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Development and application of a quantitative real-time PCR assay to detect feline leukemia virus RNA

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Abstract

We previously defined four categories of FeLV infection, designated as abortive, regressive, latent, and progressive. To determine if detectable viral DNA is transcriptionally active in the absence of antigenemia, we developed and validated a real-time viral RNA qPCR assay. This assay proved to be highly sensitive, specific, reproducible, and allowed reliable quantitation. We then applied this methodology, together with real-time DNA qPCR and p27 capsid antigen capture ELISA, to examine cats challenged with FeLV. We found that circulating viral RNA and DNA levels were highly correlated and the assays were almost in perfect agreement. This indicates that the vast majority of viral DNA is transcriptionally active, even in the absence of antigenemia. The real-time qPCR assays are more sensitive than the most commonly used FeLV diagnostic assay, the p27 capsid antigen capture ELISA. Application of qPCR assays may add greater depth in understanding of FeLV-host relationships.

Keywords

FeLV; real time PCR; immune response; latent

Introduction

Feline leukemia virus (FeLV) was first identified as a naturally occurring viral infection of cats more than 40 years ago by electron microscopy (EM) (Jarrett et al., 1964a; Jarrett et al., 1964b). Since that time, advances in sensitivity, specificity, and speed of FeLV diagnosis have prompted greater insight into the complexity in the FeLV-host relationship. The clone 81 cell line used to detect infectious virus (VI) by focus formation (Fischinger et al., 1974; de Noronha et al., 1977) and the direct immunofluorescent antibody (IFA) assay used to detect intracellular

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Conflict of Interest Statement

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled "Development and application of a quantitative real-time PCR assay to detect feline leukemia virus RNA".

gag proteins (Hardy et al., 1973; Hardy and Zuckerman, 1991a) provided the first major insights into FeLV detection and led to recognition of progressive and regressive host-virus relationships (Hoover et al., 1981). Development of an antigen capture ELISA using monoclonal antibodies directed against different epitopes of the p27 capsid protein (Lutz et al., 1983a) provided a rapid and sensitive diagnostic assay applicable to testing animals on site at low cost.

Use of the VI, IFA, and ELISA assays in combination led to recognition of cats with 'discordant' results (Lutz et al., 1980; Jarrett et al., 1982; Lutz et al., 1983b; Hardy and Zuckerman, 1991b), which in turn provided the first indication of more complex host-virus relationships among FeLV infections (Hoover and Mullins, 1991). With the advent of molecular diagnostics, conventional PCR to detect FeLV DNA was employed in attempt to better understand these discrepant results (Jackson et al., 1996; Miyazawa and Jarrett, 1997; Herring et al., 2001), but the assay did not appear to have increased sensitivity.

We, and other investigators, developed a quantitative real-time FeLV DNA PCR assay (qPCR) as means to both diagnose FeLV infection and seek further insight into the determinants of the virus-host relationship (Hofmann-Lehmann et al., 2001; Flynn et al., 2002; Tandon et al., 2005; Torres et al., 2005). When we applied DNA qPCR, together with the antigen capture ELISA, in vaccinated and unvaccinated cats challenged with FeLV-61E-A, we identified a spectrum of host-virus relationships in FeLV-exposed cats which did not develop persistent antigenemia (Torres et al., 2005). Some animals experienced *abortive* infections—i.e. infections marked not only by undetectable antigenemia at all timepoints, but also the absence of infected cells in both circulation and tissues. Because these animals appeared not to maintain a tissue reservoir, it would be impossible to distinguish these animals from those never exposed to FeLV. By contrast, two additional groups of FeLV-exposed cats which did not develop persistent antigenemia were found to maintain either low or moderate levels of infected cells in circulation and tissues—and were designated as having experienced *regressive* or *latent* infections, respectively, depending on whether transient antigenemia was recognized. Finally, cats which developed overt persistent antigenemia with persistent high circulating and tissue viral DNA burdens represented those with *progressive* infection.

