Comparative Immunology, Microbiology and Infectious Diseases 50 (2017) 88–96

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Comparison of three feline leukaemia virus (FeLV) point-of-care antigen test kits using blood and saliva

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form
22 November 2016
Accepted 22 November 2016

Keywords:
Feline leukaemia virus
FeLV diagnosis
Antigen testing
PCR
Whole blood
Saliva
Cats

ABSTRACT

Feline leukaemia virus (FeLV) can be a challenging infection to diagnose due to a complex feline host-pathogen relationship and occasionally unreliable test results. This study compared the accuracy of three point-of-care (PoC) FeLV p27 antigen test kits commonly used in Australia and available commercially worldwide (SNAP FIV/FeLV Combo, Witness FeLV/FIV and Anigen Rapid FIV/FeLV), using detection of FeLV provirus by an in-house real-time polymerase chain reaction (qPCR) assay as the diagnostic gold standard. Blood ($n = 563$) and saliva ($n = 419$) specimens were collected from a population of cats determined to include 491 FeLV-uninfected and 72 FeLV-infected individuals (45 progressive infections [p27 and qPCR positive], 27 regressive infections [p27 negative, qPCR positive]). Sensitivity and specificity using whole blood was 63% and 94% for SNAP Combo, 57% and 98% for Witness, and 57% and 98% for Anigen Rapid, respectively. SNAP Combo had a significantly lower specificity using blood compared to the other two kits ($P = 0.004$ compared to Witness, $P = 0.007$ compared to Anigen Rapid). False-positive test results occurred with all three kits using blood, and although using any two kits in parallel increased specificity, no combination of kits completely eliminated the occurrence of false-positive results. We therefore recommend FeLV proviral PCR testing for any cat that tests positive with a PoC FeLV antigen kit, as well as for any cat that has been potentially exposed to FeLV but tests negative with a FeLV antigen kit, before final assignment of FeLV status can be made with confidence. For saliva testing, sensitivity and specificity was 54% and 100%, respectively, for all three test kits. The reduced sensitivity of saliva testing compared to blood testing, although not statistically significant, suggests saliva testing with the current generation of PoC FeLV antigen kits is unsuitable for screening large populations of cats, such as in shelters.
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1. Introduction

Feline leukaemia virus (FeLV) was discovered in 1964 in a cluster of cats with lymphoma [1]. The development of molecular methods for FeLV diagnosis, specifically real-time polymerase chain reaction (qPCR) testing to detect low levels of proviral DNA and real-time reverse transcriptase PCR (qRT-PCR) to detect low levels of viral RNA in blood, has enriched our understanding of the feline host-FeLV relationship, adding nuances to the diagnosis and categorization of FeLV infection.

Currently, three main outcomes are defined for cats following FeLV challenge: (i) Some cats mount a timely and appropriate immune response and eliminate the virus before it progresses beyond local replication in oropharyngeal tissue – so called abortive infections (20–30% of cases under laboratory conditions using specific pathogen free [SPF] cats); (ii) some cats are transiently viraemic before mounting a partial immune response to eliminate the viraemia after 2–16 weeks, but not before a latent infection is established as DNA provirus in haematopoietic precursor cells in the bone marrow (regressive infections; 30–40% of experimentally inoculated SPF cats); and (iii) some cats become persistently viraemic (progressive infections; 30–40% of experimental infections). [2–4]. Such an array of possible outcomes following FeLV exposure makes defining the expectations of FeLV diagnostic testing important, including before attempting to evaluate the performance of a given point-of-care (PoC) kit.

Antigen testing (including enzyme-linked immunosorbent assay [ELISA], immunochromatography [IC] and immunofluorescent antibody [IFA] methodologies) for the detection of viral capsid

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http://dx.doi.org/10.1016/j.cimid.2016.11.014
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protein (p27) has been used for over 40 years to identify cats with progressive FeLV infections using whole blood, plasma or serum as the diagnostic specimen. Prior to the commercial availability of qPCR assays, studies of four commercially available PoC ELISA FeLV antigen test kits (1989–1991) reported almost 100% sensitivity and 100% specificity for all kits compared to IFA testing [5,6] but reduced sensitivity compared to virus isolation (VI) [6]. Furthermore, discordant results were observed between test results for whole blood and serum due to a combination of false-positive (whole blood) and false-negative (serum) results [6]. Later research (1998–2001) reported 5/6 PoC FeLV antigen kits tested with serum (including SNAP FIV/FeLV Combo[1] and Witness FeLV/FIV[2]) to have good sensitivity and specificity using VI as the 'gold standard' [7], and SNAP Combo and Witness both performed well when tested side-by-side [8]. In one of the studies, SNAP Combo testing using whole blood instead of serum increased the number of equivocal results and was therefore advised against by the authors, despite the manufacturer's recommendation that whole blood could be used [7]. More recently (2007–2010), testing of eight of the latest generation PoC FeLV antigen test kits (including SNAP Combo and Witness) with serum found all but one kit had similarly high sensitivity and specificity when compared to VI [9], while a separate study reported Anigen Rapid FIV/FeLV[3] to have comparable diagnostic accuracy to SNAP Combo using proviral PCR testing as the gold standard [10]. Witness was found to have 'a high number of tests that were difficult to interpret' (14%) [9], although exactly what was meant by this is impossible to determine. As qPCR has become the new gold standard for FeLV diagnosis, replacing VI (which is rarely available to veterinarians in the field) and IFA [3], a contemporary investigation of the most commonly used PoC FeLV antigen test kits using whole blood (the preferred specimen for patient-side testing) and considering the current complexity of FeLV infection categories, is overdue.

Diagnosis of FeLV infection using samples other than blood has been investigated using both laboratory-based ELISA testing and commercial PoC FeLV test kits. Saliva is the obvious alternative diagnostic specimen, as it is often easier to collect than blood, especially from fractious cats, and saliva contains on average five-times more FeLV per mL than plasma [11]. Results from saliva testing have been contradictory. One study of experimentally infected and sick client-owned cats using laboratory-based ELISA testing found false-positive FeLV results rarely occurred (5/1117; 0.4%) but false-negative results occurred in 23% (39/167) of FeLV-infected sick cats [12]. A later study from Switzerland, using the same sandwich ELISA, reported false-positive FeLV results in 5% (19/367) of FeLV-uninfected cats [13]. Other studies have investigated FeLV diagnosis using PoC ELISA saliva testing, finding a concordance of 98% (552/564) between saliva and serum tested concurrently using ViraChek FeLV[4] [14], and 92% (94/102) between saliva and plasma using ClinEase-Virastat[4] [15]. Both studies concluded saliva testing was a useful rapid screening procedure using these kits, but recommended confirmatory testing [14,15]. Despite these promising results, the American Association of Feline Practitioners (AAFP) declared in 2008 that 'antigen tests should not be performed on tears or saliva because these tests are prone to more errors' [16]. To the authors' knowledge, none of the latest generation of PoC FeLV antigen kits have been evaluated using saliva.

