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Rapid and sensitive insulated isothermal PCR for point-of-need feline leukaemia virus detection

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Abstract

Objectives Feline leukaemia virus (FeLV), a gamma retrovirus, causes diseases of the feline haematopoietic system that are invariably fatal. Rapid and accurate testing at the point-of-need (PON) supports prevention of virus spread and management of clinical disease. This study evaluated the performance of an insulated isothermal PCR (iiPCR) that detects proviral DNA, and a reverse transcription (RT)-iiPCR that detects both viral RNA and proviral DNA, for FeLV detection at the PON.

Methods *Mycoplasma haemofelis*, feline coronavirus, feline herpesvirus, feline calicivirus and feline immunodeficiency virus were used to test analytical specificity. In vitro transcribed RNA, artificial plasmid, FeLV strain American Type Culture Collection VR-719 and a clinical FeLV isolate were used in the analytical sensitivity assays. A retrospective study including 116 clinical plasma and serum samples that had been tested with virus isolation, real-time PCR and ELISA, and a prospective study including 150 clinical plasma and serum samples were implemented to evaluate the clinical performances of the iiPCR-based methods for FeLV detection.

Results Ninety-five percent assay limit of detection was calculated to be 16 RNA and five DNA copies for the RT-iiPCR, and six DNA copies for the iiPCR. Both reactions had analytical sensitivity comparable to a reference real-time PCR (qPCR) and did not detect five non-target feline pathogens. The clinical performance of the RT-iiPCR and iiPCR had 98.82% agreement (kappa[κ] = 0.97) and 100% agreement (κ = 1.0), respectively, with the qPCR (n = 85). The agreement between an automatic nucleic extraction/RT-iiPCR system and virus isolation to detect FeLV in plasma or serum was 95.69% (κ = 0.95) and 98.67% (κ = 0.85) in a retrospective (n = 116) and a prospective (n = 150) study, respectively.

Conclusions and relevance These results suggested that both RT-iiPCR and iiPCR assays can serve as reliable tools for PON FeLV detection.

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Introduction

Feline leukaemia virus (FeLV) is a gamma retrovirus that causes malignant, proliferative and degenerative disorders of the feline haematopoietic system.[1] The most common outcome of FeLV infection is a regressive infection, whereby cats clear infectious virus from the blood but retain proviral DNA in infected cells and their progeny.[2–4] Alternatively, in a progressive infection, FeLV replication goes unchecked, and infectious virus or viral RNA can be recovered persistently from the bloodstream. Progressively infected cats provide the major reservoir of infection, and most die within 3 years from a haematopoietic disorder.[5]

The prevalence of FeLV is in decline worldwide,[6–8] owing to the widespread use of effective vaccines, as

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well as test and removal policies. Accurate and rapid identification of FeLV-infected cats at the point-of-need (PON) can support strategies towards virus eradication. Many diagnostic tests are already available, including immunoassays that detect circulating FeLV p27 antigen, virus isolation (VI) assays that recover circulating infectious virus, and molecular assays that detect either viral RNA (reverse transcription real-time PCR [RT-qPCR]) or integrated proviral DNA (qPCR).

The p27 immunoassays are predominantly used as rapid screening tests at PON. However, given the declining prevalence of FeLV and consequent increase in false-positive results, and the significance of a positive result, it is currently recommended that antigen-positive samples are retested using a second method.[9,10] This can delay decision-making, with welfare and cost implications, particularly for shelters. VI, the gold standard in FeLV infection diagnosis, is not readily available to private practitioners; furthermore, the test is laborious with long turnaround times. Increasingly, sensitive molecular assays (RT-qPCR and qPCR) are viewed as practical alternatives to antigen and VI testing.[3,9,11,12] RT-qPCR assays detect viral RNA, which correlates well with viraemia and progressive infection,[9] whereas qPCR assays detect proviral DNA that has integrated into the genome of infected cells.[11,13] Both progressively and regressively infected cats harbour proviral DNA, but quantitative assays can assist in distinguishing between these populations.[3] Molecular assays have been recommended for the detection of FeLV-exposed cats, to investigate obscure clinical cases and to screen potential blood-donor cats.[3,9–12] However, RT-qPCR and qPCR assays are currently restricted to commercial diagnostic laboratories owing to their requirement for sophisticated instruments and skilled technicians. A rapid, affordable and user-friendly platform is needed for PON detection of FeLV viral RNA or proviral DNA.

