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Evaluation of a quantitative enzyme-linked immunosorbent assay for feline leukemia virus p27 antigen and comparison to proviral DNA loads by real-time polymerase chain reaction

Melissa J. Beall*, Jesse Buch, Roberta J. Cahill, Genevieve Clark, Jancy Hanscom, Marko Estrada, Christian M. Leutenegger, Ramaswamy Chandrashekar

IDEXX Laboratories, Inc., 1 IDEXX Drive, Westbrook, ME 04092, USA

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ABSTRACT

Feline leukemia virus (FeLV) is an oncogenic retrovirus of cats. While higher viral RNA and proviral DNA loads have been correlated with progressive infections and disease, a similar correlation has been suggested for p27 antigen concentrations. This analytical study compared the results of a quantitative ELISA for p27 antigen with quantitative real-time PCR results for FeLV proviral DNA in patient samples. A significant positive correlation between copies of proviral DNA and the concentration of p27 antigen was identified ($r = 0.761$, $P < 0.0001$). Samples with high proviral DNA loads, at least 1×10^6 copies/mL of whole blood, typically had p27 antigen concentrations greater than 30 ng/mL in plasma. Samples with proviral DNA loads below this level all had concentrations of p27 antigen in plasma that were less than 10 ng/mL. Given this correlation, it is hypothesized that the concentration of p27 antigen at a given point in time may help to indicate the likelihood of a progressive or regressive infection similar to what has been demonstrated for proviral DNA loads.

1. Introduction

Feline leukemia virus (FeLV) infections are heterogeneous in their biologic behavior and can lead to several different outcomes for an infected cat [1]. In some cases, the infection can lead to cytoproliferative diseases with the development of lymphoma and leukemia while in others it can result in cytosuppressive states and associated immune compromise. For some infected cats, those with progressive infections, it may mean persistent viremia and a shorter lifespan due to a greater risk of FeLV-related disease [2,3]. Others, with regressive infections, will lack any evidence of viremia and yet remain infected without any impact on outcome until much later in life [4]. The cat's immune response to the virus has been implicated in influencing the course of infection [5–7]. While these different outcomes have been given various names to help classify cats into specified stages, there are several reports where cats have had a change in stage over time demonstrating that the course of infection may be contingent upon the overall health and immune status of the cat [4,8,9].

Both domestic cats and related wild felids are susceptible to FeLV, a gammaretrovirus that demonstrates a complex pathogenesis in its mammalian host [1,2]. Cats are typically infected by means of horizontal transmission from infected cats. Contact with virus that is shed most commonly in the saliva usually occurs through a nasal or oropharyngeal route. Lymphocytes in local and draining lymph nodes are targets for infection and subsequent proviral DNA integration during cellular proliferation. These lymphocytes along with macrophages are responsible for a primary viremia and distribution of the virus to secondary sites, including additional lymphoid organs, gut-associate lymphoid tissue, and bone marrow, where further viral amplification may occur. Infection of nonlymphoid, hematopoietic progenitor cells in the bone marrow can lead to a secondary viremia with infected neutrophils and platelets present in the peripheral circulation. In those cats that fail to establish an effective immune response, this infection can extend to mucosal and glandular epithelial tissues where infectious virus may be shed [1].

Some cats are able to mount an immune response following the primary viremia but before the secondary viremia and control the infection within the bone marrow as well as other lymphoid sites, thereby

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FeLV, feline leukemia virus; IFA, immunofluorescent assay; PCR, polymerase chain reaction

* Corresponding author.
E-mail addresses: melissa-beall@idexx.com (M.J. Beall), jesse-buch@idexx.com (J. Buch), roberta-cahill@idexx.com (R.J. Cahill), genevieve-clark@idexx.com (G. Clark), jancy-hanscom@idexx.com (J. Hanscom), marko-estrada@idexx.com (M. Estrada), cmleutenegger@yahoo.com (C.M. Leutenegger), chandra-chandrashekar@idexx.com (R. Chandrashekar).

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limiting viremia and antigenemia [4,10–12]. Cats with this regressive disease and latent viral infection still have integrated, exogenous FeLV proviral DNA within lymphoid and myeloid cells that may never be entirely eliminated [8]. These cats have nonproductive infections and remain at risk of FeLV reactivation [4,8,11,13].