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

2. Material and methods

2.1. Sample population

A total of 563 cats were recruited, comprised of three distinct groups: (i) **Group 1** ($n = 440$) consisted of healthy client-owned cats that were part of a case-control study into the effectiveness of the feline immunodeficiency virus (FIV) vaccine, and contained both FIV-vaccinated and FIV-unvaccinated cats [17,18]; (ii) **Group 2** ($n = 72$) consisted of predominantly sick cats that had blood sent to our laboratory[5] for confirmatory FeLV testing following a positive FeLV antigen test result (either an in-clinic PoC kit or a laboratory microwell assay[6]; $n = 53$), sick cats with haematologic abnormalities (most commonly non-regenerative anaemia) that had FeLV testing requested by a New South Wales veterinary laboratory[7] ($n = 13$) and healthy cats that had been in-contact with a progressively FeLV-infected cat during the previous 12 months ($n = 6$); and (iii) **Group 3** ($n = 51$) consisted of semi-feral cats housed at a rescue facility in western Sydney that were tested in response to recent unexplained deaths. The age of some cats in Group 3 was undeterminable.

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

2.2. Blood collection, DNA extraction and proviral qPCR testing

Blood was collected by jugular or cephalic venipuncture from conscious cats, as described [17]. DNA was extracted from whole EDTA blood using a kit (QIAamp DNA Mini Kit)[8], as per the manufacturer's instructions. The concentration and quality of extracted DNA was measured using a spectrophotometer (Nanodrop 1000)[9]. DNA was stored at −80 °C.

PCR testing for FeLV provirus was chosen as the gold standard for the current study. PCR testing was performed according to a published protocol, using primers designed to amplify a section of the unique region (U3) of the long terminal repeat (LTR) of subtypes FeLV −A, −B and −C, but not endogenous retroviral sequences [19]. Each 25 μL PCR reaction was composed of 0.125 μL Taq DNA polymerase[8], 2.5 μL 10x PCR Buffer[8], 0.5 μL 10 mM dNTP mix, a final concentration of 480 nM of each primer[10], 160 nM of a dual-labelled fluorogenic probe (labelled at the 5′ end with the fluorescent reporter dye FAM [6-carboxyfluorescein] and at the 3′ end with the fluorescent quencher dye Black Hole Quencher-1™)[10], 3 μL of DNA (approximately 100 ng), and nuclease-free water. Following 15 min of denaturation at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 60 s were carried out. PCR reactions were performed using a Bio-Rad CFX96™ Real-Time System Thermocycler PCR

[1] IDEXX Laboratories, Westbrook, ME, USA.
[2] Zoetis Animal Health, Lyon, France.
[3] BioNote, Gyeonggi-do, Korea.
[4] Synbiotics Corporation, San Diego, CA, USA.
[5] Veterinary Pathology Diagnostic Services (VPDS), The University of Sydney, Sydney, NSW, Australia.
[6] ViraCHEK FeLV, Synbiotics Corporation, San Diego, CA, USA (testing performed at Vetnostics).
[7] Vetnostics, 60 Waterloo Road, North Ryde, NSW, Australia.
[8] Qiagen, Valencia, CA, USA.
[9] Thermo Fisher Scientific, Waltham, MA, USA.
[10] Biosearch Technologies, Navato, CA, USA.

machine[11], and fluorescence was detected during each annealing step (60 °C) at 515–530 nm. All samples were run in duplicate.

Construction and production of a FeLV DNA standard for absolute quantitation of the PCR assay (precision and sensitivity) was done by molecular cloning. PCR amplification of the 131 bp section was performed, the purified product cloned into a vector (pCRII-TOPO®, 3973 bp)[11], grown in *E. coli* cells (Rapid One Shot®)[11] and DNA extracted using a kit (QIAprep Spin Miniprep Kit)[8]. Amplicon sequencing and the Basic Local Alignment Search Tool (BLAST) in Genbank® was used to ensure the extracted plasmid contained the same FeLV sequence as the initial PCR product (99% alignment with FeLV strain Glasgow-1 accession number KP728112.1). Within-run and between-run precision of the FeLV qPCR assay were assessed using three dilutions of the DNA standard (1.46×10^3, 1.46×10^5 and 1.46×10^7 copies per reaction). Within-run precision was evaluated with 10 replicates of each dilution, and between-run precision was evaluated using 10 replicates of each dilution in three separate experiments. For both sets of experiments, coefficients of variation for the cycle threshold (C_T) values (CV_{CT}) were calculated using the formula (CV_{CT} = C_T SD/arithmetic mean C_T, where SD is standard deviation). Absolute copy numbers were calculated for each run using the formula ($X_o = 10^{([C_T - b]/m)}$, where X_o is the absolute copy numbers per reaction, *b* is the *y*-intercept of the standard curve and *m* is the slope), and coefficients of variation for the absolute copy numbers (CV_{abs}) also determined using the same formula (CV_{abs} = X_o SD/X_o of arithmetic mean C_T).

The CV_{CT} and CV_{abs} of the within-run precision experiments were 0.54–0.8% and 8.4–12.8%, respectively. The CV_{CT} and CV_{abs} of the between-run precision experiments were 0.45–0.85% and 6.7–17.2%, respectively. A sensitivity experiment was performed using 10-fold serial dilutions of the DNA standard to determine the diagnostic sensitivity of the assay. The lower limit of detection was 100 copies of DNA standard per 25 μL reaction (10/10 reactions positive). For the next lower dilution (90 copies per 25 μL reaction), 9/10 reactions were positive. At the lowest dilution tested (40 copies per 25 μL reaction), 5/10 reactions were still positive.

