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Diagnosing feline immunodeficiency virus (FIV) infection in FIV-vaccinated and FIV-unvaccinated cats using saliva

Mark E. Westman[a,*], Richard Malik[b], Evelyn Hall[a], Jacqueline M. Norris[a,*]

[a] Faculty of Veterinary Science, The University of Sydney, NSW 2006, Australia
[b] Centre for Veterinary Education, The University of Sydney, NSW 2006, Australia

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ABSTRACT

We recently showed that two immunochromatography point-of-care FIV antibody test kits (Witness FeLV/FIV and Anigen Rapid FIV/FeLV) were able to correctly assign FIV infection status, irrespective of FIV vaccination history, using whole blood as the diagnostic specimen. A third FIV antibody test kit, SNAP FIV/FeLV Combo (an enzyme-linked immunosorbent assay [ELISA]), was unable to differentiate antibodies produced in response to FIV vaccination from those incited by FIV infection. The aim of this study was to determine if saliva is a suitable diagnostic specimen using the same well characterized feline cohort. FIV infection status of these cats had been determined previously using a combination of serology, polymerase chain reaction (PCR) testing and virus isolation. This final assignment was then compared to results obtained using saliva as the diagnostic specimen utilizing the same three point-of-care FIV antibody test kits and commercially available PCR assay (FIV RealPCR). In a population of cats where one third (117/356; 33%) were FIV-vaccinated, both immunochromatography test kits accurately diagnosed FIV infection using saliva via a centrifugation method, irrespective of FIV vaccination history. For FIV diagnosis using saliva, the specificity of Anigen Rapid FIV/FeLV and Witness FeLV/FIV was 100%, while the sensitivity of these kits was 96% and 92% respectively. SNAP FIV/FeLV Combo respectively. SNAP FIV/FeLV Combo had a specificity of 98% and sensitivity of 44%, while FIV RealPCR testing had a specificity of 100% and sensitivity of 72% using saliva. A revised direct method of saliva testing was trialed on a subset of FIV-infected cats ($n = 14$), resulting in 14, 7 and 0 FIV positive results using Anigen Rapid FIV/FeLV, Witness FeLV/FIV and SNAP FIV/FeLV Combo, respectively. These results demonstrate that saliva can be used to diagnose FIV infection, irrespective of FIV vaccination history, using either a centrifugation method (Anigen Rapid FIV/FeLV and Witness FeLV/FIV) or a direct method (Anigen Rapid FIV/FeLV). Collection of a saliva specimen therefore provides an acceptable alternative to venipuncture (i) in fractious cats where saliva may be easier to obtain than whole blood, (ii) in settings when a veterinarian or trained technician is unavailable to collect blood and (iii) in shelters where FIV testing is undertaken prior to adoption but additional blood testing is not required.

1. Introduction

Feline immunodeficiency virus (FIV) and human immunodeficiency virus (HIV) are retroviruses of the genus *Lentivirus*. Both cause life-long infections, resulting in persistently high antibody titres which are useful diagnostically for identifying infected patients [1–4].

Serologic testing for FIV infection is commonly undertaken by veterinarians for patients with severe stomatitis, sequential or persistent opportunistic infections, lymphoma and other malignancies, or signs of non-specific illness when a cause is not apparent after preliminary investigations. Veterinarians in shelters typically perform FIV screening of cats prior to admission into a shelter, or prior to re-homing [5,6]. The introduction of a FIV vaccine[1] in 2002 complicated the serologic diagnosis of FIV infection because the most widely used point-of-care antibody test kit available at the time and western blot analyses were unable to differentiate antibodies produced by FIV-vaccinated and FIV-infected cats [7]. Consequently, in FIV-vaccinated cats and cats of unknown FIV

* Corresponding authors.
E-mail addresses: mark.westman@sydney.edu.au (M.E. Westman), jacqui.norris@sydney.edu.au (J.M. Norris).

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

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0147-9571/

vaccination status, diagnosis of FIV required the use of more expensive molecular methods to demonstrate the presence of the virus, such as nucleic acid amplification, with variable results in terms of accuracy and reliability [8–13]. Recently, we reported that two point-of-care FIV antibody test kits (Anigen Rapid FIV/FeLV[2] and Witness FeLV/FIV[3]) were able to accurately diagnose FIV infection in cats, irrespective of FIV vaccination history, using whole blood as the diagnostic specimen [14]. A third point-of-care FIV antibody test kit (SNAP FIV/FeLV Combo[4]) could not distinguish FIV-vaccinated from FIV-infected cats. Since each antibody kit uses a different panel of viral epitopes, we hypothesized that the humoral response to different viral antigens (proteins and glycoproteins) within the formalin-inactivated killed-virus vaccine was more complex than had been appreciated [14].

