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Determining the feline immunodeficiency virus (FIV) status of FIV-vaccinated cats using point-of-care antibody kits

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ABSTRACT

This study challenges the commonly held view that the feline immunodeficiency virus (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 immunochromatography 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.

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1. Introduction

Feline immunodeficiency virus (FIV) is a retrovirus of the genus *Lentivirus*. It was discovered in 1986 following investigation of an immunodeficiency syndrome in a household of cats in California, USA [1] and shown subsequently to have worldwide distribution. Healthy client-owned cat populations have been reported with infection rates of approximately 3% in Germany [2] and the USA [3], 6% in Canada [4] and the United Kingdom [5], 8% in Australia [6], 10% in New Zealand [7] and 23% in Japan [8]. The prevalence of FIV infection is higher in entire male cats, castrated male cats and feral cats compared to the general client-owned domestic cat population [3,6].

The FIV genome is comprised of three main structural genes, *gag*, *pol* and *env*, which encode internal structural proteins, viral enzymes and envelope glycoproteins, respectively. Six distinct FIV subtypes (A to F) have been identified based on genetic diversity in

the variable V3–5 region of the *env* gene [9,10], while an additional subtype has been detected in New Zealand cats [11]. Subtypes A, B and C are most commonly encountered worldwide [12], with subtype A predominant in Australia [9,13]. Nucleotide sequence may vary up to 15% within a subtype and up to 38% between subtypes [14,15]. Subtyping of FIV infections in each geographic area is important as the sole commercially available FIV vaccine contains only subtypes A and D¹ and heterologous challenge may lower vaccine effectiveness [16,17], although subtyping alone appears insufficient to predict vaccine performance [18].

Regardless of the FIV subtype, point-of-care testing to identify antibodies directed against FIV has been the mainstay of diagnostic testing for over 20 years, supplemented by western blot analysis and virus isolation in research settings. Point-of-care test kits are inexpensive, easy to use and reliably diagnose FIV infection in FIV-unvaccinated cats [19]. There is variation between commercially available antibody test kits in the methodology and target viral antigen for antibody detection. SNAP FIV/FeLV Combo² is a lateral

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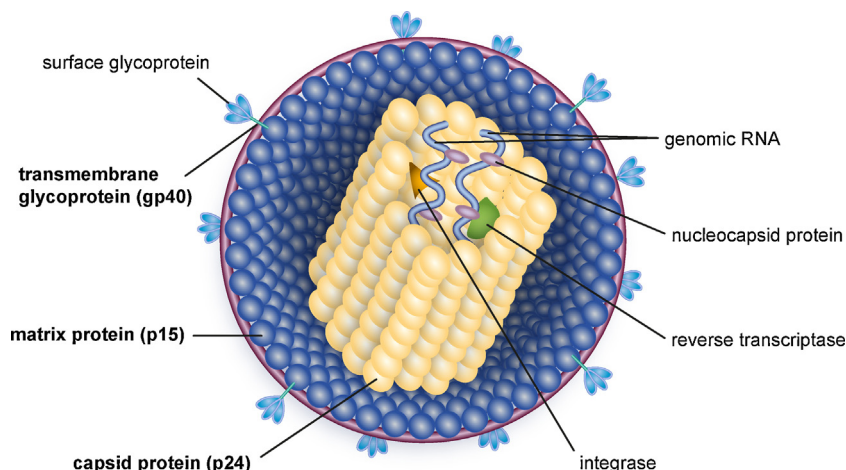


Fig. 1. Schematic of FIV emphasising the different target antigens for antibody testing.

Table 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 by Hartmann et al. [19].

flow enzyme-linked immunosorbent assay (ELISA) kit that detects antibodies to p15 (matrix protein) and p24 (capsid protein), Witness FelV/FIV³ is a lateral flow immunochromatography kit that detects antibodies to gp40 (transmembrane glycoprotein), while Anigen Rapid FIV/FelV⁴ is a lateral flow immunochromatography kit that detects antibodies to p24 and gp40 (Fig. 1 and Table 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 (<https://www.idexx.com/files/small-animal-health/products-and-services/snap-products/snap-fiv-felv-combo/snap-combo-test-accuracy.pdf>), [35] 100% and 100% for SNAP FIV/FelV Combo Plus, 95% and 99% for Witness FelV/FIV [19], and 89% and 100% for Anigen Rapid FIV/FelV [20].