Known positive and negative FeLV samples were included as internal controls in each run, as well as a 'no template' control. Any sample with two C_T values less than 40 was recorded as a positive result. Any sample with two C_T values greater than 40 was recorded as a negative result. Samples with mixed C_T values (*i.e.* one $C_T < 40$ and one $C_T > 40$) were re-run in quadruplicate, with a positive result assigned if a sample recorded two or more C_T values less than 40 in the second run, and a negative result assigned if a sample recorded three or four C_T values greater than 40. Nine samples (out of a total of 12) declared PCR positive following sample testing in quadruplicate had amplicons sequenced and Genbank® BLAST used to confirm they were genuine FeLV sequences (93–99% alignment with FeLV strain Glasgow-1 accession number KP728112.1).

PCR testing for genomic mammalian DNA was also performed using a published protocol to ensure the quality of the thawed DNA [20,21]. This protocol uses primers designed to amplify a region of the feline 28S rDNA gene. Each 25 μL PCR reaction was composed of 0.125 μL Taq DNA polymerase[8], 2.5 μL 10x PCR Buffer[8], 0.5 μL 10 mM dNTP mix, 200 nM of each primer[10], 200 nM of a dual-labelled fluorogenic probe (5′ end fluorescent reporter dye CAL Fluor Orange 560™, 3′ end fluorescent quencher dye Black Hole Quencher-1™)[10], 3 μL of 25 mM $MgCl_2$, 3 μL of DNA (approximately 100 ng), and nuclease-free water. The same cycling conditions and PCR machine were used as for FeLV proviral testing, except fluorescence was detected during each annealing step (60 °C) at 560–580 nm. Samples were run singularly. Known positive samples and a no template control were included in each run. Samples with a C_T value of less than 37 recorded a positive result.

2.3. FeLV PoC testing using blood

FeLV antigen testing was performed as per manufacturers' instructions within 24 h of sampling (Groups 1 and 3) or within 24 h of receiving the blood sample (Group 2) using whole EDTA blood. Thirteen samples not able to be tested within this time period were stored at −20 °C, either as whole EDTA blood ($n = 10$) or plasma ($n = 3$), then thawed and tested at a later date. Three FeLV test kits marketed to detect p27 in whole blood, plasma or serum were tested concurrently. SNAP FIV/FeLV Combo[1] is a lateral flow ELISA kit, while Witness FeLV/FIV[2] and Anigen Rapid FIV/FeLV[3] are lateral flow kits that use colloidal gold IC methodology. When determining the results of the test kits, a faint spot (SNAP Combo) or a faint band (Witness, Anigen Rapid) was recorded as a positive result. Although the manufacturers' instructions for both Witness and Anigen Rapid contain no guidelines for interpreting faint results, instructions for SNAP Combo advise that any colour development in the FeLV sample spot should be considered significant [22].

2.4. Categories of FeLV infection

The following outcomes were described: (i) FeLV-uninfected (qPCR negative), either due to an abortive infection or non-exposure to FeLV; (ii) FeLV-infected with progressive infection (qPCR positive and at least one positive p27 test using blood); and (iii) FeLV-infected with regressive infection (qPCR positive but three negative p27 tests using blood).

FeLV proviral qPCR testing determined 72 cats to be FeLV-infected and 491 cats to be FeLV-uninfected. Of 72 FeLV-infected cats, 45 were suspected of being progressively infected and 27 regressively infected. FeLV C_T values for progressively FeLV-infected cats (median 22, geometric mean 23, range 14–37, IQR 20–25) were significantly lower (*i.e.* higher proviral load) compared with regressively FeLV-infected cats (median 35, geometric mean 35, range 29–40, IQR 34–37) ($P < 0.001$ for both median and geometric C_T values; Fig. 1). Seven regressively infected cats recorded FeLV C_T values of 37–40, of which five had sequencing performed to confirm that the amplified product was exogenous FeLV material (*i.e.* they were true-positive FeLV qPCR results). Progressively FeLV-infected cats were younger (median age 3.4 years) than regressively FeLV-infected cats (median age 7.6 years; $P = 0.007$).

All samples tested positive for the DNA quality control feline 28S rDNA gene (median C_T value 29, range 27–36, IQR 28–30). The median and geometric mean 28S C_T values for progressively and regressively FeLV-infected cats were not significantly different (33 *vs.* 33, 29 *vs.* 30, $P = 0.58$ and 0.59, respectively).

2.5. Saliva collection and FeLV PoC testing using saliva

Of 563 cats recruited for blood testing, 419 were available for saliva sampling at the time blood was collected. This included 26 FeLV-infected cats (17 progressively infected, 9 regressively infected) and 393 FeLV-uninfected cats. Saliva was collected immediately following blood collection, as described [23]. Briefly, a sterile individually packaged cotton swab mounted on a plastic rod[12] was rubbed against the buccal mucosa on each side of the mouth, with the cheek pressed gently against the upper dental arcade while slowly twisting the swab, for approximately 10 s per side. The plastic rod was cut approximately 2 cm from the cotton tip, the tip

[11] Bio-Rad, Hercules, CA, USA.

[12] Sarstadt, Mawson Lakes, SA, Australia (Plastic Stem Cotton Tip Catalogue No. 80.625).

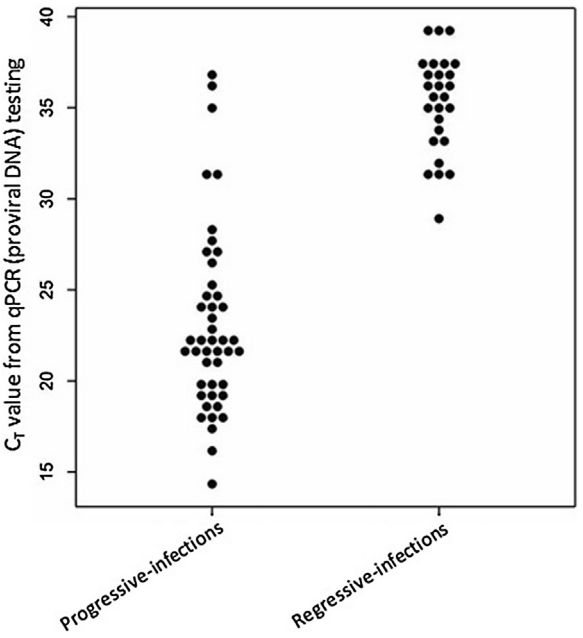

Fig. 1. Scatter plot of C_T values (*y* axis) from FeLV proviral PCR testing of 563 cats. Progressively FeLV-infected cats ($n = 45$) recorded a significantly lower median C_T value (*i.e.* higher proviral load) compared with regressively FeLV-infected cats ($n = 27$) (*x* axis).

transferred to a sterile microcentrifuge tube[13] (plastic rod at the bottom of the tube), 450 μL of sterile phosphate buffered saline (PBS) added and the tube shaken vigorously by hand for 10 s. The tube, still containing the cut cotton swab, was then centrifuged for 30 s at 10,000*g*[14]. Afterwards the swab was removed from the tube using forceps and the supernatant tested using the same three PoC kits as used for testing with whole blood. Testing was performed as per manufacturers' instructions except that an equivalent volume of saliva-containing supernatant was substituted for blood in the test protocol. None of the manufacturers endorses using saliva as a diagnostic specimen for their FeLV test kits. The primary author performed FeLV PoC testing using saliva immediately following FeLV PoC testing using blood, meaning samples were not blinded for saliva testing.