In general, it is easier and less invasive to collect a saliva specimen than a blood specimen from feline patients. Indeed, venipuncture is impossible in some cats without sedation or skilled manual restraint. Antibody testing using saliva accurately detects HIV infection in people; a *meta*-analysis of the OraQuick Advance Rapid HIV-1/2 In-Home HIV Test[5] identified similar specificity, and only a 2% reduction in sensitivity, when saliva was used instead of whole blood [15]. As a result, this test kit has been approved by the USA Food and Drug Administration for self-testing using saliva[6] [16]. Surprisingly, despite IgG being reliably detectable in cat saliva [17], only three studies have investigated using saliva to diagnose FIV infection in cats. Poli and colleagues reported that detection of FIV antibodies in saliva using ELISA was extremely unreliable, with a high frequency of false-positive and false-negative results, although the exact numbers and details of the commercial ELISA kits used were not provided [18]. In contrast, an indirect immunofluorescence assay and Western blot testing (WB-IgG) detected FIV antibodies in the saliva of 15/16 (94%) FIV-seropositive cats and no false-positive results were recorded amongst the 16 FIV-seronegative cats [18]. Matteucci et al. [19] attempted to isolate FIV from the saliva, plasma and peripheral blood mononuclear cells (PBMC) of naturally FIV-infected cats; the isolation rate of FIV from saliva was considerably lower than from PBMC (18% versus 81% of cats). The third study investigating saliva testing to diagnose FIV infection was a prevalence survey of client-owned cats using a later generation of a commercially available ELISA kit (SNAP FIV/FeLV Combo) to detect FIV antibodies in addition to utilizing nucleic acid amplification (polymerase chain reaction [PCR] testing) to detect proviral DNA [20]. Although blood was not obtained in the main study, preliminary evaluation using three FIV-infected and two FIV-uninfected cats found results for FIV antibody testing using the ELISA kit to be identical when blood and saliva from the same cat were tested concurrently. There was also good correlation between the ELISA antibody test kit and combined results from the three PCR assays using saliva (Kappa value 0.76; 95% confidence interval [CI] 0.64–0.87) [20].

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

2. Material and methods

2.1. Sample population

Client-owned cats were recruited as part of another study into FIV diagnostic testing using whole blood [14]. Briefly, cats of known FIV vaccination history were recruited through veterinary clinics and classified as 'FIV-vaccinated' (had received at least one FIV vaccine at any time in their life) or 'FIV-unvaccinated' (had never received a FIV vaccine). Clinical records were interrogated to enforce this criterion. Practices where the prevalence of FIV infection was perceived to be high were targeted. Animal ethics approval was granted by The University of Sydney (Approval number N00/1-2013/3/5920).

2.2. Blood collection, blood testing and defining FIV infection status

The procedures for blood collection, FIV antibody testing of whole blood using three point-of-care test kits, nucleic acid amplification of blood (FIV RealPCR), use of virus isolation (VI) in rare discrepant cases and final assignment of FIV status have been described previously [14]. In summary, consideration of all four FIV test results (three antibody tests and PCR testing) led to FIV status being assigned when there was a majority, either of negative or positive FIV results (i.e. 3–1 or 4–0). In seven cases, where test results were equally split (i.e. 2–2), VI was undertaken as the 'tie-breaker'. VI was also undertaken to confirm FIV-vaccinated/FIV-infected cats, even though in all cases there was a clear FIV-positive test majority.