Quantitative molecular analyses of both viral RNA and integrated, proviral DNA have identified a correlation between viral loads and different FeLV disease states [8,12,14–17]. In the most recent publication by Helfer-Hungerbuehler, et al. [2015], cats with persistent viremia and antigenemia, and considered to have progressive infections, had higher levels of viral RNA and proviral DNA in tissues when compared to those cats with regressive infections. Likewise, it was observed that when regressive infections became reactivated, there was an associated increase in the detectable levels of soluble p27 antigen in circulation.

In order to investigate a possible correlation between proviral loads and p27 antigen, the current analytical study validated a quantitative enzyme-linked immunosorbent assay (ELISA) for FeLV p27 antigen. Using samples submitted to a commercial reference laboratory for real-time polymerase chain reaction (PCR), quantitative results for both FeLV p27 antigen and FeLV proviral DNA copies in whole blood were compared.

2. Materials and methods

2.1. FeLV PCR

Real-time PCR (FeLV RealPCR™, IDEXX Laboratories, Inc.) was performed at IDEXX Reference Laboratories on EDTA-anticoagulated whole blood samples. Total nucleic acid was extracted and tested for FeLV proviral DNA using primers and probes specific for exogenous FeLV based on previously published methods [18]. Serial dilutions of a 100 μM synthetic DNA positive control (IDT DNA, Coralville, IA) were tested in triplicate and used to generate a standard curve. Real-time PCR was performed on a standardized platform (LightCycler®, Roche Diagnostics, Indianapolis, IN) with a master mix calibrated to align with the standard curve and employing six quality controls, including a quantitative PCR-positive control [19]. Crossing points (i.e., threshold cycle or Ct) were recorded for all samples. Taken together, this method allowed for absolute quantification of FeLV proviral load using previously published protocols [20,21].

2.2. FeLV p27 antigen

The p27 antigen concentration in plasma was measured using an ELISA (PetChek FeLV 15, IDEXX Laboratories, Inc., Westbrook, ME) as previously described [18]. The accuracy of this assay was shown to be 100% using samples characterized as positive or negative using the combined results of FeLV PCR and a FeLV p27 ELISA developed by a different manufacturer [18]. A half-log dilution series of recombinant p27 (ICL, Portland, OR) in fetal bovine serum (FBS) was used to generate a set of master calibrators across a concentration range of 0.03 ng/mL to 33.3 ng/mL and establish a standard curve with each level run in duplicate. Dilutional linearity of the assay was evaluated using inactivated FeLV (Advanced Biotechnologies, Eldersburg, MD) in FBS with each level run in triplicate. Stock concentrations of protein and viral particle counts were provided by the respective manufacturer in each product's certificate of analysis.

2.3. Samples

A total of 353 domestic feline whole blood samples submitted to a commercial reference laboratory for retroviral, real-time PCR (RealPCR™, IDEXX Laboratories, Inc.) from July 2010 through November 2015 were utilized for the study. Samples were obtained and submitted by veterinarians in practice during the course of a normal diagnostic workup. Following completion of the requested testing, ownership of the samples transferred to IDEXX as per terms of the service contract. Plasma from these samples was separated and stored frozen (-80 C) until tested for the presence of p27 antigen. The study was performed in accordance with guidelines established by the institutional Animal Welfare Committee.

2.4. Statistics

Linear regression was used to evaluate the dilutional linearity of both the real-time PCR assay and the p27 antigen ELISA. McNemar's test was used to assess significant differences between the qualitative results from the two methodologies in this sample population. After confirming Normality of the population using D'Agostino & Pearson omnibus K2, a Pearson correlation coefficient was used to evaluate a linear relationship between the log-transformed results for copies of FeLV proviral DNA in whole blood and p27 antigen concentration in plasma. A Mann Whitney U Test was used to assess differences in proviral DNA loads relative to age. Statistical analyses were performed using GraphPad Prism (version 7.0e, GraphPad Software, La Jolla California USA) with significance assessed at $P < 0.05$.

3. Results

Based on a serial dilution of synthetic DNA constructs, a standard curve was generated and used to convert real-time PCR crossing points for FeLV proviral DNA to quantitative values of proviral loads. This standard curve had a 7 orders of magnitude dynamic range with the highest dilution, on average, resulting in one copy of the standard per reaction. The limit of detection and the limit of quantification was 300 proviral DNA equivalents/mL of whole blood (Fig. 1).

