tested for inclusion as positive controls (Chapter 3). Plasma stored at -80^{o}C was transported on ice to Veterinary Diagnostic Services, The University of Glasgow for a laboratory-based gp40 ELISA. Approval was granted by The University of Sydney Animal Ethics Committee (Approval number N00/1-2013/3/5920).

Figure 5.1 Categorization of FIV-uninfected cats from Study 1 based on time (days) elapsed since last FIV vaccination (n = 114). Of the 114 FIV-uninfected cats, 114 had tested FIV-positive with SNAP Combo, six with Witness and zero with Anigen Rapid. The six FIV false-positive results obtained with Witness occurred at the following intervals after FIV vaccination: 0–30 days (1), 121–150 days (1), 181–210 days (1), 241–270 days (1) and 331–360 days (2).



5.3.2 Sample population (Study 2)

Four FIV-unvaccinated/FIV-uninfected kittens (< 6 months-of-age) and 12 FIV-unvaccinated/FIV-uninfected cats (> 6 months) were recruited from two veterinary clinics and two animal shelters in Sydney, Australia. The median age of all recruited cats was two years (range 0.3–8 years, IQR 1–4 years), significantly younger than cats in study 1 (P < 0.001; Mann-Whitney U-test). Recruited cats were given a primary course of three FIV vaccines subcutaneously, four weeks apart (weeks 0, 4 and 8), in accordance with the

manufacturer's recommendations, and antibody-tested regularly (up to 11 times) using four rapid FIV antibody test kits for 34 weeks (238 days; Table 5.2). Antibody testing at weeks 14, 16 and 20 was only pursued in cats that tested FIV-positive with Anigen Rapid or Witness at the previous sampling, given the high likelihood of negative results with Anigen Rapid/Witness and positive results with SNAP Combo/VetScan in the other cats; only one of these cats was lost to follow-up and unable to be tested at week 16 and 20. PCR testing was performed by a commercial laboratory (FIV RealPCR)[37] at the start of the study (week 0; prior to the first FIV vaccine being given), and at the end of the study (week 34), to ensure FIV infection had not occurred during the course of vaccinations and period of antibody-testing.

Owners were offered free testing and FIV vaccination in return for enrolling their cat in the study. Cats were housed with their owners for the duration of the study; outdoor access was not regulated and was at the owners' discretion. One cat tested FIV-positive with an antibody test kit at week 0 (Anigen Rapid)[38] and was ultimately withdrawn at the conclusion of the study owing to uncertainty regarding its FIV status, and three other cats were withdrawn during the study for various reasons unrelated to blood sampling or FIV vaccination (one cat was hit by a car and died between week 0 and week 2; one was withdrawn at the owner's request after week 4 due to transport difficulties; and one cat was euthanased by the shelter after week 20 as the cat was re-surrendered following an incident of human-directed aggression at home). All cats tested FeLV-negative with the four kits. Approval was granted by The University of Sydney Animal Ethics Committee (Approval number N00/1-2015/858).

5.3.3 Detection of antibodies to gp40 using a laboratory ELISA

A peptide ELISA, using a nine amino acid sequence (CNQNQFFCK)[39] from the highly conserved immunodominant TM2 domain of gp40, was used to detect antibodies (Avrameas et al., 1993). Plasma samples were first complement inactivated by incubation at 56^{o}C for 30 min. The wells of 96-well microtitre plates[40] were coated with 250 ng/well of lyophilized gp40 epitope,[41] diluted in sodium carbonate bicarbonate binding buffer (0.2M anhydrous sodium carbonate, 0.2M sodium carbonate and deionized water at a ratio of 1:11.5:4, respectively). The plates were incubated at 4^{o}C overnight whilst being agitated at 30 rpm. The following day the wells were aspirated and washed five times with 200 µL of phosphate buffered saline (PBS) supplemented with 0.1% Tween (PBST). Unabsorbed sites were blocked following incubation with 200 µL of 2% low fat milk powder in PBST (MP/PBST) for one hour at room temperature. The wells were then aspirated and washed five times with 200 µL of PBST, and 100 µL of plasma added to the wells at a dilution of 1/200 (MP/PBST). The plates were sealed and incubated at room temperature for one hour before being washed five times with 200 µL of PBST, after which 100 µL of biotinylated goat anti-cat secondary antibody[42] was added to each well at a dilution of 1/1000 (MP/PBST). The plates were sealed and incubated at room temperature for one hour. Wells were then aspirated and washed five times with 200 µL of PBST, and 100 µL of horseradish peroxidase conjugated to streptavidin[g] added to each well at a dilution of 1/1000 (MP/PBST). The plates were sealed and incubated at room temperature for 20 min, aspirated and washed five times with 200 µL of PBST, and then 100 µL of 3',3',5',5'-tetramethylbenzidine liquid (TMB)[43] added to each well. Plates were again sealed and incubated at room temperature for 30 min before being read at 650 nm using a microplate reader[44] and OD values recorded.

Positive and negative controls were included on each test plate. The positive control plasma was collected from a cat, infected experimentally with the biological isolate of FIV_{GL8}, which tested FIV-positive by Western blot and virus isolation. The negative control plasma was collected from an uninfected, specific pathogen free cat that had been confirmed FIV-negative by Western blot and virus isolation. ELISA results were not categorised as 'positive' or 'negative', but rather the range of antibody responses against gp40 were compared amongst the FIV-vaccinated cats tested.

5.3.4 Detection of antibodies using FIV point-of-care test kits

Blood was collected via jugular or cephalic venipuncture and stored in an EDTA tube at 4^{o}C. FIV antibody-testing was performed using four commercially available point-of-care kits within 24 hours of sampling, in accordance with the manufacturers' recommendations. The kits tested were SNAP FIV/FeLV Combo,[45] Anigen Rapid FIV/FeLV[46], Witness FeLV/FIV[47] and VetScan FeLV/FIV Rapid.[48] SNAP Combo is a lateral-flow ELISA kit while the other three kits use IC to detect different FIV antibodies (Table 5.1). The fourth kit (VetScan Rapid) was added to the three kits tested previously (Chapter 3) to include a methodology detecting antibodies to p24 alone. The results panel for each cat was photographed digitally at the time of testing. It is important to note that all four kits are marketed for the diagnosis of FIV infection, rather than the detection of antibodies produced in response to FIV vaccination as used in the current study.

Table 5.1 FIV target antigen for the antibodies detected using the four different point-of-care FIV antibody kits tested in Study 2.

FIV Antibody Detection Kit	FIV Target Antigen		
	p15	p24	gp40
SNAP FIV/FeLV Combo (Australia, NZ, North America)[a]	●	●	
Anigen Rapid FIV/FeLV		●	●
Witness FeLV/FIV			●
VetScan FeLV/FIV Rapid		●	

[a]SNAP FIV/FeLV Combo sold in Europe has an additional target antigen (gp40) included.

5.3.5 Statistical analysis

Numerical analyses were performed at the conclusion of the study using statistical software (Genstat 16th Edition).[49] Significance was considered at $P < 0.05$. A Shapiro-Wilk test was used to assess data for normality; since data was not normally distributed (age of cats in study 1 and study 2, days post FIV vaccination in study 1 and gp40 ELISA OD values) medians were reported and Mann-Whitney U-tests used for comparisons. ANOVA testing was used on $\log_e$ transformed data to compare gp40 ELISA OD values grouped according to months since last annual FIV vaccination (0–3, 3–6, 6–9 and 9–15 months), number of annual booster FIV vaccinations administered (2–8) and age of cat at testing (grouped < 5 years, 5–10 years, > 10 years). Simple linear regression modelling was also performed with $\log_e$ OD values as the outcome and days since last annual FIV vaccination, or age of cat at testing, as explanatory variables. Multivariate regression modelling was performed to consider the combined effect of days elapsed since last vaccination and number of annual booster vaccinations administered.

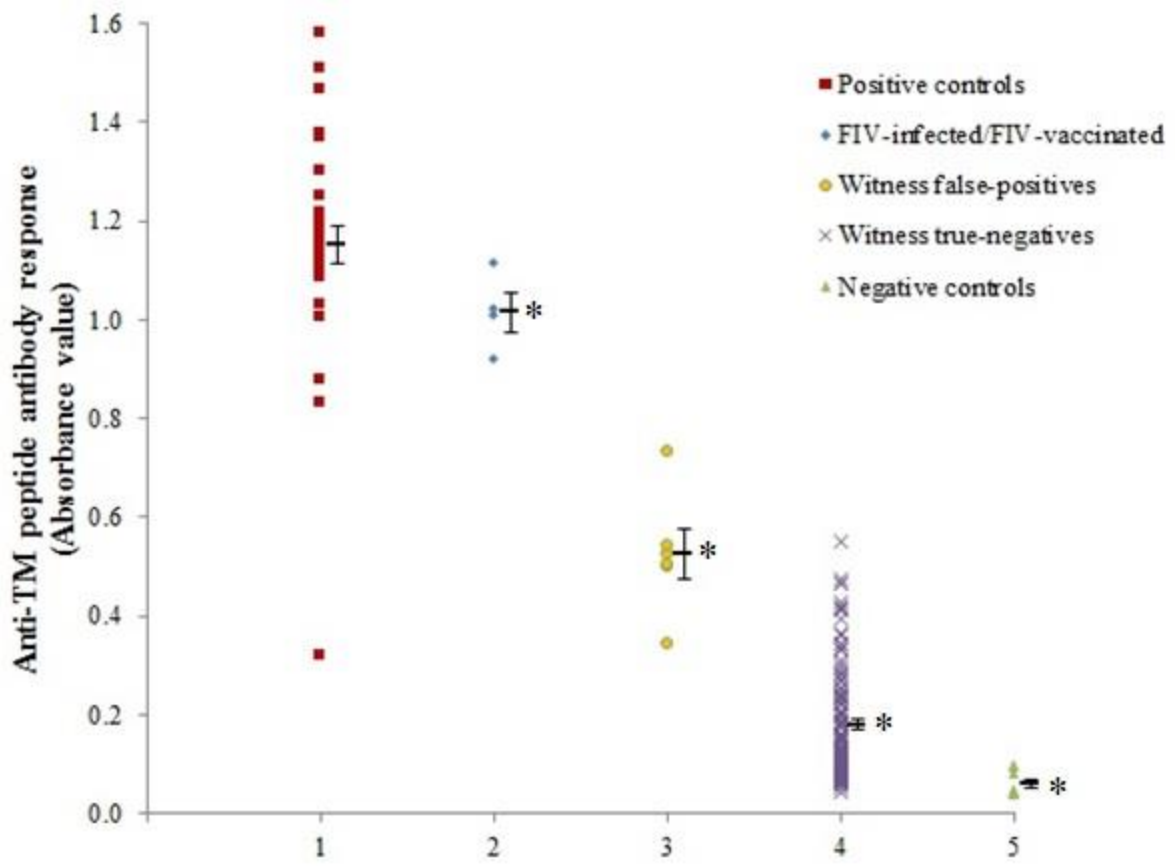
[37] IDEXX Laboratories, East Brisbane, Queensland, Australia.
[38] This cat was inexplicably FIV-negative on PCR testing at week 0 and week 34, and remained FIV-positive with Anigen Rapid throughout the 34 weeks. Antibody-testing at week 0 was negative with SNAP Combo, Witness and VetScan.
[39] Cysteine-asparagine-glutamine-asparagine-glutamine-phenylalanine-phenylalanine-cysteine-lysine
[40] Immulon 2 HB, Thermo Fisher Scientific, Waltham, MA, USA.
[41] AltaBioscience, Birmingham, UK.
[42] Vector Laboratories, Peterborough, UK.
[43] TMB Super Slow, Sigma Aldrich, St. Louis, MO, USA.
[44] MultiSkan Ascent Plate Reader (spectrophotometer), MTX Lab Systems, Bradenton, FL, USA.
[45] IDEXX Laboratories, Westbrook, ME, USA.
[46] BioNote, Gyeonggi-do, Korea.
[47] Zoetis Animal Health, Lyon, France.
[48] Abaxis, Union City, CA, USA
[49] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.

5.4 RESULTS

5.4.1 FIV gp40 laboratory quantitative ELISA (Study 1)

FIV-infected cats (n = 4) tested positive for antibodies to gp40, irrespective of FIV vaccination status (P = 0.93 compared to [cf:] positive control, $P < 0.001$ cf: negative control). FIV-vaccinated/FIV-uninfected cats that tested FIV true-negative with Witness kits in the original study (n = 108) also tested antibody positive ($P < 0.001$ cf: negative control), but antibody levels for these cats were lower compared to FIV-infected cats ($P < 0.001$). FIV-vaccinated/FIV-uninfected cats that tested FIV false-positive with Witness kits in the original study (n = 6) tested antibody positive (P = 0.001 cf: negative control), with higher antibody concentrations compared to the 108 Witness true-negative group (P = 0.007) (Figure 5.2). This distinction, however, was not crisp; for example, the upper range of the 108 Witness true-negative cats encompassed the six Witness false-positive cats apart from one individual. When time since last vaccination was analysed as a possible factor in the 114 FIV-vaccinated/FIV-uninfected cats, no significant effect was found (P = 0.42 [days], P = 0.071 [grouped by month]) (Figure 5.3). When age of cat at testing was considered as a possible factor in the 114 FIV-vaccinated/FIV-uninfected cats, no significant effect was found (P = 0.21 [years], P = 0.20 [grouped by category < 5, 5–10, > 10]) (Figure 5.4). There was no significant difference found when cats were grouped according to number of annual FIV vaccinations administered (P = 0.43) Figure 5.5). When days since last vaccination and number of annual vaccinations were considered together, there was no significant effect observed ($P \geq 0.61$).

Figure 5.2 Results from ELISA testing for antibodies against gp40 peptide (Study 1), including 4 FIV-vaccinated/FIV-infected cats, 6 FIV-vaccinated/FIV-uninfected cats (FIV false-positive with Witness) and 108 FIV-vaccinated/FIV-uninfected cats (FIV true-negative with Witness). Positive controls (FIV-infected/FIV-unvaccinated) and negative controls (FIV-uninfected/FIV-unvaccinated) are shown. The OD is displayed on the *y*-axis. Mean and SEM bars are shown.



*represents significant difference ($P < 0.01$).

Figure 5.3 Results from ELISA testing for antibodies against gp40 peptide (Study 1) of 114 FIV-vaccinated/FIV-uninfected cats according to time (days) elapsed since last annual FIV vaccination. Positive controls (FIV-infected/FIV-unvaccinated) and negative controls (FIV-uninfected/FIV-unvaccinated) are shown. The OD is displayed on the *y*-axis. Mean and SEM bars are shown. No significant effect was found ($P = 0.42$).

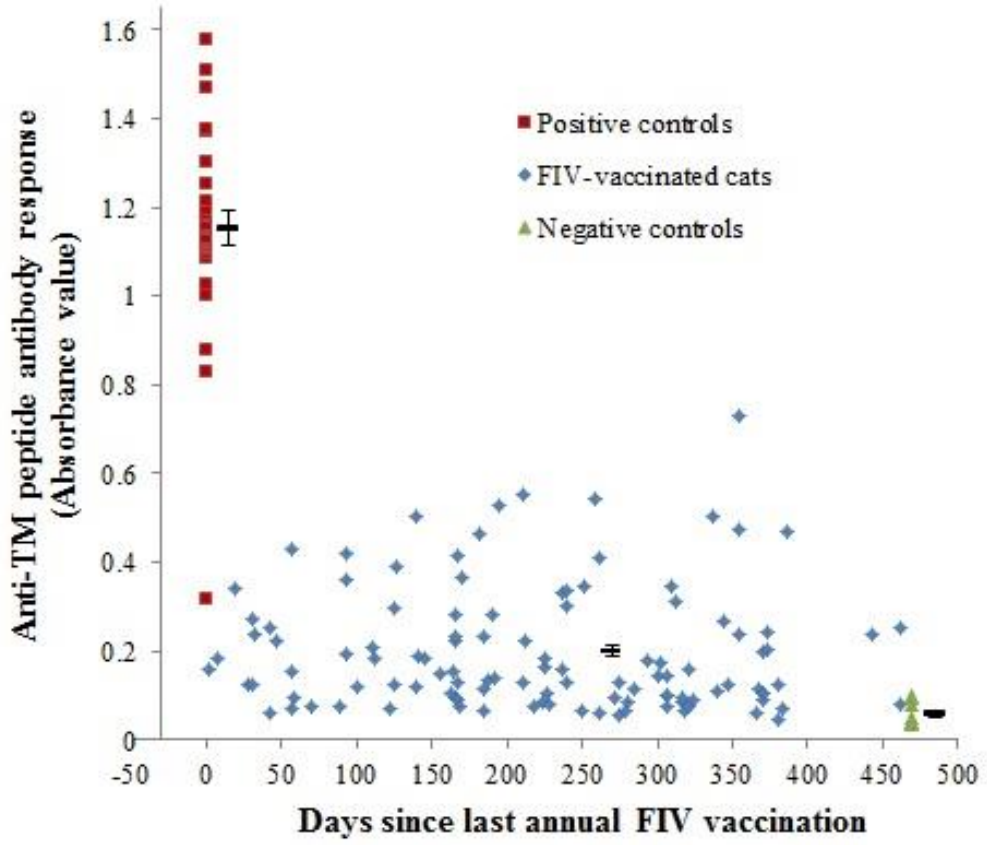


Figure 5.4 Results from ELISA testing for antibodies against gp40 peptide (Study 1) of 114 FIV-vaccinated/FIV-uninfected cats according to age of cat (years) at the time of sampling. Positive controls (FIV-infected/FIV-unvaccinated) and negative controls (FIV-uninfected/FIV-unvaccinated) are shown. The OD is displayed on the *y*-axis. Mean and SEM bars are shown. No significant effect was found ($P = 0.21$).

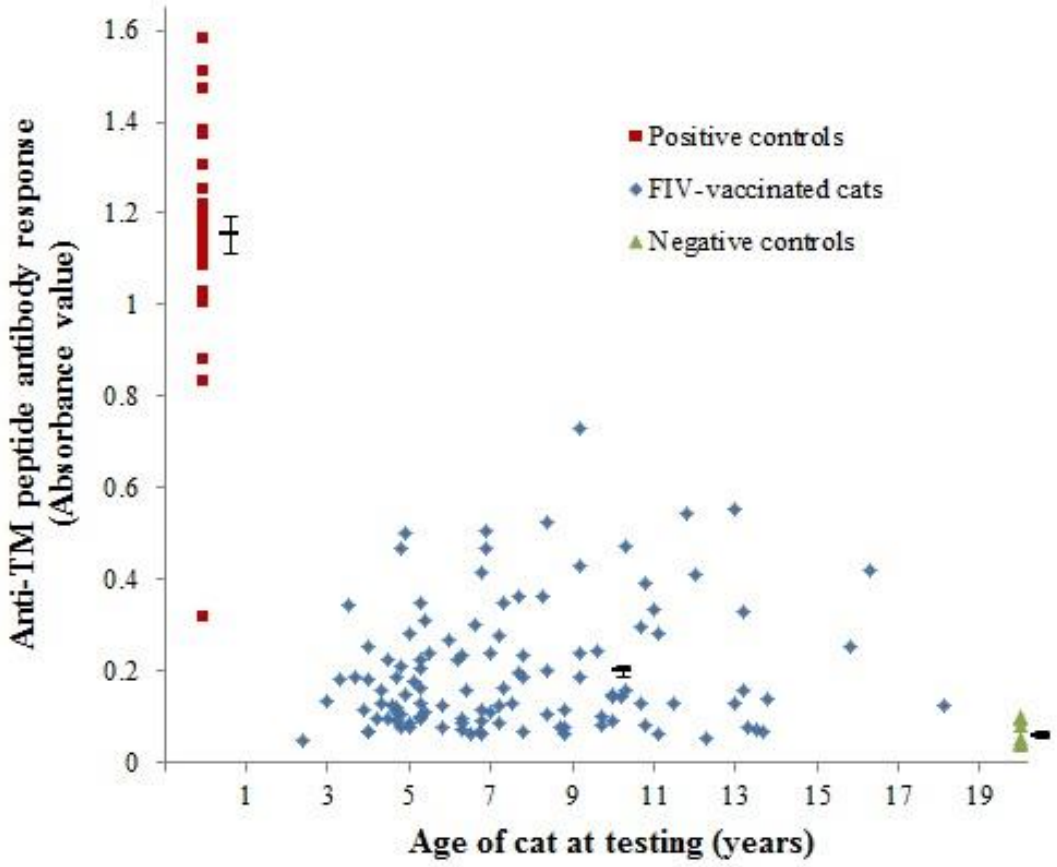
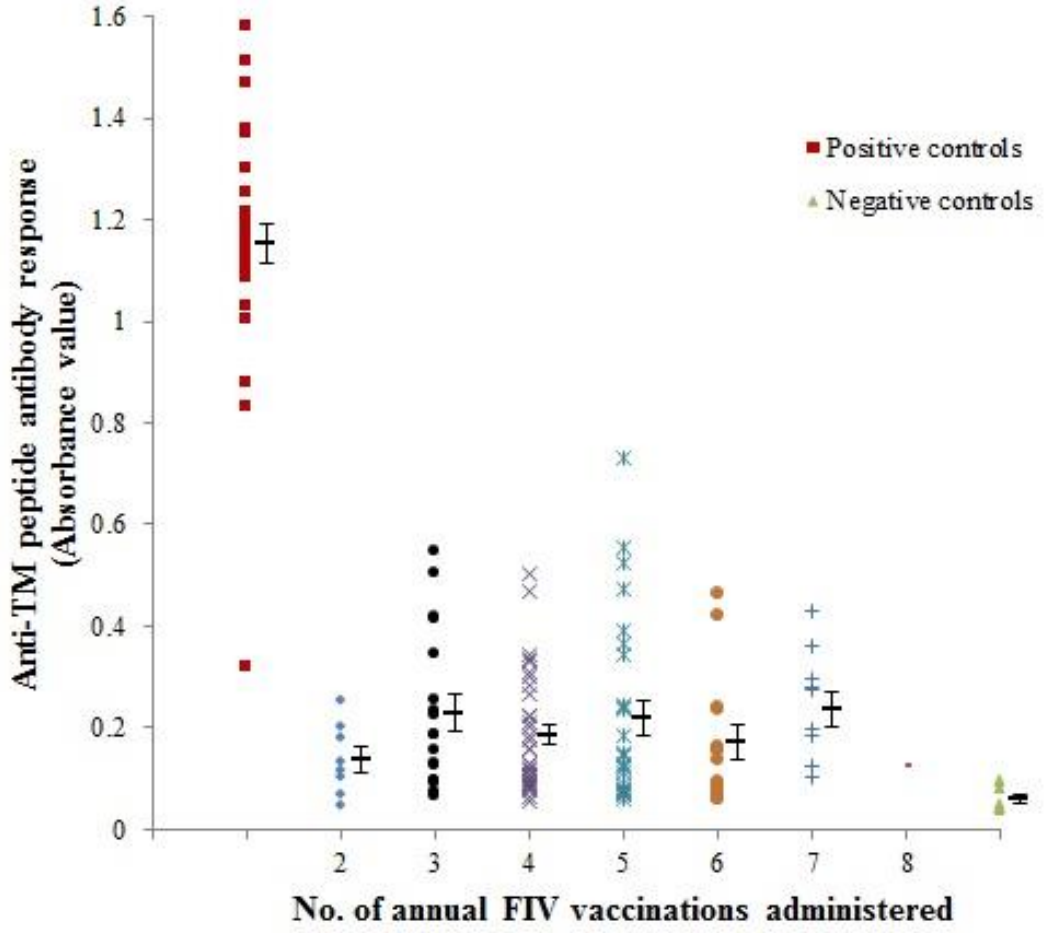


Figure 5.5 Results from ELISA testing for antibodies against gp40 peptide (Study 1) of 114 FIV-vaccinated/FIV-uninfected cats according to number of annual booster FIV vaccinations received. Positive controls (FIV-infected/FIV-unvaccinated) and negative controls (FIV-uninfected/FIV-unvaccinated) are shown. The OD is displayed on the *y*-axis. Mean and SEM bars are shown. No significant effect was found ($P = 0.43$).