The finding of previously covert viral DNA in some cats which ostensibly totally resisted FeLV infection was not restricted to FeLV-61E-A, as other investigators found a similar phenomenon in cats challenged with the FeLV-A/Glasgow-1 (Lehmann et al., 2001; Flynn et al., 2002; Tandon et al., 2005; Cattori et al., 2006; Gomes-Keller et al., 2006a; Gomes-Keller et al., 2006b; Hofmann-Hofmann-Lehmann et al., 2006). It remained unknown, however, whether the viral DNA we detected by our qPCR assay represented intact, replication-competent provirus or replication-defective viral DNA sequences. To determine whether this viral DNA is transcriptionally active in the absence of antigenemia we developed a qPCR assay to quantitate FeLV RNA in feline plasma, the validation and application of this assay is described here.

Materials and methods

2.1. Experimental animals and challenge virus

Forty specific-pathogen-free (SPF) cats were obtained from Harlan Sprague Dawley, Inc. (Mt. Horeb, WI). The cats were randomly housed up to 5 cats per enclosure at Charmany Instructional Facility at the University of Wisconsin-Madison School of Veterinary Medicine (Madison, WI). The animals were housed in accordance with the university animal care and use committee regulations. At 34–35 weeks of age, all cats were challenged intraperitoneally with 200 μ L of 5×10^4 TCID₅₀/mL FeLV-61E-A. Cats were observed twice daily for signs of

illness after virus inoculation. Sample collections were performed on cats anesthetized with an intramuscular administration of ketamine hydrochloride (11 mg/kg).

2.2. Sample collection and processing

Blood samples were collected at challenge and every week thereafter through 8 weeks post-challenge (PC). Whole blood was shipped overnight on ice to Colorado State University (Ft. Collins, CO) where they were immediately processed upon arrival. Buffy coat cell pellets were stored at -70°C until analysis for FeLV DNA by qPCR. Plasma samples were separated into 1 mL aliquots and stored at -70°C until analysis for FeLV RNA by qPCR and FeLV p27 capsid antigen by capture ELISA.

DNA was extracted from buffy coat cells using a QIAamp DNA blood mini kit (QIAGEN, Inc., Valencia, CA), eluted in 100 μL of elution buffer, and DNA concentrations determined spectrophotometrically. RNA was extracted from 140 μL of plasma using a QIAamp viral RNA mini kit (QIAGEN). On-column digestion of DNA during RNA purification was performed using the RNase-free DNase set (QIAGEN). The RNA was eluted in 80 μL of elution buffer.

2.3. Primers and probe for RNA and DNA qPCR assays

We designed a primer/probe set to amplify exogenous and not endogenous FeLV sequences within the U3 region of FeLV-61E-A (Casey et al., 1981; Berry et al., 1988) as previously described (Torres et al., 2005). These primers and probe were used to detect both FeLV RNA and FeLV DNA.

2.4. RNA standard preparation for absolute quantification

The plasmid p61E-FeLV, an *EcoRI* fragment containing the full-length FeLV-61E-A provirus subcloned into pUC18 (Donahue et al., 1988; Overbaugh et al., 1988), was used to construct an RNA standard. This plasmid was provided as ampicillin-resistant transformed *E. coli* JM109 cells through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. James Mullins. The transformed *E. coli* cells were grown on LB media containing 50 $\mu\text{g/mL}$ ampicillin. Plasmid DNA was isolated from the bacterial cells using the QIAfilterTM plasmid midi kit (QIAGEN). The plasmid was then double digested with *EcoRI* and *BglIII*. The 1909 bp fragment was confirmed by agarose gel electrophoresis with GelStar[®] (Cambrex, Corp., East Rutherford, NJ) staining and gel purified using the QIAquick[®] gel extraction kit (QIAGEN). The pGEM[®]-3Z vector (Promega, Corp., Madison, WI) was double digested with *EcoRI* and *BamHI* and the linearized vector, minus 21 bp, was confirmed by agarose gel electrophoresis. The purified 1909 bp fragment from p61E-FeLV was directly ligated into the linearized pGEM[®]-3Z using a Quick LigationTM kit (New England BioLabs, Inc., Ipswich, MA). The constructs were transformed into chemically competent *E. coli* JM109 cells (Promega) and the cells were grown on LB media with 50 $\mu\text{g/mL}$ ampicillin using blue/white screening. Plasmid DNA was isolated from the bacterial cells, double digested with *EcoRI* and *HindIII*, and the insert confirmed by agarose gel electrophoresis. The recombinant plasmid (named pGEM-3Z-61E) was sequenced by Davis Sequencing, Inc. (Davis, CA) to verify the insert orientation and length, and the primer/probe target site within the U3 region.