Calibrators consisting of half-log dilutions of recombinant FeLV p27 were used to create a standard curve that ranged from 0.03 ng/mL to 33.0 ng/mL. A five-parameter logistic (5 PL) regression model with best fit parameters was used to establish a dose response curve and quantify results [22]. The limit of detection (LoD) for the assay was determined to be 0.2 ng/mL [23]. A limit of quantitation (LoQ) was set at 1.0 ng/mL in the screening protocol to allow for definitive neutralization of ≥50% of the ELISA signal (OD) in the confirmatory protocol [18]. Dilutional linearity of the assay was evaluated using inactivated FeLV diluted over a 34-fold concentration range from 0.2 ng/mL to 7.7 ng/mL. The assay demonstrated a linear dilution response when comparing the recovered concentration to that of the actual concentration with a slope of 0.984 and a R^2 of 1.0. This accurate quantitation of diluted

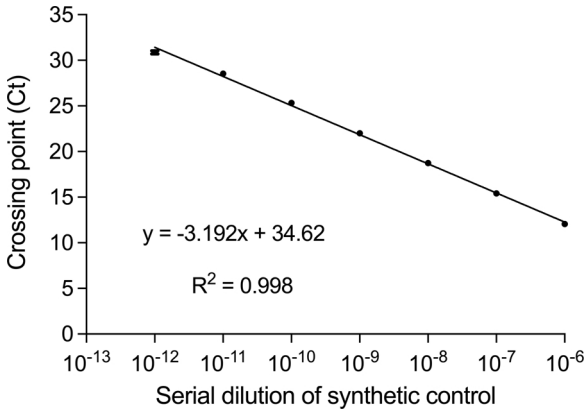

Fig. 1. Feline leukemia virus standard curve generated with synthetic DNA constructs. The starting quantity (copies per reaction) of a 10-fold serial dilution of the synthetic DNA is plotted vs. the measured crossing point (threshold cycle or Ct). The standard curve extended over 7 orders of magnitude and was run in triplicate. Standard deviations are invisible due to high reproducibility except for the last dilution, where the CV was at 0.6%.

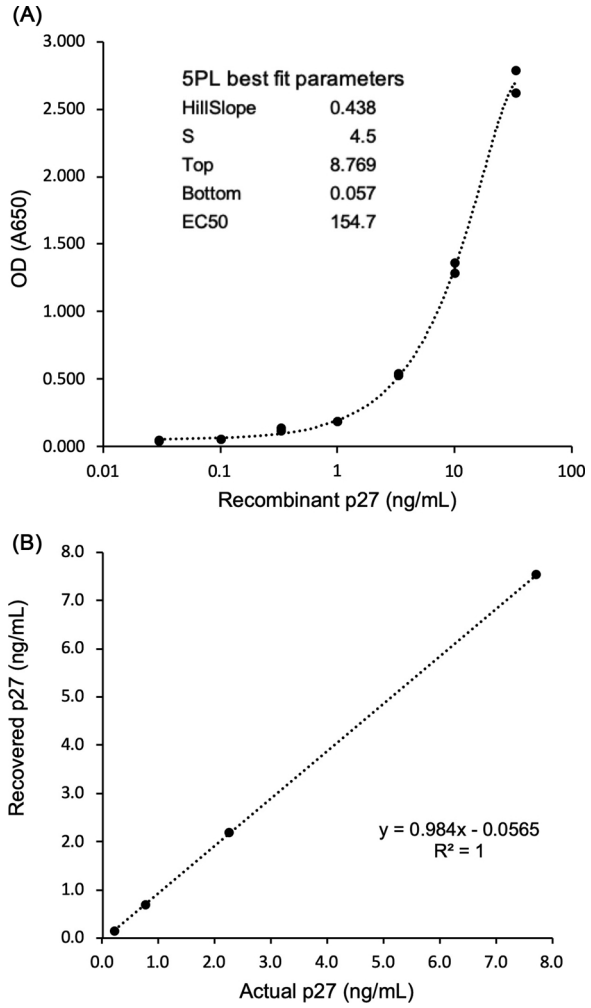

Fig. 2. Recombinant p27 standard curve (A) and assay dilutional linearity (B). A five-parameter logistic regression (5 PL) model fit was applied to the standard curve for interpolation of p27 concentrations in feline samples. Accurate quantitation of p27 concentrations was demonstrated across a 34-fold range based on dilution of inactivated FeLV.

virus enabled an extended dynamic range of the assay from 0.2 ng/mL to 1400 ng/mL (Fig. 2). A sample was determined to be positive when it had an antigen concentration of 1.0 ng/mL or greater when tested using the screening protocol, and it had greater than or equal to 50% neutralization of ELISA signal (OD) when tested using the confirmatory protocol [18].

