ORIGINAL ARTICLE

Analytical validation of an ELISA for the measurement of feline pancreas-specific lipase and re-evaluation of the reference interval and decision threshold for diagnosing pancreatitis

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Abstract

Background: The diagnosis of feline pancreatitis can be challenging. The clinical presentation often includes mild, nonspecific clinical signs, such as vomiting, anorexia, and weight loss. Measurement of feline pancreatic lipase immunoreactivity (fPLI) concentration in serum has been reported to be sensitive and specific for a diagnosis of pancreatitis in cats. However, analytical validation for a widely available commercial assay for the measurement of fPLI concentration has not been published.

Objective: We aimed to analytically validate the Spec fPL assay (IDEXX Laboratories, Westbrook, ME), a commercial ELISA for the measurement of fPLI concentration, and re-evaluate its reference interval and decision threshold for diagnosing pancreatitis in cats.

Methods: Dilutional linearity, accuracy, precision, and the effect of interfering substances were assessed. The upper limit of the reference interval was calculated based on the 95th percentile of results from clinically healthy cats (n = 107), and a decision threshold for diagnosing pancreatitis was calculated with an expected specificity of 99%.

Results: Analytical validation demonstrated good linearity, accuracy, and precision, as well as the absence of interference from lipemia, hemolysis, or icterus. The upper limit of the reference interval for Spec fPL was determined to be 4.4 μg/L, and the decision threshold (a theoretical cut-off) for diagnosing pancreatitis was determined to be 8.8 μg/L based on a desired specificity of 99%.

Conclusions: The Spec fPL assay is analytically valid, and results suggest that a decision threshold of 8.8 μg/L would have high diagnostic specificity for excluding clinically healthy cats.

KEYWORDS
cat, ELISA, fPLI, pancreatic lipase immunoreactivity, pancreatitis, spec fPL

1 | INTRODUCTION

Pancreatitis has been reported to be more common in cats than previously believed,[1] but the antemortem diagnosis of pancreatitis in cats is challenging due to the vague clinical signs that overlap with those of other diseases.[2] Hematology and biochemistry could show abnormalities in cats with pancreatitis, but usually only reflect systemic complications and do not provide any specific guidance as to a diagnosis of the disease.[3] Several other modalities have been employed to arrive at a diagnosis of pancreatitis in cats, including various imaging modalities, such as abdominal ultrasound or advanced imaging. These have variable diagnostic sensitivities and specificities depending on the chosen modality, disease severity, disease chronicity, and user experience. Histopathology is currently still considered the gold standard for diagnosing pancreatitis in cats. However, pancreatic lesions tend to be focal to multifocal, and therefore, a normal histopathological result would not be sufficient to rule out pancreatitis. Also, a lesion observed in a single pancreatic biopsy might not be representative of the whole pancreas. Furthermore, many cats with acute pancreatitis, especially when associated with systemic complications, are poor anesthetic candidates. As a result, a readily available, specific biomarker for pancreatitis is required.

The utility of several biomarkers for diagnosing pancreatitis in cats has been studied. Serum amylase and lipase activities have been shown to have low diagnostic utility.[4,5] Pancreatic lipase is expressed exclusively by pancreatic acinar cells and can be valuable in evaluating cats for pancreatitis and has been used in several studies using various assays.[6-13] Assays for the measurement of pancreatic lipase concentrations in serum have been developed and analytically validated, but the radioimmunoassay[14] described in the literature was not suitable for commercialization and broad-based use. The Spec fPL assay is a quantitative enzyme-linked immunosorbent assay (ELISA) for measuring the concentration of classical pancreatic lipase in serum from cats. Its diagnostic utility has been reported in several studies,[8-13] using the reference interval determined from an abstract published in 2009.[8] The reported sensitivity varies from 42% to 79%, with a reported specificity from 63% to 100%.[8-13] The variation of the sensitivity and specificity might partly be due to differences in study design. Serum lipase activity, as measured by assays using 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as a substrate, has been evaluated for its diagnostic utility in pancreatitis in cats and was shown to have a good agreement with the Spec fPL assay.[9,10,15] However, studies focused on agreement need to be interpreted cautiously for several reasons: (1) DGGR is not a specific substrate for hydrolysis by pancreatic lipase but is also broken down by other enzymes[16] and as a result, lipase of extra-pancreatic sources could impact the results of the DGGR lipase activity assay; (2) agreement between different biomarkers is not equivalent to these biomarkers having the same sensitivity and specificity[17] and thus differentiation between health and disease; (3) agreement of

