Duration of antibody response following vaccination against feline immunodeficiency virus (FIV)

ME Westman[1], R Malik[2], E Hall[1], M Harris[3], M Hosie[3], JM Norris[1]

[1]Faculty of Veterinary Science, The University of Sydney, NSW Australia 2006

[2]Centre for Continuing Veterinary Education, The University of Sydney, NSW Australia 2006

[3]Centre for Virus Research, The University of Glasgow, Scotland, United Kingdom G61 1QH

Abstract

Aim: Recently, two point-of-care FIV antibody test kits (Witness and Anigen Rapid) were reported as being 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) [1]. 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.

Methods: First, residual plasma from the original study was tested using a laboratory-based enzyme-linked immunosorbent assay (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], Witness FeLV/FIV [gp40], Anigen Rapid FIV/FeLV [p24/gp40] and VetScan FeLV/FIV Rapid [p24]).

Results: The laboratory based ELISA 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 (Witness and Anigen Rapid kits). The prospective study showed that antibody-testing with SNAP Combo and VetScan Rapid was positive in all cats two

weeks after the second FIV vaccination, and remained positive for the duration of the study (12/12 and 10/12 cats positive, respectively). Antibody-testing with Witness and Anigen Rapid was also positive in a high proportion of cats two weeks after the second FIV vaccination (8/12 and 7/12, respectively), but antibody levels declined below the level of detection in most cats (10/12) by one month after the third (final) FIV vaccination. All cats tested negative using Witness and Anigen Rapid six months after the third FIV vaccination.

Conclusions and relevance: This study has shown that a primary course of FIV vaccination does not interfere with FIV antibody-testing in cats using Witness and Anigen Rapid, provided primary vaccination has not occurred within the previous six months. Consequently, Witness and Anigen Rapid antibody test kits can be used reliably to determine FIV infection status at the time of annual booster vaccination to help detect 'vaccine breakthroughs' and in cats that have not received a primary course of FIV vaccination within the preceding six months. The duration of antibody response following annual booster FIV vaccination and the resulting effect on antibody testing using point-of-care kits needs to be determined by further research. The mechanism(s) for the variation in FIV antibody test kit performance remains unclear.

Key words

Feline immunodeficiency virus; antibodies; testing; FIV vaccination; cats

Introduction

Feline immunodeficiency virus (FIV) can infect domestic cats and may cause, after a long asymptomatic phase, variable clinical disease due to its immunosuppressive and oncogenic properties [2,3]. A FIV vaccine[a], released for use in domestic cats (2002 in the USA; 2004 in Australia), was used as a 'proof of concept' for the development of sterilizing immunity against lentiviruses, including human immunodeficiency virus (HIV-1) [4-6]. 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, such antibodies being the target for all point-of-care test kits [7]. Recently, we showed that some FIV antibody detection kits could differentiate FIV-vaccinated and FIV-infected cats under field conditions, reinforcing the complexity of antibody responses that occurs following FIV vaccination [1,8].

The FIV genome is composed of approximately 9,500 nucleotides, comprising three main open reading frames (*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 [9]. Areas of the 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 [4,10]. 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 [8,11]. Antibody production in FIV-infected cats persists for life, although antibody levels (particularly to p15 and p24) may wane in the terminal stage of infection [4]. The complex nature of this antibody cascade has resulted in variable definitions of FIV positivity based on Western blots, 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 [11,12].

The only commercial FIV vaccine (Fel-O-Vax FIV) available consists of formalin-inactivated whole cells (IWC) and whole virus (IWV) suspended together in an adjuvant [5]. 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 [13]. However, by using three point-of-care antibody tests detecting antibodies to different target FIV antigens, it was shown that FIV-vaccinated/FIV-uninfected cats consistently tested seropositive with SNAP FIV/FeLV Combo (p15 &

p24), but seronegative with Witness FeLV/FIV (gp40) and Anigen Rapid FIV/FeLV (p24 & gp40) for 0 to 7 years following FIV vaccination. Consequently, there was speculation that p15 retains immunogenicity during FIV vaccine production to a greater extent than p24 or gp40 [1]. 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 [14]. Another larger study (*n* = 104), however, confirmed the ability of the Witness and Anigen Rapid 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 [15]. 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 Witness FeLV/FIV and Anigen Rapid FIV/FeLV kits, using a laboratory well-based enzyme-linked immunosorbent assay (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, Witness FeLV/FIV , Anigen Rapid FIV/FeLV and VetScan FeLV/FIV Rapid).

[a] Fel-O-Vax® FIV, Boehringer Ingelheim, Fort Dodge, IA, USA.

Material and methods

Sample Population (Study 1)

Residual blood from Westman *et al.* (2015) was utilized for the first arm of this study [1]. 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, interquartile range [IQR] 5–10 years) and the procedure for final assignment of FIV status, which included a combination of antibody testing, polymerase chain reaction (PCR) testing and occasionally virus isolation, was previously described [1]. All 118 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 1). Seven cats from the original study overdue for their annual FIV vaccination by 3–7 years were not tested [1]. 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, determined by antibody and PCR testing and comprising a median age of six years (range 3–16 years, IQR 5–10 years), were also tested [1]. 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).

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 1). Antibody testing at weeks 14, 16 and 20 was only pursued in cats that tested FIV-positive with Witness or Anigen Rapid at the previous sampling, given the high likelihood of negative results with Witness/Anigen Rapid and positive results with SNAP Combo/VetScan Rapid in the other cats; one of these cats was lost to follow-up and unable to be tested at weeks 16 and 20. PCR testing was performed by a commercial laboratory (FIV Real PCR)[b] at the start of the study (week 0; prior to primary FIV vaccination commencing), 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 FIV testing and 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)[c] 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).