5.4.2 FIV point-of-care testing (Study 2)

Sixteen cats commenced the study and were vaccinated against FIV, with 12/16 cats completing the study. Table 5.3 provides a summary of results for these 12 cats. Table 5.4 provides a summary of results for all 16 cats (i.e. including the 4 exclusions), Table 5.5 results for kittens < 6 months-of age (n = 4) and Table 5.6 results for cats > 6 months (n = 8).

Considering the 12 cats, FIV antibodies were detected as early as two weeks after the first vaccination using SNAP Combo and Witness, and as early as four weeks using Anigen Rapid and VetScan Rapid. Two weeks after the second vaccination (week 6), all cats (12/12) tested FIV-positive with SNAP Combo and VetScan Rapid, 7/12 (58%) tested FIV-positive with Anigen Rapid and 8/12 (67%) tested FIV-positive with Witness. At the completion of the study, six months after the third vaccination (week 34), all cats (12/12) were FIV sero-positive with SNAP Combo, two cats had become FIV-negative with VetScan Rapid and all cats were FIV-negative with Anigen Rapid and Witness (Figure 5.6).

Three cats were tested between weeks 14 and 20 as a consequence of testing FIV-positive with Anigen Rapid and/or Witness at week 12:

(i) One cat tested FIV-positive with Anigen Rapid at week 12 and 14, but became sero-negative using this kit at week 20;

(ii) One cat tested FIV-positive with Witness and Anigen Rapid at week 12, 14, and 16, but FIV-negative with both kits at week 20;

(iii) One cat tested FIV-positive with Witness at week 12 and 14, then was lost to follow-up until week 34 when it tested FIV-negative with Witness.

Table 5.2 Outline of the prospective study design (Study 2), showing time points for three primary FIV vaccinations (week 0, 4 and 8), PCR testing (week 0 and 34) and antibody-testing (up to 11 times between week 0 and 34). Antibody-testing was not performed at weeks 14, 16 or 20 for cats that were FIV-negative at week 12 with Anigen Rapid and Witness. *T* = time.

	First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	second FIV vaccine, 6 weeks after first FIV vaccine	Third FIV vaccine	final FIV vaccine, 10 weeks after first FIV vaccine	after final FIV vaccine, 12 weeks after first vaccine	after final FIV vaccine, 14 weeks after first vaccine	after final FIV vaccine, 16 weeks after first vaccine	after final FIV vaccine, 20 weeks after first vaccine	after final vaccination, 34 weeks after first vaccination
In weeks T=	*0*	*2*	*4*	*6*	*8*	*10*	*12*	*14*	*16*	*20*	*34*
In days T =	*0*	*14*	*28*	*42*	*56*	*70*	*84*	*98*	*112*	*140*	*238*
Diagnostic antibody testing											
SNAP Combo	•	•	•	•	•	•	•	•	•	•	•
Anigen Rapid	•	•	•	•	•	•	•	•	•	•	•
Witness	•	•	•	•	•	•	•	•	•	•	•
VetScan Rapid	•	•	•	•	•	•	•	•	•	•	•
IDEXX PCR	•										•
FIV vaccine - 3 boosters 4 weeks apart	•		•		•						

Table 5.3: Summary of FIV PCR and FIV antibody test results from the prospective study (Study 2) at various time points (n = 12). Four cats were withdrawn from the study (see text for details); results from these cats are not included here but are provided in Table 6.4. T = time.

	First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine, 6 weeks after first FIV vaccine	Third FIV vaccine	final FIV vaccine, 10 weeks after first FIV vaccine	4 weeks after final FIV vaccine, 12 weeks after first vaccine	6 weeks after final FIV vaccine, 14 weeks after first vaccine	8 weeks after final FIV vaccine, 16 weeks after first vaccine	after final FIV vaccine, 20 weeks after first vaccine	final vaccination, 34 weeks after first vaccination
In weeks T=	*0*	*2*	*4*	*6*	*8*	*10*	*12*	*14*	*16*	*20*	*34*
In days T =	*0*	*14*	*28*	*42*	*56*	*70*	*84*	*98*	*112*	*140*	*238*
Antibody testing (positive results in brackets)											
SNAP Combo	0/12	7/12	11/12	12/12	12/12	12/12	12/12	3/3	1/1	1/1	12/12
Anigen Rapid	0/12	0/12	1/12	7/12	5/12	4/12	2/12	1/3	1/1	0/1	0/12
Witness	0/12	2/12	7/12	8/12	6/12	6/12	2/12	2/3	1/1	0/1	0/12
VetScan Rapid	0/12	0/12	3/12	12/12	12/12	12/12	12/12	3/3	1/1	1/1	10/12
IDEXX PCR	0/12										0/12

Table 5.4 Summary of FIV PCR and FIV antibody test results from the prospective study (Study 2) at various time points (n = 16), including four cats withdrawn from the study at various time points (after week 0, 4, 20 and 34). T = time.

	First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine, 6 weeks after first FIV vaccine	Third FIV vaccine	final FIV vaccine, 10 weeks after first FIV vaccine	4 weeks after final FIV vaccine, 12 weeks after first vaccine	6 weeks after final FIV vaccine, 14 weeks after first vaccine	8 weeks after final FIV vaccine, 16 weeks after first vaccine	after final FIV vaccine, 20 weeks after first vaccine	final vaccination, 34 weeks after first vaccination
In weeks T=	*0*	*2*	*4*	*6*	*8*	*10*	*12*	*14*	*16*	*20*	*34*
In days T =	*0*	*14*	*28*	*42*	*56*	*70*	*84*	*98*	*112*	*140*	*238*
Antibody testing (positive results in brackets)											
SNAP Combo	0/16	10/15	14/15	14/14	14/14	14/14	14/14	5/5	3/3	3/3	13/13
Anigen Rapid	1/16	1/15	2/15	9/14	7/14	6/14	4/14	3/5	2/3	1/3	1/13
Witness	0/16	3/15	9/15	10/14	8/14	8/14	3/14	3/5	2/3	1/3	0/13
VetScan Rapid	0/16	1/15	4/15	14/14	14/14	14/14	14/14	5/5	3/3	3/3	11/13
IDEXX PCR	0/16										0/13

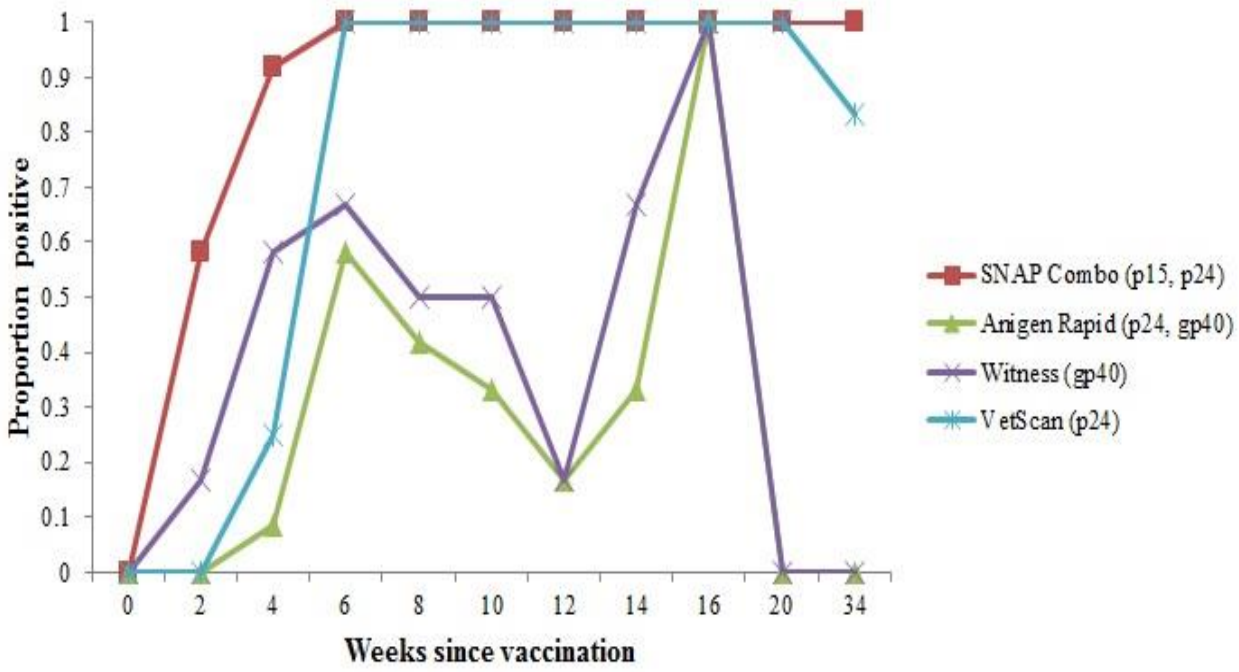
Table 5.5 Summary of FIV PCR and FIV antibody test results from the prospective study (Study 2) at various time points for kittens < 6 months-of-age (n = 4). T = time.

	First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine, 6 weeks after first FIV vaccine	Third FIV vaccine	final FIV vaccine, 10 weeks after first FIV vaccine	4 weeks after final FIV vaccine, 12 weeks after first vaccine	6 weeks after final FIV vaccine, 14 weeks after first vaccine	8 weeks after final FIV vaccine, 16 weeks after first vaccine	after final FIV vaccine, 20 weeks after first vaccine	final vaccination, 34 weeks after first vaccination
In weeks T=	*0*	*2*	*4*	*6*	*8*	*10*	*12*	*14*	*16*	*20*	*34*
In days T =	*0*	*14*	*28*	*42*	*56*	*70*	*84*	*98*	*112*	*140*	*238*
Antibody testing (positive results in brackets)											
SNAP Combo	0/4	3/4	4/4	4/4	4/4	4/4	4/4	1/1	0/0	0/0	4/4
Anigen Rapid	0/4	0/4	0/4	1/4	1/4	1/4	1/4	0/1	0/0	0/0	0/4
Witness	0/4	1/4	1/4	2/4	2/4	2/4	0/4	0/1	0/0	0/0	0/4
VetScan Rapid	0/4	0/4	0/4	4/4	4/4	4/4	4/4	1/1	0/0	0/0	4/4
IDEXX PCR	0/4										0/4

Table 5.6 Summary of FIV PCR and FIV antibody test results from the prospective study (Study 2) at various time points for cats > 6 months-of-age (n = 8). T = time.

	First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine, 6 weeks after first FIV vaccine	Third FIV vaccine	final FIV vaccine, 10 weeks after first FIV vaccine	4 weeks after final FIV vaccine, 12 weeks after first vaccine	6 weeks after final FIV vaccine, 14 weeks after first vaccine	8 weeks after final FIV vaccine, 16 weeks after first vaccine	after final FIV vaccine, 20 weeks after first vaccine	final vaccination, 34 weeks after first vaccination
In weeks T=	*0*	*2*	*4*	*6*	*8*	*10*	*12*	*14*	*16*	*20*	*34*
In days T =	*0*	*14*	*28*	*42*	*56*	*70*	*84*	*98*	*112*	*140*	*238*
Antibody testing (positive results in brackets)											
SNAP Combo	0/8	4/8	7/8	8/8	8/8	8/8	8/8	2/2	1/1	1/1	8/8
Anigen Rapid	0/8	0/8	1/8	6/8	4/8	3/8	1/8	1/2	1/1	0/1	0/8
Witness	0/8	1/8	6/8	6/8	4/8	4/8	2/8	2/2	1/1	0/1	0/8
VetScan Rapid	0/8	0/8	3/8	8/8	8/8	8/8	8/8	2/2	1/1	1/1	6/8
IDEXX PCR	0/8										0/8

Figure 5.6 Summary of FIV antibody test results from the prospective study (Study 2) at various time points (*n* = 12). The FIV target capture antigen/s for each point-of-care antibody test kit is included in brackets. A primary FIV vaccination course was administered at 0, 4 and 8 weeks.



5.5 DISCUSSION

The complexity of the antibody response following vaccination with a commercial IWC/IWV FIV vaccine was further described in this study. Laboratory-based ELISA quantification of antibodies to gp40 demonstrated FIV-vaccinated cats had a detectable humoral response to gp40 for at least 15 months after FIV vaccination, despite a gp40 point-of-care test kit (Witness) testing negative in 95% of these samples (108/114) (Chapter 3). It was surprising not to find a quantitative decrease in gp40 antibody concentration over time since last FIV vaccination as determined by the ELISA OD value, especially when the Witness results from the second arm of the study are considered. The explanation for this is unknown, and may relate to the older age of cats in Study 1 compared to Study 2 as well as reduced immunogenicity of the FIV vaccine with repeated booster vaccinations. Serial antibody-testing using four different kits showed that six months after a primary course of FIV vaccines was administered, Anigen Rapid FIV/FeLV and Witness FeLV/FIV tested FIV-negative in 100% of cats, while SNAP FIV/FeLV Combo and VetScan FeLV/FIV Rapid tested FIV-positive in 100% and 83% of cats, respectively.

At first glance, the Witness gp40 antibody results from Study 2 and the original study (Chapter 3) appear contradictory. Study 2 found that a proportion of immunologically naïve cats administered a primary course of FIV vaccines produced levels of antibodies to gp40 detectable by Witness for up to six months following vaccination, yet the original study found a very low level of FIV-positive results with Witness (6/114) in a cohort of FIV-vaccinated cats, including a FIV-positive rate of only 1/16 in recently inoculated cats (vaccinated within the previous 12 weeks). Results obtained from the current study with

Witness testing were similar to results obtained by another researcher who concluded that Witness testing alone could not be relied on to distinguish natural FIV infection and FIV vaccination shortly after a primary FIV vaccination course (Lappin, 2015). In that study (Lappin 2015), it was reported that 100% of FIV-vaccinated cats during their primary course tested FIV-positive with Witness four weeks after the second FIV vaccination, 50% tested FIV-positive five weeks after the second vaccination and 0% tested FIV-positive 30 weeks after the second vaccination (Lappin, 2015).

The explanation for this seeming discrepancy with Witness testing is possibly two-fold. Firstly, age may be a factor; cats recruited for Chapter 3 (which became the cats in Study 1 in this manuscript) were substantially older than cats in Study 2 (median age 7 *versus* 2 years; $P < 0.001$) due to the large proportion of kittens (4/12), and Lappin only tested kittens (Lappin, 2015). DOI studies are sparse in the veterinary literature, and most are only concerned with protection from challenge rather than antibody quantitation for diagnostic purposes (Schultz, 2006). Flow cytometry studies have demonstrated an age-related remodeling of the immune system in cats, with a gradual decline in relative percentage of lymphocytes (Heaton et al., 2002), and an absolute reduction in B-cells in senior cats (10–14 years) compared to young cats (2–5 years) (Campbell et al., 2004). DOI studies are more common in the human literature, where it is generally accepted that older people sometimes have a lower humoral response after vaccination than younger people. For example, one study investigating antibody response in people administered an inactivated H1N1 vaccine found pre- and post-vaccination titres were generally lower in the elderly (> 70 years-of-age) than the young (< 30 years) (de Bruijn et al., 1999). Secondly, some studies have reported a lower antibody response in people being re-vaccinated compared to those being vaccinated for the first time. Govaert and colleagues

found older people (> 60 years) re-vaccinated with an inactivated influenza vaccine had a 'strikingly' lower humoral immune response compared to people who had not previously been vaccinated (Govaert et al., 1994). We postulate that the accuracy of the Witness kit to correctly assign FIV infection status in FIV-vaccinated cats reported previously may be explained by a relatively low level of gp40 antibodies in older cats following booster FIV vaccination, compared to the younger cats in both Study 2 and Lappin's study, whereby they were vaccinated against FIV for the first time. Contrary to this theory is the absence of a trend in Study 1 for gp40 antibody concentration to diminish with increased age of cat at testing and/or number of annual FIV booster vaccinations administered (Figures 6.4 and 6.5), nor was there a noticeable trend for kittens to test false-positive with Witness more often than adult cats in Study 2 (Tables 5.5 and 5.6). Inadequate sample sizes for both studies may have been responsible.

Sequential semi-quantitative antibody testing following FIV vaccination with Anigen Rapid and Witness showed peak antibody production occurred during and shortly after a primary course of FIV vaccination (three injections at four week intervals). Two weeks after the second vaccination (week 6), 58% (7/12) and 67% (8/12) cats tested seropositive for FIV antibodies using Anigen Rapid and Witness, respectively. By four weeks after the third vaccination (week 12), only 17% (2/12) cats tested seropositive with Anigen Rapid or Witness, and by six months after the third vaccination (week 34) p24 and gp40 antibody levels had decreased below the detection limit for both kits. Peak antibody production to p24 and gp40 six to 12 weeks after the first primary FIV vaccination (in a series of three), as demonstrated by these results, supports results from previous studies (Huang et al., 2004; Kusuhara et al., 2005; Huang et al., 2010). The reason why ELISA gp40 testing in the current study (Study 1) did not show a peak (and subsequent fall) in antibody

production (Figure 5.3), similar to ELISA testing by Huang *et al*. and Kusuhara *et al*., is uncertain. It may also relate to a reduced antibody response in older cats following annual booster FIV vaccination, rather than younger cats receiving a primary course of FIV vaccination (Govaert et al., 1994; de Bruijn et al., 1999).

The study by Lappin had some design concerns which made the results difficult to interpret, including (i) only two FIV vaccinations were administered (instead of the recommended three) and (ii) there was a high dropout rate during the study. Furthermore, of the 19 kittens that were enrolled, only four kittens were tested at four weeks, eight kittens were tested at five weeks and 11 kittens were at 30 weeks post-vaccination (Lappin, 2015). In response, we set out to carefully determine the antibody response following FIV vaccination in cats using systematic sequential sampling and multiple test kits (rather than just one), including the Witness kit used by Lappin. Our results did confirm that care needs to be exercised in the period immediately following primary FIV vaccination using Anigen Rapid and Witness, with seropositive results occurring using both. In light of our findings, we suggest an amendment to our previous conclusion (Chapter 3) and recommend that antibody testing to detect FIV infection in FIV-vaccinated cats is reliable using Anigen Rapid and Witness, providing primary vaccination against FIV has not occurred within the preceding six months. If a positive FIV-antibody result is obtained in a cat where recent primary vaccination is possible, submitting seropositive specimens for confirmatory FIV PCR testing is recommended. A negative FIV test result with either Anigen Rapid or Witness remains robustly reliable and is recommended as the screening test of choice, except in cases of recent infection, when repeat testing two months later is recommended (Levy et al., 2008a).

Results from sequential antibody testing in this study challenge our previous notion that p15 is more immunogenic than p24 and gp40 in the FIV vaccine. SNAP Combo (which detects antibodies to both p15 and p24) gave a seropositive result in 12/12 vaccinated cats from six weeks after the first FIV vaccination and all 12 cats remained FIV-positive for the duration of the study (34 weeks). Additionally, VetScan Rapid (which detects antibodies to p24 but not p15) tested seropositive in 12/12 vaccinated cats from six weeks after the first FIV vaccination and 10/12 (83%) remained seropositive at the end of the study. If the difference in performance between SNAP Combo and Anigen Rapid/Witness was solely attributable to p15 being more immunogenic in the FIV vaccine, then VetScan Rapid would have performed comparably well to Anigen Rapid or Witness in the current study. The differing performance of VetScan Rapid infers that the difference in kit performance may rely more on factors related to testing methodology (e.g. ELISA *versus* IC, the antibody threshold at which the test is set and how the capture antigen is prepared) than factors related to the FIV vaccine (i.e. immunogenicity of different epitopes). For this reason, care must be taken when selecting an appropriate antibody kit to avoid false-positive results in FIV-vaccinated cats, and our findings cannot be extrapolated to other antibody kits without appropriate additional testing being performed.

5.6 CONCLUSION

The complexity of antibody production following FIV vaccination was further described using both laboratory-based ELISA and an extended range of point-of-care test kits. Antibodies to p15, p24 and gp40 were detectable early (within four weeks of the first FIV vaccination) using various test kits. SNAP Combo and VetScan Rapid tested persistently positive for six months in cats given a primary course of FIV vaccination, while Anigen Rapid and Witness tested negative in all cats by six months following primary FIV vaccination. The limit of detection at which these antibody kits are calibrated appears to be the critical factor, since antibodies to gp40 (and likely p15 and p24) persist for at least 15 months after FIV vaccination and kits that are biased towards sensitivity (e.g. SNAP Combo, VetScan Rapid) will detect these antibodies in addition to those produced by natural FIV infection. An advantage of Anigen Rapid and Witness over SNAP Combo and VetScan is that their detection of seropositivity following primary FIV vaccination is transient (up to six months), permitting annual testing for FIV infection prior to annual FIV vaccination boosters; although this must be balanced by the risk of missing early FIV infection and FIV infection in cats with low antibody production.