Convective PCR or insulated isothermal PCR (iiPCR) represents a new generation of PCR technologies.[14–17] The methodology is simple, cost-effective and rapid, with the potential to be field-deployable. One iiPCR system, the portable POCKIT Nucleic Acid Analyzer (GeneReach USA), is commercially available. This device is equipped with a simple heating source to establish natural thermal convection in a capillary tube. A hydrolysis probe (eg, a TaqMan probe) generates optical signals that are converted to S/N ratios ($signal_{after}/signal_{before}$) and automatically reported as positive/negative results on a digital screen by a data-pr°Cessing module. Accumulated evidence is available to support the assertion that the clinical performance of the POCKIT system is comparable to that of various laboratory qPCR or nested PCR assays for a number of veterinary-significant pathogens, including feline immunodeficiency virus (FIV), canine parvovirus, canine distemper virus, equine influenza virus H3N8 subtype, classical swine fever virus and blue tongue virus.[18–23]

Taking advantage of the POCKIT system, a commercial RT-iiPCR that detects both viral RNA and proviral DNA (POCKIT FeLV Reagent Set; GeneReach USA), and an iiPCR that only detects proviral DNA (POCKIT FeLV Reagent Set [-RT]; GeneReach USA) have been developed to help PON identification of FeLV-exposed cats. Field-deployable manual and automatic nucleic acid extraction methods are available to work with the POCKIT system for PON applications (PetNAD Nucleic Acid Co-prep kit and taco mini Nucleic Acid Automatic Extraction System [taco mini], respectively [GeneReach USA]).

This study evaluated the analytical sensitivity and specificity of the FeLV RT-iiPCR and iiPCR reagent sets. Their diagnostic performance was verified by comparison with the routine methods, a proviral qPCR assay and VI, used in two diagnostic laboratories, one in the USA and one in the UK, respectively, with l°Cally collected clinical samples. The performance of the automated nucleic acid extraction system in combination with the RT-iiPCR for FeLV nucleic acid detection was also evaluated.

Materials and methods

Sample collection

FeLV VR-719 strain (ST-FeLV VR-719, American Type Culture Collection [ATCC]) and a FeLV-A isolated in Crandell-Rees feline kidney cells (CRFK CCL-94; ATCC) from EDTA whole blood in the Clinical Virology Laboratory, University of Tennessee Veterinary Medical Center (UTVMC), Knoxville, TN, USA, were used for sensitivity testing. *Mycoplasma haemofelis* DNA (Clinical Immunology Laboratory, University of Tennessee), feline coronavirus (FCoV; WSU 79-1683, ATCC VR-989 [ATCC]), feline herpesvirus (FHV; FVR-SGE [US Department of Agriculture, National Veterinary Services Laboratory]), feline calicivirus (FCV; F-9 strain, VR-782 [ATCC]) and FIV (NCSU VR-2333 [ATCC]) were used for specificity testing. In addition, 85 clinical samples (blood in EDTA) submitted to the UTVMC for FeLV testing were used for functionality testing.

Residual plasma or serum from clinical samples submitted to Veterinary Diagnostic Services, University of Glasgow (VDS-UG), UK, by veterinary practitioners for FeLV VI testing were included in the retrospective study. The 116 samples (52 VI-positive and 64 VI-negative) had all been tested previously using VI, FeLV proviral qPCR, and an in-house p27 antigen ELISA (described below). For the prospective study, all whole-blood EDTA and heparinised plasma or serum samples submitted to VDS-UG for routine diagnostic testing during the study period were eligible for inclusion, provided sufficient residual volumes were available. A total of 150 samples were collected over a period of 6 months in 2015.