The pGEM-3Z-61E plasmid was linearized with *HindIII* and purified by the QIAquick[®] gel extraction kit. RNA transcripts (1943 nt) were produced via *in vitro* transcription using the T7 RiboMAXTM express large scale RNA production system (Promega). Residual plasmid DNA was removed using one RQ1 RNase-free DNase (Promega) and two TURBO DNaseTM (Ambion, Inc., Austin, TX) treatments. After each DNase treatment, the resulting RNA transcripts were purified using the MEGAclearTM kit (Ambion). The absence of contaminating DNA template was confirmed by RNA qPCR of the RNA standard with and without the

addition of reverse transcriptase (RT) to the reaction. The RNA standard copy number was calculated from optical density measurements at 260 nm. The RNA standard was diluted to 10^9 copies/ μ L in THE RNA storage solution (Ambion) with 30 ng/ μ L transfer RNA (Sigma-Aldrich, Corp., St. Louis, MO) as a carrier. This RNA stock was aliquoted and frozen immediately at -70°C . Each aliquot was used for making a single-use 10-fold dilution series. The starting quantities of the samples were determined by comparing the threshold cycle (C_T) value of the samples' RNA with the standard curve of the co-amplified standard template RNA.

2.5. Detection of FeLV RNA in plasma by a one-tube qPCR assay

The 25 μ L one-tube reaction consisted of 400 nM of each primer, 80 nM of fluorogenic probe, 12.5 μ L of TaqMan® One-Step RT-PCR Master Mix (Applied Biosystems), 0.625 μ L of MultiScribe™ Reverse Transcriptase and RNase Inhibitor Mix (Applied Biosystems), 2.875 μ L of PCR-grade H_2O , and 5 μ L of sample or RNA standard. The master mix was supplied at a 2X concentration and contained AmpliTaq® Gold DNA Polymerase, dNTPs with dUTP, and optimized buffer components. Reactions were performed in triplicate using an iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Every reaction plate contained a negative control (FeLV-naïve SPF cat RNA), a template control (no RNA, PCR-grade H_2O), and an extraction control (extracted PCR-grade H_2O). Thermal cycling conditions were 30 minutes at 48°C for the RT reaction, 10 minutes at 95°C to activate AmpliTaq® Gold DNA Polymerase and to denature the template cDNA, followed by 40 cycles of 15 seconds at 95°C for denaturation and 60 seconds at 60°C for annealing/extension.

2.6. Analytical specificity and sensitivity of RNA qPCR assay

Following agarose gel electrophoresis confirmation, the 68 bp PCR products from two separate reactions were sequenced to verify analytical specificity. The TOPO TA Cloning® kit (with pCR®2.1-TOPO® vector) (Invitrogen, Corp., Carlsbad, CA) was used for cloning the amplicons prior to sequencing. Briefly, the PCR products were directly ligated into the linearized pCR®2.1-TOPO® vector, the constructs were transformed into One Shot® TOP chemically competent *E. coli* cells (Invitrogen), and the cells grown on LB media with 50 μ g/mL ampicillin using blue/white screening. Plasmid DNA was isolated from the bacterial cells, linearized with *Eco*RI, and the insert confirmed by agarose gel electrophoresis. The cloned inserts were sequenced by Davis Sequencing. The sequences of the PCR products were then aligned with FeLV-61E-A using MacVector™ software version 7.0 for Macintosh, copyright 2000 (Oxford Molecular, Ltd., Madison, WI).