Detection of antibodies to gp40 using a laboratory ELISA

A peptide ELISA, using a nine amino acid sequence (CNQNQFFCK)[d] from the highly conserved immunodominant TM2 domain of gp40, was used to detect antibodies [16]. Plasma samples were first complement inactivated by incubating at 56°C for 30 min. The wells of 96-well microtitre plates[e] were coated with 250 ng/well of lyophilized gp40 epitope,[f] 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°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[g] 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)[h] 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[i] and optical density (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.

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°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,[j] Witness FeLV/FIV[k], Anigen Rapid FIV/FeLV[l] and VetScan FeLV/FIV Rapid.[m] SNAP Combo is a lateral-flow ELISA kit while the other three kits use immunochromatography to detect different FIV antibodies (Table 2). The fourth kit (VetScan Rapid) was added to the three kits tested in a previous study [1] 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.

Statistical analysis

Numerical analyses were performed at the conclusion of the study using statistical software (Genstat 16th Edition).[n] 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.

[b] IDEXX Laboratories, East Brisbane, Queensland, Australia.
[c] 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 Rapid.
[d] Cysteine-asparagine-glutamine-asparagine-glutamine-phenylalanine-phenylalanine-cysteine-lysine
[e] Immulon 2 HB, Thermo Fisher Scientific, Waltham, MA, USA.
[f] AltaBioscience, Birmingham, UK.
[g] Vector Laboratories, Peterborough, UK.
[h] TMB Super Slow, Sigma Aldrich, St. Louis, MO, USA.
[i] MultiSkan Ascent Plate Reader (spectrophotometer), MTX Lab Systems, Bradenton, FL, USA.
[j] IDEXX Laboratories, Westbrook, ME, USA.
[k] Zoetis Animal Health, Lyon, France.
[l] BioNote, Gyeonggi-do, Korea.
[m] Abaxis, Union City, CA, USA
[n] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.

Results

FIV gp40 laboratory quantitative ELISA (Study 1)

FIV-infected cats tested positive for antibodies recognising gp40, irrespective of FIV vaccination status ($P < 0.001$ compared to [cf:] negative control). FIV-unvaccinated/FIV-infected cats showed a similar antibody response to the positive control ($P = 0.20$), as expected, while FIV-vaccinated/FIV-infected cats showed a weaker antibody response compared to the positive control ($P = 0.01$). No significant difference in the magnitude of antibody response was observed between FIV-unvaccinated/FIV-infected and FIV-vaccinated/FIV-infected cats ($P = 0.07$) (Figure 2A). 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 than in 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 levels compared to the 108 Witness true-negative cats ($P <$

0.001). This distinction, however, was not clear-cut; for example, the upper range of the 108 Witness true-negative cats encompassed the six Witness false-positive cats apart from one individual (Figure 2A). When time since last vaccination was analysed as a potential factor in the antibody response of the 114 FIV-vaccinated/FIV-uninfected cats, no significant effect was found (P = 0.42 [days], P = 0.07 [grouped by month]) (Figure 2B). When age of cat at testing was considered as a possible factor affecting the antibody response of 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 years]) (Figure 2C). There was no significant difference in antibody response when cats were grouped according to the number of annual FIV vaccinations administered (P = 0.43; Figure 2D). Similarly, when the time since the last vaccination and the number of annual vaccinations were considered together, no significant effect was observed ($P \geq 0.61$).

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 3 provides a summary of results for these 12 cats. Online Supplement 1 provides a summary of results for all 16 cats (i.e. including the 4 exclusions), online Supplement 2 results for kittens < 6 months-of age (n = 4) and online Supplement 3 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, 8/12 (67%) tested FIV-positive with Witness and 7/12 (58%) tested FIV-positive with Anigen Rapid. At the completion of the study, six months after the third vaccination (week 34), all cats (12/12) were FIV-positive with SNAP Combo, two cats had become FIV-negative with VetScan Rapid and all cats were FIV-negative with Witness and Anigen Rapid (Figure 3).

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

(i) 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.

(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 Anigen Rapid at week 12 and 14, but FIV-negative using this kit at week 20;

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 (Study 1) 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) [1]. 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 (Study 2) were 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, Witness FeLV/FIV and Anigen Rapid FIV/FeLV 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 gp40 antibody results from Study 2 and the original study [1] appear contradictory. Study 2 found that a finite proportion of immunologically naïve cats administered a primary course of FIV vaccination 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 false-positive FIV results with Witness (6/114) in a cohort of cats vaccinated against FIV annually for at least two years, including a false-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 group who concluded that Witness testing alone could not be relied on to distinguish natural FIV infection and FIV vaccination shortly after a primary vaccination course [14]. 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. There was a high dropout rate, however; 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. Furthermore, only two FIV vaccinations were administered to kittens (instead of the recommended three), which made the results more difficult to interpret [14]. A similar longitudinal study to the current design is required in adult cats prior to and following annual vaccination to determine whether this period of detectable antibody response extends beyond primary vaccination.

The explanation for this seeming discrepancy with Witness testing is possibly two-fold. Firstly, 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 [17]. A longitudinal study of elderly people (56–79 years) administered a primary pneumococcal polysaccharide vaccine, and given a booster vaccination six years later, found the antibody titres after re-vaccination were about half the

titres after primary vaccination [18]. The trend for a weaker antibody response following booster vaccination compared to primary vaccination, however, is not steadfast; for example, one study that investigated the vaccine-induced antibody response against hepatitis virus B in people reported higher antibody levels following booster vaccination compared to at the end of the primary vaccination course six years earlier [19]. Factors related to the nature of the pathogen and the antigen(s) and adjuvants presented during vaccination are likely to play a crucial role in determining the ongoing humoral response. Secondly, age may be a factor; cats recruited for the original Westman *et al.* (2015) study (which became the cats in Study 1) 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). Likewise, Lappin (2015) tested only kittens [14]. Duration of immunity (DOI) studies are sparse in the veterinary literature, and most are only concerned with protection from challenge rather than antibody quantitation for diagnostic purposes [20]. Flow cytometry studies have demonstrated an age-related remodeling of the immune system in cats, with a gradual decline in relative percentage of lymphocytes [21], and an absolute reduction in B-cells in senior cats (10–14 years) compared to young cats (2–5 years) [22]. DOI studies are more common in the human literature, where it is generally accepted that older people have a weaker humoral response following 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) [23]. 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 the study reported by Lappin that 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 2C and 2D), nor was there a noticeable trend for kittens to test false-positive with Witness more often than adult cats in Study 2 (online