Of the 353 samples included in this study, 171 were from male and 94 were from female cats (sex not available for 88 samples). The majority of cats (n = 197) were domestic short, medium or long hair (breed not available for 102 samples). The average age of the population (n = 257) was 6 years with a range from 2 months to 18 years (age not available for 96 samples).

Over 85% of samples had concordant results between PCR for proviral DNA and ELISA for p27 antigen; 64 samples were positive and 240 samples were negative (Table 1). In total the real-time PCR for FeLV proviral DNA identified 89 samples as positive with proviral DNA loads of 1.3×10^3 copies/mL to 1.8×10^{10} copies/mL of whole blood, and the quantitative p27 antigen ELISA identified 88 samples as positive with antigen concentrations ranging from 1 ng/mL to 756 ng/mL. Samples with discordant results between proviral DNA and p27 antigen were equally divided between those that were PCR negative but antigen positive (n = 24) and those that were PCR positive but antigen negative (n = 25). McNemar's test found no significant dfference (P = 1.00) between the results of proviral DNA and p27 antigen-based classification of each sample, thus, indicating no bias between these two diagnostic methods.

Table 1
Results of proviral DNA PCR and p27 antigen ELISA testing for the 353 feline samples evaluated in the study.

		Proviral DNA PCR	
		Positive	Negative
p27 Antigen	Positive (≥ 1 ng/mL)	64	24
	Negative (< 1 ng/mL)	25	240

The samples in this population were evaluated for a correlation between FeLV proviral DNA loads and p27 antigen concentrations (Fig. 3). A Pearson correlation coefficient was determined to assess the linear relationship between the log transformed results for each measure. A significant positive correlation between copies of proviral DNA and the concentration of p27 antigen was found (r = 0.761, P < 0.0001). Samples with high proviral DNA loads, those with at least 1×10^6 copies/mL, typically had p27 antigen concentrations greater than 30 ng/mL. Samples with low proviral DNA loads, those falling below 1×10^6 copies/mL, all had p27 antigen concentrations less than 10 ng/mL. For those samples with p27 antigen below the LoQ but positive by PCR, most had fewer than 1×10^6 copies/mL of proviral DNA (Fig. 3). And for those samples that were below the limits of quantitation (BLQ; PCR negative) for FeLV proviral DNA but did have measurable p27 antigen, all had concentrations of p27 that were less than or equal to 30 ng/mL (Fig. 4). Based on these observations a threshold of 1×10^6 copies/mL of FeLV proviral DNA was selected to differentiate samples with low vs. high proviral DNA loads, and a threshold of 30 ng/mL of p27 antigen was selected to discriminate low vs. high concentrations of p27 antigen.

Applying this threshold for proviral DNA loads and p27 antigen concentration to the age of the patients in this population, samples containing either high proviral loads (n = 51) or high antigen concentrations (n = 46) were from feline patients with a median age of 11 months (IQR = 4 months – 3 years). These cats were significantly younger (P < 0.05) than the patients whose samples had low proviral DNA loads (n = 23, median = 4 years; IQR = 2–8 years) or low antigen concentrations (n = 29, median = 3 years; IQR = 1.5–8 years).

4. Discussion

This analytical study of feline patient samples was able to demonstrate a significant positive correlation between copies of FeLV proviral DNA in whole blood and the concentration of p27 antigen in plasma using quantitative real-time PCR and ELISA assays. A positive correlation between FeLV provirus and quantity of p27 antigen has previously been suggested in the literature based on observations of clinical outcome or correlations between provirus and optical density on ELISA [4,8,12,14,17,24,25]. One of the original descriptions of a p27 antigen ELISA also noted a correlation between results of a FeLV immunofluorescence assay (IFA) and p27 antigen concentration [26]. Thus, the results from the two quantitative assays used in this study are consistent with the observations reported in previous studies.