CHAPTER 6. EFFECTIVENESS OF THE FIV VACCINE IN THE FIELD IN AUSTRALIA

6.1 ABSTRACT

A case-control field study was undertaken to determine the level of protection conferred to client-owned cats in Australia against FIV using a commercial vaccine (Fel-O-Vax FIV®). 440 cats with outdoor access from five Australian states/territories underwent testing, comprising 139 potential cases (complete course of primary FIV vaccinations and annual boosters for three or more years), and 301 potential controls (age, sex and postcode matched FIV-unvaccinated cats). FIV status was determined using a combination of antibody testing (using point-of-care test kits) and PCR testing, as well as VI in cases where results were discordant and in all suspected FIV-vaccinated/FIV-infected cats ('vaccine breakthroughs'). Stringent inclusion criteria were applied to both 'cases' and 'controls'; 89 FIV-vaccinated cats and 212 FIV-unvaccinated cats ultimately satisfied the inclusion criteria. Five vaccine breakthroughs (5/89; 6%), and 25 FIV-infected controls (25/212; 12%) were identified, giving a vaccine protective rate of 56% (95% CI -20 to 84). The difference in FIV prevalence rates between the two groups was not significant (P = 0.14). Findings from this study raise doubt concerning the effectiveness of Fel-O-Vax FIV® under field conditions. Screening for FIV infection may be prudent before annual FIV re-vaccination and for sick FIV-vaccinated cats. Owners should not rely on vaccination alone to protect cats against the risk of acquiring FIV infection; other measures such as cat curfews, the use of 'modular pet parks' or keeping cats exclusively indoors, are recommended.

6.2 INTRODUCTION

An estimated 14.5 million pet cats are infected with FIV worldwide, and 33.5 million if feral cats are included (Yamamoto et al., 2007), which is similar to the estimated number (35 million) of individuals infected with HIV-1 globally (www.who.int/gho/hiv/en/). The FIV-cat model is advocated as a 'test-bed' for HIV infection and HIV-1 vaccine development, and Australia, which has one of the highest FIV prevalence rates in the world (8-15% in client-owned cats with outdoor access; 20-25% in feral cats), is an excellent setting to study FIV transmission and its prevention by vaccination (Malik et al., 1997; Norris et al., 2007; Chapter 2).

To date, a total of 262 cats (139 FIV-vaccinated, 123 FIV-unvaccinated) have been tested using the current commercial FIV vaccine in laboratory-based efficacy studies (including 105 cats from two pre-registration studies), with reported vaccine efficacy of between 0 and 100%, and an overall preventable fraction of 66% (Huang et al., 2004; Kusuhara et al., 2005; Pu et al., 2005; Dunham et al., 2006b; Yamamoto et al., 2007; Huang et al., 2010; Yamamoto et al., 2010; Coleman et al., 2014) (Table 6.1). Despite uncertain efficacy, millions of FIV vaccine doses have been sold worldwide, with no unequivocal 'vaccine breakthroughs' reported following in-field use in Australia (personal communication, Dr. Phillip McDonagh [Head of Regulatory Affairs for Animal Health, Boehringer Ingelheim Australia] and Dr. Elvira Currie [Australian Pesticides and Veterinary Medicines Authority]) or elsewhere (Yamamoto et al., 2007; Yamamoto et al., 2010).

The aim of this study was to determine the 'protective rate' (effectiveness) for the Fel-O-Vax FIV® vaccine in the field in Australia.

Table 6.1 Summary of laboratory-based efficacy studies in which Fel-O-Vax FIV® was given according to the manufacturer's guidelines (three subcutaneous injections 2-4 weeks apart, followed by a single annual booster in the long-term studies). Experimental vaccine efficacy (preventable fraction) = ([percentage viraemia in controls – percentage viraemia in vaccinates] / percentage viraemia in controls) (Yamamoto et al., 2007). Studies where Fel-O-Vax FIV® was modified before administration, where Fel-O-Vax FIV® was administered via non-registered routes (e.g. intranasally) and where non-commercial vaccines (e.g. single subtype FIV vaccines) were trialed are excluded (with one exception, see notes beneath table). FDAH = the parent company that developed and registered Fel-O-Vax FIV® (the FDAH vaccine range has since been acquired in Australia by BI). CID_{50} = cat infectious dose 50, which is equivalent to the amount of virus required to cause infection in half of susceptible subjects. IM = intramuscular, IV = intravenous. Origins of homologous challenges: FIV_{Pet} (A) = California, USA; FIV_{Shi} (D) = Shizuoka, Japan, FIV_{UK8} (A) = Glasgow, UK. Origins of heterologous challenges: $FIV_{FD/US}$ (A) = California, USA; FIV_{FC1} (B) = Florida, USA; FIV_{Ao2} (B) (Aomori) = Aomori, Japan; FIV_{NZ1} (F'/C) = Auckland, New Zealand (prime sign represents that a full sequence of subtype F has yet to be identified)(Yamamoto et al., 2010); $FIV_{FD/DutA}$ (A) = Netherlands; FIV_{Bang} (A/B) = Massachusetts, USA. NA = not available.

Author	Challenge Virus, clade, % difference from vaccine *env* sequence (FIV_{Pet} and FIV_{Shi})	Source	Dose (x CID_{50}), route	Time after final vaccination	Viraemia in FIV-vaccinated cats	Viraemia in placebo controls	Vaccine efficacy (Preventable fraction, %)
Study 1 for USDA license approval[a] (Pu et al., 2005; Yamamoto et al., 2010)	$FIV_{FD/US}$, A, 9% and 20%	*In vitro*	x 1.47, IM	1 year	9/27 (PCR)	25/34 (PCR)	55
Study 2 for USDA license approval (Huang et al., 2004; Yamamoto et al., 2010)	$FIV_{FD/US}$, A, 9% and 20% (overall 11% difference in sequence)	*In vitro*	x 1.79/11[b], IM	375 days	4/25 (PCR)	17/19 (PCR)	82

Pu et al., 2005	FIV_{FC1}, B, 19% and 19.2%	*In vivo*	x 15, IV	21 days	0/8 (VI)	9/9 (VI)	100
Kusuhara et al., 2005	FIV_{Ao2}, B, 18.5% and 19.6%	*In vitro*	Natural, biting	21 days-19 months	0/6 (nested PCR)	4/8 (nested PCR)	100
Dunham et al., 2006b	FIV_{UK8}, A, NA	NA	x 10, IM	28 days	5/5 (VI, RT-PCR)	6/6 (VI, RT-PCR)	0
Pu et al., 2005; Yamamoto et al., 2007	FIV_{FC1}, B, 19% and 19.2%	NA	x 100, IV	3-4 weeks	¾	4/4	25
Yamamoto et al., 2010	$FIV_{FD/DutA}$, A, NA	NA	x 1.73, IM	NA	3/24	13/15	86
Pu et al., 2005; Huang et al., 2010	FIV_{FC1}, B, 19% and 19.2%	*In vivo*	x 1000 PMBC, IV	54 weeks	4/14 (PCR, RT-PCR)	5/5 (PCR, RT-PCR)	71
Pu et al., 2005; Coleman et al., 2014	(i) FIV_{Bang}, A/B, NA	*In vivo*	NA, IV	3-4 weeks	3/4 (VI, PCR)	4/4 (VI, PCR)	25
	(ii) FIV_{FC1}, B, 19% and 19.2%	*In vivo*	NA, IV	3-4 weeks	0/8 (VI, PCR)	4/4 (VI, PCR)	100
	(iii) FIV_{FC1}, B, 19% and 19.2%	*In vivo*	NA (higher than [iii]), IV	3 weeks	7/9 (VI, PCR)	5/5 (VI, PCR)	22
	(iv) FIV_{NZ1}, F'/C, NA	*In vivo*	NA, IV	3-4 weeks	3/5 (VI, PCR)	10/10 (VI, PCR)	40
TOTAL					**41/139**	**106/123**	**66%**

[a]Fel-O-Vax FIV® used in the first trial for USDA (United States Department of Agriculture) approval was a slightly different version to what was eventually registered and released commercially[a] (Uhl et al., 2002; Kusuhara et al., 2005).
[b]Conflicting CID_{50} doses are both presented[b] (Huang et al., 2004; Yamamoto et al., 2010).

6.3 MATERIALS AND METHODS

6.3.1 Sample population

Criteria for recruitment were described in Chapter 3. Briefly, client-owned cats were recruited through veterinary clinics in Australia during 2013-15, most commonly at the same time as an annual health check or routine procedure (e.g. dental procedures). Two groups of cats were recruited: a FIV-vaccinated group ('cases') and a FIV-unvaccinated group matched to cases for age, sex and postcode ('controls'). Cats in the FIV-vaccinated group had been FIV antibody-tested before FIV vaccination was commenced (unless younger than six months-of-age when first vaccinated, due to the low risk of FIV infection and the possibility of false-positive antibody results from maternal antibodies) (Callanan et al., 1991), given a primary course of three FIV vaccinations 2-4 weeks apart, and vaccinated annually against FIV for at least three years. Cats were excluded from the FIV-vaccinated group if FIV nucleic acid amplification (PCR) testing had been performed instead of FIV antibody-testing before FIV vaccination was commenced (due to the PCR assay's lower sensitivity) (Hartmann et al., 2007; Litster et al., 2012; Chapter 3), if any primary FIV vaccinations were more than two weeks overdue (i.e. greater than 6 weeks interval between vaccinations), and if any of the annual FIV vaccinations were more than three months overdue (i.e. greater than 15 months interval between vaccinations). Cats included in the FIV-unvaccinated group had never been given the FIV vaccine. Outdoor access was a requirement for cats in both groups. Information pertaining to outdoor access, as well as number of suspected cat fights based on medical records and owner recollection, was collected at the time of sampling via a questionnaire. Owners of cats meeting the criteria of either group were offered free FIV testing in return for enrolling their cat in the

study, and participating clinics were given free vaccines (FIV and/or non-FIV core vaccines) as an inducement, in return for their assistance recruiting cats.

Animal ethics approval was granted by the University of Sydney (Approval number N00/1-2013/3/5920).

6.3.2 Blood collection and determining FIV infection status

Procedures for venipuncture, FIV antibody testing of EDTA blood using point-of-care test kits (SNAP FIV/FeLV Combo[50], Witness FeLV/FIV[51] and Anigen Rapid FIV/FeLV[52] concurrently), nucleic acid amplification of blood using a commercial PCR assay that detects proviral DNA and viral RNA by targeting a conserved region of the *gag* gene (FIV RealPCR)[53], collection of blood for VI[54,55] and final assignment of FIV status were described in Chapter 3. All FIV-vaccinated/FIV-infected cats ('vaccine breakthroughs') were confirmed by VI, RT assay and proviral PCR testing using primers targeting the *env* gene. For cats where FIV was isolated in cell culture, sequencing of the e*nv* product was performed and compared to sequences in GenBank to determine the clade of breakthrough FIV isolate.[56] For all FIV-infected cats, FIV subtype was determined by FIV RealPCR testing using subtype-specific primer pairs for clades -A, -B, -D and -F (www.idexx.com.au/pdf/en_au/smallanimal/education/realpcr-test-for-fiv.pdf).

6.3.3 Statistical analysis

Sample size calculations were made using statistical software (Minitab 16th Edition)[57] based on projected FIV prevalence rates of 3% and 16% in the FIV-vaccinated and FIV-unvaccinated groups, respectively, and statistical power of 80%. A study design aiming for a 1:3 vaccinate (case) to control ratio was chosen to improve the power (Grimes and Schulz, 2005). Numerical analyses were performed at the conclusion of the study using

commercial software (Genstat 16th Edition).[58] Significance was considered at $P < 0.05$ and 95% CIs were calculated using Microsoft Excel.[59] Shapiro-Wilk tests were used to assess data for normality. When data was normally distributed, means were reported and a two-sample *t*-test (two-sided) used (days between last FIV vaccination and sampling, breakthroughs *versus* FIV-uninfected cases; C_T value from FIV RealPCR testing, breakthroughs *versus* controls). When data was not normally distributed, medians were reported and Mann-Whitney U-tests used (age, cases *versus* controls). Fisher's exact tests (two-tailed) were used to investigate whether there was a significant difference in recruitment criteria (sex, breed, outdoor access and number of suspected cat fights) or FIV prevalence rate between the FIV-vaccinated and FIV-unvaccinated groups. Protective rate (PR; effectiveness) of the FIV vaccine was calculated using the formula, where OR is the odds ratio:

$$\mathbf{PR = (1 - OR) \times 100}$$

(Orenstein et al., 1985; Weinberg and Szilagyi, 2010; Marcus et al., 2015).

[50] IDEXX Laboratories, Westbrook, ME, USA.
[51] Zoetis Animal Health, Lyon, France.
[52] BioNote, Gyeonggi-do, Korea.
[53] IDEXX Laboratories, East Brisbane, Queensland, Australia.
[54] Yamamoto Laboratory, The University of Florida, Gainesville, FL, USA.
[55] Veterinary Diagnostic Services, The University of Glasgow, Scotland, UK.
[56] www.blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch
[57] Minitab 16th Edition for Windows, State College, PA, USA.
[58] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.
[59] Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.

6.4 RESULTS

6.4.1 Sample population

Blood samples were obtained from 440 client-owned cats recruited from 13 clinics distributed over five jurisdictions within Australia (NSW, VIC, QLD, SA and ACT) (Tables 6.2.1 and 6.2.2). There were 139 FIV-vaccinated cats (cases) and 301 FIV-unvaccinated cats (controls). 139 cats were excluded from further analysis for various reasons (Table 6.3). All cats recruited from VIC and QLD (n = 92) were excluded because FIV infection was not detected in any cats, removing the presumption of meaningful FIV exposure. 301 cats remained for final analysis (89 FIV-vaccinated, 212 FIV-vaccinated; case: control ratio of 1:2.4).

6.4.1.1 Cases (n = 89)

The 89 FIV-vaccinated cats recruited ranged from 3 to 18 years (median 8 years; IQR 5–11 years). These cats comprised 46 castrated males and 43 spayed females. Most had been antibody-tested prior to vaccination (60/89; 67%), and a summary of the number of annual FIV vaccines administered to cases is provided in Table 6.4. All cats had been vaccinated against FIV within the previous 15 months (range 2 to 443 days; mean 224 days; IQR 141–307 days). Most cats were described by their owner as having mainly day-time outdoor access (70/89; 79%), with fewer described as having unlimited outdoor access (17/89; 19%) or mainly night-time outdoor access (2/89; 2%). The majority of cases were suspected of having been in at least one cat fight (64/89; 72%), with 36/89 (40%) involved in more than three fights (Table 6.5).

6.4.1.2 Controls (n = 212)

The 212 FIV-unvaccinated cats ranged from 3 to 20 years (median 7 years; IQR 6–11 years). The cats comprised 102 castrated males and 110 spayed females. 120 cats had mainly day-time outdoor access (120/212; 57%), 90 cats had unlimited outdoor access (90/212; 42%) and one cat had mainly night-time outdoor access (1/212; 0.5%). The majority of controls were suspected of having been in at least one fight (144/212; 68%), with 78/212 (37%) involved in more than three fights.

Controls matched cases when age ($P = 0.83$), sex ($P = 0.61$), breed ($P = 1.00$) and number of fights ($P = 0.58$ for at least one fight) were compared between groups (Table 6.5). The only statistical difference between groups was in relation to outdoor access; cases were more likely than controls to have day-time only outdoor access, while controls were more likely to have unlimited outdoor access ($P < 0.001$).

Table 6.2.1 List of eight recruited veterinary clinics including clinic address and number of cats tested.

Name of clinic	Clinic details	FIV-vaccinated cats recruited	FIV-unvaccinated cats
Great Western Animal Hospital	469 Great Western Hwy, Pendle Hill NSW 2145 (02) 9631 9322	19	75
Elizabeth Drive Animal Hospital	Cnr Elizabeth Drive and Woodlands Rd, Liverpool NSW 2170 (02) 9602 7018	19	23
Campbelltown Animal Hospital	15 Chamberlain St, Campbelltown NSW 2560 (02) 4626 4222	13	20
Inner South Veterinary Hospital	47 Jerrabomberra Ave, Narrabundah ACT 2604 (02) 6295 0770	12	15
Bankstown Veterinary Hospital	14 Marshall St, Bankstown NSW 2200 (02) 9790 1101	11	27
Fulham Gardens Veterinary Surgery	441 Tapleys Hill Rd, Fulham Gardens SA 5024 (07) 8355 5475	10	26
Casula Veterinary Hospital	674 Hume Hwy, Casula NSW 2170 (02) 9602 9863	5	12

Mt Annan Veterinary Hospital	17/2-4 Main St, Mt Annan NSW 2567 (02) 4647 7722	0 (used to recruit controls for Campbelltown Animal Hospital)	14
TOTAL:		**89**	**212**

Table 6.2.2 List of five veterinary clinics excluded owing to an absence of FIV infection in controls (first three clinics) or an inability to appropriately match cases to controls (last two clinics), including clinic address and number of cats tested.

Name of clinic	Clinic details	Number of cats excluded
Newtown Veterinary Clinic	121 West Fyans St, Newtown VIC 3220 (03) 5221 5333	48
The Cat Clinic	189 Creek Road, Mt Gravatt, QLD 4122 (07) 3349 0811	38
Kardinia Veterinary Clinic	355 Moorabool St, Geelong VIC 3220 (03) 5221 5122	6
Sydney Animal Hospital (Inner West)	1A Northumberland Ave, Stanmore NSW 2048 (02) 9516 1466	3
Sylvania Veterinary Hospital	335 Princes Hwy, Sylvania NSW 2224 (02) 9522 7088	1

Table 6.3 Summary of reasons for excluding 139 cats from the final analysis.

Reason for exclusion	Total no. of cats (*n* = 139)	
	FIV-vaccinated (*n* = 49)	FIV-unvaccinated (*n* = 90)
FIV not found in vaccinates or controls (VIC)	14	40
FIV not found in vaccinates or controls (QLD)	12	26
No outdoor access	6	5
FIV vaccinations not given according to current manufacturer guidelines	10	NA
Unable to match control to vaccinate (either neutering status or postcode)	NA	8[a]
More than two cats sampled from same household	3	4
Too young	1	6
FIV PCR testing performed instead of FIV antibody testing prior to vaccination	3[b]	NA
Questionnaire not completed and unable to contact owner	0	1

NA = not applicable.
[a]including one FIV-infected, entire male cat.
[b]including one FIV-infected cat # 19; see Table 6.7 for a more detailed explanation.

Table 6.4 Summary of number of annual FIV vaccinations received by FIV-vaccinated cats (cases) recruited for the study and included in the final analysis (*n* = 89). The FIV-vaccinated/FIV-infected cats (vaccine breakthroughs) are identified in brackets.

Years vaccinated/potentially exposed to FIV	Total no. of FIV-vaccinated cats (*n* = 89)	No. of FIV-infected cats (vaccine breakthroughs; *n* = 5)
3	15	1 (# 415)
4	28	1 (# 106)
5	24	0
6	9	2 (# 1, # 404)
7	12	1 (# 152)
8	1	0

Table 6.5: Summary of criteria used to match controls to cases. All cats recruited had been neutered. All control cats sampled lived in the same or an adjacent postcode to the matching cases. Only level of outdoor access was statistically significant between groups; cases were more likely than controls to have day-time only outdoor access, while controls were more likely to have unlimited outdoor access ($P < 0.001$)[a]. Number of suspected cat fights was estimated using a combination of medical records and owner recollection.

Category	FIV-vaccinated (Cases) (*n* = 89)	FIV-unvaccinated (Controls) (*n* = 212)
Total age range (years)	3–18	3–20
Age IQR (years)	5–11	6–11
Male : female ratio	52 : 48	48 : 52
Proportion of domestic crossbred cats (%)	88	88
Outdoor access 'mainly day-time' (%) [a]	79	57
Outdoor access 'mainly night-time' (%)	2	0.5
Outdoor access 'unlimited' (%) [a]	19	42
'0' cat fights (%)	28	32
'1' cat fight (%)	16	16
'2' cat fights (%)	11	9
'3' cat fights (%)	4	6
'More than 3' cat fights (%)	40	37

6.4.2 FIV testing

6.4.2.1 Cases (n = 89)

The prevalence of FIV infection in the FIV-vaccinated cohort was 6% (5/89). The five FIV-vaccinated/FIV-infected cats were 6 to 8 years-of-age, comprising four castrated males and one spayed female. Of these vaccine breakthroughs, 4/5 had received their first vaccination when they were older than six months-of-age, and thus had been FIV antibody-tested before vaccination commenced; the fifth cat (# 404) was 16 weeks-of-age when first vaccinated and antibody-testing had therefore not been performed. A summary of cat fight incidents requiring veterinary intervention, in relation to timing of FIV vaccination, is provided in Table 6.6.

Subtyping results from both VI and FIV RealPCR testing for the five vaccine breakthroughs are presented in Table 6.7. FIV subtype A infection was identified in all cases. None of the five cats were co-infected with other clades of FIV. The mean C_T value from FIV RealPCR testing for vaccine breakthroughs was 31.1.

Two additional cats that were possible vaccine breakthroughs were excluded from further analysis because they did not meet the strict inclusion criteria. Information for these two cats is provided below, and subtyping results from virus isolation and FIV RealPCR testing is provided in Table 6.8.

(i) Cat (case) # 10 tested FIV-negative during the study and was re-tested 2.5 years later at the request of the attending veterinarian and owner following a chronic history of weight loss, lethargy and diarrhoea. On this second occasion of FIV testing this cat tested FIV-positive with all three point-of-care antibody test kits, FIV-negative with PCR testing and FIV-positive with VI. The annual FIV vaccine had not been administered in the year

following initial recruitment for the study and only recommenced four months prior to testing FIV-positive with VI (26 months interval between last annual FIV vaccination and recommencing primary course of FIV vaccinations). No FIV testing was performed before FIV vaccination was recommenced. The cat was ultimately euthanased due to suspected disseminated intestinal lymphoma that was non-responsive to chemotherapy.

(ii) Cat (case) #19 was excluded from the study since it had received an incomplete primary course of FIV vaccinations; FIV PCR testing was performed approximately 12 months later (negative result), and then FIV vaccinations recommenced. Five annual FIV vaccines were given before this cat was sampled for the present study. One month following the first annual FIV vaccine this cat presented with spinal pain, mild pyrexia (39.3^0C) and hyperaesthesia due to suspected bacterial meningitis which responded to treatment with clindamycin.

6.4.2.2 Controls (n = 212)

The FIV prevalence rate in the FIV-unvaccinated cohort was 12% (25/212). The 25 FIV-unvaccinated/FIV-infected cats ranged from 3 to 16 years-of-age (median 7 years; IQR 5–10 years), comprising 18 castrated males and 7 spayed females. FIV RealPCR testing identified two subtypes (i.e. co-infection) in over half of FIV-infected controls (13/25; 52%). FIV subtype A infection was most common (20/25 cats; 80%), followed by subtype F (5/20 cats; 25%) and subtype D (4/25 cats; 16%) (Table 6.9). The mean C_T value from FIV RealPCR testing for FIV-infected controls was 31.0 (using the lower C_T value when two subtypes were identified simultaneously in the same cat).