Table 1 Primers and probes used in the reference real-time PCR assay

Primers/probes	Sequence (5′-3′)	Location (nucleotide)*
U3-exo-F	AACAGCAGAAGTTTCAAGGCC	8151–8171
U3-exo-R	TTATAGCAGAAAGCGCGCG	8281–8263
U3-P	CCAGCAGTCTCCAGGCTCCCCA	8176–8197

*Nucleotides were numbered based on GenBank accession number KP728112.1

Virus isolation

QN10 cells, a clone of feline fibroblasts into which Moloney murine sarcoma provirus had been introduced,[24] were routinely used for FeLV VI at VDS-UG. A sub-confluent layer of QN1O cells in a 12-well plate was in°Culated with 200 µl heparinised plasma or serum. The in°Culum was absorbed for 2 h then replaced with fresh tissue culture medium (Dulbecco's modified Eagle medium containing glutamine, sodium pyruvate and penicillin-streptomycin [Life Technologies]). The cells were examined for characteristic cytopathic effects daily for 4–6 days; negative cells were sub-cultured until day 15. Positive (whole infectious virus) and negative (tissue culture medium) controls were included on each plate.

Plasmid DNA and in vitro transcribed RNA preparation

A plasmid containing a fragment of the U3 long terminal repeat (LTR) region of FeLV was used to evaluate analytical sensitivity of the assays and to generate the RNA templates by in vitro transcription (IVT) using the MAXIscript T7 kit (Ambion). For IVT RNA, residual DNA was removed using the Ambion Turbo DNA-free kit (Applied Biosystems). Concentration of DNA and RNA was measured using a spectrophotometer (NanoDrop 2000; Thermo Scientific). Single-use aliquots were stored at –80°C. Serial dilutions were made in 40 ng/µl yeast tRNA.

Nucleic acid extraction

For all tests at UTVMC and the proviral qPCR at VDS-UG, DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's prot°Col. Both sites extracted nucleic acid from 200 µl buffy coat obtained from EDTA blood. Elution was performed with 100 µl nuclease-free water at UTVMC and with 70 µl AE buffer at VDS-UG, as per proprietary prot°Cols.

For nucleic acid extraction on the taco mini using the taco DNA/RNA Extraction Kit (GeneReach USA), 150 µl of heparinised plasma or serum was subjected to the extraction steps as described in the manufacturer's manual. Total nucleic acids were eluted in 200 µl of elution buffer, and either tested immediately or stored at –80°C.

FeLV real-time PCR

The proviral qPCR described by Tandon et al (Table 1)[25] was used routinely for the detection of FeLV proviral DNA at both UTVMC and VDS-UG. The 25 µl reaction was run on a real-time thermal cycler at UTVMC (Smart Cycler 2; Cepheid). The programme included an initial denaturation step at 95°C for 10 mins, followed by 45 cycles of 95°C for 15 s and 60°C for 35 s. At VDS-UG, a 20 µl reaction was run on a different real-time thermal cycler (ABI 7500; Applied Biosystems); the programme ran for an initial 2 mins at 50°C and 10 mins at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

FeLV RT-iiPCR and iiPCR reagents

The FeLV RT-iiPCR and iiPCR reagent sets (POCKIT FeLV Reagent Set and POCKIT FeLV Reagent Set [-RT], respectively) were designed to target a well-conserved FeLV U3 LTR region and exclude all known FeLV-like endogenous retroviral sequences. Briefly, the lyophilised premix was rehydrated with 50 µl Premix Buffer B before a 5 µl nucleic acid sample was added. Subsequently, 50 µl of the final mixture was transferred to an R-tube, which was spun briefly in a mini-centrifuge (Cubee; GeneReach USA) and placed into a POCKIT device. The turnaround time was less than 1 h. With the default programme of POCKIT, the results were shown as '+' (positive) or '–' (negative). For each run, both positive (manufacturer supplied) and negative (PCR-grade water) template controls were used.