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 [21]. Consequently, for FIV-vaccinated cats and cats of unknown vaccination status, FIV diagnostics shifted towards molecular methods such as nucleic acid amplification [22,23]. 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 [24,25], and by calculating the CD4:CD8^{low} T-lymphocyte ratio [26].

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.

2. Material and methods

2.1. 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 guidelines¹. 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 [27].

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

2.2. 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 °C. Testing for FIV antibodies was performed within 24 hours of blood collection⁵ 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 Combo², Witness FelV/FIV³, and Anigen Rapid FIV/FelV⁴ (Table 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

³ Zoetis Animal Health, Lyon, France.

⁴ BioNote, Gyeonggi-do, Korea.

⁵ Faculty of Veterinary Science, The University of Sydney, Sydney, NSW, Australia.

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

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	-

examination.⁶ The second EDTA tube was centrifuged within 6 h of collection for 3 min at 12,000 × g and the plasma transferred to a plain tube using a sterile pipette. Plasma specimens and EDTA cell pellets were stored subsequently at –80 °C for confirmatory testing of discrepant samples at a later time. The third EDTA tube was sent for FIV nucleic acid amplification (FIV RealPCR™),⁷ 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 (http://www.idexx.com.au/pdf/en_au/smallanimal/education/realpcr-test-for-fiv.pdf) [34].

2.3. Defining FIV infection status (Table 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% [28]. 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™ 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/)⁸ [37] 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 virus isolation (VI).⁹ 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 heparinised tube and sent refrigerated to a fourth laboratory for VI,¹⁰ with the VI result taken as being definitive.

⁶ Veterinary Pathology Diagnostic Services, The University of Sydney, Sydney, NSW, Australia.

⁷ IDEXX Laboratories, East Brisbane, Queensland, Australia.

⁸ Gribbles Veterinary Pathology, Melbourne, Victoria, Australia.

⁹ Yamamoto Laboratory, The University of Florida, Gainesville, FL, USA.

¹⁰ Veterinary Diagnostic Services, The University of Glasgow, Scotland, UK.

Table 3
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°C . += positive, – = negative, NP = not performed.

Category	SNAP combo	Witness	Anigen Rapid	PCR (initial)	Virus isolation
FIV-vaccinated/FIV-uninfected ($n = 107$)	+	-	-	-	NP
FIV-vaccinated/FIV-infected ($n = 3$)	+	+	+	+	+(2/3)
FIV-vaccinated/FIV infected ($n = 2$)	+	+	+	-	+(2/2)
<u>FIV-vaccinated/FIV-uninfected discordant cats ($n = 7$)</u>					
Cat #97	+	Faint +	-	-	-
Cat #173	+	Faint +	-	-	NP ¹³
Cat #340	+	Faint +	-	-	-
Cat #341	+	Faint +	-	-	-
Cat #345	+	Faint +	-	-	-
Cat #350	+	Faint +	-	-	-
Cat #102	+	-	-	+	NP

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 (<https://www.idexx.com/resource-library/smallanimal/snap-combo-package-insert-en.pdf>) [36]. Antibody testing was repeated using stored plasma thawed from -80°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.

2.4. Statistical analysis

Numerical analyses were performed using a commercial programme (Genstat 16th Edition).¹¹ Statistical significance was considered at $P < 0.05$ and 95% confidence intervals (CI) were calculated using Microsoft Excel.¹² 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

¹¹ GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.

¹² Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.

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.

3. Results

3.1. Sample population

Blood samples were obtained from 358 client-owned cats recruited from 12 veterinary clinics distributed over four states of Australia (New South Wales, Victoria, Queensland and South Australia) (Online Supplement 1).