There was no significant difference in age or C_T value from FIV RealPCR testing when FIV-infected cases and controls were compared (P = 0.54 and 0.89, respectively).

6.4.3 Vaccine effectiveness (protective rate)

A summary of results by clinic and group (FIV-vaccinated *versus* FIV-unvaccinated) is provided in Table 6.10. The overall protective rate for Fel-O-Vax FIV® was 56% (95% CI -20 to 84). The difference in FIV prevalence rates between the two groups (i.e. 5/89; 6% *versus* 25/212; 12%) failed to reach significance ($P = 0.14$).

A *post hoc* power analysis identified that the higher than predicted rate of FIV infection in cases and lower rate of FIV infection in controls reduced the power to detect a significant difference between groups to 40%. Had the intended 1:3 vaccinate (case) to control ratio been achieved, the power to detect a significant difference between groups would have only increased to 43%. When the entire data set ($n = 440$) was analysed (i.e. including the cats excluded as not meeting the stringent criteria), the protective rate was 52% (95% CI 0 to 81), and there was no appreciable impact on the difference in FIV prevalence between groups (6/139; 4.3% *v* 26/301; 8.6%; P = 0.12). Given the prevalence rates reported in the current study, to have achieved a statistically significant effect of the vaccine (assuming one exists) with power of 80% and 1:3 case to control ratio would have required 207 FIV-vaccinated cats and 621 matching FIV-unvaccinated controls.

Table 6.6 FIV vaccination and suspected cat fight history (based on retrieved medical records only) of the five vaccine breakthroughs. Ab = FIV antibody test, Y = yes, N = no, P = primary vaccine, A = annual vaccine, d = days since last vaccination, m = months since last vaccination. P1 (first primary FIV vaccine) is taken as time = 0. Fight history (requiring veterinary intervention and verified using clinic medical records) is displayed as the time elapsed since the previous vaccination when the fight occurred. A negative symbol (cat # 415) indicates the fights occurred before FIV vaccinations had commenced. Cat # 106 did not have any fight history[a]. As per the manufacturer's guidelines, antibody-testing was not performed prior to commencing FIV vaccination in kittens less than six months-of-age at the time of the first vaccination (cat # 404).

Cat (case) no.	Ab	P1	P2	P3	A1	A2	A3	A4	A5	A6	A7
# 1	Y	0d	21d	21d	12m	11m	11m	14m	12m	13m	-
FIGHT HISTORY					**9m**		**8m**		**5m**		
# 106	Y	0d	14d	14d	12m	8m	14m	12m			
FIGHT HISTORY[a]									-	-	-
# 152	Y	0d	12d	18d	11m	11m	11m	11m	12m	12m	12m
FIGHT HISTORY							**7m**	**11m**			
# 404	N	0d	14d	21d	11m	12m	12m	13m	12m	12m	-

FIGHT HISTORY						**3m**										
# 415		Y	0d	27d	29d		15m		12m		12m					
FIGHT HISTORY	**-48d, -156d**						**9m**		**7m**		-		-		-	-

Table 6.7 FIV *env* sequencing results following virus isolation and subtyping results from FIV RealPCR testing for the five vaccine breakthroughs.

Cat (case) no.	Subtyping results (VI)	Subtyping results (FIV RealPCR), C_T
# 1 (FIV-vaccinated)	FIV-Dixon (A)	FIV-A, 31.7
# 106 (FIV-vaccinated)	FIV-Sendai 1 (A)	FIV-A, 32.0
# 152 (FIV-vaccinated)	FIV-Dixon (A)	FIV-A, 29.7
# 404 (FIV-vaccinated)	FIV-UK8 (A)	FIV-A, 31.5
# 415 (FIV-vaccinated)	FIV-Dixon (A)	FIV-A, 30.5

Env sequences were compared to stored sequences in GenBank to determine subtype. The FIV RealPCR assay includes primer pairs for FIV subtypes –A, -B, -D and –F.

Table 6.8 Subtyping results for two possible additional vaccine breakthroughs.

Cat (case) no.	Subtyping results (VI)	Subtyping results (FIV RealPCR), C_T
# 10 (excluded)	FIV-FD Sydney (A)	Negative
# 19 (excluded)	FIV-Sendai 1 (A)	FIV-A, 32.2

Env sequences were compared to stored sequences in GenBank to determine subtype. The FIV RealPCR assay includes primer pairs for FIV subtypes –A, -B, -D and -F.

Table 6.9 Subtyping results from FIV RealPCR testing for 25 FIV-unvaccinated cats (controls).

FIV subtype	Frequency
FIV A only	11/25 = 44%
FIV B only	0
FIV D only	0
FIV F only	1/25 = 4%
FIV A/F	9/25 = 36%
FIV D/F	4/25 = 16%

Primers pairs for FIV subtypes -A, -B, -D and -F were included in the PCR reaction. VI was not performed for FIV-infected controls.

Table 6.10 FIV prevalence by veterinary clinic (n = 301). In total, 89 FIV-vaccinated cats and 212 FIV-unvaccinated cats were recruited. GWAH = Great Western Animal Hospital, EDAH = Elizabeth Drive Animal Hospital, CAH = Campbelltown Animal Hospital, MAVH = Mt Annan Veterinary Hospital, ISVH = Inner South Veterinary Hospital, BVH = Bankstown Veterinary Hospital, FGVS = Fulham Gardens Veterinary Surgery, CVH = Casula Veterinary Hospital. CAH and MAVH were pooled together as they are located in adjacent suburbs and cats recruited were from the same area. Cats excluded from final analysis (n = 139) are not shown. The difference in FIV prevalence rates between groups did not reach statistical significance using a Fisher's exact test (P = 0.14).

Veterinary Clinic	FIV prevalence (FIV-vaccinated cats)	FIV prevalence (FIV-unvaccinated cats)
GWAH (NSW)	1/19 = 5%	8/75 = 11%
EDAH (NSW)	1/19 = 5%	2/23 = 9%
CAH/MAVH (NSW)	0/13 = 0%	4/34 = 12%
ISVH (ACT)	1/12 = 8%	1/15 = 7%
BVH (NSW)	2/11 = 18%	5/27 = 19%
FGVS (SA)	0/10 = 0%	3/26 = 12%
CVH (NSW)	0/5 = 0%	2/12 = 17%
TOTAL	**5/89 = 6%**	**25/212 = 12%**

6.5 DISCUSSION

The nominal protective rate for Fel-O-Vax FIV® in this study, the first field trial conducted for this vaccine anywhere in the world, was 56%. Five confirmed vaccine breakthroughs were detected, as well as two additional cases which became FIV-infected but where there were lapses in timing of vaccine administration. The FIV vaccine was shown not to significantly reduce the risk of client-owned cats becoming infected with FIV, although there was a trend for some protection. Owners wanting to prevent their cat acquiring FIV infection should consider measures in addition to vaccination, such as cat curfews, 'modular pet parks' or keeping their cat(s) exclusively indoors. Cats vaccinated against FIV should undergo annual testing prior to booster FIV vaccination, e.g. using a Witness FeLV/FIV or Anigen Rapid FIV/FeLV antibody test kit (Chapter 3), to check infection has not occurred in the preceding year.

The benefit of field studies is that they involve natural challenge in terms of dose, route, and type (i.e. a selection of genetically different viruses, with a range of pathogenicities and *env* sequences). There are, however, some disadvantages to field studies compared to experimental studies for evaluating vaccine effectiveness. The frequency and extent of viral challenge cannot be predicted, since challenge relies on bite(s) from FIV-infected cat(s) (hence use of the terms 'vaccine protective rate' and 'effectiveness' for the current study, instead of 'preventable fraction' and 'efficacy'). It is possible (based on the FIV prevalence rate in controls) that many of the FIV-uninfected cats in the study were never exposed to FIV, although the retrospective quantification of cat fight incidents documents at least possible exposure for many cats. The need to exclude cats recruited from VIC and QLD due to an absence of FIV infection in controls was surprising considering previous

studies, including a large recent serosurvey from Australia documenting FIV prevalence rates of 10-16% in healthy client-owned cats in these states (Chang-Fung-Martel et al., 2013; Chapter 2). Differences in housing conditions and lifestyles between groups was unavoidable (e.g. amount of time spent outdoors). Thus despite our best efforts to match controls to cases on the basis of age, sex and postcode it is possible there was some mismatch in relation to level of exposure to FIV between groups. Since this was a retrospective field study, there were no housing restrictions to eliminate the risk of exposure to FIV during the primary course of FIV vaccination. Furthermore, it was impossible to determine when cats became FIV-infected (cases or controls), and it was also impossible to ensure controls were FIV-negative at the start of the study period with antibody-testing (as was done for vaccinates). This may have introduced a slight bias towards the FIV vaccine showing a protective effect, although this seems unlikely, given the comparable FIV seroprevalence in the control group to previous Australian studies (Malik et al., 1997; Norris et al., 2007; Chapter 2).

Medical records of the five FIV-vaccinated/FIV-infected cats suspected of representing vaccine breakthroughs were scrutinized to investigate the prospect that some vaccine breakthroughs occurred before the primary course of FIV vaccination had been completed. No cats had received veterinary treatment for possible cat fight wounds during the primary course of FIV vaccination (Table 6.6). Cat # 415, when one year-of-age, was in a suspected cat fight 48 days prior to FIV antibody testing (SNAP FIV/FeLV Combo) and commencement of the primary course of FIV vaccination. Most cats produce detectable antibodies to FIV within four weeks of experimental inoculation (O'Connor et al., 1989; Fevereiro et al., 1991), although in rare cases this response may be delayed (Yamamoto et al., 1988). Consequently, current recommendations are to retest cats with possible recent

retrovirus exposure after 56 or 60 days (Barr, 1996; Levy et al., 2008a). It is therefore possible this cat was already infected before FIV vaccination commenced. In retrospect, it would have been helpful to have retested this cat (antibody and/or PCR testing) (Chapter 3) at the end of the primary course of FIV vaccination to investigate this possibility. Given the short duration of time in relation to the overall study period (12 weeks *versus* minimum 156 weeks) and the ages of these five cats when first vaccinated (all one year-of-age or younger) we think it unlikely that vaccine breakthroughs occurred during the primary course of FIV vaccination. This is because young cats lack confidence and are thus less inclined to fight than older cats; they are therefore much less likely to be FIV-infected than a mature, territorial cat older than three years (Chapter 2). In addition, we contend that owners are less likely to allow prolonged periods of outdoor access to kittens (< 6 months-of-age), reducing further the possibility of FIV exposure in the one breakthrough cat that was vaccinated as a kitten (# 404).

The exact mechanism(s) by which the FIV vaccine provides sterilizing immunity against certain subtypes is still unclear. In experimental studies, protection appeared to rely on both cell-mediated and antibody-mediated immunity (Uhl et al., 2002; Coleman et al., 2014). Humoral immunity, specifically the production of antibodies directed against the hypervariable V5 region of the FIV envelope, is important for homologous challenge (Uhl et al., 2002; Yamamoto et al., 2007; Hosie et al., 2011). Passive-transfer studies (using pooled serum from FIV-vaccinated cats) have consistently conferred good protection against homologous (FIV_{Pet}) challenge, but not heterologous (FIV_{FC1}) challenge (Yamamoto et al., 2010; Coleman et al., 2014). In contrast, CMI is important for both homologous and heterologous challenge (Uhl et al., 2002; Yamamoto et al., 2007; Hosie et al., 2011). T-cell responses likely important in CMI include T-helper 1 activity mediated

by specific cytokines (IL-2 and IFNγ), as well as cytotoxic lymphocyte activity, in particular the increased production of the cytotoxic-effector molecule perforin (Omori et al., 2004). Adoptive-transfer studies (using B-cell depleted, T-cell enriched preparations from MHC-matched FIV-vaccinated donor cats) have demonstrated good protection against both homologous (FIV_{Pet}) and heterologous (FIV_{FC1} and FIV_{NZ1}) challenge. Accordingly, it is believed that CMI is more critical for protection against FIV than humoral immunity (Yamamoto et al., 2007; Yamamoto et al., 2010; Coleman et al., 2014). The importance of CMI in providing protection against FIV was shown again recently in adoptive-transfer studies which found increased cytokine, cytolysin (perforin) and cytoxin production in donor cats that conferred protection, with the researchers concluding that efforts should be focused on developing a T-cell based FIV (and HIV) vaccine (Aranyos et al., 2016). The failure of the FIV vaccine to protect against multiple strains of subtype A in the current study (as well as in other studies involving homologous challenge with FIV_{UK8} (Dunham, 2006a; Dunham et al., 2006b), but possible protection against subtypes D and F, supports the notion that any sterilizing protection induced by the FIV vaccine is reliant predominantly on CMI rather than antibody-based immunity.

The release of the FIV vaccine in 2002 was the culmination of ten years of collaborative work and heralded as a triumph of veterinary vaccinology. Many approaches to FIV vaccine design were tried, including IWC, IWV, recombinant (e.g. p24), gene-deletion, vector-based and DNA-based vaccines, formulated with a range of adjuvants and administered in different prime and boost protocols (Uhl et al., 2002; Yamamoto et al., 2007; Yamamoto et al., 2010; Bienzle, 2014). Vaccinating cats against FIV using an IWC vaccine was found to induce higher VNA levels than a IWV vaccine, although the duration of protection following IWC vaccination may be shorter (Uhl et al., 2002). The prototype

FIV vaccine, which contained only IWV (no IWC), outperformed the commercial FIV vaccine in several studies (Pu et al., 2001; Yamamoto et al., 2010; Coleman et al., 2014). Ultimately, the lower production cost of IWC over IWV led to the compromise of the combined IWC/IWV formulation in Fel-O-Vax FIV® (Yamamoto et al., 2010).

The identification of five vaccine breakthroughs, and a further two equivocal cases, casts doubt over the ability to induce solid protection against an immunodeficiency virus through vaccination and is a setback in the quest to develop a uniformly effective HIV-1 vaccine. Research is already underway towards the development of a FIV epitope vaccine targeting T-cell immunity (Yamamoto et al., 2010). It is possible that for both FIV and HIV-1, sterilizing immunity is unattainable. A more realistic aim might be a vaccine that reduces viral load to a level that delays or prevents the onset of clinical signs (Hosie and Beatty, 2007). If the aim of FIV vaccine development shifts from sterilizing to protective immunity, like the core vaccines against feline calicivirus and feline rhinotracheitis, then future research will need to focus on reduction of FIV-associated disease in vaccinated individuals rather than the prevention of infection.

6.6 CONCLUSION

A field study into the effectiveness of a commercial FIV vaccine determined a protective rate of 56% in client-owned Australian cats and documented the first convincing in-field vaccine breakthroughs. FIV infection rate was not significantly different between FIV-vaccinated and FIV-unvaccinated cats, although there was a trend for some protection. The result is disappointing for veterinarians wanting to use the vaccine in high risk situations, as well as for researchers working on developing a HIV-1 vaccine, but is a reminder of the difficulties associated with vaccinating against any *Lentivirus*. Recently, the World Small Animal Veterinary Association upgraded the FIV vaccine classification from 'Not Recommended' to 'Non-Core', a change which may encourage more veterinarians to administer this vaccine (www.wsava.org/sites/default/files/WSAVA%20Vaccination%20Guidelines%202015%20Full%20Version.pdf). We recommend FIV-vaccinated cats should undergo annual testing to ascertain whether they are still FIV-uninfected before administering the booster FIV vaccine, with testing commencing at the end of the primary course of FIV vaccination, to check infection has not already occurred. Complete protection from FIV infection is only possible by eliminating FIV exposure through the use of 'modular pet parks' or keeping cat exclusively indoors. Further research needs to be conducted where the FIV vaccine is available, FIV prevalence is high and other FIV subtypes are present (e.g. Taiwan, Japan) to establish the protective rate of Fel-O-Vax FIV® against the full range of FIV subtypes.

CHAPTER 7. DIAGNOSIS OF FeLV INFECTION IN THE DOMESTIC CAT USING BLOOD AND SALIVA

7.1 ABSTRACT

FeLV can be a challenging infection to diagnose due to a complex feline host-pathogen relationship and occasionally unreliable test results. This study compared the accuracy of three point-of-care FeLV antigen test kits commonly used in Australia and available commercially worldwide (SNAP FIV/FeLV Combo, Witness FeLV/FIV and Anigen Rapid FIV/FeLV), using detection of FeLV provirus by an in-house qPCR assay as the diagnostic gold standard. Blood (n = 563) and saliva (n = 419) specimens were collected from a population of cats determined to include 491 FeLV-uninfected and 72 FeLV-infected individuals (45 progressive infections, 27 regressive infections). Sensitivity and specificity using whole blood was 63% and 94% for SNAP FIV/FeLV Combo, 57% and 98% for Witness FeLV/FIV, and 57% and 98% for Anigen Rapid FIV/FeLV, respectively. SNAP FIV/FeLV Combo had a significantly lower specificity using blood compared to the other two kits (P = 0.004 compared to Witness FeLV/FIV, P = 0.007 compared to Anigen Rapid). False-positive test results occurred with all three kits using blood, and although using any two kits in parallel increased specificity, no combination of test kits completely eliminated the occurrence of false-positive results. We therefore recommend FeLV provirus PCR testing for any cats testing positive with a point-of-care FeLV antigen test kit, as well as for any cat that has been potentially exposed to FeLV but tests negative with a FeLV antigen kit, before final assignment of FeLV status can be made with confidence. For saliva testing, sensitivity and specificity was 52% and 100%, respectively, for all three

test kits. The reduced sensitivity of saliva testing compared to blood testing suggests saliva testing is unsuitable for screening large populations of cats, such as in shelters.

7.2 INTRODUCTION

Antigen testing (including ELISA, IC and IFA methodologies) has been used for over 40 years to detect cats with progressive FeLV infections using whole blood, plasma or serum as the diagnostic specimen. As qPCR has become the new gold standard for FeLV diagnosis, replacing VI (which was never available to veterinarians in the field) and IFA (Lutz et al., 2009), a contemporary investigation of the most commonly used rapid FeLV antigen test kits using whole blood (the preferred specimen for patient-side testing) and considering the current complexity of FeLV infection categories, is overdue. Furthermore, to the author's knowledge, none of the latest generation of rapid FeLV antigen kits have been evaluated using saliva.

The aim of this study was to compare the performance of the three most commonly used rapid FeLV test kits in Australia to diagnose FeLV infection, using qPCR testing on blood to detect FeLV provirus as the gold standard, using both whole blood and saliva as diagnostic specimens.

7.3 MATERIALS AND METHODS

7.3.1 Sample population

A total of 563 cats were recruited, comprised of three distinct groups: (i) **Group 1** (*n* = 440) consisted of healthy client-owned cats that were part of a case-control study into the effectiveness of a commercially available FIV vaccine, and contained both FIV-vaccinated and FIV-unvaccinated cats (Chapter 6); ii) **Group 2** (*n* = 72) consisted of cats that had blood sent to our laboratory[60] for FeLV testing to either confirm a positive in-clinic or laboratory microwell[61] FeLV antigen result (*n* = 53), to further investigate haematologic abnormalities (most commonly non-regenerative anaemia), sent either directly by the attending clinician or referred by a commercial New South Wales veterinary laboratory[62] (*n* = 13) or to test cats that had been in-contact with a progressively FeLV-infected cat during the previous 12 months (*n* = 6); and (iii) **Group 3** (*n* = 51) consisted of semi-feral cats housed at a rescue facility in western Sydney that were tested in response to recent unexplained deaths. The age of some cats in group 3 was undeterminable.

Recruited cats across all three groups ranged from 3 months to 20 years-of-age (median 7 years; IQR 5-10 years; age data available for 522 cats). These cats comprised 289 (51%) castrated males, 262 (47%) spayed females, 8 (1%) entire males and 4 (0.7%) entire females. Most were domestic crossbred cats (485/563; 86%), the remainder comprised a range of pedigree breeds.

7.3.2 Blood collection, DNA extraction and real-time PCR (qPCR) testing

Blood was collected by jugular or cephalic venipuncture from conscious cats, as described in Chapter 3. DNA was extracted from whole EDTA blood using a kit (QIAamp DNA Mini Kit)[63], as per manufacturer's instructions. The concentration and quality of extracted

DNA was measured using a spectrophotometer (Nanodrop 1000)[64]. DNA was stored at -80^{o}C.

PCR testing for FeLV provirus was chosen as the gold standard for the current study. PCR testing was performed according to a published protocol, using primers designed to amplify a 131 bp section of the unique region (U3) of the LTR of all three subtypes of FeLV (-A, -B and -C), but not endogenous retroviral sequences (Tandon et al., 2005). Each 25 µL PCR reaction was composed of 0.125 µL Taq DNA polymerase[63], 2.5 µL 10x PCR Buffer[63], 0.5 µL 10 mM dNTP mix, a final concentration of 480 nM of each primer[65], 160 nM of a dual-labelled fluorogenic probe (labelled at the 5' end with the fluorescent reporter dye FAM [6-carboxyfluorescein] and at the 3' end with the fluorescent quencher dye Black Hole Quencher-1^{TM})[65], 3 µL of DNA (approximately 100 ng), and nuclease-free water. Following 15 min of denaturation at 95^{o}C, 45 cycles of 95^{o}C for 15 s and 60^{o}C for 60 s were carried out. PCR reactions were performed using a Bio-Rad CFX96^{TM} Real-Time System Thermocycler PCR machine[66], and fluorescence was detected during each annealing step (60^{o}C) at 515-530 nm. All samples were run in duplicate. Known positive and negative FeLV samples were included as internal controls in each run, as well as a 'no template' control. Any sample with two C_T values less than 45 was recorded as a positive result. Any sample with two C_T values greater than 45 was recorded as a negative result. Samples with mixed C_T values (i.e. one C_T < 45 and one C_T > 45) were re-run in quadruplicate, with a positive result assigned if a sample recorded two or more C_T values less than 45 in the second run, and a negative result assigned if a sample recorded three or four C_T values greater than 45. Nine samples declared PCR positive following sample testing in quadruplicate had amplicons sequenced and BLAST searches performed in

Genbank® to confirm they were genuine FeLV sequences (93–99% alignment with FeLV strain Glasgow-1 accession number KP728112.1).