The average weight of ten swabs after saliva sampling was determined using an electronic balance (Precision Plus)[15] and compared to the average weight of ten unused swabs. The median weight of saliva collected per swab was 70 mg (IQR 30–110 mg).

At the conclusion of the study a subset of cats with progressive FeLV-infection ($n = 15$) was resampled using three new cotton swabs and the aforementioned technique. However, instead of using PBS and centrifugation to extract a supernatant sample, a single cotton swab was used exclusively for each FeLV test kit (randomly ordered), using a simpler method. For each PoC kit, the saliva swab was directly applied to the sample well spot and the cotton tip soaked with twice the volume of conjugate (SNAP Combo) or buffer (Witness, Anigen Rapid) recommended in the manufacturers' instructions, rolling the cotton tip on the sample spot for 10 s while conjugate/buffer was added. The result was read 10 min later. This revised 'direct' technique for FeLV antigen testing using saliva was investigated to determine the accuracy of a quicker, simpler method for patient-side use [23].

2.6. Statistical analysis

Numerical analyses were performed using statistical software (Genstat 16th Edition)[16]. Statistical significance was considered at $P < 0.05$ and 95% confidence intervals (CIs) were calculated for test kit performance based on a normal approximation and the Wald method using Microsoft Excel[17]. 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). Binomial logistic regression with a logit link function was conducted on the test results to compare sensitivity and specificity between test kits and between blood and saliva results for the same test kit. Cohen's Kappa Index Value (κ) was calculated to assess agreement between blood and saliva results for each test kit using the standard formula ($\kappa = 1-(1-P_o)/(1-P_e)$), where P_o was the observed agreement and P_e was the expected agreement (0.5). Since age and C_T values for both FeLV and feline 28S assays were not normally distributed according to Shapiro-Wilk testing, median was reported for each (as well as geometric mean for C_T values) and Mann-Whitney *U*-tests used for comparisons.

3. Results

3.1. FeLV PoC testing using blood ($n = 563$)

The performance of the three PoC FeLV antigen kits compared to proviral qPCR testing, using blood as the diagnostic specimen, is shown in Table 1. SNAP Combo produced more false-positive results than either Witness or Anigen Rapid, resulting in significantly lower specificity (94% *vs.* 98%, $P = 0.004$ and 0.007, respectively). SNAP Combo recorded less false-negatives than the other two kits, although its sensitivity was not significantly higher (63% *vs.* 57%, $P = 0.50$ for both). The specificity and sensitivity of Witness and Anigen Rapid were comparable to each other ($P = 0.83$ and 1.00, respectively). A summary of test kit results in FeLV-infected (qPCR positive) cats, grouped by proviral C_T value, is provided in Table 2 to highlight the decreasing sensitivity of all three kits as FeLV C_T increased (*i.e.* as the amount of FeLV provirus present decreased). True-positive p27 results occurred more commonly in younger animals (median age 3.4 years) while false-positive p27 results occurred more commonly in older animals (median age 7.5 years; $P < 0.001$). Of the 35 cats that recorded a false-positive p27 result with at least one of the kits, 15 had presented to a veterinarian displaying clinical signs and/or haematologic abnormalities consistent with progressive FeLV infection (*e.g.* severe anaemia and lymphadenopathy). There was no statistical difference between 28S C_T values of p27 true-positive and p27 false-positive samples ($P = 0.14$), suggesting that negative FeLV qPCR results in the 35 false-positive samples were due to an absence of FeLV provirus rather than an inadequate amount of DNA in the initial PCR reaction (*i.e.* they were true-negative qPCR results).

When the results of two different p27 kits were considered in parallel PPV increased (highest PPV was 91% using Witness and Anigen Rapid together), but the occurrence of false-positive results was not completely eliminated (Table 3). Of the 35 cats that recorded a false-positive p27 result with at least one of the test kits, false-positive results were obtained with more than one kit in

[13] Sarstadt, Mawson Lakes, SA, Australia (1.5 mL Micro Tube Catalogue No. 72.706.400).
[14] Eppendorf AG, Hamburg, Germany (Model 5424).
[15] Ohaus, Parsippany, NJ, USA.
[16] GenStat 16th Edition for Windows, VSN International, Hemel Hempstead, United Kingdom.
[17] Microsoft Excel 2010 for Windows, Microsoft, Redmond, WA, USA.

Table 1
Results of whole blood testing using three point-of-care (PoC) FeLV antigen test kits ($n = 563$, comprising 45 FeLV-infected cats with progressive infections, 27 FeLV-infected cats with regressive infections and 491 FeLV-uninfected cats).

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

PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

Table 2
Results of whole blood testing using three point-of-care (PoC) FeLV antigen test kits, arranged by cycle threshold (C_T) values from FeLV proviral PCR testing. The number recorded for each PoC kit refers to the number of true-positive results observed for that C_T category. FeLV-infected cats ($n = 72$) with both progressive ($n = 45$) and regressive ($n = 27$) infections are included.

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

Table 3
Results from point-of-care (PoC) FeLV testing using whole blood and considering results in parallel ($n = 563$, comprising 45 FeLV-infected cats with progressive infections, 27 FeLV-infected cats with regressive infections and 491 FeLV-uninfected cats). If both kits tested p27 positive, the cat was assigned FeLV-positive; conversely, if both kits tested p27 negative, the cat was assigned FeLV-negative. When the kits had differing results (*i.e.* one positive and once negative), a discordant result was assigned. Considering the results of two kits in combination increased the specificity and PPV of p27 testing to identify cats with progressive FeLV infections. Proviral PCR testing, rather than a second antigen test, should always be pursued for FeLV confirmatory testing where possible, particularly when results of two different FeLV antigen kits are discordant.