Supplements 2 and 3). Inadequate sample sizes for both studies may have been responsible. To further investigate the role of age and re-vaccination, we plan to monitor cats in Study 2 for several years and determine their gp40 antibody response following booster FIV vaccination, to see if the antibody response following annual vaccination is less than their antibody response following initial (primary) vaccination.

Sequential semi-quantitative antibody testing following FIV vaccination with Witness and Anigen Rapid 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), 67% (8/12) and 58% (7/12) cats tested seropositive for FIV antibodies using Witness and Anigen Rapid, respectively. By four weeks after the third vaccination (week 12), only 17% (2/12) cats tested seropositive with Witness or Anigen Rapid, 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, including results from p24 ELISA determinations in experimental kittens vaccinated three times 2-3 weeks apart (unpublished data, Boehringer Ingelheim). 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 [24,25]. 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 [26]. Western blot analysis of four FIV-vaccinated cats confirmed antibody production to p15, p24 and gp40 three weeks after second vaccination, which persisted for at least 12 months following third vaccination [13]. The reason why ELISA gp40 testing in the current study (Study 1) did not show a peak (and subsequent fall) in antibody production according to time elapsed since last vaccination (Figure 2B), similar to ELISA testing by Huang *et al*. and Kusuhara *et al*.,

335 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 [17,23].

Our results confirmed that care needs to be exercised in the period immediately following primary FIV vaccination using Witness and Anigen Rapid, with false-positive FIV results occurring using both. In light of these findings, we suggest an amendment to our previous conclusion [1] and recommend
340 that antibody testing to detect FIV infection in FIV-vaccinated cats is reliable using Witness and Anigen Rapid, providing primary vaccination against FIV has not occurred within the preceding six months. As discussed above, more research needs to be performed to investigate whether this recommendation also applies to cats receiving annual booster FIV vaccinations. In a shelter situation, where large scale FIV screening is being undertaken, it is highly unlikely cats will have received a
345 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 [27]. 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. If a positive FIV-antibody result is obtained in a cat where recent primary
350 vaccination is possible, submitting seropositive specimens for confirmatory FIV PCR testing is recommended. A negative FIV test result with either Witness or Anigen Rapid 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 [28].

Results from sequential antibody testing in this study challenge our previous notion that p15 is more
355 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 FIV-

positive at the end of the study. If the difference in performance between SNAP Combo and Witness/Anigen Rapid was solely attributable to p15 being more immunogenic in the FIV vaccine, then VetScan Rapid would have performed comparably to Witness/Anigen Rapid. 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* immunochromatography, 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. Our findings cannot be extrapolated to other antibody kits without testing being performed. Future research should ideally quantitate the antibody response directed against each epitope (p15, p24 and gp40) 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.

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 testing 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 FIV-positive for six months in cats given a primary course of FIV vaccination, while Witness and Anigen Rapid tested FIV-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. In jurisdictions where FIV vaccination is practiced, testing for potential FIV break-through infection prior to annual FIV vaccination is prudent [29]. In this setting, Witness and Anigen Rapid

are the FIV antibody test kits of choice since fewer false-positive results would be anticipated compared to the SNAP Combo and VetScan Rapid tests.

Acknowledgements

This study was supported financially by the Australian Companion Animal Health Foundation (ACAHF), Feline Health Research Fund (FHRF), and Boehringer Ingelheim, Australia. We are grateful to the significant in-kind contribution of diagnostic kits by IDEXX Laboratories, Zoetis Animal Health, BioNote and Abaxis. Mark Westman was supported by an Australian Postgraduate Award (APA), the Neil and Allie Lesue Scholarship and the Herbert Johnson Travel Grant Scholarship. Richard Malik was supported by the Valentine Charlton Bequest. Alan Marcus and Phillip McDonagh (Head of Regulatory Affairs for Animal Health, Boehringer Ingelheim Australia) provided helpful comments to the manuscript. Finally, we thank all of the cats and owners who supported this study.

Conflict of interest

The authors have no conflicts of interest to declare.

References:

1. Westman, M.E., R. Malik, E. Hall, P.A. Sheehy, and J.M. Norris, Determining the feline immunodeficiency virus (FIV) status of FIV-vaccinated cats using point-of-care antibody kits. Comp. Immun. Microbiol. Infect. Dis., 2015. **42**:43-52.
2. Pedersen, N., E. Ho, M. Brown, and J. Yamamoto, Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science, 1987. **235**(4790):790-793.
3. Shelton, G.H., C.K. Grant, S.M. Cotter, M.B. Gardner, W.D. Hardy, Jr., and R.F. DiGiacomo, Feline immunodeficiency virus and feline leukemia virus infections and their relationships to lymphoid malignancies in cats: a retrospective study (1968-1988). Journal of Acquired Immune Deficiency Syndromes, 1990. **3**(6):623-630.
4. Lecollinet, S. and J. Richardson, Vaccination against the feline immunodeficiency virus: The road not taken. Comp. Immun. Microbiol. Infect. Dis., 2008. **31**(2-3):167-190.
5. Yamamoto, J.K., R.Y. Pu, E. Sato, and T. Hohdatsu, Feline immunodeficiency virus pathogenesis and development of a dual-subtype feline-immunodeficiency-virus vaccine. AIDS, 2007. **21**(5):547-563.
6. Bienzle, D., FIV in cats - a useful model of HIV in people? Vet. Immunol. Immunopathol., 2014. **159**(3-4):171-179.