The quantitative ELISA for p27 antigen is based on a qualitative assay which demonstrated 100% accuracy when distinguishing positive and negative samples characterized by concordant results between proviral DNA PCR and a p27 ELISA developed by a different manufacturer [18]. The qualitative ELISA was shown to have similar analytical sensitivity with either recombinant p27 antigen or inactivated FeLV. The p27 ELISA used in this study is similar to other published assays in that it uses a combination of monoclonal antibodies to create a

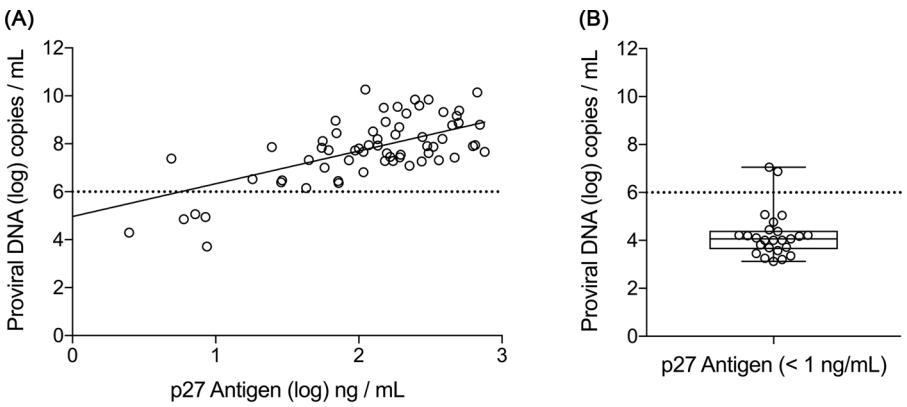

Fig. 3. Correlation between proviral DNA loads by quantitative PCR and the concentration of p27 antigen by quantitative ELISA. A significant Pearson correlation coefficient was identified ($r = 0.761$, $P < 0.0001$) using the log transformed results of each assay suggesting a correlation between the measures (A). For those samples with p27 antigen below the limit of quantitation (< 1 ng/mL), the distribution of quantitative proviral DNA PCR results is shown in panel B. The solid line represents the linear regression and the dotted horizontal line represents a provirus load of 1×10^6 copies/mL.

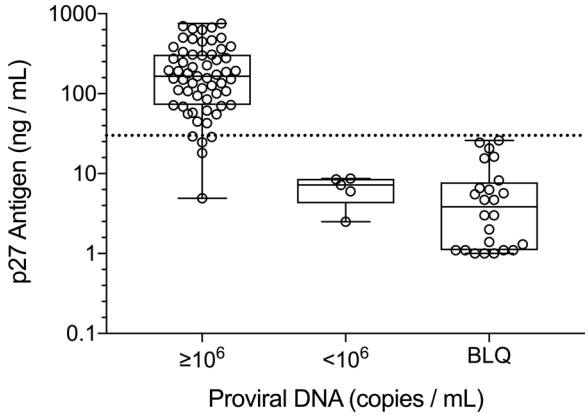

Fig. 4. Concentration of p27 antigen in samples grouped by quantitative proviral DNA loads. Provirus loads are grouped by $\geq 1 \times 10^6$ copies/mL (high), $< 1 \times 10^6$ copies/mL (low) and below the limit of quantitation (BLQ or negative). The dotted horizontal line represents a p27 antigen concentration of 30 ng/mL.

sandwich ELISA. However, it differs based on the use of a fixed cut-off in the assay for distinguishing a positive from negative sample and the use of orthogonal screening and confirmatory protocols to reduce the risk of false positives which may occur on a microtiter plate ELISA [27]. Antigen assays described in the literature often use a cut-off for the sample which may vary from 4 to 10% of a positive control sample and generally do not include a confirmatory protocol with a neutralization step [8,28].