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

PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

10/35 instances (6 instances where 2/3 kits tested false-positive, 4 instances where 3/3 kits tested false-positive; median age 6 years, range 11 months to 12 years, IQR 4–10 years). Seven of these 10 cases where multiple false-positive p27 results were obtained were in cats displaying clinical signs consistent with FeLV disease, while three were in clinically well cats. Testing in parallel was able to identify all progressively infected cats, provided SNAP Combo was one of the kits used and proviral qPCR testing was pursued when results were discordant (*i.e.* one positive p27 result, one negative p27 result). If Witness and Anigen Rapid had been used in combination, three progressively infected cats would not have been identified (*i.e.* both kits tested p27 negative).

FeLV test kit performance using blood, with the exclusion of regressive infections from final analyses, is provided in online supplement 1.

3.2. FeLV PoC testing using saliva ($n = 419$)

Table 4 shows the comparative performance of the three PoC FeLV antigen kits using saliva as the diagnostic specimen. The sensitivity (54%) and specificity (100%) of each of the three kits was identical when saliva was used, although there was not complete agreement with test results for two cats. Each kit recorded three false-negative results; two progressively infected cats tested p27 negative with all three kits using saliva, while another two had discordant results with saliva (one was positive with SNAP Combo and Anigen Rapid only, the other was positive with Witness only). No false-positive p27 results were recorded with any of the kits using saliva.

FeLV test kit performance using saliva, with the exclusion of regressive infections from final analyses, is provided in online supplement 1.

3.3. Repeat PoC FeLV saliva testing using a revised 'direct' technique ($n = 15$)

Table 5 shows the results from re-testing 15 progressively FeLV-infected cats using the simpler patient-side technique described earlier. Despite SNAP Combo recording three more false-negative results than both Witness and Anigen Rapid, owing to the small sample size there was no significant difference in sensitivity (67%

Table 4
Results of saliva testing using three point-of-care (PoC) FeLV antigen test kits and a centrifugation method (n = 419, comprising 17 FeLV-infected cats with progressive infections, 9 FeLV-infected cats with regressive infections and 393 FeLV-uninfected cats).

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

PPV = positive predictive value, NPV = negative predictive value. Confidence intervals (95%) are given in brackets.

Table 5
Results of saliva testing using three point-of-care (PoC) FeLV antigen test kits and a revised 'direct' technique (n = 15, comprising 15 FeLV-infected cats with progressive infections). Only sensitivity was able to be calculated since no FeLV-uninfected cats were tested with this technique.

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

Confidence intervals (95%) are given in brackets.

vs. 87%) between the three kits using saliva and the direct technique (P = 0.21).

3.4. Comparing PoC FeLV testing using blood and saliva

Table 6 compares the sensitivity and specificity of blood and saliva testing for each FeLV antigen test kit. All comparisons were statistically similar. Cohen's Kappa Index Value (κ) confirmed excellent concordance between blood and saliva test results per individual (n = 419): SNAP Combo κ = 0.95, Witness κ = 0.98, Anigen Rapid κ = 0.97.

4. Discussion

The performance of three FeLV antigen kits was found to be similar when compared with proviral qPCR testing, irrespective of whether whole EDTA blood or saliva was used for PoC testing, except more false-positive FeLV results occurred with SNAP Combo compared with the other two kits using blood. Since whole blood is currently the recommended specimen for patient-side FeLV testing, these results provide an important reminder that PCR detection of DNA provirus remains the gold standard confirmatory test to definitely diagnose FeLV infection. Thus, PCR testing should be pursued to confirm any positive p27 antigen test result.

The rationale of screening for FeLV infection by p27 testing of blood, followed by confirmatory PCR testing [3], is to (i) correctly identify progressively infected cats and (ii) avoid unnecessary euthanasia due to false-positive p27 results. Progressively infected cats are 62 times more likely to develop leukaemia or lymphoma [24] and are also more likely develop disorders of haematopoiesis (including aplastic anaemia, thrombocytopenia, neutropenia and lymphocytosis) compared to FeLV-uninfected cats [25]. The survival rate for progressively FeLV-infected cats is estimated at 50% by two years and 20% by three years after infection [2]. In general, false-positives become more common as disease prevalence diminishes [26–28], such as in Australia where the prevalence of progressive FeLV infection is likely less than 1% [29].

False-positive FeLV results are said to occur as a result of anti-mouse IgG antibodies present in a small proportion (estimated ≤ 0.5%) of the domestic cat population (as well as in people and other domestic animals) [30]. The reason why anti-mouse IgG can be present in cat sera is not well understood, but might relate to predation of mice and subsequent ingestion. Whatever the mechanism of their generation, anti-mouse IgG bind to the murine-derived monoclonal antibodies (MAbs) in FeLV test kits that capture p27, causing a false-positive reaction [30]. It has previously been reported that an older version of the IDEXX Laboratories FeLV test kit[18] did not produce false-positive results when samples containing (or spiked with) cat anti-mouse antibodies were tested [5]. Based on communications between the authors of that study [5] and the manufacturer at the time, it was confirmed that the test kit contained reagents to correct for cat anti-mouse antibodies. All three manufacturers were contacted during the final preparation of this manuscript to request a comment regarding this potential cause of false-positive results, with the following information supplied by the relevant company representatives (November 2016). IDEXX Laboratories continues to use reagents in the current SNAP Combo liquid conjugate to help block cat anti-mouse antibodies in the blood sample from binding to murine-derived anti-FeLV antibodies used in the assay, thereby helping avoid false-positive reactions. SNAP Combo conjugate is mixed with the patient's sample in a separate tube prior to addition to the test kit, allowing this blocking activity to occur before exposure to the solid phase murine-derived MAbs. The SNAP Combo conjugate also contains the second murine-derived anti-FeLV MAbs conjugated to horseradish peroxidase to facilitate amplification and improve test sensitivity. Moreover, FeLV testing performed at IDEXX Reference Laboratories uses a microwell plate ELISA protocol with a confirmatory 'neutralisation' step to rule out non-specific activity as the cause of a positive test result (PetChek® FeLV). For this laboratory-based ELISA, polyclonal antiserum (containing non-murine anti-FeLV polyclonal antibodies) which specifically binds FeLV proteins and makes them unavailable for detection in the assay, is mixed with the patient's positive sample prior to testing. Loss of colour (>50%) in the test well compared to an untreated sample well confirms a true-positive FeLV result, while if the colour remains it is assumed to be a non-specific reaction one cause of which could be cat anti-mouse antibodies [30,31]. Zoetis Animal Health claims the type of biologics used in the Witness FeLV test kit make anti-mouse antibody cross-reaction unlikely. BioNote claims the Anigen Rapid FeLV test kit contains a substance inside the buffer to prevent any non-specific binding of various proteins that might be present in the cat's blood to the murine-derived anti-FeLV MAbs used in the test kit, thereby avoiding non-specific responses and false-positive results. To the authors' knowledge, external validation of the Witness and Anigen Rapid claims does not appear in the peer-reviewed literature. It remains possible that occasionally