7. Hosie, M.J., D. Addie, S. Belak, C. Boucraut-Baralon, H. Egberink, T. Frymus, T. Gruffydd-Jones, K. Hartmann, A. Lloret, H. Lutz, F. Marsilio, M.G. Pennisi, A.D. Radford, E. Thiry, U. Truyen, and M.C. Horzinek, Feline Immunodeficiency: ABCD Guidelines on Prevention and Management. J. Feline Med. Surg., 2009. **11**(7):575-584.
8. Yamamoto, J.K., E. Sparger, E.W. Ho, P.R. Andersen, T.P. Oconnor, C.P. Mandell, L. Lowenstine, R. Munn, and N.C. Pedersen, Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. Am. J. Vet. Res., 1988. **49**(8):1246-1258.
9. Olmsted, R.A., V.M. Hirsch, R.H. Purcell, and P.R. Johnson, Nucleotide sequence analysis of feline immunodeficiency virus - Genome organisation and relationship to other lentiviruses. Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(20):8088-8092.
10. Yamamoto, J.K., M.P. Sanou, J.R. Abbott, and J.K. Coleman, Feline immunodeficiency virus model for designing HIV/AIDS vaccines. Curr. HIV Res., 2010. **8**(1):14-25.
11. O'Connor, T.P., S. Tanguay, R. Steinman, R. Smith, M.C. Barr, J.K. Yamamoto, N.C. Pedersen, P.R. Andersen, and Q.J. Tonelli, Development and evaluation of immunoassay for detection of antibodies to the feline T-lymphotropic lentivirus (feline immunodeficiency virus). J. Clin. Microbiol., 1989. **27**(3):474-479.
12. Hosie, M.J. and O. Jarrett, Serological responses of cats to feline immunodeficiency virus. AIDS, 1990. **4**(3):215-220.
13. Uhl, E.W., T.G. Heaton-Jones, R. Pu, and J.K. Yamamoto, FIV vaccine development and its importance to veterinary and human medicine: FIV vaccine 2002 update and review. Vet. Immunol. Immunopathol., 2002. **90**(3-4):113-132.
14. Lappin, M.R., Detection of feline immunodeficiency virus antibodies in serum of vaccinated cats using a commercially available kit (2015 ACVIM Forum Research Abstract Program). J. Vet. Intern. Med., 2015. **29**(4):1122-1256.
15. Crawford, C., Does a DIVA test exist for differentiating FIV infection from FIV vaccination? (2016 ACVIM Forum Research Abstract Program). J. Vet. Intern. Med., 2016. **30**(4):1407-1519.
16. Avrameas, A., A.D. Strosberg, A. Moraillon, P. Sonigo, and G. Pancino, Serological diagnosis of feline immunodeficiency virus infection based on synthetic peptides from *env* glycoproteins. Research in Virology, 1993. **144**(3):209-218.
17. Govaert, T.M.E., M.J.W. Sprenger, G.J. Dinant, K. Aretz, N. Masurel, and J.A. Knottnerus, Immune response to influenza vaccination of elderly people. A randomized double-blind placebo-controlled trial. Vaccine, 1994. **12**(13):1185-1189.
18. Mufson, M.A., D.F. Hughey, C.E. Turner, and G. Schiffman, Revaccination with pneumococcal vaccine of elderly persons 6 years after primary vaccination. Vaccine, 1991. **9**(6):403-407.
19. Trivello, R., M. Chiaramonte, T. Ngatchu, V. Baldo, S. Majori, M.E. Moschen, I. Simoncello, G. Renzulli, and R. Naccarato, Persistence of anti-HBs antibodies in health care personnel vaccinated with plasma-derived hepatitis B vaccine and response to recombinant DNA HB booster vaccine. Vaccine, 1995. **13**(2):139-141.
20. Schultz, R.D., Duration of immunity for canine and feline vaccines: A review. Vet. Microbiol., 2006. **117**(1):75-79.
21. Heaton, P.R., D.G. Blount, S.J. Mann, P. Devlin, S. Koelsch, B.H.E. Smith, J. Stevenson, E.J. Harper, and J.M. Rawlings, Assessing age-related changes in peripheral blood leukocyte phenotypes in domestic shorthaired cats using flow cytometry. Journal of Nutrition, 2002. **132**(6):1607S-1609S.
22. Campbell, D.J., J.M. Rawlings, S. Koelsch, J. Wallace, J.J. Strain, and B.M. Hannigan, Age-related differences in parameters of feline immune status. Vet. Immunol. Immunopathol., 2004. **100**(1-2):73-80.
23. de Bruijn, I.A., E.J. Remarque, C.M. Jol-van der Zijde, M.J.D. van Tol, R.G.J. Westendorp, and D.L. Knook, Quality and Quantity of the Humoral Immune Response in Healthy Elderly and

Young Subjects after Annually Repeated Influenza Vaccination. Journal of Infectious Diseases, 1999. **179**(1):31-36.

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

25. Huang, C., D. Conlee, M. Gill, and H.-J. Chu, Dual-subtype feline immunodeficiency virus vaccine provides 12 months of protective immunity against heterologous challenge. J. Feline Med. Surg., 2010. **12**(6):451-457.

26. Kusuhara, H., T. Hohdatsu, M. Okumura, K. Sato, Y. Suzuki, K. Motokawa, T. Gemma, R. Watanabe, C.J. Huang, S. Arai, and H. Koyama, Dual-subtype vaccine (Fel-O-Vax FIV) protects cats against contact challenge with heterologous subtype BFIV infected cats. Vet. Microbiol., 2005. **108**(3-4):155-165.