Of the 353 samples tested in this study, over 85% demonstrated concordant results between the real-time PCR for proviral DNA in whole blood and the ELISA for FeLV p27 antigen in plasma. However, 7% of samples tested positive for FeLV provirus but did not have quantifiable p27 antigen, and these samples had low proviral DNA loads (median of 1.2×10^4 copies/mL). Both experimental and field studies have previously reported this type of discrepancy noting that the discordant samples had significantly lower proviral loads than samples that were concordant between provirus and antigen [12]. These cats likely have regressive infections. It is interesting to note that in the current study, 7% of samples had FeLV proviral DNA below the limit of quantification but p27 antigen was detected at low concentrations (< 30 ng/mL). While fewer reports of this type of discrepancy exist within the literature, it has been previously described [16]. Based on the low concentrations of p27 antigen and proviral DNA below the limits of quantitation, these cats may also have regressive infections.

In this study, the samples that were discordant between PCR and ELISA occurred when proviral DNA loads were low or p27 antigen concentrations were low. Samples at the threshold of detection in any assay may be at risk of lower repeatability due to sampling or other sources of variation, and this may have contributed to the discordant results that were observed. Real-time PCR methods based on the unique region (U3) of the long terminal repeat (LTR) of exongenous FeLV have previously demonstrated high analytical and field specificity, and therefore a low risk of false positive PCR results [12,17,28]. False positive p27 antigen results are minimized, as described previously, by using the confirmatory protocol which is unique to this particular p27 ELISA [18]. Samples which test positive on the screening assay must also demonstrate at least 50% neutralization following incubation with an anti-FeLV polyclonal antibody in the confirmatory protocol to be considered positive for specific p27 reactivity. The confirmatory protocol helps to ensure the high specificity of this assay.

The correlation between low provirus and low antigen may reflect the biology of the disease and the spectrum of outcomes. A correlation between proviral DNA and viral RNA loads in blood and tissues and the outcome of an FeLV infection has been described [4,8,12,15]. In experimental infection studies, cats with higher viral loads were at higher risk of FeLV-related disease and had a shorter life expectancy, on average 3 years (max 6 years). Cats with lower viral loads, and considered to have regressive infections, had longer survival times averaging 12 years but were still at risk for reactivation to an infectious status and FeLV-related disease [8]. In the current study, the samples with lower quantities of proviral DNA and antigen tended to be from cats of older age. Therefore, these types of discordant results may be more common in cats with regressive infections where an effective immune response limits viremia and results in a nonproductive infection in the bone marrow or lymphoid tissues [11]. Previous reports have described regressively infected cats with reactivated infections where gradual increases in detectable p27 antigen appear to occur prior to the concentration exceeding the threshold of positivity in the qualitative ELISA [4,8]. A similar observation may be made from experimental infection studies where antigen was detected in cats with regressive infections but at a level below the threshold of positivity for the ELISA [14,28]. Based on the performance of the quantitative p27 ELISA described in this study, not only may it be possible to detect regressive infections when proviral DNA loads are below the limit of quantitation, but it may also be possible to detect early reactivation of regressive infections. Future studies are needed to address these possibilities.

The purpose of this study was to evaluate a quantitative p27 ELISA relative to a well-established method of real-time PCR for FeLV proviral DNA and validate the performance using a population of laboratory samples from owned cats. Limitations associated with this approach included the lack of clinical or staging information for these samples. It was not known what proportion of these patients were healthy, sick, or suspected of a retroviral infection. Additionally, it was not possible to follow these patients for outcome, or to collect additional samples to evaluate concordance or the consistency of quantitative results over time.

5. Conclusion

The results of this analytical study demonstrated a significant positive correlation between FeLV p27 antigen concentrations and proviral DNA loads, with some of the most interesting findings arising in samples with low quantities of both p27 antigen and copies of proviral DNA. The diversity of outcomes in FeLV-infected cats suggests that neither a single test method, nor a single point in time test, may be sufficient for accurately diagnosing or staging these patients. Future prospective studies are needed to understand how quantitative measures of FeLV p27 antigen and proviral DNA loads could be used to help indicate the current stage of infection and evaluate how these measures could be used over time to monitor outcome.

Author contributions

MJB, JB, RC, CML contributed to the conceptualization and methodology of the study. RJC, GC, JB, ME, and CML performed the investigation and analyses. JH was responsible for resourcing reference laboratory samples used in the study. MJB wrote the manuscript. RC provided review and editing of the manuscript.

Data statement

Relevant data set available.

Declaration of Competing Interest

This study was performed by IDEXX Laboratories, Inc. All authors at the time the study was performed were employees of IDEXX.

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