Construction and production of a FeLV DNA standard for absolute quantitation of the PCR assay (precision and sensitivity) was done by molecular cloning. Firstly, PCR amplification using DNA from a known progressively FeLV-infected cat (i.e. positive control) and the aforementioned cycling conditions was performed, the product run on a 2% agarose gel for 60 min at 100 V and the product cut out with a scalpel and purified (Qiaquick Gel Extraction Kit)[63]. The purified product was cloned into a vector (pCR2.1-TOPO®, 3931 bp)[64] and grown in *E. coli* cells (Rapid One Shot®)[64], the transformed cells plated onto kanamycin infused SBA plates and the plates incubated overnight at 37°C. The following day, PCR testing was performed on a selection of colonies using FeLV primers and M13 primers, the latter that amplify a section of the plasmid containing the inserted PCR product. The products were run on a 2% agarose gel for 60 min at 100 V to confirm the presence of the expected amplicons (131 bp for the FeLV primers, 375 bp for the M13 primers) and the colonies representing the most intensely staining bands subcultured onto new kanamycin infused SBA plates and incubated overnight at 37°C. The following day, PCR testing using both sets of primers and gel electrophoresis were repeated, the six colonies representing the most intensely staining bands used to inoculate kanamycin infused liquid LB and the cultures placed in a shaking incubator overnight at 37°C. DNA was then extracted by performing plasmid extraction on each culture (Plasmid Spin Miniprep Kit)[63], spectrophotometer readings were taken to assess DNA quality and quantity, and PCR analysis using both sets of primers and gel electrophoresis were repeated. Finally, amplicon sequencing and BLAST searches in Genbank® were performed to ensure the extracted plasmid contained the same FeLV sequence as the initial PCR

product (99% alignment with FeLV strain Glasgow-1 accession number KP728112.1). The six DNA standards were stored at -20^{o}C.

Within-run and between-run precision of the FeLV qPCR assay were assessed using three dilutions of the DNA standard (10^3, 10^5 and 10^7 molecules per reaction, as determined by the average of four spectrophotometer readings (Nanodrop 1000)[64] and standard calculation involving Avogadro's constant (see below). Within-run precision was evaluated with 10 replicates of each dilution, and between-run precision was evaluated using 10 replicates of each dilution in three separate experiments. For both, mean C_T value and standard deviation (SD) were used to calculate coefficients of variation (CV) for the C_T values (CV = SD / mean C_T). Diagnostic sensitivity for the PCR assay was determined by 10-fold serial dilutions of the DNA standard. Mean within-run and between-run precisions (CV) were both 0.66% (within-run 0.54–0.8%, between-run 0.45–0.85%), while the lower limit of detection for the assay was 100 copies of DNA standard per 25 µL reaction (10 out of 10 reactions positive). For the next lower dilutions (90, 80, 70, 60, 50 and 40 copies per 25 µL reaction), 9/10, 7/10, 4/10, 5/10, 4/10 and 5/10 reactions were positive, respectively.

$$\textbf{Number of copies (molecules)} = \frac{\textbf{A x (6.0221 x } 10^{23} \textbf{ molecules/mole)}}{\textbf{B x 660g/mole x (1 x } 10^{9} \textbf{ ng/g)}}$$

A = amount of plasmid per reaction (64.95 ng, based on the average of four spectrophotometer readings)

B = length of dsDNA plasmid (i.e. number of nucleotides; 4062 including the inserted 131 bp FeLV amplicon)

660g/mole = average mass of 1 bp dsDNA

PCR testing for genomic mammalian DNA was also performed using a published protocol to ensure the quality of the thawed DNA (Helps et al., 2005; Pinches et al., 2007b). This protocol uses primers designed to amplify a region of the feline 28S rDNA gene. Each 25 µL PCR reaction was composed of 0.125 µL Taq DNA polymerase[63], 2.5 µL 10x PCR Buffer[63], 0.5 µL 10mM dNTP mix, 200 nM of each primer[65], 200 nM of a dual-labelled fluorogenic probe (labelled at the 5' end with the fluorescent reporter dye CAL Fluor Orange 560 ^{TM} and at the 3' end with the fluorescent quencher dye Black Hole Quencher-1^{TM})[65], 3 µL of 25 mM $MgCl_2$, 3 µL of DNA (approximately 100 ng), and nuclease-free water. The same cycling conditions and PCR machine were used as for FeLV proviral testing, except fluorescence was detected during each annealing step (60^{o}C) at 560–580 nm. Samples were run singularly. Known positive samples and a no template control were included in each run. Samples with a C_T value of less than 45 recorded a positive result.

7.3.3 Blood collection and FeLV antigen testing

FeLV antigen testing using whole blood was performed as per manufacturers' instructions within 24 hours of sampling (Groups 1 and 3) or within 24 hours of receiving the blood sample (Group 2) using whole EDTA blood. Thirteen samples not able to be tested within this time period were stored at -20^{o}C, either as whole EDTA blood (n = 10) or plasma (n = 3), then thawed and tested at a later date. Three FeLV antigen test kits were used in parallel to detect viral capsid protein p27 in whole blood. SNAP FIV/FeLV Combo is a lateral flow ELISA kit, while Witness FeLV/FIV and Anigen Rapid FIV/FeLV use IC methodology.

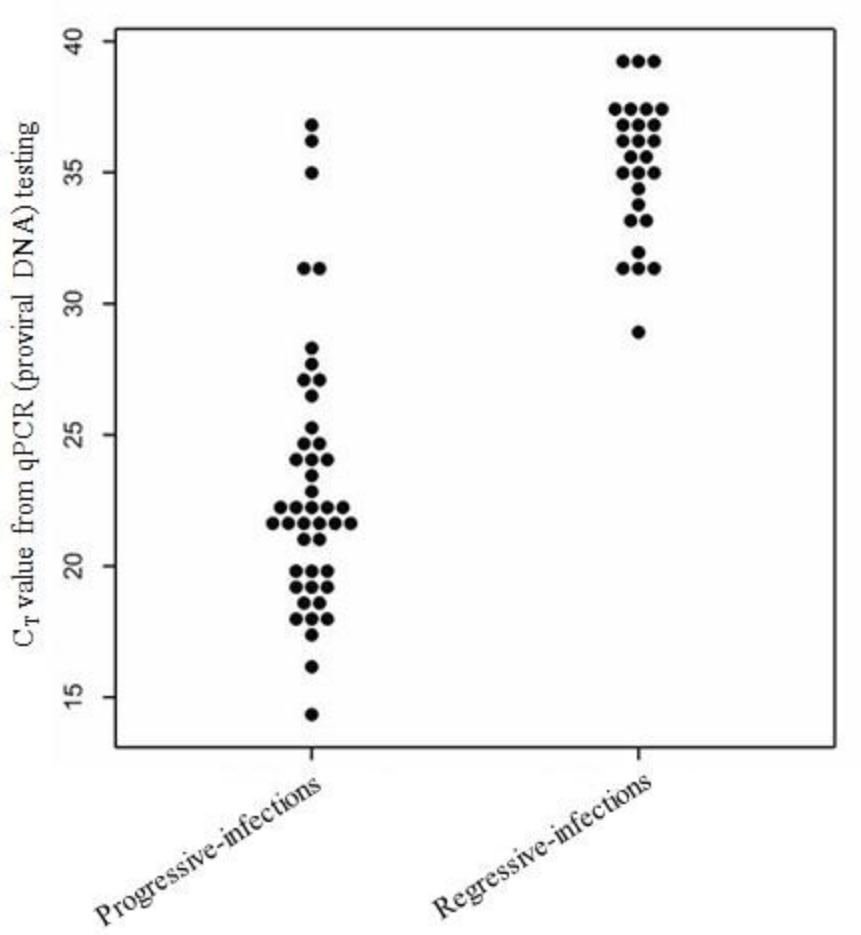
7.3.4 Categories of FeLV infection

The following outcomes were described: (i) FeLV-uninfected (PCR negative), either due to an abortive-infection or non-exposure to FeLV; (ii) FeLV-infected with progressive-infection (PCR positive and at least one positive p27 test using blood); and (iii) FeLV-infected with regressive-infection (PCR positive but three negative p27 tests using blood). Atypical-infections were unable to be diagnosed in this study owing to testing only being performed at a single time point (serial testing was not possible).

FeLV provirus PCR testing determined 72 cats to be FeLV-infected and 491 cats to be FeLV-uninfected. Of 72 FeLV-infected cats, 45 were suspected of being progressively-infected and 27 regressively-infected. FeLV C_T values for progressively FeLV-infected cats (median 22, range 14–37, IQR 20–25) were significantly lower (i.e. higher proviral load) compared with regressively FeLV-infected cats (median 35, range 29–40, IQR 34–37) ($P < 0.001$; Figure 7.1), with 40/45 progressive infections recording a C_T value < 30. Progressively FeLV-infected cats were younger (median age 3.4 years) than regressively FeLV-infected cats (median age 7.6 years; $P = 0.007$).

The median C_T value for feline 28S testing was 29 (range 27–36, IQR 28-30). The median 28S C_T value for progressively and regressively FeLV-infected cats was not significantly different ($P = 0.58$).

Figure 7.1 Scatter plot of C_T values (*y* axis) from FeLV provirus PCR testing of 563 cats. Progressively FeLV-infected cats (*n* = 45) recorded a significantly lower median C_T value (i.e. higher proviral load) compared with regressively FeLV-infected cats (*n* = 27) (*x* axis).



7.3.5 Saliva collection and FeLV antigen testing

Of 563 cats recruited for blood testing, 419 were available for saliva sampling at the time blood was collected. This included 26 FeLV-infected cats (17 progressively-infected, 9 regressively-infected) and 393 FeLV-uninfected cats. Saliva was collected immediately following blood collection, as described in Chapter 4. Briefly, a sterile individually cased cotton swab mounted on a plastic rod[67] was rubbed against the buccal mucosa on each side of the mouth, with the cheek pressed gently against the upper dental arcade while slowly twisting the swab, for approximately 10 s per side. The plastic rod was cut approximately 2

cm from the cotton tip, the tip transferred to a sterile microcentrifuge tube[68] (plastic rod at the bottom of the tube), 450 µL of sterile phosphate buffered saline (PBS) added and the tube shaken vigorously by hand for 10 s. The tube, still containing the cut cotton swab, was then centrifuged for 30 s at 10,000 g[69]. The swab was then removed from the tube using forceps and the supernatant tested using the same three FeLV antigen test kits as used for testing with whole blood. FeLV p27 testing was performed as per manufacturers' instructions except that an equivalent volume of saliva-containing supernatant was substituted for blood in the test protocol. None of the manufacturers endorses using saliva as a diagnostic specimen for their FeLV test kits. The primary author performed FeLV antigen testing using saliva immediately following FeLV antigen testing using blood, meaning samples were not blinded for saliva testing.

The average weight of ten swabs after saliva sampling was determined using a Precision Plus electronic balance[70] and compared to the average weight of ten unused swabs. The median weight of saliva collected per swab was 70 mg (IQR 30–110 mg).

At the conclusion of the study a subset of cats with progressive FeLV-infection (n = 15) was resampled using three new swabs and the aforementioned technique. However, instead of using PBS and centrifugation to extract a supernatant sample, a single cotton swab was used exclusively for each FeLV antigen test kit (randomly ordered), using a simpler method. For each FeLV antigen test kit, the saliva swab was directly applied to the sample well spot, then the cotton tip was soaked with twice the volume of buffer recommended in the manufacturers' instructions, rolling the cotton tip on the sample spot for 10 s while buffer was added. The result was read 10 min later. This revised 'direct' technique for FeLV antigen testing using saliva was investigated to determine the accuracy of a quicker, simpler method for patient-side use (Chapter 4).

7.3.6 Statistical analysis

Numerical analyses were performed using statistical software (Genstat 16th Edition)[71]. Statistical significance was considered at $P < 0.05$ and 95% CIs were calculated for test kit performance based on a normal approximation and the Wald method using Microsoft Excel[72]. A Shapiro-Wilk test was used to assess data for normality; since age and C_T value (for both FeLV and feline 28S assays) were not normally distributed, median was reported for each and a Mann-Whitney U-test used for any age or C_T comparisons. PPV and NPV were calculated using the standard formulas (PPV = 'number of true positives' / ('number of true positives' + 'number of false positives'); NPV = 'number of true negatives' / ('number of true negatives' + 'number of false negatives')). Overall test accuracy was determined by the formula (('number of true positives' + 'number of true negatives') / total number of cats sampled). Binomial logistic regression with a logit link function was conducted on the test results to compare sensitivity and specificity between test kits and between blood and saliva results for the same test kit. Cohen's Kappa Index Value (κ) was calculated to assess agreement between blood and saliva results for each test kit using the standard formula ($\kappa = 1 - (1 - P_o) / (1 - P_e)$), where P_o was the observed agreement and P_e was the expected agreement (0.5).

[60] Veterinary Pathology Diagnostic Services (VPDS), The University of Sydney, Sydney, NSW, Australia.
[61] ViraCHEK FeLV, Synbiotics Corporation, San Diego, CA, USA (testing performed at Vetnostics).
[62] Vetnostics, 60 Waterloo Road, North Ryde, NSW 2113 AUSTRALIA.
[63] Qiagen, Valencia, CA, USA.
[64] Thermo Fisher Scientific, Waltham, MA, USA.
[65] Biosearch Technologies, Navato, CA, USA.
[66] Bio-Rad, Hercules, CA, USA.
[67] Sarstadt, Mawson Lakes, South Australia, Australia (Plastic Stem Cotton Tip Catalogue No. 80.625).
[68] Sarstadt, Mawson Lakes, South Australia, Australia (1.5 mL Micro Tube Catalogue No. 72.706.400).
[69] Eppendorf AG, Hamburg, Germany (Model 5424).
[70] Ohaus, Parsippany, NJ, USA.
[71] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.
[72] Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.

7.4 RESULTS

7.4.1 FeLV point-of-care testing using blood (*n* = 563)

The performance of the three point-of-care FeLV antigen kits, compared to qPCR for FeLV provirus, using blood as the diagnostic specimen is shown in Table 7.1. SNAP FIV/FeLV Combo produced more false-positive FeLV results than either Witness FeLV/FIV or Anigen Rapid FIV/FeLV, resulting in significantly lower specificity (94% *versus* 98%, $P = 0.004$ and 0.007, respectively). SNAP FIV/FeLV Combo recorded less false-negatives than the other two kits, although its sensitivity was not significantly higher (63% *versus* 57%, $P = 0.50$ for both). The specificity and sensitivity of Witness FeLV/FIV and Anigen Rapid FIV/FeLV were comparable to each other ($P = 0.83$ and 1.00, respectively). A summary of FeLV p27 results grouped by FeLV C_T value is provided in Table 7.2 to highlight the decreasing sensitivity of all three test kits as C_T rose (i.e. as the amount of FeLV provirus present decreased). True-positive p27 results (i.e. progressive-infections) were more common in younger animals (median age 3.4 years) with lower C_T values, while false-positive p27 results occurred more commonly in older animals (median age 7.5 years) ($P < 0.001$).

When two different p27 test kits were used in parallel this increased PPV (highest was 91% using Witness FeLV/FIV and Anigen Rapid FIV/FeLV), but did not completely eliminate the occurrence of false-positive FeLV results (Table 7.3). Of the 35 cats that recorded a false-positive p27 result with at least one of the test kits, false-positive FeLV results were obtained with more than one test kit in 10/35 instances (6 instances where 2/3 kits were positive and 4 instances where 3/3 kits were positive, despite a negative FeLV qPCR result; median age 6 years, range 11 months to 12 years, IQR 4–10 years). Seven of

these 10 cases where multiple false-positive p27 results were obtained were in cats displaying clinical signs consistent with FeLV disease, while three were in clinically well cats. Testing in parallel was able to identify all cats with progressive FeLV infections, so long as SNAP FIV/FeLV Combo was one of the test kits used and provirus testing was pursued when results were discordant (i.e. one positive p27 result, one negative p27 result). If Witness FeLV/FIV and Anigen Rapid FIV/FeLV were used in combination, three cats progressively infected with FeLV were not identified (i.e. both kits tested p27 negative).

We wrestled with whether or not to include suspected regressive-infections in the final analyses of test kit performance (sensitivity, specificity, PPV and NPV). Ultimately we decided to retain regressive infections in all analyses and consider FeLV provirus PCR testing as a true gold standard for diagnosis of FeLV infection. However, we appreciate that it could be considered unfair to declare a false-negative p27 result for an antigen test kit if there was actually no antigen present to detect. Analyses with the exclusion of likely regressive-infections are therefore also provided below in Table 7.4.

Table 7.1 Results of testing using three point-of-care FeLV antigen test kits using blood (*n* = 563), comprising 45 FeLV-infected cats with progressive-infections, 27 FeLV-infected cats with regressive-infections and 491 FeLV-uninfected cats.

FeLV test kit	SNAP Combo	Witness	Anigen Rapid
True positive	45	41	41
False negative	27	31	31
True negative	463	481	480
False positive	28	10	11
Sensitivity (%)	45/72 = **63** (51–74)	41/72 = **57** (46–68)	41/72 = **57** (46–68)
Specificity (%)	463/491 = **94** (92–96)	481/491 = **98** (97–99)	480/491 = **98** (96–99)
PPV (%)	45/73 = **62** (50–73)	41/51 = **80** (69–91)	41/52 = **79** (68–90)
NPV (%)	463/490 = **94** (92–97)	481/512 = **94** (92–96)	480/511 = **94** (92–96)
Overall accuracy (%)	**508/563 = 90**	**522/563 = 93**	**521/563 = 93**

CIs (95%) are given in brackets.

Table 7.2 Results of testing using three point-of-care FeLV antigen test kits using blood, arranged by C_T values from FeLV proviral PCR testing using blood. FeLV-infected cats (*n* = 72) with both progressive (*n* = 45) and regressive (*n* = 27) infections are included.

C_T values	Type of infection		SNAP Combo	Witness	Anigen Rapid
	Progressive	Regressive			
< 20 (*n* = 14)	14	0	14	14	14
20 – 24.99 (*n* = 20)	20	0	2	19	20
25 – 29.99 (*n* = 7)	6	1	6	6	5
30 – 34.99 (*n* = 11)	2	9	2	2	2
> 35 (*n* = 20)	3	17	3	0	0
TOTAL (*n* = 72)	**45**	**27**	**45/72**	**41/72**	**41/72**

Table 7.3 Results of the point-of-care FeLV antigen test kits using blood when considered in parallel. If both kits tested p27 positive, the cat was assigned FeLV-positive; conversely, if both kits tested p27 negative, the cat was assigned FeLV-negative. When the kits had differing results (i.e. one positive and once negative), a discordant result was assigned. Considering the results of two kits in combination increased the specificity and positive predictive value of p27 testing to identify cats with progressive FeLV infections, although no combination completely eliminated the occurrence of false-positive or false-negative results. Therefore provirus PCR testing, rather than a second antigen test, should always be pursued for FeLV confirmatory testing where possible.

FeLV test kit	SNAP Combo / Witness	SNAP Combo / Anigen Rapid	Witness / Anigen Rapid
True positive	41	41	40
False negative	27	27	30
True negative	461	457	476
False positive	8	5	4
Discordant results	26	33	13
Sensitivity (%)	41/68 = **60** (49–72)	41/68 = **60** (49–72)	40/70 = **57** (46–69)
Specificity (%)	461/469 = **98** (97–99)	457/462 = **99** (98–100)	476/480 = **99** (98–100)
PPV (%)	41/49 = **84** (73–94)	41/46 = **89** 80–98)	40/44 = **91** (82–99)
NPV (%)	461/488 = **94** (92–96)	457/484 = **94** (92–96)	476/506 = **94** (92–96)
Overall accuracy (%)	**502/537 = 93**	**498/530 = 94**	**516/550 = 94**

CIs (95%) are given in brackets.

Table 7.4 Results of testing using three point-of-care FeLV antigen test kits using blood (*n* = 536, comprising 45 FeLV-infected cats with progressive-infections and 491 FeLV-uninfected cats). FeLV-infected cats with regressive-infections (*n* = 27) were excluded from final analysis.

FeLV test kit	SNAP Combo	Witness	Anigen Rapid
True positive	45	41	41
False negative	0	4	4
True negative	463	481	480
False positive	28	10	11
Sensitivity (%)	45/45 = **100**	41/45 = **91** **(83–99)**	41/45 = **91** **(83–99)**
Specificity (%)	463/491 = **94** **(92–96)**	481/491 = **98** **(97–99)**	480/491 = **98** **(96–99)**
PPV (%)	45/73 = **62** **(50–73)**	41/51 = **80** **(69–91)**	41/52 = **79** **(68–90)**
NPV (%)	463/463 = **100**	481/485 = **99** **(98–100)**	480/484 = **99** **(98–100)**
Overall accuracy (%)	**508/536 = 95**	**522/536 = 97**	**521/536 = 97**

CIs (95%) are given in brackets.

7.4.2 FeLV point-of-care testing using saliva (*n* = 419)

Table 7.5 shows the comparative performance of the three point-of-care FeLV antigen kits using saliva as the diagnostic specimen. The sensitivity (54%) and specificity (100%) of each of the three test kits was identical when saliva was used, although there was not complete agreement with test results between kits for two cats. Each kit recorded three false-negative FeLV results; two progressively FeLV-infected cats tested p27 negative with all three kits using saliva, while another two had discordant results with saliva (one was p27 positive with SNAP FIV/FeLV and Anigen Rapid FIV/FeLV only, the other was p27 positive with Witness FeLV/FIV only). No false-positive FeLV results were recorded with any of the kits using saliva.

FeLV test kit performance using saliva, with the exclusion of suspected regressive-infections from final analyses, is provided in Table 7.6.

7.4.3 Repeat FeLV saliva testing using revised 'direct' technique (*n* = 15)

Table 7.7 shows the results from re-testing 15 progressively FeLV-infected cats using the simpler patient-side technique described above. Despite SNAP FIV/FeLV recording three more false-negative results than both Witness FeLV/FIV and Anigen Rapid FIV/FeLV, owing to the small sample size there was no significant difference in sensitivity (67% *versus* 87%) between the three FeLV test kits using saliva and the direct technique (P = 0.21).

Table 7.5 Results of testing using three point-of-care FeLV antigen test kits using saliva and a centrifugation method (*n* = 419), comprising 17 FeLV-infected cats with progressive-infections, 9 FeLV-infected cats with regressive-infections and 393 FeLV-uninfected cats.

FeLV test kit	SNAP Combo	Witness	Anigen Rapid
True positive	14	14	14
False negative	12	12	12
True negative	393	393	393
False positive	0	0	0
Sensitivity (%)	14/26 = **54** (35–73)	14/26 = **54** (35–73)	14/26 = **54** (35–73)
Specificity (%)	393/393 = **100**	393/393 = **100**	393/393 = **100**
PPV (%)	14/14 = **100**	14/14 = **100**	14/14 = **100**
NPV (%)	393/405 = **97** (95–99)	393/405 = **97** (95–99)	393/405 = **97** (95–99)
Overall accuracy (%)	**407/419 = 97**	**407/419 = 97**	**407/419 = 97**

CIs (95%) are given in brackets.