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

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

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

2.3. Saliva collection and saliva testing

Saliva collection was performed immediately following blood collection. Two sterile, individually cased cotton swabs mounted on plastic rods[7] were used to obtain saliva. Each swab was rubbed one after the other against the buccal mucosa on each side of the mouth, with the cheek pressed gently against the upper dental arcade while slowly twisting the swab, for approximately 10 s per side. Swabs

[2] BioNote, Gyeonggi-do, Korea.
[3] Zoetis Animal Health, Lyon, France.
[4] IDEXX Laboratories, Westbrook, ME, USA.
[5] OraSure Technologies Inc., PA, USA.
[6] www.fda.gov/ForConsumers/ConsumerUpdates/ucm310545.htm

[7] Sarstadt, Mawson Lakes, South Australia, Australia (Plastic Stem Cotton Tip Catalogue No. 80.625; 1.5 mL Micro Tube Catalogue No. 72.706.400).

with frank blood on the cotton tip due to gingivitis were noted. The average weight of a total of ten saliva swabs from five cats after sampling was determined using a Precision Plus electronic balance[8] and compared to the average weight of ten unused swabs. Both saliva swabs were then refrigerated at 4 °C. One of the saliva swabs (selected randomly) was used for FIV antibody testing[9] within 24 h of collection by cutting the plastic rod approximately 2 cm from the cotton tip and transferring it to a sterile microcentrifuge tube.[7] After placing the cotton tip in the tube, with the plastic rod at the bottom of the tube, 450 μL of sterile phosphate buffered saline (PBS) was added and the tube shaken vigorously by hand for 10 s. The tube, still containing the cut cotton swab, was centrifuged for 30 s at 10,000 g.[10] The swab was then removed from the tube using forceps and the supernatant tested using three FIV antibody test kits designed for FIV antibody detection in whole blood, plasma or serum (SNAP FIV/FeLV Combo[4], Witness FeLV/FIV[3] and Anigen Rapid FIV/FeLV[2]). Testing was performed per manufacturers' instructions except that an equivalent volume of saliva-containing supernatant was substituted for blood in the test protocol. None of the manufacturers endorses using saliva as a diagnostic specimen for their test kits. The primary author performed FIV antibody testing using saliva immediately following FIV antibody testing using blood, meaning samples were not blinded for saliva antibody testing.

The manufacturer's instructions for SNAP FIV/FeLV Combo advise that any colour development in the FIV sample spot should be considered significant.[11] Although the manufacturers' instructions for Anigen Rapid FIV/FeLV and Witness FeLV/FIV contain no guidelines for interpreting faint results using whole blood, plasma or serum, a faint band (immunochromatography) or spot (ELISA) was recorded as a positive result. This was the same criteria used for our previous study using blood [14].

The second saliva sample was stored at −80 °C within 24 h of collection. At the conclusion of the study, stored samples were shipped on dry ice for nucleic acid extraction and FIV testing using a commercially available qPCR assay (FIV RealPCR).[12] Primers for this assay target the conserved *gag* region in both viral RNA (using cDNA following a reverse transcription step) and proviral DNA. FIV subtype was determined using subtype specific primer pairs for subtypes A, B, D and F.[13] The laboratory does not endorse using saliva as a diagnostic specimen for FIV RealPCR testing. Laboratory technicians performing FIV RealPCR testing using saliva were blind to saliva FIV antibody test results.

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

2.4. Statistical analysis

Numerical analyses were performed using a commercial program (Genstat 16th Edition).[14] Statistical significance was considered at $P < 0.05$ and 95% CI were calculated based on a normal approximation and the Wald method using Microsoft Excel.[15] Positive predictive value (PPV) and negative predictive value (NPV) were calculated using the standard formulas (PPV = 'number of true positives'/('number of true positives' + 'number of false positives'); NPV = 'number of true negatives'/('number of true negatives' + 'number of false negatives')). Overall test accuracy was determined by the formula ('number of true positives' + 'number of true negatives'/total number of cats sampled). Two-tailed Fisher's exact tests were used to investigate whether false-positive and false-negative FIV results were more common in FIV-vaccinated cats than FIV-unvaccinated cats, and whether false-positive FIV results were more common in cats returning a blood-tinged saliva swab than a non-bloody swab. Binomial logistic regression with a logit link function was conducted on the test results to determine if there was a significant difference in sensitivity between test methodologies using saliva, and also to compare sensitivity and specificity between blood and saliva for each test methodology.

3. Results

3.1. Sample population

Blood and saliva samples were obtained from 356 client-owned cats recruited from eleven veterinary clinics distributed over four states of Australia (New South Wales, Victoria, Queensland and South Australia).