End-point dilution experiments of the RNA standard were performed to assess analytical sensitivity. A dilution series of 500, 100, 50, 10, 5, and 1 copies of the RNA standard, each in triplicate, was tested.

2.7. Amplification efficiency and reproducibility of RNA qPCR assay

To assess amplification efficiencies, serial dilutions (1:10, 1:100, and 1:1000) of plasma RNA from an experimentally FeLV-61E-A-infected cat and of the RNA standard were amplified in triplicate and the difference in the slopes (Δ s) of the regression lines (C_T vs. dilution) was evaluated.

To assess assay reproducibility, dilutions of the RNA standard (5×10^7 , 5×10^6 , and 5×10^5 copies) and of RNA from an experimentally FeLV-61E-A-infected cat (neat, 1:10, and 1:100) were evaluated for within-run and between-run precision. Each dilution was run 10 times within the same reaction plate and between 10 different reaction plates to test the within-run and between-run precision, respectively. The coefficients of variations (CV) of the threshold cycles (C_T) were calculated: $\text{CV}(C_T)$.

2.8. Detection of FeLV DNA in buffy coat cells by qPCR assay

FeLV DNA was detected in buffy coat cells as previously described (Torres et al., 2005).

2.9. Detection of FeLV p27 capsid antigen in plasma by capture ELISA

FeLV p27 capsid antigen was detected in plasma by capture ELISA using the monoclonal antibodies (mAbs) anti-p27 A2 and G3 (Lutz et al., 1983a) (kindly provided by Niels C. Pedersen; University of California, Davis, CA) as previously described with minor modifications (Torres et al., 2005). Briefly, we adjusted the concentration of the primary mAb, G3, to 0.25 µg/well and the secondary horseradish peroxidase-conjugated mAb, A2, to 1:1000. In addition, SERUM was assessed by the IDEXX SNAP® FeLV antigen diagnostic test (IDEXX Laboratories, Inc., Westbrook, ME). In rare instances of incongruous results, the most sensitive finding of the two assays was chosen.

2.10. Statistical analysis

The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the RNA and DNA qPCR assays and between the RNA qPCR assay and the p27 capture ELISA. Pearson correlation coefficients were determined to assess the linear relationship between viral RNA vs. DNA levels and between viral RNA vs. p27 levels. After a Fisher's *r* to *z* transformation, *p* values were obtained. A statistically significant difference between tests was considered to have occurred when a *p* value was < 0.05. Undetectable results by both qPCR assays were corrected to a value of one and then all viral RNA and DNA levels were log transformed. The Pearson correlation coefficient was performed using StatView® version 5.0.1 for Macintosh, copyright 1999 (Abacus Concepts, Inc., Berkeley, CA).

Results

3.1. RNA qPCR specificity

The analytical specificity of the FeLV RNA qPCR assay was confirmed by sequencing two amplicons after agarose gel confirmation (data not shown). Using MacVector™ software, the amplicon sequence from the RNA standard was identical to that of FeLV-61E-A and the amplicon sequence from an FeLV-61E-A-infected cat contained one base mismatch (data not shown). Plasma RNA from FeLV-naïve, SPF cats were consistently negative for FeLV RNA (43/43 samples from 9 cats; data not shown). Consequently, diagnostic specificity was 100% in FeLV-61E-A-infected animals.

3.2. RNA qPCR sensitivity

The analytical sensitivity of the FeLV RNA qPCR assay was assessed in end-point dilution experiments. These studies consistently detected 10 copies and inconsistently detected 5 copies of the RNA standard (data not shown). Negative samples never crossed threshold: negative control (FeLV-naïve SPF cat RNA), template control (no RNA, PCR-grade H₂O), extraction control (extracted PCR-grade H₂O), DNA control (no RT added to RNA standard), and samples containing 1 copy of the RNA standard. All FeLV-61E-A-infected cats that tested positive for p27 capsid antigen also were positive by qPCR (88/88 samples from 19 cats) (Table 1). Thus, diagnostic sensitivity in the animals studied was 100%.