Table 7.6 Results of testing using three point-of-care FeLV antigen test kits using saliva and a centrifugation method (*n* = 410, comprising 17 FeLV-infected cats with progressive-infections and 393 FeLV-uninfected cats). FeLV-infected cats with regressive-infections (*n* = 9) were excluded from final analysis.

FeLV test kit	SNAP Combo	Witness	Anigen Rapid
True positive	14	14	14
False negative	3	3	3
True negative	393	393	393
False positive	0	0	0
Sensitivity (%)	14/17 = **82** **(64–100)**	14/17 = **82** **(64–100)**	14/17 = **82** **(64–100)**
Specificity (%)	393/393 = **100**	393/393 = **100**	393/393 = **100**
PPV (%)	14/14 = **100**	14/14 = **100**	14/14 = **100**
NPV (%)	393/396 = **99** **(98–100)**	393/396 = **99** **(98–100)**	393/396 = **99** **(98–100)**
Overall accuracy (%)	**407/410 = 99**	**407/410 = 99**	**407/410 = 99**

CIs (95%) are given in brackets.

Table 7.7 Results of testing using three point-of-care FeLV antigen test kits using saliva and a revised 'direct' testing technique (*n* = 15, comprising 15 FeLV-infected cats with progressive-infections). Only sensitivity was able to be calculated since no FeLV-uninfected cats were tested with the direct technique.

Test kit	SNAP Combo	Witness	Anigen Rapid
True positive	10	13	13
False negative	5	2	2
Sensitivity (%)	10/15 = **67** **(43–91)**	13/15 = **87** **(69–100)**	13/15 = **87** **(69–100)**

CIs (95%) are given in brackets.

7.4.4 Comparing FeLV point-of-care testing for blood and saliva (*n* = 419)

Table 7.8 compares the sensitivity and specificity of blood and saliva testing for each FeLV antigen test kit. All comparisons were statistically similar. Cohen's Kappa Index Value (κ) confirmed excellent concordance between blood and saliva test results per individual: SNAP FIV/FeLV Combo κ = 0.95, Witness FeLV/FIV κ = 0.98, Anigen Rapid FIV/FeLV κ = 0.97.

Table 7.8 Comparison of overall sensitivity and specificity for three point-of-care FeLV antigen test kits using whole blood and saliva (*n* = 563 for blood, *n* = 419 for saliva). There were no significant differences between blood and saliva testing for any of the test kits (*P* values shown).

	SNAP Combo		Witness		Anigen Rapid	
	BLOOD	**SALIVA**	**BLOOD**	**SALIVA**	**BLOOD**	**SALIVA**
Sensitivity	**63** (51 – 74)	**54** (35 – 73)	**57** (46 – 68)	**54** (35 – 73)	**57** (46 – 68)	**54** (35 – 73)
	(*P* = 0.39)		(*P* = 0.78)		(*P* = 0.57)	
Specificity	**94** (92 – 96)	**100**	**98** (97 – 99)	**100**	**98** (97 – 99)	**100**
	(*P* = 0.70)		(*P* = 0.73)		(*P* = 0.71)	

CIs (95%) are given in brackets.

7.5 DISCUSSION

The performance of three point-of-care FeLV antigen kits was found to be similar when compared with proviral qPCR testing, irrespective of whether whole EDTA blood or saliva was tested, except for more false-positive FeLV results occurring with SNAP FIV/FeLV Combo compared with the other two kits using blood. Since whole blood is currently the recommended specimen for patient-side FeLV testing, these results provide an important reminder that PCR detection of DNA provirus remains the gold standard confirmatory test to definitely diagnose FeLV infection. Thus, PCR testing should be pursued to confirm any positive p27 antigen test result. Furthermore, veterinarians should consider C_T value from FeLV qPCR testing as a possible predictor of cats more likely to become regressively-infected; cats with progressive FeLV infections had lower C_T values (i.e. higher proviral load), with 40/45 progressively-infected cats recording a C_T of < 30.

The rationale of screening for FeLV infection using p27 testing of blood, followed by confirmatory PCR testing (Lutz et al., 2009), is to identify cats progressively infected with FeLV and avoid unnecessary euthanasia due to false-positive p27 results. False-positives become more common as prevalence diminishes (Hawkins, 1991; Beatty et al., 2011; Möstl et al., 2013), such as in Australia where the prevalence of progressive FeLV infection is likely less than 1% (Chapters 2 and 8). False-positive FeLV results are said to occur as a result of anti-mouse IgG antibodies present in a small proportion (estimated ≤ 0.5%) of the cat population (as well as in people and other domestic animals). The reason why anti-mouse IgG is present in cat sera is not well understood, but might relate to predation of mice and subsequent ingestion. Whatever the mechanism of their generation, anti-mouse IgG bind to murine-derived monoclonal antibodies in FeLV test kits that

capture p27 (Lopez and Jacobson, 1989a). It is possible that some false-positive p27 results occurred in truly infected cats with different LTR sequences to the primers chosen. Future studies could consider repeating provirus PCR testing at another facility with a methodologically distinct assay, or performing VI or IFA in these cases.

Using two different p27 kits in parallel, as some authors have recommended (Hartmann et al., 2007), increased PPV and should be considered in cases where an urgent diagnosis is necessary or where PCR testing is unavailable. Parallel testing, however, did not completely eliminate the occurrence of false-positive p27 results in between four and eight FeLV-uninfected cats (depending on the combination of test kits employed). Thus, confirmatory PCR testing is still recommended for any cat (healthy or sick) that tests p27 positive with multiple rapid kits, as decisions regarding treatment or euthanasia will be much better informed by the PCR result.

Several studies have reported populations of regressively FeLV-infected cats, including 5-10% of cats tested in Switzerland (24/445 and 6/597, respectively), 10% in UK (45/465), 3% (2/75) in Australia and 1% in Germany (6/495) (Hofmann-Lehmann et al., 2001; Gomes-Keller et al., 2006; Pinches et al., 2007b; Beatty et al., 2011; Englert et al., 2012). Regressively FeLV-infected cats eliminate the viraemia within 2-16 weeks of exposure, and a limitation of our study was that some of the cats defined as progressively-infected may have been in the process of clearing the viraemia, thereby becoming regressively-infected. This limitation could be overcome in the future by serial p27 testing. For this reason, and until the role of regressive-infections in disease is explored further, we do not recommend solely using qPCR testing for FeLV diagnosis or FeLV screening (including blood donors). Instead, a combination of p27 antigen and provirus PCR testing should be undertaken in all cases.

FeLV provirus PCR testing was chosen as the gold standard as it can detect very low levels of nucleic acid from progressive, regressive and, to a variable degree, atypical infections (Gomes-Keller et al., 2006; Sand et al., 2010). PCR testing is not without its challenges. Since it is able to detect as little as 1 to 10 copies of provirus in a given sample extreme care must be taken in the laboratory to avoid DNA contamination and false-positive results (Crawford and Levy, 2007), while primer design must allow detection of FeLV-A, -B and -C sequences to ensure FeLV-infected cats do not test provirus PCR-negative (Tandon et al., 2005; Pinches et al., 2007b). It could be argued that it was unreasonable for this study to record a false-negative p27 result using proviral PCR as the benchmark, thereby lowering test kit sensitivity, when by definition there was no FeLV antigen present to detect (i.e. that it was unfair to include regressive infections in the analysis). We contend that the purpose of FeLV point-of-care test kits is to screen for FeLV infection, and therefore the sensitivities reported (Tables 7.1 and 7.5) highlight the inability of test kits to identify a potentially important subcategory of FeLV infection. A recent study using a similar methodology also reported comparable (low) sensitivity for SNAP FIV/FeLV Combo and Anigen Rapid FIV/FeLV (53% and 40%, respectively) to the current study (Sand et al., 2010). The development of molecular techniques such as RT-PCR have demonstrated that some cats defined as being regressively-infected are actually transcriptionally active and still shed low levels of virus. One group found 5/14 cats, that would have traditionally been classified as regressively-infected (p27 negative, qPCR positive), tested RT-PCR positive and were thus ‘regressive infections with transient antigenaemias’ (Hofmann-Lehmann et al., 2007).

The performance of all three FeLV test kits using saliva as the diagnostic specimen was comparable to testing with blood, in disagreement with the 2008 AAFP guidelines (Levy et

al., 2008a). When venipuncture is not possible without skilled physical restraint or sedation, collecting and testing saliva for the presence of FeLV p27 antigen should be considered. In one previous study, saliva testing was actually considered to have identified seven FeLV-infected cats with FeLV-related disease that tested FeLV-negative with serum; although definitive FeLV diagnosis was not pursued in these cats, it was proposed that the cats were truly infected and the discordant test results were due to viral replication being higher in salivary glands than other tissues such as blood (Lutz and Jarrett, 1987). This potential added benefit of saliva testing was replicated in one cat using a Witness FeLV/FIV test kit in the current study, using both methods of saliva testing. No false-positive results were recorded with any of the FeLV test kits using saliva, but of concern were 3/17 (18%) false-negative FeLV results occurring in progressively-infected cats with each test kit.

7.6 CONCLUSION

FeLV antigen testing using whole blood remains justifiable since it is inexpensive and produces rapid results which facilitate clinical decision making while waiting for definitive confirmatory testing at a PCR facility. Where PCR testing is unavailable, or rapid confirmation of a positive p27 result is required, repeat p27 testing with a different rapid kit reduces (but doesn't completely eliminate) the occurrence of false-positive results. Consideration of the patient's age may help direct clinical judgment (progressive FeLV infection more common in younger cats, false-positive p27 results more common in older cats), while requesting the proviral qPCR C_T value may provide another indication as to whether the infection is progressive ($C_T < 30$) or regressive ($C_T > 30$). In addition to confirming a positive p27 result, PCR testing for FeLV provirus should also be undertaken to investigate animals with signs consistent with FeLV-related disease that test p27 negative, in case a regressive-infection is present and contributing to the clinical picture. All blood donor cats should be screened by qPCR for FeLV provirus. Although saliva testing with point-of-care kits shows promise, more research is needed before it can be recommended as a reliable screening tool for detecting FeLV infection.

CHAPTER 8. EPIDEMIOLOGY OF FeLV INFECTION IN THE DOMESTIC CAT IN AUSTRLIA

8.1 ABSTRACT

A field study was undertaken to explore the outcomes of natural FeLV exposure in three well defined cohorts of cats in Australia (*n* = 529). Group 1 (*n* = 440) consisted of healthy client-owned cats with outdoor access recruited as part of a FIV vaccine effectiveness study (Chapter 6), while Groups 2 (*n* = 38) and 3 (*n* = 51) consisted of a mixture of apparently healthy and sick cats being group-housed in two separate rescue facilities on the outskirts of Sydney (35km apart). Cats in Group 1 were prospectively recruited using clinical medical records, while cats in Groups 2 and 3 were tested in response to recent outbreaks of illness and sudden death in individual cats sourced from these facilities. Diagnostic testing for FeLV infection included antigen (p27) testing using three different point-of-care FeLV/FIV kits, qPCR testing for the detection of proviral DNA and qPCR testing for the detection of viral RNA following a reverse transcription step (qRT-PCR). In total, FeLV-infection was identified in 52/529 cats (21 progressively-infected, 31 regressively-infected). This was comprised of 11/440 Group 1 cats (2 progressively-infected [0.5%], 9 regressively-infected [2%]), 14/38 Group 2 cats (7 progressively-infected [18%], 7 regressively-infected [18%]) and 27/51 Group 3 cats (12 progressively-infected [24%], 15 regressively-infected [29%]). Progressively-infected cats had significantly lower qPCR C_T values (i.e. higher levels of proviral DNA) compared to regressively-infected cats ($P < 0.001$). Viral RNA was detected in 16/21 cats with progressive FeLV-infections and in 1/31 cats with regressive-infections. Cats with progressive-infections tended to be younger than those with regressive-infections (median

age 5 *versus* 10 years; $P = 0.064$). Five cats were FeLV/FIV co-infected. FeLV infection remains a risk for Australian cats, although in the general population it would appear the majority of infections are regressive. FeLV infection was widespread among the two different rescue populations tested, a reminder of the importance of retroviral testing (FeLV and FIV) on entry prior to co-habitation with other cats and the need to reduce group-size to facilitate improved infectious disease threat minimization. It is advisable to test and vaccinate any young cat in Australia with outdoor access against FeLV to reduce the risk of infection.

8.2 INTRODUCTION

Development of molecular methods for FeLV diagnosis such as qPCR to detect proviral DNA and qRT-PCR to detect vRNA in blood has enhanced our ability to appreciate the complexity of FeLV infection. Consequently, contemporary investigations of FeLV prevalence should utilize these techniques to adequately appraise FeLV infection. In places such as Australia where the overall prevalence of FeLV in the general cat population is low, the resulting low positive predictive value of point-of-care antigen testing creates an added challenge, despite generally excellent sensitivity and specificity of the current generation of kits (Hartmann et al., 2007; Adam and Dandrieux, 2011; Beatty et al., 2011). Without confirmatory provirus PCR testing, estimates of FeLV prevalence are likely to be unreliable, prone to error and certain to underestimate the extent of subclinical infection (Chapter 7).

Recent studies utilizing molecular diagnostic techniques have begun to report the full gamut of possible outcomes following FeLV exposure (Table 8.1). To date, only one such study has been conducted in Australia, although vRNA testing (to detect transcriptional activity) was not performed, and cats were only recruited from one city in Australia (Sydney), thereby limiting the scope of the report (Beatty et al., 2011).

The aim of the current study was to report the range of possible outcomes following natural FeLV exposure in one widespread group of client-owned cats living predominantly in eastern Australia and two groups of rescue cats, also living in eastern Australia (Sydney, NSW), using a combination of antigen (p27) and nucleic acid (qPCR and qRT-PCR) testing. Unfortunately, considering the higher prevalence of FeLV infection in WA client-owned cats reported in Chapter 2, samples were only available from eastern Australia.

Table 8.1 Summary of previous FeLV studies where categories of FeLV infection were investigated using nucleic acid amplification (proviral qPCR +/- vRNA qRT-PCR testing). Where FeLV-antibody testing was not performed, not exposed/abortive-infections are grouped together. Where qRT-PCR testing to detect vRNA was not performed, regressive-infections with or without detectable vRNA are grouped together. Focal-infections are not represented as none of the studies shown performed the necessary testing for detection.

		----------Outcome of FeLV exposure----------					
Author (chronological order)/country	Recruitment criteria	Not exposed	Abortive infection	Regressive infection (without detectable vRNA)	Regressive infection (with detectable vRNA)	Progressive infection	Ratio progressive: regressive infections
Hofmann-Lehmann *et al.* (2001) Switzerland	Not specified	495/597 (83%)		61/597 (10%)		41/597 (7%)	0.7
Gomes-Keller *et al.* (2006) Switzerland	Mainly multi-cat households (not representative of the general cat population)	367/445 (82%)		23/445 (5%)	1/445 (0.2%)[a]	54/445 (12%)	2.3
Pinches *et al.* (2007b) UK	Samples sent to a diagnostic laboratory (presumably	364/465 (78%)		45/465 (10%)		56/465 (12%)	1.2

	from sick cats)						
Beatty *et al.* (2011) Sydney, Australia	Mixed population comprising sick cats presenting to a university veterinary hospital, FeLV in-contact cats and juvenile (< 12 months) healthy cats	243/248 (98%)		3/248 (1%)		2/248 (1%)	0.7
Englert *et al.* (2012) Germany	Mixture of randomly selected cats presenting to a university veterinary hospital and healthy shelter cats	458/495 (93%)	22/495 (4%)	6/495 (1%)	0/495 (0%)[b]	9/495 (2%)	1.5

[a]evaluated by testing both blood and saliva with RT-PCR assay.
[b]evaluated by testing saliva only with RT-PCR assay.

8.3 MATERIALS AND METHODS

8.3.1 Sample population

Three groups of cats were recruited in Australia: (i) **Group 1** (*n* = 440) consisted of healthy client-owned cats that were part of a case-control study into the effectiveness of a commercially available FIV vaccine[73] (Chapter 6). Both FIV-vaccinated cats (cases) and FIV-unvaccinated cats (controls) were recruited using medical records from participating veterinary clinics located in four states and one territory; (ii) **Group 2** (*n* = 38) consisted of a mixture of healthy and sick rescue cats group-housed in a facility on the outskirts of Sydney, NSW; and (iii) **Group 3** (*n* = 51) comprised a mixture of healthy and sick rescue cats group-housed in a different facility on the outskirts of Sydney, NSW (35km from Group 2; no transfer of cats between two facilities) (Figure 8.1). Cats in Group 2 and 3 were sampled and tested at the request of each facility manager in response to recent unexplained illnesses and deaths in the cat populations. Approval was granted by the University of Sydney Animal Ethics Committee (Approval number N00/1-2013/3/5920).

Recruited cats from Group 1 (*n* = 440) ranged from two to 20 years-of-age (median 7 years; IQR 5–10 years), comprising 229 (52%) castrated males, 207 (47%) spayed females and four (1%) entire males. Most were domestic crossbred cats (371/440; 84%), the remainder comprising a range of pedigree breeds. Most cats were described by their owner as having some level of outdoor access (429/440; 98%).

Recruited cats from Group 2 (*n* = 38) ranged from one to 14 years-of-age (median 4 years; IQR 3–10 years), comprising 15 (39%) castrated males and 23 (61%) spayed females. Most were domestic crossbred cats (36/38; 95%). The age of one cat in group 2 was unknown. Recruited cats from Group 3 (*n* = 51) ranged in age from three months to five years

(median 1 year; IQR 0.4–2 years), although the age of only 10 cats in group 3 was known (the other 41 were adult cats > 6 months). This comprised 23 (45%) castrated males, 27 (53%) spayed females and one entire female (2%). Most were domestic crossbred cats (49/51; 96%). Cats in groups 2 and 3 were predominantly free-roaming, with some group-housing of the more tractable cats exercised at night (Figure 8.2 provides images of the first rescue facility (Group 2) to illustrate the free-roaming, group-housing conditions).

A complete vaccination history was available for cats in Group 1 only: 146/440 (33%) had been vaccinated against FeLV with an IWV vaccine[74], of which 108/146 (74%) had been vaccinated within the past 12 months and 38/146 (26%) were overdue for FeLV vaccination (more than 12 months since last vaccination; median time 3.5 years, range 1–11 years, IQR 2–6 years).

8.3.2 FeLV/FIV point-of-care testing

Three commercially available dual FeLV-antigen/FIV-antibody kits were used to test whole blood (EDTA anti-coagulated). All three kits (SNAP FIV/FeLV Combo[75], Witness FeLV/FIV[76] and Anigen Rapid FIV/FeLV[77]) detect FeLV viral capsid protein p27 in whole blood, plasma, serum and saliva (Chapter 7).

8.3.3 FeLV qPCR testing

DNA extraction from white cells within whole blood and the qPCR assay used for detection of FeLV proviral DNA is described in detail in section 7.3.2.

8.3.4 FeLV qRT-PCR testing

Plasma was thawed from -80^{o}C and RNA extracted using a standard kit (Qiagen RNeasy)[78] as per manufacturer's instructions. A standard RT protocol was used to create complementary DNA (cDNA; RevertAid)[79], the resulting cDNA being measured using a spectrophotometer (Nanodrop 1000)[79]. The FeLV qPCR assay described in section 7.3.2 was run to detect vRNA, using cDNA as the template (instead of genomic DNA), with a slightly altered primer/probe concentration as recommended by the original authors (900 nM of forward primer, 300 nM of reverse primer and 200 nM of probe (Tandon et al., 2005).

8.3.5 Determination of FeLV status

Table 8.2 describes the possible outcomes to FeLV exposure that were considered (Englert et al., 2012). Cats that tested (i) p27 with at least one point-of-care kit and qPCR positive were classified as progressively-infected; (ii) p27 negative with all three kits and qPCR positive were classified as regressively-infected, with the qRT-PCR result dividing regressive-infections further into with or without detectable vRNA; and (iii) p27 negative and PCR negative were classified as FeLV-uninfected (which may have included non-exposure to FeLV and abortive-infections). Focal- infections have variable p27 and qPCR results and therefore, if present, may have been classified as progressive-infections, regressive-infections or FeLV-uninfected (FeLV PCR testing of tissue samples would have been required to determine) (Hartmann, 2012). Due to an absence of sequential testing, it is possible that cats that were viraemic as a result of recent exposure to FeLV may have cleared the viraemia if retested 2–16 weeks later, and therefore might have been incorrectly categorized as progressive rather than regressive-infections.

8.3.6 Determination of FIV status

Since all three FeLV antigen kits used simultaneously tested for the presence of FIV antibodies, the presence of dual retroviral infections was investigated. Defining FIV positivity in Group 1 was complicated by 139 cats having received at least one FIV-vaccination previously, meaning some false-positive FIV antibody test results were encountered (Uhl et al., 2002). The algorithm used to overcome this issue and determine FIV infection status was described in Chapter 3 and involved both FIV PCR testing (using a commercially available assay) as well as VI in rare discrepant cases. Diagnosis of FIV infection in Groups 2 and 3 was not complicated by recent FIV vaccination and a positive FIV status was assigned when all three test kits tested positive for FIV antibodies. No discordant FIV results from Groups 2 and 3 were recorded (i.e. the three FIV antibody tests were always in agreement, either all positive or all negative).

8.3.7 Statistical analysis

Numerical analyses were performed using statistical software (Genstat 16th Edition)[80]. Statistical significance was considered at $P < 0.05$. A Shapiro-Wilk test was used to assess data for normality; where data was normally distributed REML testing was performed (comparing age between groups), and where data was not normally distributed Mann-Whitney U-tests were used (comparing age between progressive and regressive-infections, C_T values between progressive and regressive-infections, and C_T values between groups). Two-sample binomial tests were used to compare sex and breed proportions between groups, FeLV outcome between groups and the likelihood of a single retroviral infection (FeLV or FIV) *versus* co-infection (FeLV and FIV). Two-tailed Fisher's exact tests were used to investigate the relationship between sex and pedigree status on the prevalence of

FeLV and FIV infection. Potential geographic 'hot spots' of FeLV infection in Group 1 based on postcode were investigated using the scan statistic (SaTScan version 7). A Bernoulli model (case-control) was used and both circular and elliptical scanning windows of up to 50% of the study area size were investigated.

[73] Fel-O-Vax® FIV, Boehringer Ingelheim, Fort Dodge, IA, USA.
[74] All cats in this study vaccinated against FeLV had been given Fel-O-Vax LvK (Boehringer Ingelheim, Fort Dodge, IA, USA).
[75] IDEXX Laboratories, Westbrook, ME, USA.
[76] Zoetis Animal Health, Lyon, France.
[77] BioNote, Gyeonggi-do, Korea.
[78] Qiagen, Valencia, CA, USA.
[79] Thermo Fisher Scientific, Waltham, MA, USA.
[80] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.