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

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

3.2. Saliva testing

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

3.3. FIV-vaccinated cohort (n = 117)

Of the four FIV-vaccinated/FIV-infected cats, three tested FIV-positive with SNAP FIV/FeLV Combo (i.e. there was one false-

[8] Ohaus, Parsippany, NJ, USA.
[9] Faculty of Veterinary Science, The University of Sydney, Sydney, NSW, Australia.
[10] Eppendorf AG, Hamburg, Germany (Model 5424).
[11] https://www.idexx.com/resource-library/smallanimal/snap-combo-package-insert-en.pdf.
[12] IDEXX Laboratories, East Brisbane, Queensland, Australia.
[13] http://www.idexx.com.au/pdf/en_au/smallanimal/education/realpcr-test-for-fiv.pdf.
[14] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.
[15] Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.

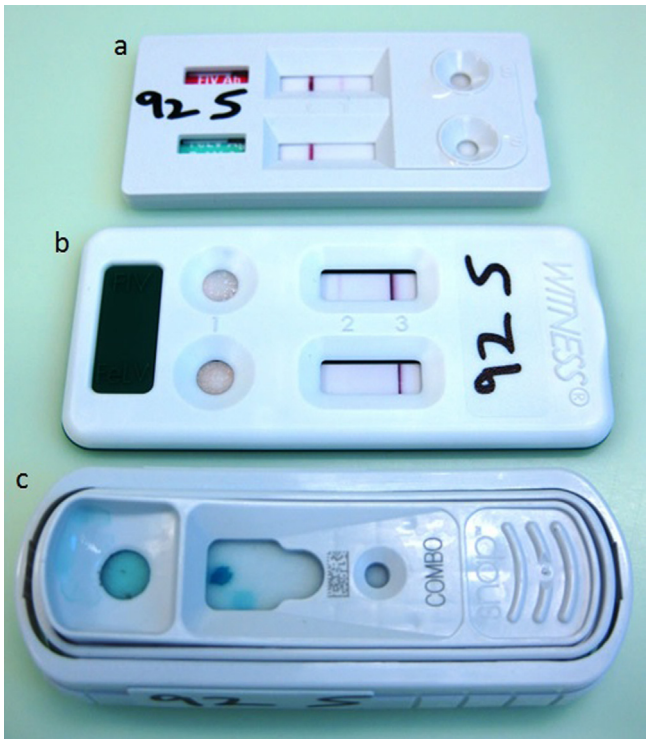

Fig. 1. Photograph of data from a FIV-infected cat (cat #92) using saliva as the diagnostic specimen, showing three positive FIV antibody test kit results using: (a) Anigen Rapid FIV/FeLV, (b) Witness FeLV/FIV, and (c) SNAP FIV/FeLV Combo. For both immunochromatography kits, the top strip is for FIV antibody testing and the bottom strip is for FeLV antigen testing. One band in the FIV or FeLV strip indicates a negative result, two bands in the FIV or FeLV strip indicates a positive result. For the SNAP FIV/FeLV Combo kit, two spots in the illustrated conformation indicates a positive FIV result. This result was found in 11/25 FIV-infected cats.

negative result), two tested FIV-positive with Witness FeLV/FIV and all four tested FIV-positive with Anigen Rapid FIV/FeLV. Two of the four cats tested FIV-positive with FIV RealPCR; the same two cats that were initially FIV-negative with FIV RealPCR testing using blood were also FIV-negative using saliva.

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

A summary of results for each test methodology in FIV-vaccinated cats, including sensitivity, specificity, PPV and NPV, is provided in Table 1.

3.4. FIV-unvaccinated cohort (n = 239)

Of the 21 FIV-unvaccinated/FIV-infected cats, eight tested FIV-positive with SNAP FIV/FeLV Combo (i.e. there were 13 false-negative results), 21 tested FIV-positive with Witness FeLV/FIV and 20 tested FIV-positive with Anigen Rapid FIV/FeLV. A photograph of one of the eight FIV-unvaccinated/FIV-infected cats that tested positive with all three antibody kits (cat #92) is shown in Fig. 1. A total of 16/21 cats tested FIV-positive with FIV RealPCR.

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

A summary of results for each test methodology in FIV-unvaccinated cats, including sensitivity, specificity, PPV and NPV, is provided in Table 2.

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

Combined results for saliva testing of both cohorts, including overall accuracy of each test methodology, are summarized in Table 3.