p27 antigen ELISA

In the double antibody sandwich ELISA,[26] micro-ELISA plates were coated with two custom-made anti-p27 mon°Clonal antibodies that recognised distinct epitopes. A biotinylated anti-p27 polyclonal antibody (Aviva Systems Biology) was then added to the plates, followed by clinical samples. These were mixed and incubated for 45 mins at 37°C, followed by an incubation for 30 mins with phosphatase-labelled streptavidin conjugate (Sigma-Aldrich). A final 30 min incubation with phosphatase substrate (Sigma-Aldrich) preceded quenching of the phosphatase activity with sodium hydroxide (Sigma-Aldrich). Absorbance was measured using a spectrophotometer (Multiskan microplate reader; Thermo Scientific). High and low positive samples, as well as a negative sample were included as controls; a sample was considered positive when the absorbance was equal to, or exceeded, 150% of the negative control value.

Table 2 Analytical sensitivity of the feline leukaemia virus reverse transcription insulated isothermal PCR (RT-iiPCR) and iiPCR reagent sets with in vitro transcribed RNA (IVT RNA) and/or plasmid DNA

	Template	Copies/ reaction	No. positive/ No. tested	Rate (%)
iiPCR	Plasmid DNA	100	8/8	100
		50	8/8	100
		20	20/20	100
		5	19/20	95
		0	0/8	0
RT-iiPCR	IVT RNA	1000	21/21	100
		100	21/21	100
		10	9/21	43
		0	0/9	0
	Plasmid DNA	50	20/20	100
		20	20/20	100
		5	19/20	95
		0	0/10	0

Statistical analysis

Statistical probit analysis, a non-linear regression model, was performed to determine the limit of detection at 95% ($LOD_{95\%}$); that is, the lowest target concentration that could be detected with 95% confidence interval (CI), by using commercial software SPSS 14.0 (IBM). The 2 × 2 contingency tables were analysed by kappa (κ) statistic using SPSS, to determine the inter-rater agreement.

Results

Analytic specificity of the FeLV RT-iiPCR and iiPCR reagent sets

Analytic specificity of the FeLV RT-iiPCR and iiPCR reagent sets was assessed by testing significant feline pathogens (*M haemofelis*, FCoV, FHV, FCV and FIV). Neither reagent sets reacted with any members in the test panel.

Analytical sensitivity of the FeLV iiPCR reagent set

The FeLV iiPCR was designed to detect FeLV proviral DNA exclusively. Its analytical sensitivity was determined using serial dilutions of the plasmid DNA containing the target region. Positive signals were obtained from 100%, 100%, 100%, 95% and 0% of the 100, 50, 20, 5 and 0 copy reactions, respectively (Table 2). $LOD_{95\%}$ was six copies per reaction. Sensitivity of this reagent set was also compared with the reference qPCR assay,[25] which also targeted the U3 LTR region. Ten-fold serial dilutions of nucleic acids prepared from cells infected with the ATCC VR-719 strain and the FeLV-A clinical isolate were tested. The detection limit of the proviral qPCR was found at a dilution of 10^{-3} for both the VR-719 strain (cycle threshold [Ct] = 36.12 ± 0.454) and the type A isolate (Ct = 36.25 ± 0.064). Similarly, the iiPCR reached the detection limit at 10^{-2} and 10^{-3} dilutions with the VR-719 strain and the type A isolate (Table 3), respectively, indicating that the FeLV iiPCR was as sensitive as the qPCR in detecting proviral DNA.