A total of 119 FIV-vaccinated cats were recruited, ranging from six months to 18 years (median 7 years; interquartile range [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 6–10 years). These cats

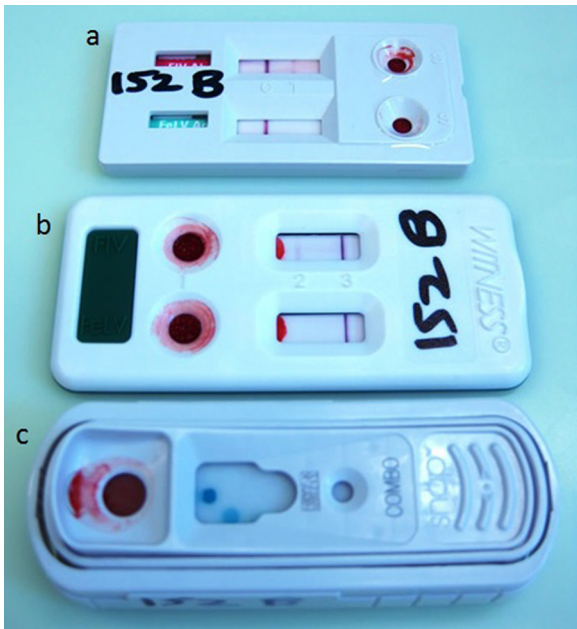


Fig. 2. Photograph of test kit results from a FIV-vaccinated/FIV-infected cat, showing three positive FIV antibody test kit results: (a) Anigen Rapid FIV/FeLV, (b) Witness FeLV/FIV, and (c) SNAP FIV/FeLV Combo. For both immunochromatography kits the top strip is for FIV antibody testing and the bottom 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.

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.

3.2. Serological and molecular detection of FIV infection

3.2.1. FIV-vaccinated cohort (n = 119, Tables 3 and 4)

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) (Figs. 2 and 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 RealPCRTM testing confirmed 3/5 cats to be FIV-infected, which was further confirmed by VI in 2 of these cats (one cat was unavailable for further sampling). The remaining two cats were initially negative with both FIV RealPCRTM and PCR testing at the second laboratory. Subsequent resampling and re-testing, however, found both cats positive with FIV RealPCRTM and VI. Thus, all five cats testing FIV positive using Anigen Rapid FIV/FeLV were truly

Table 4

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 +ve	5	5	5
False +ve	114	6	0
True -ve	0	108	114
False -ve	0	0	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

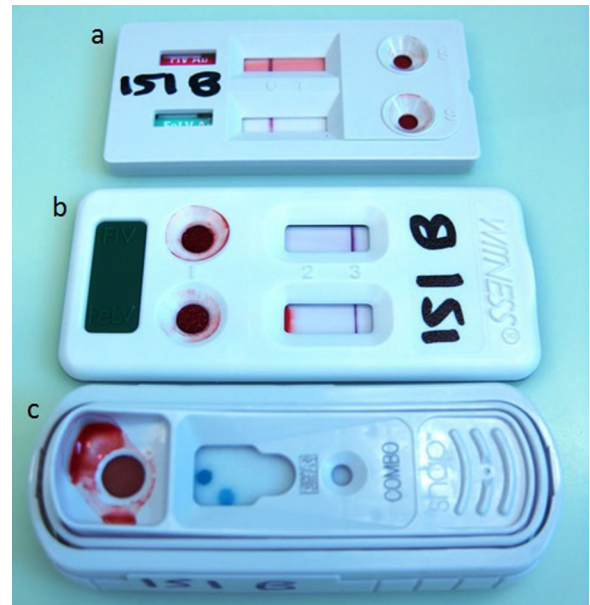


Fig. 3. Photograph of test kit results from a FIV-vaccinated/FIV-uninfected cat, showing two negative FIV antibody test kit results: (a) Anigen Rapid FIV/FeLV, and (b) Witness FeLV/FIV; and one positive FIV antibody test kit result, (c) SNAP FIV/FeLV Combo. For both immunochromatography kits the top 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.

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 RealPCRTM, were considered to be FIV-uninfected based on VI results (i.e. the positive antibody kit results were false-positives).¹³ Another FIV-vaccinated cat had FIV RealPCRTM testing repeated due to possible contamination in the PCR facility; this cat, which initially was FIV RealPCRTM positive, subsequently tested FIV RealPCRTM negative and was ultimately considered to be FIV-uninfected.