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

The specificity of all four test methodologies (SNAP FIV/FeLV Combo, Witness FeLV/FIV, Anigen Rapid FIV/FeLV and FIV RealPCR) using saliva was comparable, while a significant effect of test methodology on sensitivity was found ($P < 0.001$). Witness FeLV/FIV and Anigen Rapid FIV/FeLV had comparable sensitivities ($P = 0.55$), Anigen Rapid FIV/FeLV was significantly more sensitive than FIV RealPCR ($P = 0.038$), and all three methodologies were significantly more sensitive than SNAP FIV/FeLV Combo (Witness FeLV/FIV and Anigen Rapid FIV/FeLV [$P = 0.001$]; FIV RealPCR [$P = 0.048$]).

Subtyping results and cycle threshold (C_T) values from FIV RealPCR testing of both blood and saliva from the 25 FIV-infected cats are available online (Online Supplements 1 and 2).

3.6. Repeat saliva testing using revised technique (n = 14)

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

3.7. Comparing blood and saliva results (n = 356)

A full comparison of blood and saliva results is found in Online Supplement 3, while a comparison of sensitivity and specificity for each antibody test kit and FIV RealPCR testing using blood and saliva is found in Table 4. Using saliva instead of blood for testing with SNAP FIV/FeLV Combo significantly increased specificity ($P < 0.001$) but concurrently reduced sensitivity ($P = 0.002$), while using saliva instead of blood for FIV RealPCR testing trended towards reduced sensitivity ($P = 0.081$). All other comparisons were statistically similar.

4. Discussion

FIV infection was diagnosed in FIV-vaccinated and FIV-unvaccinated cats with >99% accuracy by using two inexpensive, fast, simple to use, antibody detection kits utilizing saliva as the diagnostic specimen (Witness FeLV/FIV and Anigen Rapid

Table 1
(FIV-vaccinated cohort): Results of testing using three point-of-care FIV antibody kits and a commercial real-time PCR assay in FIV-vaccinated cats using saliva ($n = 117$; comprising 113 FIV-uninfected and 4 FIV-infected cats). PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

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

Table 2
(FIV-unvaccinated cohort): Results of testing using three point-of-care FIV antibody kits and a commercial real-time PCR assay in FIV-unvaccinated cats using saliva ($n = 239$; comprising 218 FIV-uninfected and 21 FIV-infected cats). PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

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

Table 3
Combined results of three point-of-care FIV antibody test kits in FIV-vaccinated and FIV-unvaccinated cats using saliva ($n = 356$). Note that this composite population is biased by the inclusion of many FIV-vaccinated cats (117/356; 33%). In practice, the percentage of FIV-vaccinated cats in an area will be heavily dependent on the vaccination protocols of the local veterinary clinics and may differ considerably from this value. PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

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

Table 4
Comparison of overall sensitivity and specificity for the three antibody test kits and FIV RealPCR testing for blood [14] and saliva ($n = 356$) (see also Online Supplement 3). Note that this composite population is biased by the inclusion of many FIV-vaccinated cats (117/356; 33%). In practice, the percentage of FIV-vaccinated cats in an area will be heavily dependent on the vaccination protocols of the local veterinary clinics and may differ considerably from this value. Superscripts indicate a significant difference – (a) P = 0.002, (b) P = 0.001; or a trend towards significance – (c) P = 0.081. All other values recorded were not significantly different. Confidence intervals (95%) are given in brackets.

	SNAP Combo		Witness		Anigen Rapid		FIV ReaLPCR	
	Blood	Saliva	Blood	Saliva	Blood	Saliva	Blood	Saliva
Senstivity	100[a]	44[a] (25–64)	100	92 (81–100)	100	96 (88–100)	92[c] (82–100)	72[c] (54–90)
Specificity	64[b] (59–69)	98[b] (96–99)	98 (96–99)	100	100	100	99 (98–100)	100

FIV/FeLV). These findings will facilitate rapid and accurate determination of FIV infection status in certain scenarios, namely when a veterinarian or veterinary technician is unavailable for venipuncture, where cats are too fractious for safe venipuncture without chemical restraint, in shelters where large numbers of cats need to be assessed for FIV infection prior to rehoming and vaccination his-

tory is often unknown and for cat breeders screening new cats on entry to a cattery. This will, in some circumstances, provide a less expensive and less stressful option for owners and often a superior option for their cats.