a biomarker with a reference standard (eg, ultrasound results or histopathology) might be influenced by the imperfection of the selected reference standard (for example, the result of the reference standard might be affected by differences in disease severity, disease chronicity, and lesion distribution), as early pancreatitis might not be associated with evident morphologic changes.[18] Thus, the poor agreement between a biomarker using selected cutoffs for diagnosing pancreatitis with either ultrasonographic diagnosis of pancreatitis[10] or pancreatic histopathology[11] might reflect that patients had a reduction in the release of enzymes due to a milder form of pancreatitis, which the assay may not be sensitive enough to detect,[6] or a reduction in acinar cell mass in advanced stages of pancreatitis due to pancreatic atrophy and fibrosis.[19] Diagnosis of pancreatitis requires an integration of all available clinical data, including patient history, physical examination, complete blood count, biochemistry profile, urinalysis, abdominal ultrasound findings, specific biomarker concentrations, and, when available, cytology or histopathology findings. Examining the agreement between a biomarker and a selected reference standard might only capture a subset of cats with pancreatitis. Pancreatitis is an umbrella term for cats with heterogeneous presentations, and each diagnostic tool likely provides different information and could be complementary for informing the status of the pancreas. Instead of focusing on the research question of which diagnostic tool constitutes the single most reliable test for diagnosing pancreatitis, a consensus on the classification and/or staging of pancreatitis that incorporates different diagnostic tools is warranted, along with different levels of confidence of a diagnosis when certain diagnostics are equivocal or unavailable. Classification and diagnosis algorithms using this logic have been developed for pancreatitis in humans[20,21] and could be useful for pancreatitis in cats, too. Readers are referred to other publications for detailed discussions about lipases[22] and their use for diagnosing pancreatitis in veterinary medicine[17] and to the recent ACVIM consensus statement on pancreatitis in cats.[23]

Due to the often mild or vague clinical presentation, and its unknown relevance with respect to commonly found histological changes,[1] the clinical significance of pancreatitis or the detection of increased pancreas-specific lipase in cats might vary from patient to patient.[24] Hence the concern of over-diagnosis of this condition has been raised.[25] This concern might reflect that the current reference interval and cutoff for pancreatitis are relatively low and hence may lead to the capture of some pancreatitis cases that might not be clinically relevant. The reference interval and decision threshold for diagnosing pancreatitis with Spec fPL assay, therefore, requires re-evaluation with an aim to capture more clinically relevant pancreatitis and to avoid overinterpreting reactive or aging changes of this organ.[25]

The aims of this study were to (1) analytically validate the Spec fPL assay for the measurement of feline pancreas-specific lipase in cat serum and (2) re-evaluate the reference interval for the Spec fPL assay and propose a decision threshold for diagnosing pancreatitis that would have high specificity for excluding clinically healthy cats.

2 | MATERIALS AND METHODS

2.1 | Spec fPL assay

The Spec fPL assay is manufactured by IDEXX Laboratories (Westbrook, Maine) and is designed to measure pancreas-specific lipase in serum samples from cats. The Spec fPL assay uses a proprietary sandwich ELISA (available only through IDEXX Laboratories, Westbrook, Maine) that consists of (1) an anti-fPL monoclonal capture antibody immobilized on a 96-well microtiter plate, (2) calibrators of recombinant feline pancreatic lipase, which are used to construct a standard curve for each plate, and (3) a second anti-fPL monoclonal antibody conjugated to horseradish peroxidase (HRPO). Spec fPL concentrations are determined by measuring the intensity of the color (absorbance values) produced by patient samples and comparing these to the absorbance values produced by calibrators.