Figure 8.1 Map of Australia, showing the location of 13 veterinary clinics used to recruit cats for Group 1 (blue dots) and the location of the two rescue facilities that formed Groups 2 and 3 (red dots). An enlargement of Sydney, NSW is shown in the breakout box. The grey shading in the breakout box represents the 'Built Up Areas' of Sydney (as defined by Geosciences Australia, www.ga.gov.au/mapspecs/250k100k/appendixA_files/Habitation.html#Habitation Built Up Area Polygon) and is included to show the semi-rural location of the two rescue facilities and one veterinary clinic on the outskirts of Sydney.

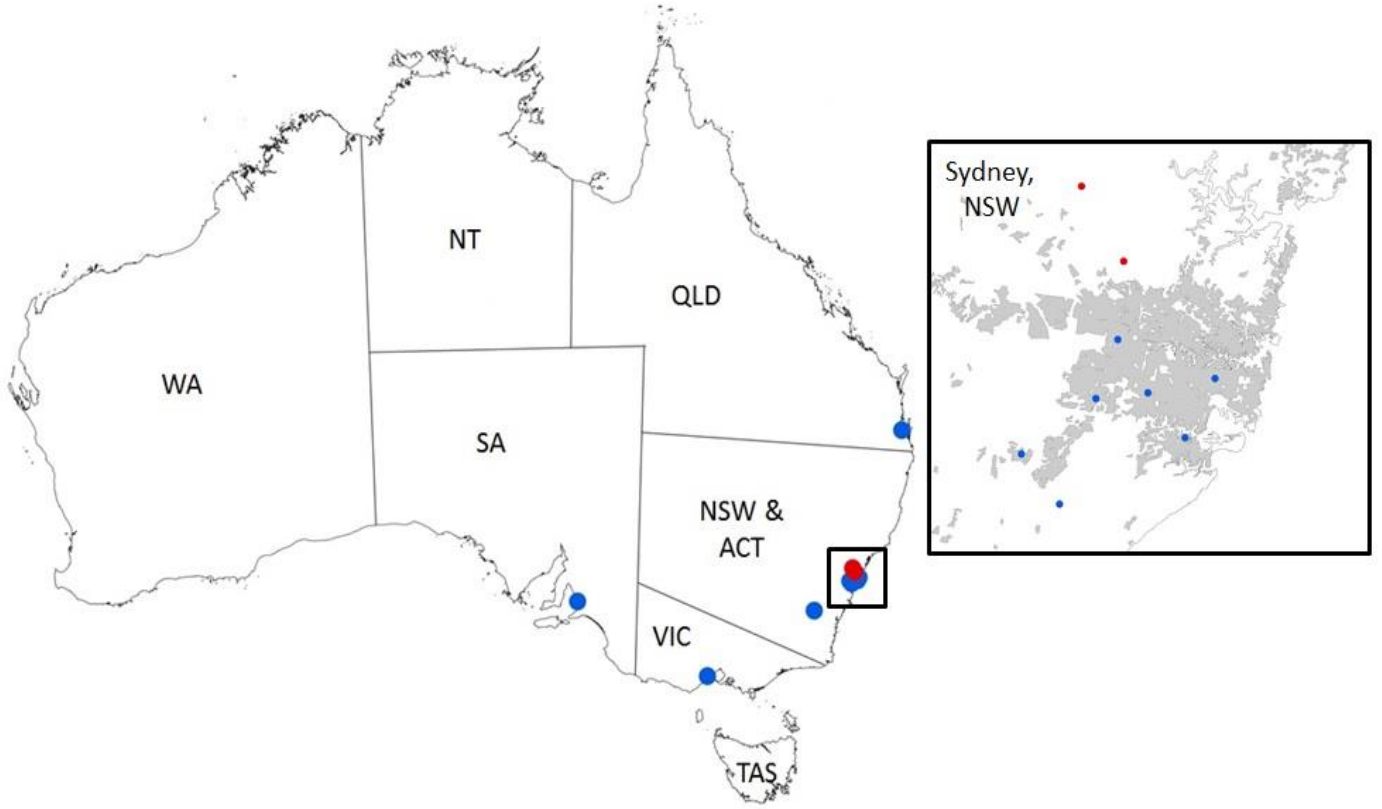


Figure 8.2 Images of the first rescue facility to illustrate the free-roaming, group-housing conditions of cats in Group 2 (*n* = 38). Images of cats in Group 3 (*n* = 51) were unavailable.

Table 8.2 Outcomes following FeLV exposure categorized by observed test results. In the current study abortive-infections could not be diagnosed as FeLV-antibody testing was not performed and focal-infections could not be determined as FeLV PCR of tissue samples was not performed.

Test performed	Outcome of FeLV exposure			
	FeLV-uninfected	Regressive infection (without detectable vRNA)	Regressive infection (with detectable vRNA)	Progressive infection
p27 antigen	-	-	-	+
qPCR (DNA)	-	+	+	+
qRT-PCR (vRNA)	NP	-	+	+

NP = not performed.

8.4 RESULTS

8.4.1 Sample population

The median age of each group was significantly different (Group 1 [median = 7 years] > Group 2 [median = 4 years] > Group 3 [median = 1 year], $P < 0.001$). The proportion of males and females in each group was similar ($P = 0.11$). Group 1 contained proportionally more pure-bred cats compared to Group 3 ($P = 0.02$) but not Group 2 ($P = 0.08$). Groups 2 and 3 contained a similar proportion of pure-bred cats ($P = 0.76$).

8.4.2 FeLV status

A summary of outcomes by group is presented in Table 8.3. Of the 529 cats tested, 52 FeLV-infected cats were identified (21 progressively-infected, 31 regressively-infected). Of the 52 FeLV-infected cats, 11 were from Group 1 (2 progressively-infected, 9 regressively-infected from a total of 440 cats), 14 were from Group 2 (7 progressively-infected, 7 regressively-infected from a total of 38) and 27 were from Group 3 (12 progressively-infected, 15 regressively-infected from a total of 51).

C_T values from qPCR testing of progressively-infected cats ranged from 17 to 37 (median 21, IQR 18–29). C_T values from qPCR testing of regressively-infected cats ranged from 28 to 40 (median 35, IQR 33–37). Progressively-infected cats had significantly lower qPCR C_T values (i.e. higher levels of proviral DNA) than regressively-infected cats ($P < 0.001$). When analysed by cohort, Group 1 cats had significantly higher qPCR C_T values (i.e. lower levels of proviral DNA; median 36) compared to cats in Group 2 (median 25) and Group 3 (median 33) ($P = 0.008$ and 0.03, respectively), but qPCR C_T values for Groups 2 and 3 were not significantly different ($P = 0.15$) (Figure 8.3).

With regards to qRT-PCR testing, 16/21 progressively-infected cats and 1/29 regressively-infected cats tested positive for vRNA (plasma not available from two regressively-infected cats). The qRT-PCR C_T values from the 16 progressively-infected cats ranged from 25 to 42 (median 34, IQR 26–38). The five progressive-infections that tested qRT-PCR negative for vRNA recorded the five highest qPCR C_T values (i.e. lowest levels of proviral DNA) of the progressively-infected cats. The qRT-PCR C_T value for the one regressively-infected cat that tested positive was 38.

A summary of the signalments of FeLV-infected cats is presented in Table 8.4. Progressively-infected cats were younger than regressively-infected cats (median age 5 *versus* 10 years), although this difference did not reach statistical significance ($P = 0.064$). There was no sex predilection for either progressive or regressive-infections ($P = 0.38$ and $P = 0.71$, respectively). When FeLV-infected cats were analysed by breed (domestic cross-bred *versus* pure-bred), there was no significant difference in proportion compared to FeLV-uninfected cats for either progressive or regressive-infections ($P = 0.10$ and $P = 1.00$, respectively). Cats in Groups 2 and 3 were significantly more likely to be FeLV-infected than cats in group 1 ($P < 0.001$). There was no significant difference in likelihood of FeLV-infection between Groups 2 and 3 ($P = 0.13$), irrespective of whether progressive ($P = 0.56$) or regressive-infections ($P = 0.23$) were analysed separately. The age of only three FeLV-infected cats in Group 3 was known.

Using a circular scanning window, two potential geographical ‘hot spots’ of FeLV infection in group 1 were identified, one in NSW (postcode 2145; 4 cases reported, 1.63 expected) and one in VIC (postcodes 3218, 3219 and 3220; 3 cases reported, 0.58 expected). Using an elliptical scanning window, two similar potential geographical ‘hot spots’ of FeLV infection were identified in NSW (postcodes 2145 and 2168; 7 cases

reported, 3.43 expected) and in VIC (postcodes 3218, 3219 and 3220; 3 cases reported, 0.58 expected). However, none of these 'hot spots' were statistically significant ($P \geq 0.60$).

One cat with a progressive FeLV infection (1/21; cat # 62) had been vaccinated against FeLV with an IWV vaccine[b] (two primary vaccinations given one month apart, 16 months before sampling, with a single annual booster administered four months before sampling). FeLV testing, however, had not been performed in this cat to ensure it was FeLV-uninfected prior to vaccination. Of the regressively FeLV-infected cats, three (3/31) had been vaccinated against FeLV (two cats within the past 12 months, one cat 11 years previously) but none had been FeLV tested prior to vaccination. There was no significant difference in qPCR C_T values between FeLV-vaccinated and FeLV-unvaccinated regressive-infections ($P = 0.11$).

8.4.3 FIV status

In total, 39/529 (7%) cats were FIV-infected, comprising 32/440 (7%) in Group 1, 3/38 (8%) of Group 2 and 4/51 (8%) of Group 3. FIV-infected cats ranged from three to 16 years-of-age (median 7 years; IQR 5–9 years), comprised 27 (79%) castrated males, 11 (28%) spayed females and one (4%) entire male. Most were domestic crossbred cats (35/39; 90%). Male cats were significantly more likely to be FIV-infected than female cats ($P = 0.008$, odds ratio 2.6). The age of only one FIV-infected cat in group 3 was known.

8.4.4 FeLV/FIV co-infection

Five cats (5/529; 1%) were FIV/FeLV co-infected (3 progressively FeLV-infected, 2 regressively FeLV-infected) (Table 8.5). One co-infection was identified in Group 1 (progressively FeLV-infected; cat # 62; FIV-unvaccinated), one in Group 2 (progressively FeLV-infected) and the remaining three were from Group 3 (one progressively FeLV-

infected, two regressively FeLV-infected). All three FIV-infected/progressively FeLV-infected cats have died since their diagnosis (survival time from testing 5 months, 5 months and 13 months). There was no statistical difference in C_T values for FeLV PCR testing between FIV/FeLV co-infected and FeLV-infected/FIV-uninfected cats, irrespective of whether regressive FeLV infections were included ($P = 0.24$) or excluded ($P = 0.60$). Cats in all three groups were more likely to be infected with one retrovirus (either FeLV or FIV) than be co-infected with both ($P < 0.001$).

8.4.5 Follow up of rescue cats (Groups 2 and 3)

All p27 negative cats in Groups 2 and 3 were vaccinated against FeLV using either an IWV vaccine or a recombinant p45 vaccine[81] (two primary vaccinations given one month apart)[82]. Cats in Group 2 were closely followed; serial testing of progressively-infected cats (tested up to six times over two years) confirmed all seven remained strongly antigenaemic with p27 testing. A summary of outcomes for progressively-infected cats in Group 2 is provided in Table 8.6. Husbandry advice and assistance with FIV/FeLV testing of new animals was provided to both rescue facilities in an effort to reduce the incidence of retroviral disease in the cat populations.

[81] Fel-O-Vax LvK (Boehringer Ingelheim, Fort Dodge, IA, USA) or Leucogen (Virbac Animal Health, Carros, France).

[82] Both FeLV-uninfected and regressively FeLV-infected cats in Group 2 were vaccinated against FeLV after PCR testing. Although data is lacking and more research needs to be done in this area, we hypothesized vaccinating regressively-infected cats might 'prime' the immune system to help prevent them reverting to a progressive state and becoming viraemic. p27 negative cats in Group 3 were vaccinated against FeLV immediately after testing (i.e. before PCR results were known), in order to protect these cats from becoming progressively-infected as quickly as possible. Consequently, both FeLV-uninfected and regressively FeLV-infected cats in Group 3 were also vaccinated against FeLV.

Table 8.3 FeLV infection status in each group from the current study (*n* = 529). Abortive-infections could not be diagnosed as FeLV-antibody testing was not performed and focal-infections could not be determined as FeLV PCR of tissue samples was not performed.

Group	Description	Outcome of FeLV exposure			
		FeLV-uninfected	Regressive infection (without detectable vRNA)	Regressive infection (with detectable vRNA)	Progressive infection
Group 1 (*n* = 440)	Case-control in-field study of a FIV vaccine	429/440 (98%)	9/440 (2%)	0/440 (0%)	2/440 (0.5%)
Group 2 (*n* = 38)	Rescue facility with group-housing	24/38 (63%)	7/38 (18%)	0/38 (0%)	7/38 (18%)
Group 3 (*n* = 51)	Rescue facility with group-housing	24/51 (47%)	14/51 (27%)	1/51 (2%)	12/51 (24%)

Figure 8.3 Scatter plot of C_T values (*y* axis) from FeLV provirus PCR testing of whole blood in 529 cats. Progressively FeLV-infected cats ($n = 21$) recorded a significantly lower median C_T value (i.e. higher proviral load, $P < 0.001$) compared with regressively FeLV-infected cats ($n = 31$) (*x* axis). C_T values for Group 1 were significantly higher (i.e. lower proviral load) than Group 2 ($P = 0.008$) and Group 3 ($P = 0.03$), but C_T values for Groups 2 and 3 were not significantly different ($P = 0.15$).

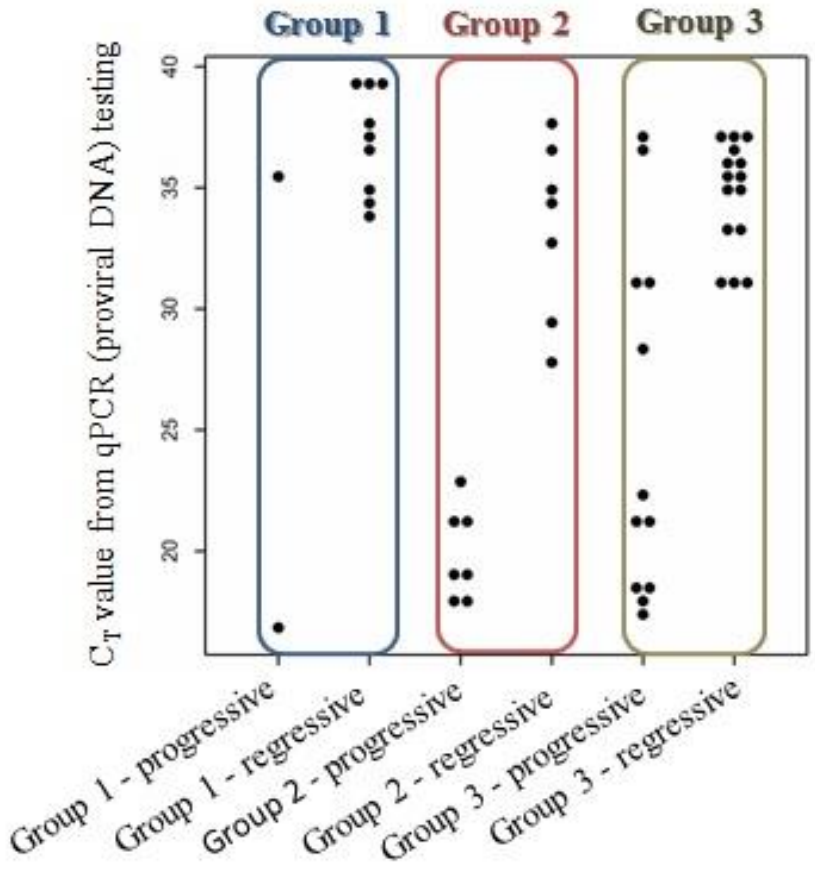


Table 8.4 Age, sex and breed of FeLV-infected cats identified in the study (*n* = 52, including 21 progressive and 31 regressive infections).

Category	Age (years)	Sex	Breed
Progressively FeLV-infected			
Group 1 (*n* = 2)	3, 6	1 MN, 1 FS	2 DCB
Group 2 (*n* = 7)	2–11 (median 8)	5 MN, 2 FS	7 DCB
Group 3 (*n* = 12)	0.4, 5[a]	7 MN, 5 FS	12 DCB
OVERALL (*n* = 21)	**0.4–11 (median 5)[b]**	**13 MN, 8 FS[c]**	**21 DCB[d]**
Regressively FeLV-infected			
Group 1 (*n* = 9)	2–16 (median 10)	7 MN, 2 FS	7 DCB, 2 PB
Group 2 (*n* = 7)	3–14 (median 10)	3 MN, 4 FS	6 DCB, 1 PB
Group 3 (*n* = 15)	4[a]	7 MN, 8 FS	14 DCB, 1 PB
OVERALL (*n* = 31)	**2–16 (median 10)[b]**	**17 MN, 14 FS[c]**	**27 DCB, 4 PB[d]**

MN = neutered (castrated), FS = female spayed, DCB = domestic cross-bred, PB = pure-bred.
[a]The age of only three FeLV-infected cats in group 3 was known.
[b]Progressively-infected cats were younger than regressively-infected cats, although this difference did not reach statistical significance ($P = 0.064$).
[c]There was no sex predilection for either progressive or regressive-infections ($P = 0.38$ and $P = 0.71$, respectively).
[d]When FeLV-infected cats were analysed by breed, there was no significant difference in proportion compared to FeLV-uninfected cats for either progressive or regressive-infections ($P = 0.10$ and $P = 1.00$, respectively).

Table 8.5 Summary of FIV antibody testing, stratified by FeLV infection status. A total of 39 cats (39/529; 7%) were FIV-infected, including five (5/529; 1%) FeLV/FIV co-infected cats. Group 1 consisted of cats recruited as part of a study into FIV vaccine effectiveness, while Groups 2 and 3 consisted of rescue cats being group-housed in different locations on the outskirts of Sydney (35km apart). Cats in all three groups were more likely to be infected with one retrovirus (either FeLV or FIV) than be co-infected with both ($P < 0.001$).

Category	Group 1	Group 2	Group 3
FIV-infected/FeLV-uninfected ($n = 34$)	31	2	1
FIV-infected/progressively FeLV-infected ($n = 3$)[a]	1 ($C_T = 17$)	1 ($C_T = 23$)	1 ($C_T = 21$)
FIV-infected/regressively FeLV-infected ($n = 2$)[a]	0	0	2 ($C_T = 31, 37$)
TOTAL ($n = 39$)	**32/440 (7%)**	**3/38 (11%)**	**4/51 (8%)**

[a]C_T = cycle threshold value from FeLV proviral PCR testing using blood.

Table 8.6 Outcomes known at the time of writing for progressively FeLV-infected cats in Group 2 (*n* = 7). All cats were domestic cross-bred.

Cat	Age at time of diagnosis	Sex	Outcome
SP-5	11	FS	Euthanased 24 months after diagnosis due to mass in abdomen, post-mortem not performed
SP-7	11	FS	Alive and healthy
SP-14	4	MN	Euthanased one month after diagnosed with feline sarcoma virus (FeSV) infection, multiple skin masses all over body, index case for group 2
SP-20	8	MN	Euthanased 38 months after diagnosis due to nasal cavity tumour, awaiting histopathology
SP-21	9	MN	Euthanased 13 months after diagnosis due to breathing difficulties, cardiac disease suspected due to severe heart murmur, post-mortem not performed, co-infected with FIV
SP-30	2	MN	Euthanased 22 months after diagnosis due to breathing difficulties, post-mortem revealed mediastinal lymphoma
SP-31	2	MN	Euthanased 6 months after diagnosis, severe non-regenerative anaemia and icterus, post-mortem revealed hepatomegaly, splenomegaly, pericardial effusion and pleural effusion

MN = male neutered (castrated), FS = female spayed.

8.5 DISCUSSION

This study reports for the first time a comprehensive analysis of outcomes following natural FeLV exposure in cats domiciled in Australia. The data suggests FeLV-infection has not decreased in prevalence over the past 20 years, and that most infections are subclinical regressive-infections, whose long term effects are not completely understood. In a cohort representative of cats with some level of outdoor access (Group 1), FeLV prevalence was 2.5% (11/440), including 9 regressively-infected cats identified with proviral qPCR testing. Investigation of closed populations with 'FeLV outbreaks' (Groups 2 and 3) revealed that FeLV was common among in-contact cats.

Previous FeLV prevalence studies in Australia have reported variable rates of infection in 'healthy' populations of cats, ranging from 0% to 7% (Chapter 2). One Australian study from 26 years ago reported FeLV seroprevalence of 7% (5/72) in a small population of healthy cats recruited by random selection of households from the electoral roll (Robertson et al., 1990b). This relatively high rate of progressive-infections compared to the current study (2/440 in Group 1; 0.5%) was probably a reflection of limited sample size, location sampled (Perth, Western Australia has since been shown to have a higher rate of FeLV-infected cats compared to the rest of the country (Chapter 2) and an era when FeLV infection was more prevalent worldwide than it is today (Lutz et al., 2009). More recent studies in Australia (1997, 2007 and 2011) have reported lower FeLV prevalence rates in healthy client-owned cats of 0–2% (Malik et al., 1997; Norris et al., 2007; Beatty et al., 2011) (Chapter 2). Only one previous study of healthy Australian cats employed proviral PCR testing to confirm any p27 positive result (Beatty et al., 2011); thus, earlier estimates of FeLV prevalence may have been falsely elevated due to the reduced positive predictive

value of point-of-care test kits associated with testing a low prevalence population (Möstl et al., 2013). Beatty *et al.* (2011) did not identify any progressive or regressive FeLV infections in a population (n = 169) comprising healthy young cats (all < 1 year), mostly re-homed from rescue organisations where single-housing was carried out (except for kittens) (Beatty et al., 2011). The current study has fulfilled the need for a comprehensive contemporary investigation of the prevalence of progressive and regressive FeLV infections in the general cat population in Australia.

Several studies have reported mixed populations of progressively and regressively FeLV-infected cats (Table 8.1) (Hofmann-Lehmann et al., 2001; Gomes-Keller et al., 2006; Pinches et al., 2007b; Beatty et al., 2011; Englert et al., 2012). The current study found 0.5% (2/440) and 2% (9/440) of the general cat population (Group 1) were progressively and regressively FeLV-infected, respectively. Despite vaccination regressive-infections can still occur, irrespective of which FeLV vaccine is administered (Torres et al., 2005; Hofmann-Lehmann et al., 2007), and indeed three regressively-infected cats in Group 1 had been given an IWV FeLV vaccine (Fel-O-Vax LvK).