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

3.2.2. FIV-unvaccinated cohort (n = 239, Tables 5 and 6)

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 RealPCRTM 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 RealPCRTM; VI was subsequently performed and confirmed the negative FIV status.

Three cats tested FIV negative with all three antibody kits but initially positive with FIV RealPCRTM with varying cycle threshold (C_T) values: cat #126 tested positive for subtype A (C_T 32), cat

¹³ 13Only 5/6 cats had VI performed due to the unfortunate death (unrelated to this study) of one of the cats.

Table 5
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°C . + = positive, - = negative, NP = not performed.

Category	SNAP Combo	Witness	Anigen Rapid	PCR (initial)	Virus isolation
FIV-unvaccinated/FIV-uninfected ($n = 209$)	-	-	-	-	NP
FIV-unvaccinated/FIV-infected ($n = 21$)	+	+	+	+	NP
<u>FIV-unvaccinated/FIV-uninfected discordant cats ($n = 9$)</u>					
Cat #60	Faint +	-	-	-	NP
Cat #263	Faint +	-	-	-	NP
Cat #280	Faint +	-	-	-	NP
Cat #326	Faint +	-	-	-	NP
Cat #335	+	-	-	-	NP
Cat #305	+	Faint +	-	-	-
Cat #126	-	-	-	+	NP
Cat #259	-	-	-	+	NP
Cat #277	-	-	-	+	NP

#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.

3.2.3. Combined FIV-vaccinated and FIV-unvaccinated cohorts ($n = 358$, Table 7)

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'

Table 6
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 +ve	21	21	21
False +ve	6	1	0
True -ve	212	217	218
False -ve	0	0	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

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°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 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 8, and C_T values are available online (Online Supplement 2). 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; 35%) and subtype D (3/26 cats; 12%). Subtype B was not identified in any FIV-infected cats.

Table 7

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 strongly 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 +ve	26	26	26
False +ve	120	7	0
True –ve	212	325	332
False –ve	0	0	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

4. 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). 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 [21]. 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 (Petcheck FIV, IDEXX Laboratories) at 14 weeks after the third primary FIV vaccine [29]. 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 [24,25]. Recently, investigation using the CD4:CD8^{low} T-lymphocyte ratio to differentiate between FIV-vaccinated and FIV-infected cats showed promise [26]. 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

Table 8

Subtyping results from FIV RealPCRTM testing. 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%

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 [30].

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 [31]. 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.

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 (Fig. 4). If results of the two immunochromatography kits were considered together there was agreement in 351/358 (98%) of cats, with FIV RealPCRTM testing and VI required to clarify the FIV status of only seven cats. In a shelter environment, where resources are limited and vaccination histories are commonly unavailable, this algorithm would result in a substantial cost saving and could influence whether shelters can afford to test for FIV.

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 [29]. 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 [22,32]. It should be noted that the reported specificity of SNAP FIV/FeLV Combo for the entire study population (64%; 95% CI 59–69%; Table 6) 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 immunochromatography test kits in FIV-unvaccinated cats.