[18] Test kit 'A', Cite/FELV, kit lot no. 9505-0824D, Agritech Systems (now known as IDEXX Laboratories), Westbrook, ME, USA.

Table 6
Comparison of overall sensitivity and specificity for three point-of-care (PoC) FeLV antigen test kits using whole blood and saliva ($n = 563$ for blood, $n = 419$ for saliva). There were no significant differences between blood and saliva testing for any of the test kits (P values shown).

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

Confidence intervals (95%) are given in brackets.

a sample may have so much anti-mouse activity that it overrides the correction reagent and gives a false-positive result [32].

Using two different p27 kits in parallel, as some authors have recommended [9], increased PPV and should be considered in cases where an urgent diagnosis is necessary or where PCR testing is unavailable. Parallel testing, however, did not completely eliminate the occurrence of false-positive p27 results in 4–8 FeLV-uninfected cats (depending on the combination of test kits employed). Consideration of other pertinent details, including lifestyle (*e.g.* higher FeLV risk in fighting cats, multi-cat households and shelters practicing group housing), age (younger cats were more likely to be progressively FeLV-infected, older cats were more likely to return a false-positive p27 result) and clinical signs of FeLV-related disease (*e.g.* macrocytic anaemia, aplastic anaemia, lymphoma) will increase a clinician's index of suspicion for FeLV-infection further. Nonetheless, confirmatory PCR testing is still recommended for any cat (healthy or sick) that tests p27 positive with a PoC kit, as decisions regarding treatment or euthanasia will be much better informed by the PCR result.

The impact of regressive FeLV infections on the health of cats is largely unknown. Some authors have suggested regressively infected cats have a similar life-expectancy to cats never exposed to FeLV and do not develop FeLV-associated disease [3,33]. However, reactivation of regressive FeLV infection contained in the bone marrow has been demonstrated experimentally, following administration of corticosteroids [34]. Furthermore, research in Australia and Canada has suggested a correlation between regressive FeLV infection and the occurrence of lymphoma [35,36], and regressively infected cats are capable of transmitting active FeLV infection to recipient cats by blood transfusion [37]. Several studies have reported populations of regressively FeLV-infected cats, including 5–10% of cats tested in Switzerland (24/445 and 6/597, respectively), 10% in UK (45/465), 3% (2/75) in Australia and 1% in Germany (6/495) [4,13,21,26,38]. Future research needs to identify regressively infected client-owned cats and follow them longitudinally to investigate health impacts in the field. Regressively FeLV-infected cats eliminate the viraemia within 2–16 weeks of exposure, and a limitation of our study was that some of the cats defined as progressively infected may have been in the process of clearing the viraemia, thereby becoming regressively infected. This limitation could be overcome in the future by serial p27 testing. Veterinarians should consider the C_T value from FeLV qPCR testing as a possible predictor of cats more likely to become regressively infected; our results showed that cats with progressive FeLV infections had a lower median C_T value (*i.e.* higher proviral load), with 40/45 progressive infections recording a C_T value < 30. It must be stressed, however, that until the role of regressive infections in disease is explored further we do not recommend solely using qPCR testing for FeLV diagnosis or FeLV screening, and instead a combination of p27 antigen and proviral PCR testing should be undertaken in all cases.

FeLV proviral PCR testing was chosen as the gold standard as it can detect very low levels of nucleic acid from progressive and regressive infections [10,13]. Nevertheless, PCR testing is not without its challenges. Since it is able to detect as little as 1 to 10 copies of provirus in a given sample extreme care must be taken in the laboratory to avoid DNA contamination and false-positive results [39], while primer design must allow detection of FeLV −A, −B and −C sequences to ensure FeLV-infected cats do not test provirus PCR-negative [19,21]. It is possible in the current study that some false-positive p27 results occurred in truly infected cats with different LTR sequences to the primers chosen. Future studies could consider repeating proviral PCR testing at another facility with a methodologically distinct assay, or performing VI in these cases. Furthermore, it is possible that some FeLV qPCR-negative cats considered to have false-positive p27 results, in particular the 10 cats that returned positive p27 results with multiple kits, may have actually represented false-negative qPCR results owing to focal FeLV infections (FeLV sequestered in non-myeloid cells outside the bone marrow, *e.g.* mammary tissue), as suggested by others [6,40]. To explore this possibility further would require following false-positive cases longitudinally and performing extensive molecular analysis of a variety of tissues at necropsy [41].

It could be argued that it was unreasonable for this study to record a false-negative p27 result using proviral qPCR as the benchmark, thereby lowering test kit sensitivity, when by definition there was no FeLV antigen present to detect *(i.e.* that it was unfair to include regressive infections in the analysis). We contend that the purpose of PoC test kits is to screen for FeLV infection, and therefore the sensitivities reported (Table 5) highlight the inability of test kits to identify a potentially important subcategory of FeLV infection. A recent study also used proviral PCR testing as the diagnostic gold standard and reported comparable sensitivities for SNAP Combo and Anigen Rapid (53% and 40%, respectively) to the current study [10].