Whole saliva is composed mainly of fluid produced by the salivary glands, which contains small amounts of locally produced immunoglobulin (Ig) molecules (mainly IgA, but also IgM and IgG), and crevicular fluid [21]. Crevicular fluid is derived from the capillary bed beneath the buccal mucosa, has an antibody content similar to that of plasma, and is responsible for most of the IgM and IgG content of whole saliva [22]. Consequently, saliva may be regarded as a transudate of plasma [23]. In people, the concentration of total IgG in whole saliva is approximately 1000 times less than in plasma [24]. Investigation of total IgG in cat saliva using healthy subjects found whole saliva contained 190 times less IgG than serum using a radial immunodiffusion assay, and 340 times less total IgG than serum using an ELISA. There were no cats in which salivary IgG could not be detected using these methodologies [17]. The same researchers found that both salivary and serum total IgG were higher in cats with chronic gingivostomatitis [25], a disease process FIV-infected cats are more likely to have and to have more severely than FIV-negative cats [26,27]. All three FIV antibody kits tested in the current study, despite differing methodology, detect IgG. SNAP FIV/FeLV Combo is a lateral flow ELISA that detects antibodies to FIV matrix protein (p15) and capsid protein (p24), Witness FeLV/FIV uses immunochromatography to detect antibodies to FIV surface glycoprotein (gp40) and Anigen Rapid FIV/FeLV uses immunochromatography to detect antibodies to p24 and gp40. Our results show that all three kits were able to detect anti-FIV IgG in whole saliva from FIV-infected cats, with varying accuracy.

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

The current study provides a large, rigorous and systematic study design for the evaluation of saliva as a diagnostic specimen for determining FIV infection status. We found it possible to accurately determine the FIV status of both FIV-vaccinated and FIV-unvaccinated cats using immunochromatography methodology, without the need for further confirmatory testing such as nucleic acid amplification. The two immunochromatography antibody test kits did not produce any false-positive FIV results (0/356) and thus a positive result with either test kit represented a true-positive result. Witness FeLV/FIV produced two false-negative test results (2/356; 0.6%), while Anigen Rapid FIV/FeLV produced only one false-negative test result (1/356; 0.3%). These three false-negative results occurred in three different cats. This slight reduction in test kit sensitivity when using saliva instead of blood (8% reduction for Witness FeLV/FIV, 4% reduction for Anigen Rapid FIV/FeLV) is similar to the 2% reduction reported with the OraQuick Advance Rapid HIV-1/2 In-Home HIV Test which is commercially available over the counter for private use [15].

Two previous studies evaluating the detection of FIV antibodies in saliva as a means of diagnosing FIV infection had contradictory findings. The first study reported a high frequency of false-positive and false-negative results [18]. Our belief is that the high number of erroneous results was attributable to limitations in the test kits used, as point-of-care test kits available in the early 1990s were far less refined than kits currently available. A subsequent study conducted more recently found a good correlation between salivary antibody and salivary PCR results [20], in accord with the present results. Chang-Fung-Martel and colleagues reported a higher proportion of false-positive FIV results in FIV-vaccinated cats using SNAP FIV/FeLV Combo (2/9; 22% ν 6/113; 5%) and a lower proportion of false-negative FIV results in FIV-infected cats (3/10; 30% ν 14/25; 56%) than the current study, although neither difference was statistically significant (Fisher's exact test; $P = 0.11$ and 0.26, respectively) [20].

The main challenge to the routine use of salivary antibody testing is the requirement to purchase specific consumables, such as microcentrifuge tubes and a centrifuge. A cotton swab mounted on a plastic rod, rather than a cotton or wooden rod, is required to avoid absorption of saline by the rod in order to collect sufficient sample for testing. The centrifugation method for salivary antibody testing outlined in the present study was chosen as it had proved effective in a previous study [20]. Unfortunately the centrifugation method is more complicated than the process required for antibody testing of whole blood and this may be off-putting for some veterinarians. Simplifying the testing process, as described in a subset of cats that were retested, potentially provides a method far more suitable for use in a busy practice or animal shelter. Preliminary results showed that an excellent outcome could be obtained using Anigen Rapid FIV/FeLV by applying the cotton swab directly to the sample well and flooding the cotton tip with buffer solution. It must be stressed, however, that this method should not be relied upon for making clinical decisions until further validation is undertaken. In contrast, the performance of both SNAP FIV/FeLV Combo and Witness FeLV/FIV deteriorated with the direct application technique and consequently this technique should not be used for saliva testing with either of these kits.

5. Conclusion

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cimid.2016.03.006.

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