3.3. RNA qPCR linearity

The linear range of the RNA standard curve was evaluated. Amplification of ten-fold serial dilutions starting at 5×10^7 copies and ending at 5×10^1 copies of the RNA standard from 16 independent experiments demonstrated linearity over 6 orders of magnitude, generated a

standard curve correlation coefficient of 0.999, and produced an amplification efficiency (Klein et al., 2001) of 105.8% (Fig. 1).

3.4. RNA qPCR amplification efficiency

The amplification efficiencies of FeLV-61E-A-infected cat RNA and the RNA standard were compared to validate quantification using the RNA standard. Equivalent amplification efficiencies are indicated by regression line slopes (s) with less than 0.1 difference (Δs) (Gut et al., 1999). The observed amplification efficiencies of the target RNA ($s=3.17$, $R^2=0.993$) vs. the RNA standard ($s=3.24$, $R^2=0.997$) had a $\Delta s=0.07$ (Fig. 2). Thus, quantification using the RNA standard was expected to be valid.

3.5. RNA qPCR reproducibility

The within-run and between-run precision of the FeLV RNA qPCR assay was evaluated. Several dilutions of the RNA standard and of FeLV-61E-A-infected cat RNA were amplified 10 times within the same reaction plate and between 10 different reaction plates. The threshold cycle coefficients of variation, $CV(C_T)$, for the within-run precision was 0.93 to 1.81% and the $CV(C_T)$ for the between-run precision was 0.9 to 2.18% (Table 2).

3.6. RNA qPCR agreement and correlation with DNA qPCR and antigen capture ELISA

The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the RNA qPCR assay and the DNA qPCR assay and between the RNA qPCR assay and the p27 capture ELISA (Table 1). The majority of samples tested by RNA and DNA qPCR had identical results; 128 samples from 23 cats were positive for viral RNA and DNA while 112 samples from 19 cats were negative for viral RNA and DNA. However, 22 samples from 9 cats had circulating viral DNA but undetectable viral RNA. Surprisingly, 2 samples from 2 cats were found to be negative by DNA qPCR but positive by RNA qPCR. The kappa statistic was 0.82 indicating an almost perfect agreement between the two tests. All samples that tested positive for p27 capsid antigen were positive for viral RNA (88 samples from 19 cats). All samples with undetectable viral RNA had undetectable antigen (134 samples from 25 cats). In no sample in which viral RNA was undetected was viral antigen detected. However, 42 samples from 22 cats were positive by RNA qPCR and negative by p27 capture. Thus, RNA qPCR had a greater sensitivity than p27 capture ELISA. The kappa statistic was 0.68, indicating a substantial agreement between the two tests.

Pearson correlation coefficients were determined to assess the linear relationship between circulating viral RNA levels, DNA levels, and p27 levels. After a Fisher's r to z transformation, p values were obtained. An extremely strong linear relationship between viral RNA and DNA levels was identified; $r = 0.939$, $p < 0.0001$ (Fig. 3). Surprisingly, the p27 levels correlated more strongly with the viral DNA load ($r = 0.776$, $p < 0.0001$) than with the viral RNA load ($r = 0.428$, $p < 0.0001$) (data not shown).

Discussion

Development of the direct immunofluorescent antibody assay by Hardy et al. (1973; 1991a) to detect FeLV *gag* antigens within leukocytes and platelets on blood films made possible elucidation of the contagious transmission of FeLV. The subsequent antigen capture ELISA of Lutz et al. (1983a) detected extracellular p27 capsid antigen in plasma or sera and made possible the rapid in-house detection of FeLV infection.