27. Salman, M.D., J.J.G. New, J.M. Scarlett, P.H. Kass, R. Ruch-Gallie, and S. Hetts, Human and Animal Factors Related to Relinquishment of Dogs and Cats in 12 Selected Animal Shelters in the United States. J. Appl. Anim. Welf. Sci., 1998. **1**(3):207-226.

28. Levy, J., C. Crawford, K. Hartmann, R. Hofmann-Lehmann, S. Little, E. Sundahl, and V. Thayer, 2008 American Association of Feline Practitioners' feline retrovirus management guidelines. J. Feline Med. Surg., 2008. **10**(3):300-316.

29. Westman, M.E., R. Malik, E. Hall, and J.M. Norris, The protective rate of the feline immunodeficiency virus vaccine: an Australian field study. Vaccine, 2016. **34**:4752-4758.

Table 1: Outline of the prospective study design (Study 2), showing time points for three FIV vaccinations (weeks 0, 4 and 8), PCR testing (weeks 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 Witness and Anigen Rapid.

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

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

Table 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 online Supplement 1.

Figure 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).

Figures 2A-D: Results from ELISA testing for antibodies recognising FIV gp40 peptide (Study 1). Positive and negative controls are shown. The optical density (OD) is displayed on the y-axis. Mean and SEM bars are shown.

Figure 2A: FIV-vaccinated/FIV-infected cats (n = 4), FIV-vaccinated/FIV-uninfected cats (FIV false-positive with Witness, n = 6) and FIV-vaccinated/FIV-uninfected cats (FIV true-negative with Witness, n = 108).

*represents significant difference ($P < 0.01$) between groups of cats.

Figure 2B: FIV-vaccinated/FIV-uninfected cats (n = 114) according to the time (days) elapsed since the last annual FIV vaccination. No significant effect was found (P = 0.42).

Figure 2C: FIV-vaccinated/FIV-uninfected cats (n = 114) according to the age of cat at the time of sampling (years). No significant effect was found (P = 0.21).

Figure 2D: FIV-vaccinated/FIV-uninfected cats (n = 114) according to number of annual booster FIV vaccinations received. No significant effect was found (P = 0.43).

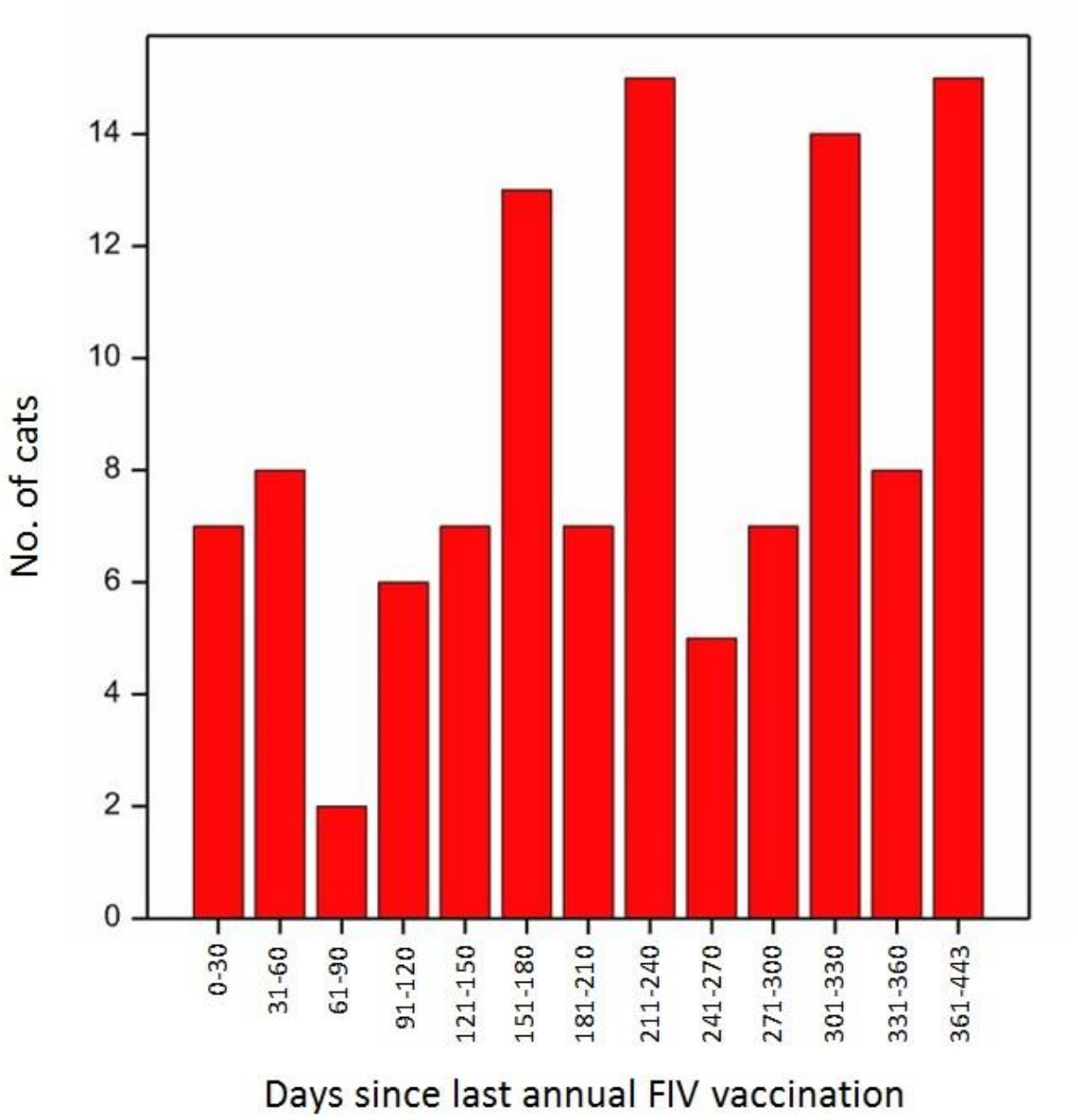
Figure 3: Summary of FIV antibody test results from the prospective study (Study 2) at various time points (n = 12). The FIV target capture antigen/s for each point-of-care antibody test kit is included in brackets. A primary FIV vaccination course was administered at 0, 4 and 8 weeks.