Analytic sensitivity of the FeLV RT-iiPCR reagent set

The FeLV RT-iiPCR could detect both viral RNA and proviral DNA. The analytical sensitivity of the FeLV RT-iiPCR for RNA and DNA was evaluated with a dilution series of the IVT RNA or plasmid containing the target sequence, respectively. Positive signals were obtained from 100%, 100%, 43% and 0% of the reactions containing 1000, 100, 10 and 0 copies of IVT RNA, respectively (Table 2). In addition, with plasmid DNA, 100%, 100%, 95% and 0% of the reactions containing 50, 20, 5 and 0 copies, respectively, were positive. Accordingly, $LOD_{95\%}$ was 16 copies of RNA and five copies of DNA per reaction. Additionally, the sensitivity of the reagent set was compared with the proviral qPCR assay by testing replicates of 10-fold dilutions of nucleic acids from cells infected with either the FeLV ATCC VR-719 strain or the FeLV-A isolate. Detection endpoints of the RT-iiPCR and qPCR were found to be 10^{-2} and 10^{-3} dilutions for the ATCC VR-719 strain, respectively. With the FeLV-A isolate, the end point of both assays was the 10^{-3} dilution (Table 3). The results indicated that the FeLV RT-iiPCR was comparable with the qPCR in detecting FeLV nucleic acids.

Clinical performance of the FeLV iiPCR reagent set

Like the reference qPCR, the FeLV iiPCR could only detect proviral DNA. Clinical performance of this reagent set was compared side by side with the proviral qPCR routinely used at the UTVMC laboratory using 85 clinical samples collected l°Cally. Nucleic acids extracted from buffy coat in EDTA blood using the QIAamp DNA Mini Kit were analysed by the reference qPCR and the iiPCR methods in parallel. Out of 85 samples tested, 32 tested positive and 53 negative by the qPCR (Table 4). All qPCR-negative samples also tested negative (53/53) by iiPCR, and 1/32 qPCR-positive samples (Ct = 35.23) generated negative signals in the iiPCR reaction. Analysis of the 2 × 2 contingency table demonstrated high agreement (98.82%, 95% CI 95.03–100; κ = 0.97) between the two methods for the detection of FeLV proviral DNA in feline blood.

Clinical performance of the FeLV RT-iiPCR regent set

The FeLV RT-iiPCR could amplify both FeLV RNA and proviral DNA, enabling detection of FeLV-infected animals at all clinical stages. Clinical performance of this reagent set was compared with the proviral qPCR at UTVMC in the USA and VI at VDS-UG in the UK. The study at UTVMC included the same 85 clinical samples

Table 3 Evaluation of analytical sensitivity of the feline leukaemia virus (FeLV) insulated isothermal PCR (iiPCR) and reverse transcription (RT)-iiPCR reagent sets using nucleic acids prepared from FeLV-infected cells

Virus	Dilution (log10)	iiPCR			RT-iiPCR			qPCR (Ct)		
FeLV ATCC VR-719 strain	1	+	+	+	ND	ND	ND	28.74	28.54	28.46
	2	+	+	+	+	+	+	32.02	32.86	32.32
	3	–	–	–	+	+	–	**36.48**	**35.61**	**36.27**
	4	–	–	–	–	–	–	NEG	NEG	NEG
	5	–	–	–	–	–	–	NEG	NEG	NEG
Type A FeLV isolate	1	+	ND	ND	+	ND	ND	27.57	27.52	27.38
	2	+	+	+	+	+	+	31.88	32.31	32.30
	3	+	+	+	+	+	+	**36.28**	**36.30**	**36.18**
	4	+	–	–	+	–	+	NEG	NEG	NEG
	5	–	–	–	–	–	–	NEG	NEG	NEG

Bold values indicate the dilution end points where all results were positive
ATCC = American Type Culture Collection; ND = not done; NEG = negative; Ct = cycle threshold; qPCR = real-time PCR

Table 4 Performance evaluation of the insulated isothermal PCR (iiPCR) to detect feline leukaemia virus in clinical samples: comparison with reference real-time PCR using feline blood samples collected in the USA

		qPCR		
		Positive	Negative	Total
iiPCR	Positive	31	0	31
	Negative	1	53	54
	Total	32	53	85

Table 5 Performance evaluation of the reverse transcription-insulated isothermal PCR (RT-iiPCR) to detect feline leukaemia virus in clinical samples: comparison with reference real-time PCR using feline blood samples collected in the USA

		qPCR		
		Positive	Negative	Total
RT-iiPCR	Positive	32	0	32
	Negative	0	53	53
	Total	32	53	85

used above in the FeLV proviral iiPCR test. The qPCR reacted positively and negatively with 32 and 53 of the samples, respectively. Results of the RT-iiPCR and the qPCR matched completely (100% agreement, 95% CI 96.91–100, $\kappa = 1.0$ [Table 5]).