Usually results of these assays correlated however, in some animals extracellular (serum, plasma) viral antigen was detected in the absence of detectable antigen in circulating cells and/or infectious virus in plasma (Lutz et al., 1980; Jarrett et al., 1982; Lutz et al., 1983b; Hardy

and Zuckerman, 1991b). Potential reasons for the 'discordant' results could reflect assay sensitivities or specificities, sample handling, and assay expertise. In addition, the basis for discrepancies could lie in the biology of FeLV (Rojko et al., 1979), including antigen detected in plasma before the stage of bone marrow cell infection, presence of latent virus capable of producing antigen but not infectious virus, or virus being produced only in atypical sequestered foci.

Application of qPCR to detect FeLV DNA in conjunction with p27 capsid capture ELISA has resulted in diagnostic sensitivity of 100% (Hofmann-Lehmann et al., 2001; Flynn et al., 2002; Tandon et al., 2005; Torres et al., 2005; Cattori et al., 2006; Gomes-Keller et al., 2006a; Gomes-Keller et al., 2006b; Hofmann-Lehmann et al., 2006). In addition we, and others, found animals in which viral DNA was detected in the absence of detectable antigenemia, demonstrating that qPCR sensitivity was greater than p27 ELISA and that viral DNA burden was an accurate predictor of FeLV infection outcome. However, these DNA qPCR assays did not differentiate unintegrated viral DNA from integrated provirus. Work by Cattori et al. (2006) addressed this issue by designing methods to detect integration of FeLV DNA into the host genome. These investigators demonstrated that proviral integration occurs not only in cats with persistent antigenemia, but also in cats without detectable antigenemia and lower proviral burdens.

The primary purpose of the present work was to develop an assay to determine whether viral DNA detected in non-antigenemic cats represented integrated provirus that was transcriptionally active. We based the RNA qPCR assay on the U3 sequence of FeLV-61E-A (the replication competent, non-acutely pathogenic component of the FeLV-FAIDS complex) (Mullins et al., 1986; Hoover et al., 1987; Donahue et al., 1988; Overbaugh et al., 1988) both to increase the probability of detecting other strains of FeLV-A, owing to the conservation of the U3 region, (Casey et al., 1981; Berry et al., 1988) and to minimize the potential for detection of RNA that may under some circumstances be transcribed from the endogenous *env* sequences present in the feline genome (Busch et al., 1983; McDougall et al., 1994). Our assay proved to be highly sensitive, specific, reproducible, and allowed reliable quantitation. Additionally, qPCR enables a high throughput of samples in a very short amount of time. The detection limit of 5 – 10 viral copies per qPCR reaction corresponds to roughly 575 – 1150 viral copies per mL plasma, comparable with the sensitivity of the RNA qPCR assay described by Tandon and colleagues (Tandon et al., 2005). While we detected viral RNA in all p27-positive cats in our study, Tandon et al. (2005) found viral RNA to be undetectable in 3 of 41 antigenemic cats.

Agreement between the RNA and DNA qPCR assays was very high ($Kappa = 0.82$) with 240 samples from a total of 264 samples having concordant results (either both positive or both negative). In addition, a strong linear relationship between the two assays was identified ($r = 0.939$, $p < 0.0001$). A similar correlation was found by Tandon et al. (2005). Consistent with other investigators, we identified 22 samples which had detectable, albeit low, viral DNA levels and undetectable viral RNA (Tandon et al., 2005; Gomes-Keller et al., 2006a; Gomes-Keller et al., 2006b; Hofmann-Lehmann et al., 2006). Several possible explanations for discordant RNA and DNA qPCR results exist. Most likely is simply that RNA qPCR is less sensitive than DNA qPCR. Viral RNA in plasma may be less stable than viral DNA within cells although the viral envelope should protect FeLV RNA from nucleases. Presuming detectable viral DNA is integrated, it is possible that the provirus is replication-defective or in a transcriptionally silent (latent) state. Because the diagnostic specificity was previously identified at 100%, it is unlikely that these DNA qPCR results are false positives (Torres et al., 2005). Surprisingly, we also identified 2 samples in which low viral RNA levels were detected without detectable viral DNA; one sample was at 1 week and one was at week 8 PC. This phenomenon was also observed by Hofmann-Lehmann (2006). The 1-week PC sample may represent the detection of a primary cell-free viremia whereby the virus is replicating in local lymphoid tissue but