Online Supplement 1: 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 weeks 0, 4, 20 and 34).

Online Supplement 2: 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).

Online Supplement 3: 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).

Figure 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).



Figures 2A-D: Results from ELISA testing for antibodies recognising FIV gp40 peptide (Study 1). Positive and negative controls are shown. The optical density (OD) is displayed on the *y*-axis. Mean and SEM bars are shown.

Figure 2A: FIV-vaccinated/FIV-infected cats (*n* = 4), FIV-vaccinated/FIV-uninfected cats (FIV false-positive with Witness, *n* = 6) and FIV-vaccinated/FIV-uninfected cats (FIV true-negative with Witness, *n* = 108).

*represents significant difference ($P < 0.01$) between groups of cats.

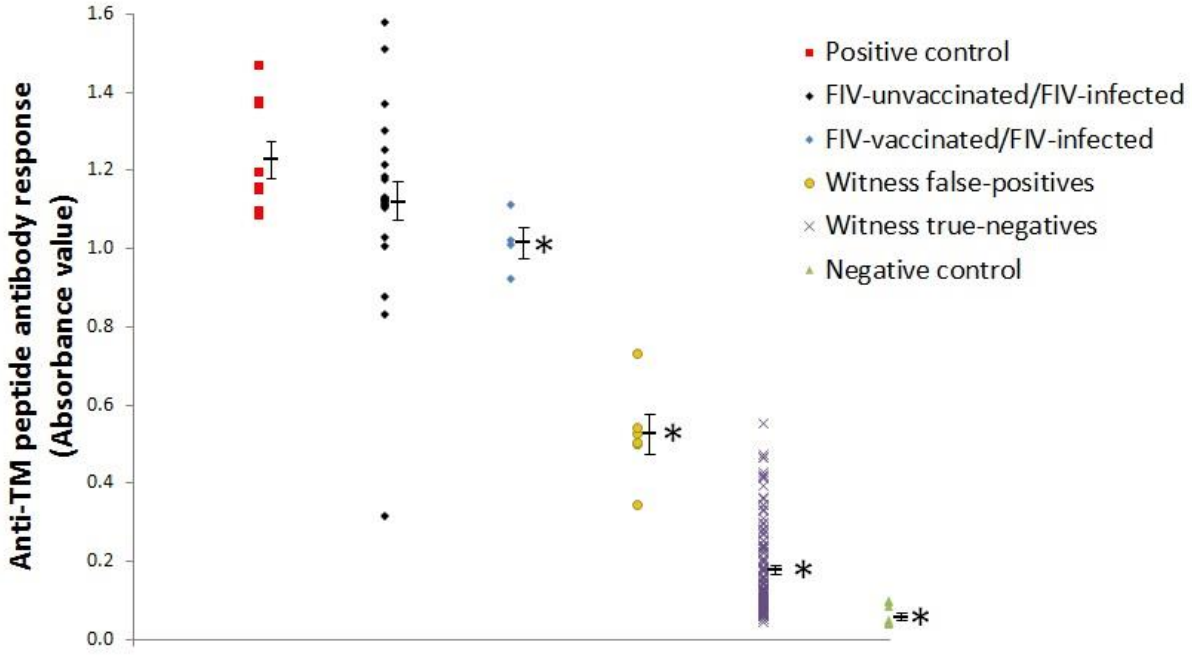


Figure 2B: FIV-vaccinated/FIV-uninfected cats (*n* = 114) according to the time (days) elapsed since the last annual FIV vaccination. No significant effect was found (*P* = 0.42).

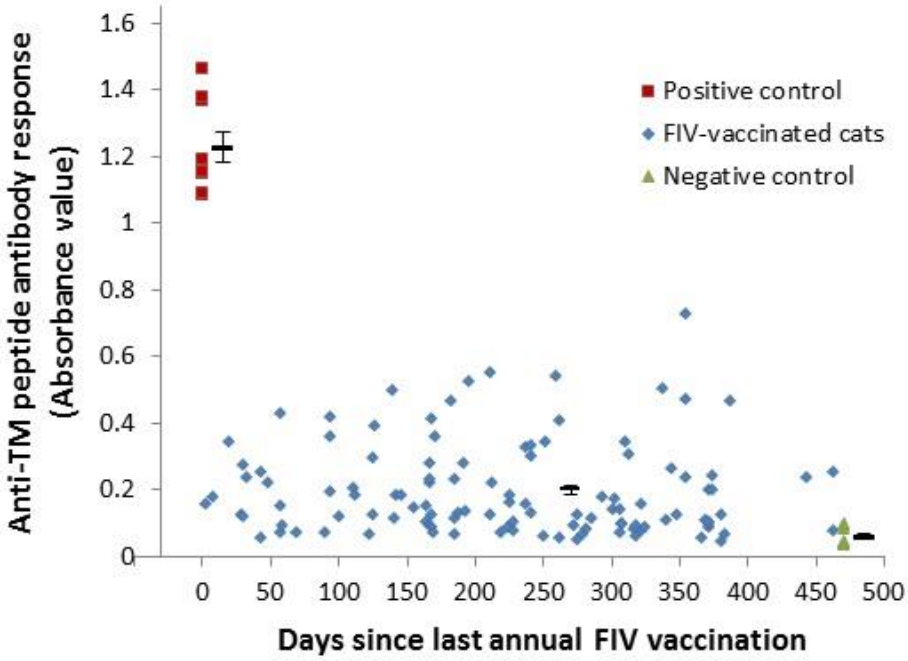


Figure 2C: FIV-vaccinated/FIV-uninfected cats (*n* = 114) according to the age of cat at the time of sampling (years). No significant effect was found (*P* = 0.21).