At VDS-UG, the clinical performance of the combination of taco mini with the FeLV RT-iiPCR (taco mini/RT-iiPCR system) was verified. VI was performed routinely for the diagnosis of FeLV viraemia at VDS-UG. The FeLV proviral qPCR and p27 antigen ELISA tests were also employed frequently to determine the clinical stage of FeLV infection.[25,26] Initially, the retrospective study was performed using 116 feline heparinised plasma or serum samples previously tested by VI, ELISA and/or the QIAamp DNA Mini/qPCR system. Among them, 52 had previously tested positive and 64 negative by VI. The taco mini/RT-iiPCR system reacted positively with 51/52 VI-positive samples and 4/64 VI-negative samples (Table 6). Three out of four VI-negative samples were positive by both the taco mini/RT-iiPCR and QIAamp DNA Mini/qPCR systems (Ct = 20, 20, 29, respectively), and all three were p27 antigen positive on ELISA (data not shown). One VI-negative sample tested positive on the taco mini/RT-iiPCR test and on the QIAamp DNA Mini/qPCR systems (Ct = 32) but was negative on the p27 antigen ELISA. The sample that tested negative on the taco mini/RT-iiPCR but positive on VI was also p27 antigen positive. qPCR was not carried out on this sample, and insufficient samples remained for further experimental analysis. The agreement between the taco mini/RT-iiPCR system and VI was 95.69% (95% CI 91.44–99.93; $\kappa = 0.95$). Compared with VI, this method had a sensitivity of 98.08% (95% CI 92.03–100) and a specificity of 93.75% (95% CI 86.84–100).

In the prospective study, 150 samples collected from UK cats within 6 months in 2015 were subjected to the taco mini/RT-iiPCR system, VI and the QIAamp DNA Mini/qPCR system in parallel. In total, six samples were positive and 144 negative by VI (Table 7), indicating that the prevalence of FeLV infection in the study region was likely relatively low. All six VI-positive and two VI-negative samples were positive by the taco mini/RT-iiPCR system; one of the VI-negative samples gave positive results with both PCR-based methods (Ct = 27) and the p27 ELISA at VDS-UG (number 7; Table 8). The second VI-negative sample tested negative on qPCR and p27 ELISA, and subsequently tested negative on POCKIT on repeat testing (number 12; Table 8). Compared with VI, the taco mini/RT-iiPCR system had a sensitivity of 100% (95% CI 68.29–100) and a specificity of 98.61% (95%

Table 6 Performance evaluation of the feline leukaemia virus (FeLV) reverse transcription-insulated isothermal PCR (RT-iiPCR) to detect FeLV in clinical samples: comparison with virus isolation (VI) using retrospective feline blood samples collected in the UK

		VI		
		Positive	Negative	Total
RT-iiPCR	Positive	51	4	55
	Negative	1	60	61
	Total	52	64	116

Table 7 Performance evaluation of the feline leukaemia virus (FeLV) reverse transcription-insulated isothermal PCR (RT-iiPCR) to detect FeLV in clinical samples: comparison with virus isolation (VI) using prospective feline blood samples collected in the UK

		VI		
		Positive	Negative	Total
RT-iiPCR	Positive	6	2	8
	Negative	0	142	142
	Total	6	144	150

Table 8 Comparison of the results from 12 prospective samples that tested positive on at least one method

	1	2	3	4	5	6	7	8	9	10	11	12
VDS-UG qPCR	Ct 20	Ct 21	Ct 22	Ct 19	Ct 24	Ct 21	Ct 27	Ct 32	Ct 36	Ct 33	Ct 38	NEG
VI	POS	POS	POS	POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG
RT-iiPCR	POS	POS	POS	POS	POS	POS	POS	NEG	NEG	NEG	NEG	POS/NEG*

VDS-UG = Veterinary Diagnostic Services, University of Glasgow; qPCR = real-time PCR; RT-iiPCR = reverse transcription-insulated isothermal PCR; NEG = negative; VI = virus isolation; POS = positive; Ct = cycle threshold
*This sample tested negative on repeat testing by RT-iiPCR

CI 95.99–100). The two methods had an agreement of 98.67% (95% CI 96.14–100%; $\kappa = 0.85$).