lymphocytes containing viral DNA are not yet circulating. The terminal 8-week PC sample could represent cell-free viremia possibly due to atypical sequestered foci of FeLV infected cells. Because the diagnostic specificity of our RNA qPCR assay was 100%, it is unlikely this sample was falsely positive.

Both the DNA and RNA qPCR assays were more sensitive than the most commonly used clinical FeLV diagnostic assay, the capsid antigen capture ELISA. Although the agreement between RNA qPCR and antigen capture ELISA was substantial (Kappa = 0.68), in 42 samples viral RNA was detected in the absence of antigenemia. In contrast to the experience of Tandon et al. we found the p27 ELISA to be more strongly correlated with DNA qPCR ($r = 0.776$) than with RNA qPCR ($r = 0.428$) (Tandon et al., 2005).

The RNA qPCR results are consistent with the concept that at least part of the detected FeLV DNA detected in previous work is integrated and initiates a transcriptionally active infection, even in the absence of detectable antigenemia. This infers that infectious virus and p27 antigen are produced but below assay sensitivity. However, the clinical relevance of detectable nucleic acids without detectable antigen or infectious virus remains unknown. While we have shown that FeLV-host relationships established by 8 weeks PC can remain unchanged for years, such “latently infected” cats may serve as a risk to susceptible cats. Application of qPCR assays may add greater depth in understanding of FeLV-host relationships.

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Fig. 1.

Linearity and sensitivity of quantitative real-time FeLV RNA PCR. Standard curve of the RNA standard is linear. Amplification of ten-fold serial dilutions between 5×10^7 copies to 5×10^1 copies of the standard demonstrated linearity over six orders of magnitude, generated a standard curve correlation coefficient of 0.9989, and produced an amplification efficiency of 105.8%. Ten copies of the standard are consistently detected. Mean $\pm$ SD for 16 independent experiments are plotted.

Fig. 2.

Amplification efficiency comparison to validate real-time RNA PCR quantification. Serial dilutions (1:10, 1:100, and 1:1000) of plasma RNA from an FeLV-61E-A-infected cat and of the RNA standard were amplified. Amplification efficiencies of the FeLV-positive RNA and the RNA standard were approximately equal ($\Delta s=0.07$) demonstrating that quantification using the RNA standard is expected to be valid. Mean $\pm$ SD are plotted.

Fig. 3.

Correlation of FeLV RNA vs. DNA levels. Plasma and buffy coat cells collected at challenge and weekly thereafter through 8 weeks PC from 40 cats were analyzed for viral RNA and DNA, respectively, by qPCR assays. The Pearson correlation coefficient was determined. Viral RNA levels strongly correlated with viral DNA levels ($r = 0.939$, $p < 0.0001$) thus, a positive linear relationship existed.

Table 1

RNA qPCR vs. DNA qPCR and p27 capsid capture ELISA for FeLV detection

		RNA qPCR		
		(+)	(-)	
DNA qPCR	(+)	128	22	Kappa value = 0.82 (almost perfect agreement)
	(-)	2	112	
p27 ELISA	(+)	88	0	Kappa value = 0.68 (substantial agreement)
	(-)	42	134	

Table 2
RNA qPCR coefficients of variations (%) of within-run and between-run precision.

	RNA standard (copies)			neat	FeLV-positive RNA (dilution)	
	5×10^7	5×10^6	5×10^5		1:10	1:100
CV (C_T) within-run	1.81	1.21	1.44	1.08	1.03	.93
CV (C_T) between-run	1.94	2.13	2.06	2.18	1.19	.90