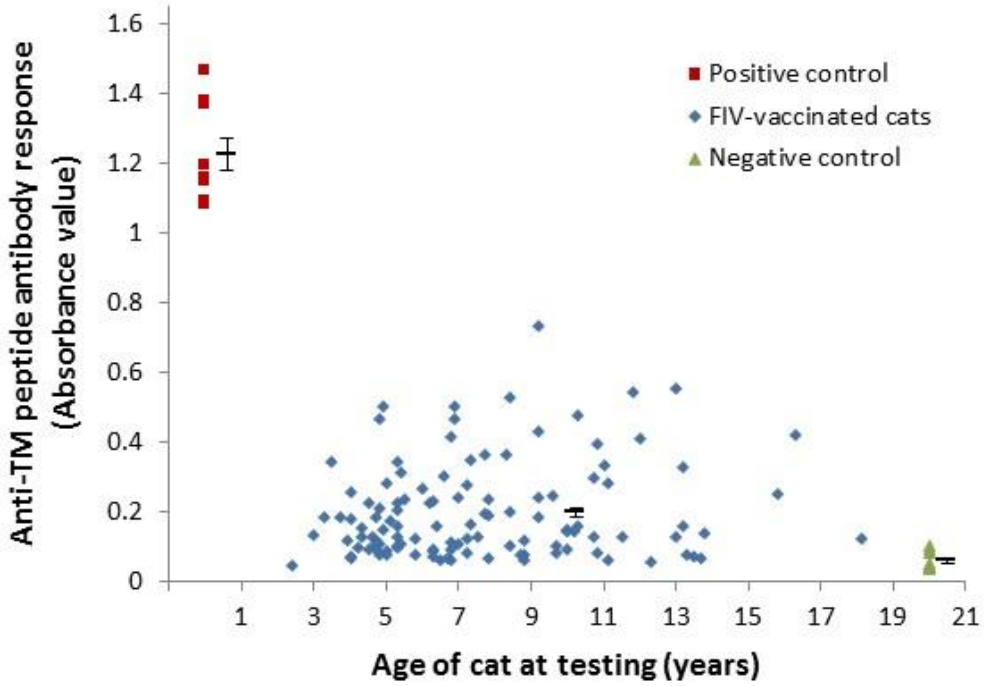


Figure 2D: FIV-vaccinated/FIV-uninfected cats (*n* = 114) according to number of annual booster FIV vaccinations received. No significant effect was found (*P* = 0.43).

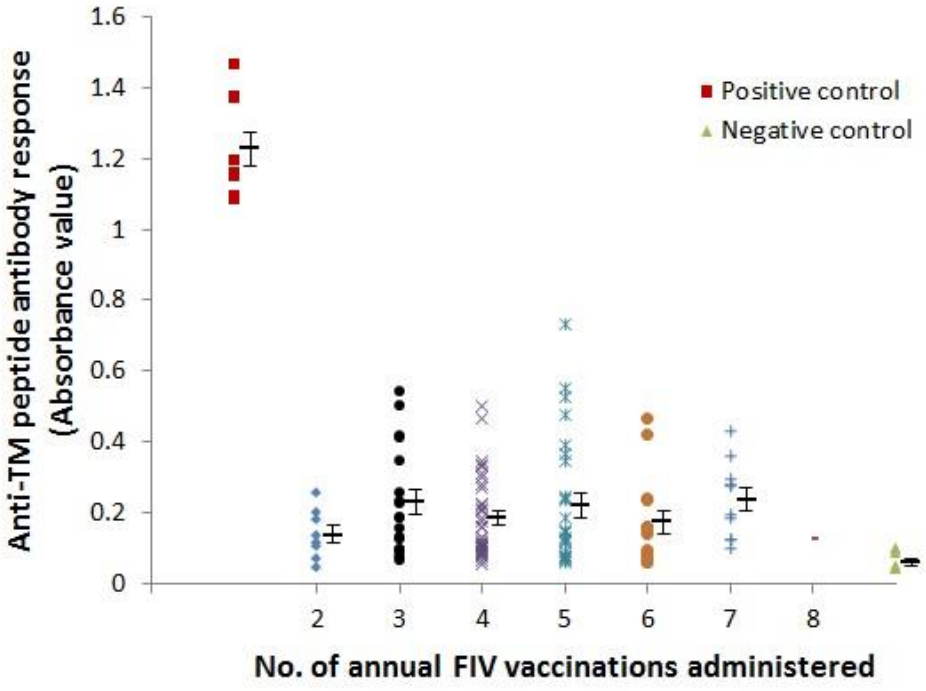


Figure 3: Summary of FIV antibody test results from the prospective study (Study 2) at various time points (*n* = 12). The FIV target capture antigen/s for each point-of-care antibody test kit is included is brackets. A primary FIV vaccination course was administered at 0, 4 and 8 weeks.