Discussion

Both FeLV iiPCR and RT-iiPCR performed similarly to the reference qPCR and VI when testing clinical samples, with few discrepancies. Three retrospective and one prospective VI-negative samples tested at VDS-UG were positive by both the taco mini/RT-iiPCR and QIAamp DNA Mini/qPCR systems (Ct = 20, 20, 29, 27, respectively), and were p27 antigen positive on ELISA. In the VDS-UG laboratory and other diagnostic laboratories,[27] low qPCR Ct values are consistent with viraemia. Additionally, in the VDS-UG laboratory, provirus-positive samples that are antigenaemic have a high probability of viraemia (data not shown). As all four samples were both provirus and p27 antigen positive, it is likely that the VI results are false negative. Although very sensitive, VI is still dependent on the successful recovery of live virus from blood samples, which may fail owing to non-viable virus or undetectable viral loads. Results from a fourth retrospective sample that tested negative on VI and p27 antigen ELISA, but positive on RT-iiPCR and qPCR (Ct = 32), suggests that FeLV virions were not actively produced from the proviral DNA; that is, this was a regressive infection. At VDS-UG, one retrospective VI-positive sample was unexpectedly negative by the taco mini/RT-iiPCR system. One possibility may be poor quality or degenerate nucleic acid; RNA is particularly fragile once extracted. Alternatively, the presence of sample inhibitors may have interfered with the reaction, or the viral loads were relatively low which may lead to <100% positive results in repeat tests.

One retrospective sample had discrepant results between the iiPCR (negative) and the proviral qPCR (positive, Ct = 35) at UTVMC (Table 4), while four prospective samples were all negative on the taco mini/RT-iiPCR system and VI but positive using the QIAamp DNA Mini/qPCR system (Ct 32, Ct 33, Ct 36, Ct 38; numbers 8–11, Table 8) at VDS-UG. This interesting finding may reflect the testing of diverse blood fractions in this study. The VDS-UG qPCR screened cells within the buffy coat for proviral DNA, whereas both VI and RT-iiPCR assays tested plasma or serum for whole infectious virions or viral RNA. The detection of proviral DNA at high Ct levels, and the failure to detect viral RNA or infectious virus in plasma or serum suggest that all four cats were regressively infected.[4] This might suggest that using the RT-iiPCR to test different blood fractions may assist in distinguishing progressively and regressively infected cats; that is, testing serum for viral RNA and testing buffy coat for both viral RNA and proviral DNA. A positive result on serum would suggest progressive infection. However, in the retrospective study, one cat with a suspected regressive infection tested positive using the RT-iiPCR on plasma or serum, so this may not be absolutely reliable.

It is highly recommended that FeLV infection status is determined before introduction of cats into group housing, before administration of the FeLV vaccine, and before blood donation, to help mitigate the spread of FeLV. On-site sensitive testing prior to blood transfusion could help reduce unintentional FeLV transmission.[28] Timely isolation or removal of a FeLV-infected cat in a multi-cat household, shelter or boarding kennel environment is also important for FeLV infection

control. It is increasingly recognised that it may be important to identify all FeLV-exposed cats irrespective of their infection status. In regions where FeLV prevalence is low, identifying all FeLV-exposed cats may be an important step towards FeLV eradication. Although regressively infected cats appear to have a low risk of developing FeLV-related disease,[29–31] and were previously not believed to pose a significant infection risk to healthy cats,[32,33] recent studies have concluded that regressive infections can be reactivated to resume virus replication, shedding and disease.[2,34]