Studies using highly sensitive qRT-PCR assays capable of detecting extremely low concentrations of vRNA in plasma have demonstrated that most regressively FeLV-infected cats are transcriptionally active, despite testing antigen (p27) and VI negative (Hofmann-Lehmann et al., 2007; Torres et al., 2010). Based on studies documenting transmission of FeLV to uninfected cats transfused with blood taken from regressively-infected cats, the low level vRNA detected in p27 negative/provirus positive cats likely represents infectious viral particles (Nesina et al., 2015). Hofmann-Lehmann and colleagues also suggested that vRNA-positive regressively FeLV-infected cats are at higher risk for reactivation of active infection than vRNA-negative regressively FeLV-

infected cats (Hofmann-Lehmann et al., 2008). We identified only one cat in this category; the remainder (28/29) of regressively FeLV-infected cats tested negative with the qRT-PCR assay. In the current study, qPCR testing for proviral DNA and qRT-PCR testing for vRNA demonstrated good agreement in progressive-infections (16/21), but consistent with other reports, five progressive-infections tested negative for vRNA with RT-PCR (Tandon *et al*. 2005 reported agreement between DNA and vRNA testing in 104/120 samples, while Torres *et al*. 2008 reported agreement in 240/264 samples) (Tandon et al., 2005; Torres et al., 2008). This discrepancy is probably a consequence of the RT-PCR assay (which detects RNA) being less sensitive than the PCR assay (which detects DNA). This notion is consistent with the five progressive-infections that tested negative for vRNA having the lowest levels of proviral DNA. Other possible explanations include RNA degradation that may have occurred during plasma harvesting and storage and difficulties with RNA extraction due to a small amount of RNA actually present in the sample. For these reasons, it is conceivable that the qRT-PCR assay was unable to detect very low levels of viral RNA in some regressively-infected cats (< 2250 viral copies per mL plasma) (Tandon et al., 2005). Additionally, serial qRT-PCR testing was required by Hofmann-Lehmann's group to identify transcriptional activity in 11/14 cats. It is therefore possible that with repeated testing, some of the regressively FeLV-infected cats in the current study may have demonstrated transcriptional activity (Hofmann-Lehmann et al., 2007). It is more likely, however, that the high rate of transcriptionally active regressive-infections reported by the Swiss group (14/14; 100%) was an experimental phenomenon, since other studies in the field have found low levels or absent vRNA in regressively FeLV-infected cats (Gomes-Keller et al., 2006; Englert et al., 2012). The risk of FeLV transmission from regressively-infected cats to in-contact cats, therefore, appears low in Australia, although the effect of

low level virus transmission via cat bites (akin to blood transmission experiments) remains to be assessed.

8.6 CONCLUSION

Despite a reduction in FeLV prevalence in pet cats, the threat of FeLV infection remains present. Veterinarians should consider vaccinating any cat with outdoor access against FeLV to reduce the risk of infection, especially in the first few years of a cat's life, and testing for FeLV prior to vaccination. Rescue facilities that allow group-housing need to be especially vigilant about screening new arrivals for FeLV during an initial quarantine period before mixing of animals is permitted. The role of regressive-infections in FeLV-associated disease and contagion, particularly those in which detectable vRNA is present in blood, needs further investigation. Specifically, the contribution of regressive FeLV-infections to the development of lymphoma must be addressed.

CHAPTER 9. CONCLUSIONS AND FUTURE DIRECTIONS

The chapters in this thesis have been ordered in the way considered to be most logical:

(i) Background prevalence of both FIV and FeLV in domestic cats in Australia;

(ii) FIV research (diagnosis of FIV infection, duration of antibody response following FIV vaccination and protective rate of the FIV vaccine), and;

(iii) FeLV research (diagnosis of FeLV infection and epidemiological considerations of FeLV in domestic cats in Australia).

As discussed in section 1.14, however, this was not the chronological order of our investigations. The discussion below recounts some of the major highlights of our research with a 'real-world' chronological approach, in order to give a truer feel as to how this research effort evolved over time, and suggests some of the future research projects that might be performed as a continuation of our lines of enquiry.

This entire body of work was born out of the aim to report, for the first time, a field protective rate of the FIV vaccine (Fel-O-Vax FIV) in Australia. Chapter 6 described the first five unequivocal FIV vaccine breakthroughs in the field (as well as two additional possible vaccine breakthroughs) and determined a protective rate (effectiveness) of 56%, with no significant difference in FIV prevalence found between the FIV-vaccinated cats (cases) and FIV-unvaccinated cats (controls). Unfortunately, for a variety of reasons discussed previously, the power of the study was ultimately 40% instead of 80% as projected in the study design. It is possible with greater study numbers that a significant effect of the vaccine would have been found (if one indeed exists). Based on our experiences, however, there is no doubt it is very difficult to recruit cases using the

retrospective approach described due to vaccine protocol compliance issues. We also found it difficult to recruit matching control (FIV-unvaccinated) cats, since most cats from the 13 participating clinics with outdoor access were vaccinated against FIV. It could be that a poor attitude to accepting a recommendation of FIV vaccination might reflect a less attentive state of mind on the part of the client (owner), which might introduce bias into the study inadvertently if they are likewise inattentive observers of curfews and border security issues.

To address some of the flaws of the retrospective study design raised in section 6.5 a prospective study would be ideal, but would take longer to generate meaningful data, probably with a timeframe of 8 to 10 years. A prospective study design would enable FIV testing of both vaccinates and controls on day 0, thereby circumventing any potential bias from recruitment of control cats already FIV-infected at the beginning of the study period. Furthermore, strict indoor housing restrictions for cats could be enforced during the 12 weeks prior to recruitment and commencement of FIV vaccination, to ensure FIV infection does not occur before vaccine-related immunity is achieved (alternatively, appropriate FIV testing could be performed after the course of primary FIV vaccination has been completed to ensure infection has not occurred during this 'window of opportunity'). Finally, a prospective study with sequential testing (prior to annual booster FIV vaccination) could be pursued to establish if and when cats become FIV-infected. This type of study design would require a considerably larger sample population to account for the inevitable losses that occur in a longitudinal study of the required duration, and would be significantly more expensive. A faster and less expensive option for future studies would be to encourage veterinarians to routinely test for FIV infection at the time of annual FIV vaccination, using Witness or Anigen Rapid. If this recommendation were embraced by a few large

veterinary clinics that routinely use the FIV vaccine, and FIV-unvaccinated controls in the area were recruited at the same time, within a few years a large database of cases and controls could be compiled to further refine the protective rate of 56% reported in Chapter 6.

In addition to further research into the overall effectiveness of the FIV vaccine in the field, both in Australia and elsewhere, the issue of cross-protection against various FIV subtypes needs to be addressed in response to results from Chapter 6. Despite 5/25 FIV-infected controls in the current study being infected with subtypes D and F, only subtype A was identified in the five vaccine breakthroughs (and both additional 'possible breakthroughs'), suggesting that the vaccine may provide superior immunity against these other subtypes, which is counterintuitive. To further investigate this prospect, a larger Australian field study needs to be conducted to increase the number of vaccinates potentially exposed to other FIV subtypes, as well as to conduct such field studies in countries where non-A subtypes are more common (e.g. Taiwan and Japan), where subtypes C and D, respectively, are more prevalent (Kakinuma et al., 1995; Uema et al., 1999; Yamamoto et al., 2007). The diverse subtyping results in the FIV-infected controls (with presence of clades A, D and F) compared to previous Australian studies (which found a marked preponderance of subtype A, with rare subtype B isolates) (Kann et al., 2006; Iwata and Holloway, 2008) was surprising and therefore needs further attention. For subtyping results from the FIV-infected controls to be considered valid, sequencing of the *env* gene of FIV isolates from all FIV-infected cats (not just vaccinates) needs to be performed in the future to confirm the accuracy of the PCR subtyping results.

It was during this investigation into the effectiveness of the FIV vaccine that a most unexpected and novel result was obtained – the observation that two point-of-care antibody

test kits (Witness and Anigen Rapid) could generally diagnose FIV infection, irrespective of FIV vaccination status (Chapter 3). It must be remembered that at the start of our research (2012) it was universally considered that FIV vaccination rendered all FIV antibody test kits 'useless' in a discriminatory sense (e.g. (www.wsava.org/sites/default/files/VaccinationGuidelines2010.pdf; Levy et al., 2008; Hosie et al., 2009). The ability of these inexpensive test kits (current price wholesale approximately $11 AUD) to quickly and easily differentiate FIV-vaccinated and FIV-infected cats will significantly change the approach veterinarians use to screen for FIV infection, especially in pounds and shelters, and the referencing of our study in the most recent World Small Animal Veterinary Association Vaccination Guidelines (2015) evidences the impact of this finding. Our study has also recently been replicated in field studies in Florida, USA, with almost identical results reported (Crawford, 2016). In a shelter environment, where resources are limited and vaccination histories are commonly unavailable, the testing algorithm we proposed (Figure 3.4) would result in a substantial cost saving and could influence whether shelters can routinely afford to test for FIV, as well as reduce the number of cats euthanased due to testing FIV false-positive with SNAP Combo (Crawford and Levy, 2007; Levy et al., 2008a).

Chapter 4 expanded on the findings in Chapter 3 by reporting that the discriminant ability of the two IC test kits (Witness and Anigen Rapid) also applied when saliva was used as the diagnostic specimen (both kits demonstrated better than 99% accuracy). This finding has the potential to facilitate rapid and accurate determination of FIV infection status in certain scenarios, e.g. when a veterinarian or veterinary technician is unavailable for venipuncture, where cats are too fractious for safe venipuncture without chemical restraint, in shelters where large numbers of cats need to be assessed for FIV infection prior to

rehoming and where vaccination history is often unknown and for cat breeders screening new cats on entry to a cattery, or prior to mating. This will, in some circumstances, provide a less expensive and less stressful option for owners and carers and thus a superior option for their cats. Preliminary results also showed that an excellent outcome could be obtained using Anigen Rapid by applying the cotton swab directly to the sample well and flooding the cotton tip with buffer solution. It must be stressed, however, that this method should not be relied upon for making clinical decisions until further validation is undertaken with a larger study. In contrast, the performance of both SNAP Combo and Witness deteriorated with the 'direct application' technique and consequently this technique should not be used for saliva testing with either of these kits.

The surprise findings from Chapters 3 and 4, and conflicting results from another researcher (in a refereed conference abstract) shortly after our results were published (Lappin, 2015), led us to design the study in Chapter 5 to attempt to characterise the 'antibody cascade' that occurs following primary FIV vaccination using point-of-care test kits. Importantly, the prospective study design showed that some care needs to be taken in the six months after a primary course of FIV vaccination has been administered, and consequently the recommendation made in Chapter 3 regarding the use of Witness and Anigen Rapid kits for FIV screening was modified to highlight this qualification. A similar longitudinal study is now required in adult cats prior to and following annual FIV vaccination 'boosters' to determine whether this period of detectable antibody responses with point-of-care test kits also applies to cats receiving their annual FIV vaccination, or whether it only applies to young cats being vaccinated against FIV for the first time. In a shelter situation, where large scale FIV screening is being undertaken, it is highly unlikely cats will have received a primary FIV vaccination series in the preceding six months as

FIV vaccination rates are generally low, and cats so vaccinated are generally well cared for and less likely to be surrendered to a shelter facility (Salman et al., 1998). Transient antibody production for up to six months after primary FIV vaccination is also not relevant when testing cats for FIV vaccine breakthrough immediately prior to the next annual FIV vaccination booster. To further investigate the role of age and re-vaccination, we plan to monitor cats in Study 2 for several years using gp40 ELISA laboratory testing to quantitatively determine the gp40 antibody response following booster FIV vaccination, particularly to see if there is a diminished antibody response following annual re-vaccination compared to initial (primary) vaccination. Future research should ideally also quantitate the antibody response directed against other epitopes (e.g. p15 and p24) over time, using a common methodology for each (e.g. ELISA testing), to further understand the breadth, magnitude and duration of the antibody cascade following FIV vaccination.

As discussed in section 6.5, the exact mechanism(s) by which the FIV vaccine provides sterilising immunity (if indeed it does at all) is still unclear. Generally speaking, it is thought that humoral immunity plays some role in protection against homologous challenge, while CMI is important for protection against both homologous and heterologous protection. This distinction is possibly slightly arbitrary, however, since nucleotide sequences can vary as much as 15% within a particular subtype (Sodora et al., 1994). Thus, in reality, a combination of humoral and CMI is probably required for protection from challenge. Consequently, investigations into the immune response following FIV vaccination need to consider both antibody production and markers of T-cell immunity, of which this thesis has focused on an understanding of the former. Chapter 5 showed a decreased level of antibodies to both p24 and gp40 six months after primary FIV vaccination as measured with Witness and Anigen Rapid, and Chapter 6 showed all

FIV-vaccinated cats (including the five vaccine breakthroughs) had detectable levels of antibodies to p15 as measured with SNAP Combo, both of which have diagnostic implications as discussed. Further (unpublished) research I have performed in the Hosie Laboratory (Centre for Virus Research, The University of Glasgow) utilising an *in vitro* neutralising antibody assay (Bęczkowski et al., 2015b) found that of 114 FIV-vaccinated/FIV-uninfected cats (sourced from Chapter 3), most (107/114) had strong neutralisation potency (81-100% neutralisation) against homologous challenge (pseudotype designated KKS, derived from a possible vaccine breakthrough SV1, the sequence of which closely resembles that of FIV_{Pet}), while none of a subset tested (0/25) had strong neutralisation potency against a different homologous challenge (FIV_{UK8}). Considering these results in tandem suggests that all FIV-vaccinated cats have some level of antibody response following vaccination, although this antibody response wanes over time, and antibody-mediated protection is highly variable even amongst different homologous challenges. Clearly more work is required to understand the relative importance of humoral and CMI, and whether it is possible to predict protection from challenge based on the presence of particular antibodies. As a result, we have been cautious about extrapolating our antibody results beyond FIV diagnosis to duration of vaccine-induced immunity, or to speculate immunologically as to why the five vaccine breakthroughs occurred. For example, without further research into the importance of antibodies in conferring protection, it would be overreaching to suggest any protection provided by the FIV vaccine lasts for less than six months, based on results from Chapter 5. Future research undertaken by our group will likely include assessing the quality of the T-cell immune response in the five vaccine breakthroughs, as well as neutralising antibody assay work using FIV pseudotypes derived from the vaccine breakthrough isolates as the

challenge, to better understand why the breakthroughs occurred and to assess whether other FIV-vaccinated cats in the study would have been susceptible to infection, has they been challenged with the same strain of FIV.

The three FIV antibody detection kits tested in Chapters 3 and 4 also tested for FeLV antigen (p27), and therefore it was logical to examine the performance of the three kits using blood and saliva in the same cohort of cats (Chapter 7). To further increase study numbers, additional samples were sourced from veterinary clinicians around Australia, a veterinary laboratory and a rescue facility. One of the kits (SNAP Combo) was determined to have a significantly lower specificity than the other two kits tested (Witness and Anigen Rapid). All three kits, however, produced a concerning number of false-positive p27 test results, even in cats with clinical signs consistent with FeLV-infection, and testing two kits in series did not completely eliminate the possibility of an incorrect diagnosis. Consequently, our conclusion was that any cat returning a positive p27 result must have confirmatory proviral PCR testing performed. Consideration of other pertinent details, including lifestyle (e.g. higher FeLV risk in fighting cats, multi-cat households and shelters practicing group-housing), age (younger cats more likely to be progressively FeLV-infected, older cats more likely to return a false-positive p27 result) and clinical signs of FeLV-related disease (e.g. macrocytic anaemia, aplastic anaemia, lymphoma) will increase a clinician's index of suspicion for FeLV-infection further, but are not enough to eliminate doubt about the validity of a positive p27 test result. Although the lower sensitivity of p27 saliva testing compared to p27 blood testing was not statistically significant, with a larger study the trend for saliva to have increased specificity at the expense of reduced sensitivity (compared with blood) may have become further apparent. Further research into the use of saliva for FeLV p27 testing needs to be undertaken, including further assessment of the

'direct application' technique trialled. In the meantime, p27 testing using whole blood as the diagnostic specimen (with confirmatory qPCR testing of kit-positive cases) remains the recommendation for rapid screening of large numbers of cats, even though the usefulness of p27 testing using saliva looks promising.

It was whilst the research for Chapters 3, 4, 6 and 7 was being conducted that we decided a current report into the prevalence of FIV and FeLV infections in domestic cats in Australia was needed. Chapter 2 reported a higher prevalence of both FIV and FeLV infections in client-owned cats in Western Australia compared to the rest of the country (Cohort 2), a finding supported by older Australian studies from this region (Robertson et al., 1990b; Thomas et al., 1993b). This disparity based on geographical location might be attributable to the significantly higher proportion of entire male cats encountered in WA (23%) compared to elsewhere (12%) amongst cats that were sampled, a trend that may actually be a true reflection of the entire owned cat population in WA rather than merely a sampling bias. Previous studies have reported a lower de-sexing rate of client-owned male cats in Perth, WA compared with Sydney, NSW (82% *versus* 96%) (Robertson et al., 1990a; Toribio et al., 2009). This disparity, however, was not supported by results from Cohort 3 (MUVH); the FIV seroprevalence of WA cats in Cohort 3 (14%) was similar to the national FIV seroprevalence of cats in Cohort 2 (15%). A similar percentage of entire male cats was sampled in Cohort 3 (10%) compared to Cohort 2 (12%). It is not clear from the current data why the high proportion of entire male cats sampled in WA in Cohort 2 was not observed in WA cats sampled in Cohort 3 (i.e. 23% *versus* 10%). This possible increased risk of retroviral infection in WA cats should be the subject of future focused regional investigations, and may indicate a true difference in risk of exposure to the virus. In the interim, it would be prudent for WA veterinarians to conduct a public awareness

campaign to highlight the need for early neutering of male and female cats prior to sexual maturity and to routinely vaccinate young cats against FeLV.

In response to the low specificity of the FeLV antigen kits reported in Chapter 7, I decided it was important to follow up the FeLV prevalence results reported in Chapter 2 with a more detailed investigation of FeLV infection that included confirmatory PCR testing (Chapter 8). PCR testing to look for FeLV provirus also enabled us to identify regressive-infections that cannot be detected using antigen testing alone. The results reported in Chapter 8 demonstrate FeLV remains a genuine threat to Australian cats under certain circumstances. In light of this finding, young cats in Australia with access to other cats of unknown retroviral status should be vaccinated against FeLV, after pre-vaccination testing, to reduce the possibility of them becoming progressively-infected (Sparkes, 2003; Lutz et al., 2009; Patel et al., 2015). Our finding also serves to reinforce previous recommendations for shelters that allow contact between cats, including testing of all incoming cats for FeLV antigen and FIV antibody, and minimization of group size when co-housing cats to reduce the risk for cross-infection (Möstl et al., 2013).

The impact of regressive FeLV infections on the health of cats is largely unknown since few studies to date have followed a cohort of regressively-infected cats longitudinally (under field conditions) to measure outcomes from infection. Future research needs to further investigate the impact of regressive-infections on feline health, and such studies need to be longer than the usual time-frame for feline research to detect late sequelae of infection, including lymphoma and myelodysplasia. Identification of regressively-infected cats (such as the 31 cats in Chapter 8) will greatly facilitate longitudinal case-control studies of this nature, and we aim to follow all cats recruited for the current study over time to investigate the occurrence of FeLV-related disease in both progressive and regressive-

infections compared to the FeLV-uninfected controls. Potential relationships between potential pathogens and the resulting impact on disease severity also needs to be explored, particularly FeLV infection (progressive and regressive), FIV infection and feline gammaherpesvirus (FcaGHV1) infection. FcaGHV1 infection was first described in 2014 in a survey of cats from the USA, Singapore and Australia, reporting that infected cats were 2.8 times more likely to be sick than uninfected controls (Troyer et al., 2014; Beatty et al., 2014). In relation to Australian cats, FcaGHV1 prevalence was 11% and FIV-infected cats had significantly higher FcaGHV1 loads than FIV-uninfected cats. As a result, a similar mechanism to the increased Epstein-Barr virus (another human gammaherpesvirus) load encountered in HIV-infected people was hypothesised. FcaGHV1 infection was also found to be significantly associated with FeLV infection in the Singapore group (Beatty et al., 2014). Recently, the complete FcaGHV1 sequence was announced, and future research will need to determine if any of the orfs identified are indeed oncogenic (Troyer et al., 2015), and whether FcaGHC1 enhances the oncogenic ability FIV and FeLV.

In summary, this thesis has shown that retroviruses have continued importance in domestic cats in Australia. FIV infection remains prevalent amongst cats with outdoor access (12-15%, Chapters 2 and 6), and although progressive FeLV infection is approximately ten times less common (0.5-1.6%, Chapters 2 and 8), FeLV-related disease is still an important cause of mortality in at-risk cats (particularly group-housed cats). Chapter 6 provided a conceptual framework that might be utilised for future retrospective studies investigating the effectiveness of the FIV vaccine, both in Australia and elsewhere, and also suggested possible study design improvements (most importantly, a prospective study design). Similar study designs (i.e. retrospective or prospective) could be utilised to determine the

effectiveness of FeLV vaccines in the field, which to the author's knowledge has not been conducted anywhere in the world, although circumstantial evidence testifies to their likely efficacy, pushing FeLV 'back into nature' in the words of Niels C. Pedersen (www.sockfip.info/about-fip/all-articles/67-about-fip). There would be challenges with FeLV vaccine trials in an Australian setting, however, including the requirement for higher study numbers to show a difference in prevalence rates between cases and controls (due to the lower prevalence of FeLV in Australia), and the requirement to do serial testing of any infected (p27 positive) cats to establish progressive *versus* regressive infections (since no FeLV vaccine claims to provide sterilising immunity, i.e. protection from regressive-infections).

Finally, this work has made a substantial contribution to understanding antibody production following FIV vaccination and the resulting impact on the diagnosis of FIV infection. Already these results have changed how many shelters and veterinary clinics in Australia test for FIV and FeLV infection (RSPCA, Animal Welfare League and Sydney Dogs and Cats Home staff, personal communication), with veterinarians now able to confidently diagnose FIV infection in cats irrespective of their FIV vaccination status. Furthermore, more veterinarians have become aware of the absolute requirement to perform FeLV PCR testing following any positive in-clinic p27 result. The importance of FIV antibodies as a correlate of protection following vaccination has been questioned, and undoubtedly in the future we will see more research attempting to understand the relative importance of humoral *versus* CMI, which in turn will influence FIV (and HIV) vaccine development.

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