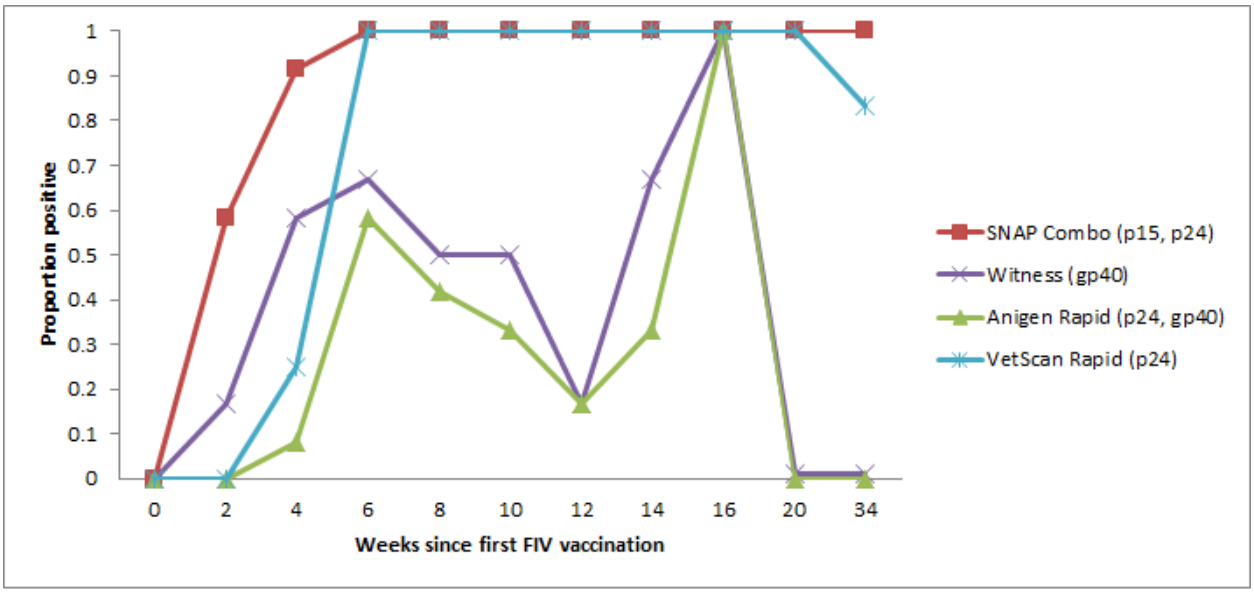


Table 1: Outline of the prospective study design (Study 2), showing time points for three FIV vaccinations (weeks 0, 4 and 8), PCR testing (weeks 0 and 34) and antibody-testing (up to 11 times between week 0 and 34). Antibody-testing was not performed at weeks 14, 16 or 20 for cats that were FIV-negative at week 12 with Witness and Anigen Rapid.

		First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine	Third FIV vaccine	2 weeks after final FIV vaccine	4 weeks after final FIV vaccine	6 weeks after final FIV vaccine	8 weeks after final FIV vaccine	12 weeks after final FIV vaccine	26 weeks after final FIV vaccine
	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		•	•	•	•	•	•	•	•	•	•	•
Witness		•	•	•	•	•	•	•	•	•	•	•
Anigen Rapid		•	•	•	•	•	•	•	•	•	•	•
VetScan Rapid		•	•	•	•	•	•	•	•	•	•	•
IDEXX PCR		•										•
FIV vaccine - 3 boosters 4 weeks apart		•		•		•						

Table 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 online Supplement 1.

		First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine	Third FIV vaccine	2 weeks after final FIV vaccine	4 weeks after final FIV vaccine	6 weeks after final FIV vaccine	8 weeks after final FIV vaccine	12 weeks after final FIV vaccine	26 weeks after final FIV vaccine
	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		0/12	7/12	11/12	12/12	12/12	12/12	12/12	3/3	1/1	1/1	12/12
Witness		0/12	2/12	7/12	8/12	6/12	6/12	2/12	2/3	1/1	0/1	0/12
Anigen Rapid		0/12	0/12	1/12	7/12	5/12	4/12	2/12	1/3	1/1	0/1	0/12
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

Online Supplement 1: 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 weeks 0, 4, 20 and 34).

		First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine	Third FIV vaccine	2 weeks after final FIV vaccine	4 weeks after final FIV vaccine	6 weeks after final FIV vaccine	8 weeks after final FIV vaccine	12 weeks after final FIV vaccine	26 weeks after final FIV vaccine
	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		0/16	10/15	14/15	14/14	14/14	14/14	14/14	5/5	3/3	3/3	13/13
Witness		0/16	3/15	9/15	10/14	8/14	8/14	3/14	3/5	2/3	1/3	0/13
Anigen Rapid		1/16	1/15	2/15	9/14	7/14	6/14	4/14	3/5	2/3	1/3	1/13
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

Online Supplement 2: 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).

		First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine	Third FIV vaccine	2 weeks after final FIV vaccine	4 weeks after final FIV vaccine	6 weeks after final FIV vaccine	8 weeks after final FIV vaccine	12 weeks after final FIV vaccine	26 weeks after final FIV vaccine
	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		0/4	3/4	4/4	4/4	4/4	4/4	4/4	1/1	0/0	0/0	4/4
Witness		0/4	1/4	1/4	2/4	2/4	2/4	0/4	0/1	0/0	0/0	0/4
Anigen Rapid		0/4	0/4	0/4	1/4	1/4	1/4	1/4	0/1	0/0	0/0	0/4
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

Online Supplement 3: 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).

		First FIV vaccine	2 weeks after first FIV vaccine	Second FIV vaccine	2 weeks after second FIV vaccine	Third FIV vaccine	2 weeks after final FIV vaccine	4 weeks after final FIV vaccine	6 weeks after final FIV vaccine	8 weeks after final FIV vaccine	12 weeks after final FIV vaccine	26 weeks after final FIV vaccine
	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		0/8	4/8	7/8	8/8	8/8	8/8	8/8	2/2	1/1	1/1	8/8
Witness		0/8	1/8	6/8	6/8	4/8	4/8	2/8	2/2	1/1	0/1	0/8
Anigen Rapid		0/8	0/8	1/8	6/8	4/8	3/8	1/8	1/2	1/1	0/1	0/8
VetScan Rapid		0/8	8/8	3/8	8/8	8/8	8/8	8/8	2/2	1/1	1/1	6/8
IDEXX PCR		0/8										0/8