Although rapid p27 antigen immunoassays can serve as the initial screening test at patient-side, they cannot identify cats with regressive infection, and a portion of p27-negative cats are regressively infected.[11,35] In addition, FeLV RNA could be detected in the circulation or in tissues of some regressively infected cats, even in the absence of detectable proviral DNA.[2,34,36] In general, PCR methods are more sensitive and specific than antigen immunoassays.[2] Therefore, the field-deployable FeLV RT-iiPCR of the POCKIT system, with excellent sensitivity for both RNA and DNA template, is the recommended method to identify all FeLV-exposed cats at patient-side; but the preferred sample is buffy coat. Testing plasma or serum with the RT-iiPCR may exclude a low number of regressively infected cats, whereas the clinical performance of this assay had excellent agreement with the reference method when buffy coat was tested (Table 4). The costs of the POCKIT Micro Plus device and the reagent, US$2000 and US$8, respectively, shall allow cost-effective on-site screening of FeLV-exposed animals even for shelter settings to facilitate eradication of FeLV infection from the community in the long run.

Integrated FeLV proviral DNA cannot be eliminated by the host's immune system;[37] therefore, both progressively and regressively infected cat populations will test positive for proviral DNA using the iiPCR reagent set. Unlike quantitative proviral qPCR assays, the iiPCR cannot be used to differentiate between these cat populations.

However, the proviral iiPCR could potentially work with a rapid p27 immunoassay as a suitable patient-side tool to aid FeLV infection confirmation and diagnosis, given that this study demonstrated an analytical and clinical performance comparable to that of the proviral qPCR. The combination of a proviral PCR method and a p27 antigen immunoassay have been shown to achieve 100% diagnostic sensitivity.[2,3,13,24] For instance, p27-positive and proviral DNA (iiPCR-positive results) suggest a progressive infection; p27-negative and the iiPCR-positive results suggest regressive infection with the potential for future viral reactivation. In addition, screening with a rapid p27 antigen immunoassay alone carries the risk of false-positive results owing to cross-reactivity (this is exacerbated in populations of low FeLV prevalence) and any positive antigen results from stand-alone rapid p27 antigen immunoassays could be followed up with the same p27 antigen immunoassay and the proviral iiPCR at PON, as suggested above.[9,10]

Providing automatic interpretation of signals from the fluorescent probe, the POCKIT method is sufficiently user-friendly to be run by animal shelter personnel or clinical technical staff with basic training. There is no requirement to open the reaction tubes at any stage, significantly reducing the risks of carry-over contamination of PCR amplicons. The POCKIT (throughput 8) and its hand-held version POCKIT Micro Plus (throughput 4; GeneReach USA), are small, lightweight and robust, and could be operated with a rechargeable battery or car battery in low-resource settings. The reagent sets are offered in a lyophilised format to allow easy shipping and storage. Moreover, the field-deployable automatic nucleic acid extraction device helps simplify the nucleic acid preparation step. The taco mini/iiPCR system allows the users to obtain results from clinical samples in <2 h. For resource-limited settings, a manual nucleic acid extraction method (PetNAD Nucleic Acid Co-prep Kit) is also available, but may be less suitable in a PON setting given the requirement for appropriate technical competency and prolonged hands-on time.

Conclusions

With performances similar to VI and the proviral qPCR, both RT-iiPCR (detecting both viral RNA and DNA) and proviral iiPCR/POCKIT systems could help rule out prior exposure to FeLV at PON. This would facilitate timely identification, care and placement of FeLV-infected animals in facilities such as veterinary clinics, shelters and customs inspection at ports of entry.

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Conflict of interest Pei-Yu A Lee, Yun-Long Tsai, Fu-Chun Lee, Hsiao-Fen G Chang and Hwa-Tang T Wang are employed by GeneReach USA, Lexington, MA, USA. However, this work does not alter our adherence to all the *Journal of Feline Medicine and Surgery*'s policies on sharing data and materials.

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