The performance of all three FeLV test kits using saliva as the diagnostic specimen was comparable to testing with blood, in disagreement with the 2008 AAFP guidelines [16]. When venipuncture is not possible without skilled physical restraint or sedation, collecting and testing saliva for the presence of FeLV p27 antigen should be considered. In one study, saliva testing was actually considered to have identified seven FeLV-infected cats with FeLV-related disease that tested FeLV-negative with serum; although definitive FeLV diagnosis was not pursued in these cats, it was proposed that the cats were truly infected and the discordant test results were due to viral replication being higher in salivary glands than other tissues such as blood [12]. This potential added benefit of saliva testing was replicated in the current study in one cat with a Witness test kit, using both methods of saliva testing. No false-positive results were recorded with any of the FeLV test kits using saliva, but of concern were 3/17 (18%) false-negative FeLV results that occurred with each kit in progressively infected cats. Although the lower sensitivity of p27 saliva testing compared to p27 blood testing was not statistically significant, we suspect with a larger study the trend for saliva to have increased specificity at the expense of reduced sensitivity (compared with blood) would become further apparent. Until further research is conducted, we remain cautious as to the usefulness of p27 testing using saliva,

and recommend p27 testing using whole blood (with confirmatory qPCR testing) for screening of large numbers of cats (*e.g.* in shelters).

5. Conclusion

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

Acknowledgements

This study was supported financially by the Australian Companion Animal Health Foundation and Boehringer Ingelheim, Australia. We are grateful to the significant in-kind contribution of diagnostic kits by IDEXX Laboratories, Zoetis Animal Health and BioNote, without which this study would not have been possible. Mark Westman was supported by an Australian Postgraduate Award (APA), an Endeavour Research Fellowship, 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 Andrea Harvey provided helpful and insightful comments. Finally, thanks to all the staff at veterinary clinics for assistance with recruiting and sampling and Vetnostics (particularly Doug Hayward) for sending samples.

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.2016.11.014.

References

[1] W.F.H. Jarrett, E.M. Crawford, W.B. Martin, F. Davie, Leukaemia in the cat: a virus-like particle associated with leukaemia (lymphosarcoma), Nature 202 (4932) (1964) 567–568.
[2] K. Hartmann, Clinical aspects of feline retroviruses: a review, Viruses 4 (11) (2012) 2684–2710.
[3] H. Lutz, D. Addie, S. Belák, C. Boucraut-Baralon, H. Egberink, T. Frymus, T. Gruffydd-Jones, K. Hartmann, M.J. Hosie, A. Lloret, F. Marsilio, M.G. Pennisi, A.D. Radford, E. Thiry, U. Truyen, M.C. Horzinek, Feline leukaemia ABCD guidelines on prevention and management, J. Feline Med. Surg. 11 (7) (2009) 565–574.
[4] T. Englert, H. Lutz, C. Sauter-Louis, K. Hartmann, Survey of the feline leukemia virus infection status of cats in Southern Germany, J. Feline Med. Surg. 14 (6) (2012) 392–398.
[5] N.A. Lopez, R.H. Jacobson, J.M. Scarlett, S.A. Center, J.F. Randolph, F.W. Scott, Sensitivity and specificity of blood test kits for feline leukemia virus antigen, J. Am. Vet. Med. Assoc. 195 (6) (1989) 747–751.
[6] D.M. Hawks, A.M. Legendre, B.W. Rohrbach, Comparison of 4 test kits for feline leukemia virus antigen, J. Am. Vet. Med. Assoc. 199 (10) (1991) 1373–1377.
[7] K. Hartmann, R.M. Werner, H. Egberink, O. Jarrett, Comparison of six in-house tests for the rapid diagnosis of feline immunodeficiency and feline leukaemia virus infections, Vet. Rec. 149 (11) (2001) 317–320.
[8] A. Robinson, K. DeCann, E. Aitken, T.J. Gruffydd-Jones, A.H. Sparkes, G. Werret, D.A. Harbour, Comparison of a rapid immunomigration test and ELISA for FIV antibody and FeLV antigen testing in cats, Vet. Rec. 142 (18) (1998) 491–492.
[9] K. Hartmann, P. Griessmayr, B. Schulz, C.E. Greene, A.N. Vidyashankar, O. Jarrett, H.F. Egberink, Quality of different in-clinic test systems for feline immunodeficiency virus and feline leukaemia virus infection, J. Feline Med. Surg. 9 (6) (2007) 439–445.
[10] C. Sand, T. Englert, H. Egberink, H. Lutz, K. Hartmann, Evaluation of a new in-clinic test system to detect feline immunodeficiency virus and feline leukemia virus infection, Vet. Clin. Pathol. 39 (2) (2010) 210–214.
[11] D.P. Francis, M. Essex, W.D. Hardy, Excretion of feline leukemia virus by naturally infected pet cats, Nature 269 (5625) (1977) 252–254.
[12] H. Lutz, O. Jarrett, Detection of feline leukemia virus infection in saliva, J. Clin. Microbiol. 25 (5) (1987) 827–831.
[13] M.A. Gomes-Keller, E. Gonczi, R. Tandon, F. Riondato, R. Hofmann-Lehmann, M.L. Meli, H. Lutz, Detection of feline leukemia virus RNA in saliva from naturally infected cats and correlation of PCR results with those of current diagnostic methods, J. Clin. Microbiol. 44 (3) (2006) 916–922.
[14] M.G. Lewis, K.A. Wright, L.J. Lafrado, P.J. Shanker, N.E. Palumbo, E.D. Lemoine, R.G. Olsen, Saliva as a source of feline leukemia virus antigen for diagnosis of disease, J. Clin. Microbiol. 25 (7) (1987) 1320–1322.
[15] N.A. Lopez, J.M. Scarlett, R.V.H. Pollock, R.H. Jacobson, Sensitivity, specificity and predictive values of Clinease-virastat saliva test for feline leukemia virus infection, Cornell Vet. 80 (1) (1990) 75–84.
[16] J. Levy, K. Crawford, S. Hofmann-Lehmann, E. Sundahl, V. Thayer, American Association of Feline Practitioners' feline retrovirus management guidelines, J. Feline Med. Surg. 10 (3) (2008) 300–316, 2008.
[17] M.E. Westman, R. Malik, E. Hall, P.A. Sheehy, 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. 42 (2015) 43–52.
[18] M.E. Westman, R. Malik, E. Hall, J.M. Norris, The protective rate of the feline immunodeficiency virus vaccine: an Australian field study, Vaccine 34 (2016) 4752–4758.
[19] R. Tandon, V. Cattori, M.A. Gomes-Keller, M.L. Meli, M.C. Golder, H. Lutz, R. Hofmann-Lehmann, Quantitation of feline leukaemia virus viral and proviral loads by TaqMan (R) real-time polymerase chain reaction, J. Virol. Methods 130 (1-2) (2005) 124–132.
[20] C.R. Helps, P. Lait, A. Damhuis, U. Bjornehammar, D. Bolta, C. Brovida, L. Chabanne, H. Egberink, G. Ferrand, A. Fontbonne, M.G. Pennisi, I. Gruffydd-Jones, D. Gunn-Moore, K. Hartmann, H. Lutz, E. Malandain, K. Mostl, C. Stengel, D.A. Harbour, E.A.M. Graat, Factors associated with upper respiratory tract disease caused by feline herpesvirus, feline calicivirus, Chlamydophila felis and Bordetella bronchiseptica in cats: experience from 218 European catteries, Vet. Rec. 156 (21) (2005) 669–673.
[21] M.D.G. Pinches, C.R. Helps, T.J. Gruffydd-Jones, K. Egan, O. Jarrett, S. Tasker, Diagnosis of feline leukaemia virus infection by semi-quantitative real-time polymerase chain reaction, J. Feline Med. Surg. 9 (1) (2007) 8–13.
[22] https://www.idexx.com/resource-library/smallanimal/snap-combo-package-insert-en.pdf.
[23] M.E. Westman, R. Malik, E. Hall, J.M. Norris, Diagnosing feline immunodeficiency virus (FIV) infection in FIV-vaccinated and FIV-unvaccinated cats using saliva, Comp. Immun. Microbiol. Infect. Dis. 46 (2016) 66–72.
[24] G.H. Shelton, C.K. Grant, S.M. Cotter, M.B. Gardner, W.D. Hardy Jr., R.F. DiGiacomo, Feline immunodeficiency virus and feline leukemia virus infections and their relationships to lymphoid malignancies in cats: a retrospective study (1968–1988), J. Acquir. Immune. Defic Syndr. 3 (6) (1990) 623–630.
[25] S. Gleich, K. Hartmann, Hematology and serum biochemistry of feline immunodeficiency virus infected and feline leukemia virus infected cats, J. Vet. Intern. Med. 23 (3) (2009) 552–558.
[26] J.A. Beatty, S. Tasker, O. Jarrett, A. Lam, S. Gibson, A. Noe-Nordberg, A. Phillips, A. Fawcett, V.R. Barrs, Markers of feline leukaemia virus infection or exposure in cats from a region of low seroprevalence, J. Feline Med. Surg. 13 (12) (2011) 927–933.
[27] K. Möstl, H. Egberink, D. Addie, T. Frymus, C. Boucraut-Baralon, U. Truyen, K. Hartmann, H. Lutz, T. Gruffydd-Jones, A.D. Radford, A. Lloret, M.G. Pennisi, M.J. Hosie, F. Marsilio, E. Thiry, S. Belák, M.C. Horzinek, Prevention of infectious diseases in cat shelters: ABCD guidelines, J. Feline Med. Surg. 15 (7) (2013) 546–554.
[28] E.C. Hawkins, Saliva and tear tests for feline leukemia virus, J. Am. Vet. Med. Assoc. 199 (10) (1991) 1382–1385.
[29] M.E. Westman, A. Paul, R. Malik, P. McDonagh, M.P. Ward, E. Hall, J.M. Norris, Seroprevalence of feline immunodeficiency virus and feline leukaemia virus in Australia: risk factors for infection and geographical influences (2011–2013), J. Feline Med. Surg. Open Rep. (2016) 2.
[30] N.A. Lopez, R.H. Jacobson, False-positive reactions associated with anti-mouse activity in serotests for feline leukemia virus antigen, J. Am. Vet. Med. Assoc. 195 (6) (1989) 741–746.
[31] J.S. Buch, G.H. Clark, R. Cahill, B. Thatcher, P. Smith, R. Chandrashekar, C.M. Leutenegger, T.P. O'Connor, M.J. Beall, Validation of reference laboratory FeLV p27 antigen ELISA, J. Vet. Diagn. Invest. (2016) (in press).