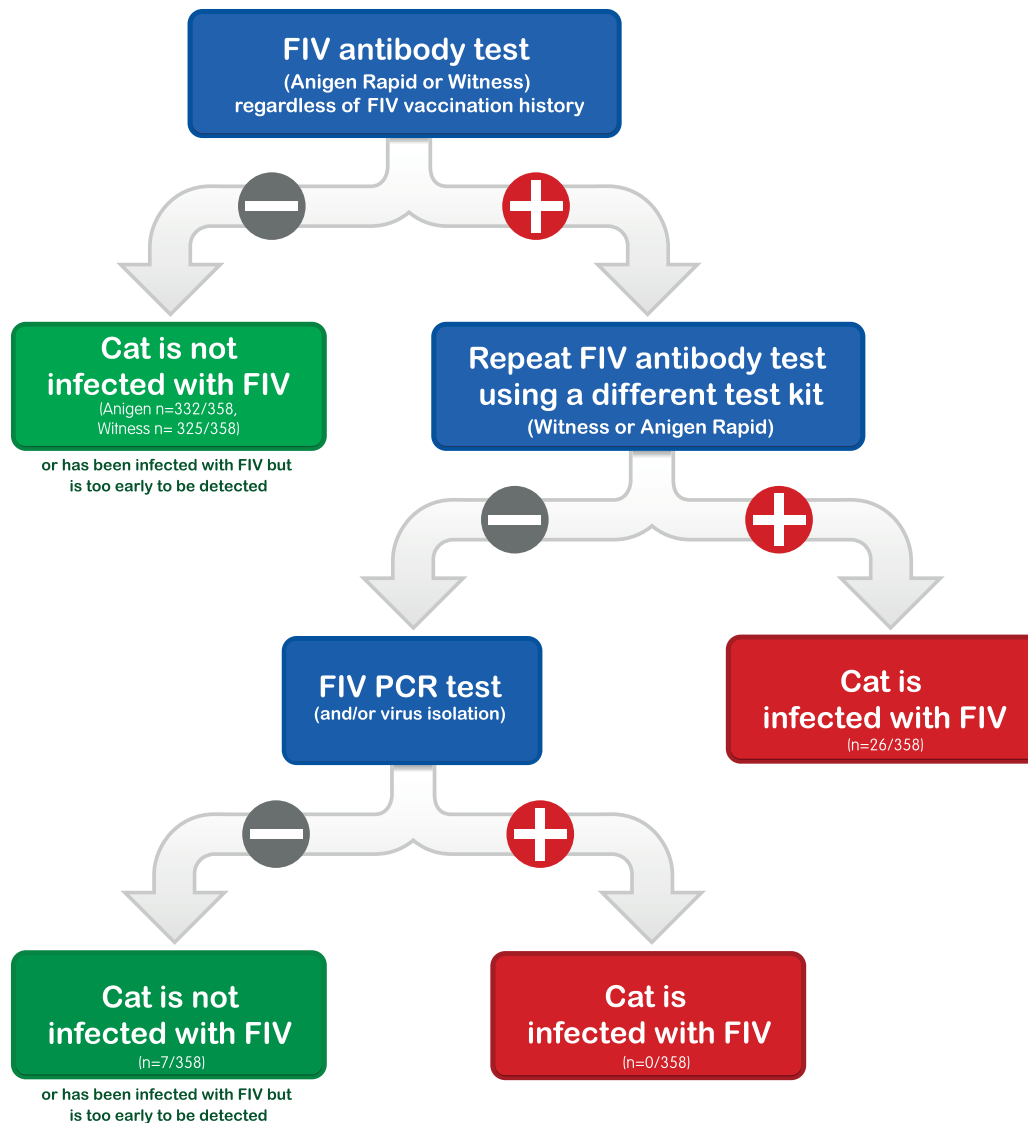


Fig. 4. Suggested algorithm for diagnosis of FIV infection. If there is the possibility of recent FIV infection, re-testing is recommended due to delays in seroconversion and potentially low viral loads early in infection. Repeat testing on negative cats should be performed at least 8 weeks later for 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.

Fel-O-Vax® FIV vaccine contains formalin-inactivated whole virus and infected Fet-J cells [33]. One possible explanation for the variation in results between the ELISA-based kit (SNAP FIV/FelV Combo) and immunochromatography 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 [24]. There are major methodological differences between western blot, ELISA and immunochromatography 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 [21].

The results of the current study may suggest a useful refinement for point-of-care FIV antibody test kits in the future. By including all three FIV antigens, but with p15 occupying a different spot or line to p24 and gp40, it may be possible to determine whether an individual cat is (i) vaccinated and uninfected (p15 positive only); (ii) infected (p15 and p24/gp40 positive); or (iii) unvaccinated and uninfected (p15 and p24/gp40 negative). Currently, differentiation of cats into these three groups is not possible using a single test methodology, including nucleic acid amplification, discriminating ELISA [24,25] or considering the CD4:CD8^{low} T-lymphocyte ratio

[26]. Such a refinement to point-of-care antibody testing, however, should not be used as rationale for reducing frequency of FIV vaccination in scenario (i) as the mechanism of vaccine induced protection involves both humoral and cell-mediated immunity [33].

The accuracy of FIV RealPCR™ testing in the current study was comparable to results for a group of FIV-unvaccinated cats [28]. 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.

5. 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cimid.2015.07.004.

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