[32] M.C. Barr, FIV, FeLV, and FIPV: interpretation and misinterpretation of serological test results. Seminars in Veterinary Medicine and Surgery, Small Anim. 11 (3) (1996) 144–153.
[33] B.J. Willett, M.J. Hosie, Feline leukaemia virus: half a century since its discovery, Vet. J. 195 (1) (2013) 16–23.
[34] J.L. Rojko, E.A. Hoover, S.L. Quackenbush, R.G. Olsen, Reactivation of latent feline leukaemia virus infection, Nature 298 (5872) (1982) 385–388.
[35] L.J. Gabor, M.L. Jackson, B. Trask, R. Malik, P.J. Canfield, Feline leukaemia virus status of Australian cats with lymphosarcoma, Aust. Vet. J. 79 (7) (2001) 476–481.
[36] M.L. Jackson, D.M. Haines, S.M. Meric, V. Misra, Feline leukemia virus detection by immunohistochemistry and polymerase chain reaction in formalin-fixed, paraffin-embedded tumor tissue from cats with lymphosarcoma, Can. J. Vet. Res. 57 (4) (1993) 269–276.
[37] S. Nesina, A.K. Helfer-Hungerbuehler, B. Riond, F.S. Boretti, B. Willi, M.L. Meli, P. Grest, R. Hofmann-Lehmann, Retroviral DNA – the silent winner: blood transfusion containing latent feline leukemia provirus causes infection and disease in naive recipient cats, Retrovirology 12 (105) (2015) (21 December 2015)-(21 December 2015).
[38] R. Hofmann-Lehmann, J.B. Huder, S. Gruber, F. Boretti, B. Sigrist, H. Lutz, Feline leukaemia provirus load during the course of experimental infection and in naturally infected cats, J. Gen. Virol. 82 (2001) 1589–1596.
[39] P.C. Crawford, J.K. Levy, New challenges for the diagnosis of feline immunodeficiency virus infection, Vet. Clin. North Am. Small Anim. Pract. 37 (2) (2007) 335–350.
[40] T. Miyazawa, O. Jarrett, Feline leukaemia virus proviral DNA detected by polymerase chain reaction in antigenaemic but non-viraemic ('discordant') cats, Arch. Virol. 142 (2) (1997) 323–332.
[41] A.K. Helfer-Hungerbuehler, S. Widmer, Y. Kessler, B. Riond, F.S. Boretti, P. Grest, H. Lutz, R. Hofmann-Lehmann, Long-term follow up of feline leukemia virus infection and characterization of viral RNA loads using molecular methods in tissues of cats with different infection outcomes, Virus Res. 197 (2015) 137–150.