2.2 | Spec fPL assay protocol

Measurement of serum fPLI concentration was performed following the protocol provided by the manufacturer and using reagents provided with the proprietary Spec fPL kit (available only through IDEXX Laboratories, Westbrook, Maine). Briefly, samples were mixed 1:5 with the conjugate. A volume of 50 μL of this mixture was added to each microtiter well and incubated for 15 minutes at 23-27°C. Following the incubation, the plate was washed, and 100 μL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for another 15 minutes at 23-27°C. The reaction was stopped with a stop solution. Optical density (OD) values were determined spectrophotometrically at a wavelength of 650 nm (Synergy 2 Plate Reader, BioTek, Winooski, VT, USA).

For each ELISA plate, a standard curve was constructed using five calibrators (0, 5, 15, 30, and 50 μg/L). The average OD produced for duplicate calibrators was used for all calculations. A second-order polynomial best-fit line was fit through all calibrator points to generate a regression formula. Concentrations of Spec fPL for samples were calculated in μg/L by solving the quadratic formula using the proprietary Spec fPL computer software (available only through IDEXX Laboratories, Westbrook, Maine).

2.3 | Dilutional linearity

Linearity was assessed by evaluating dilutional parallelism. Nine surplus serum samples were obtained from the Gastrointestinal Lab (College Station, TX, USA) following the completion of the requested testing and stored frozen until further analysis. All samples contained serum fPLI concentrations in the higher range of the assay (≥10 μg/L) and were mixed with a pool of serum of low fPLI concentration (<1.0 μg/L) to create a dilution series (1:2, 1:4, and 1:8). Dilutions of 1:2, 1:4, and 1:8 were tested for Spec fPL, and results were plotted against expected values. Results from all nine

dilutional series were used to calculate the slope of the linear best-fit line. Dilutional linearity was assessed by observed value to expected value (O/E) ratios to confirm that it met the recommended criteria of a targeted range between 80% and 120%.[26]

2.4 | Accuracy

Accuracy was assessed by spiking recovery experiment utilizing a total of nine samples—6 undiluted samples of high fPLI concentration (≥10 μg/L) from the dilutional linearity experiment and three additional serum samples with an fPLI concentration <10 μg/L. All surplus samples were obtained from the Gastrointestinal Lab (College Station, TX, USA) following the completion of the requested testing. The samples were stored frozen until further analysis and combined pairwise to create a set of 36 unique samples. Accuracy was evaluated based on the O/E ratios with acceptable criteria between 80% and 120%.[26]

2.5 | Precision

The precision of the Spec fPL assay was determined by assaying two sets of nine different surplus serum samples of various serum fPLI concentrations obtained from the Gastrointestinal Lab (College Station, TX, USA) following completion of the requested testing and stored frozen until further analysis. The mean, SD, and coefficient of variation (%CV) were calculated for 10 replicates of these samples within a plate (intra-assay variability) and individual replicates across 10 plates (inter-assay variability). Precision was assessed by %CV to confirm that it met the recommended criteria of a targeted value below 20%.[26]

2.6 | Interference study

To determine if increased concentrations of commonly occurring sample matrix components would interfere with the accuracy of the Spec fPL assay, the effects of lipid, hemoglobin, or bilirubin added to surplus serum samples obtained from the Gastrointestinal Lab (College Station, TX, USA) with low (≤3.5 μg/L), medium (3.6-5.3 μg/L), or high (≥5.4 μg/L) Spec fPL concentrations were evaluated. Lipid (up to 1200 mg/dL; Intralipid 30% IV fat emulsions, VWR, Radnor, PA, USA), hemoglobin (up to 500 mg/dL; hemolyzed red blood cells from a canine blood draw), or bilirubin (up to 22.5 mg/dL; Scripps Laboratory, San Diego, CA, USA) were added in incremental concentrations to each of nine serum samples. Each serum sample was also tested in the absence of interfering substances (neat). Spec fPL concentrations were calculated as described in the section on the Spec fPL assay protocol. The effect of interference was assessed by targeted O/E ratios between 80% and 120% or a percent change from neat fPLI concentration ≤20% to confirm that it met the recommended

criteria.[26] Linear mixed-effects models were used to assess if each of the interferents had an effect on the fPLI concentration, using serum fPLI concentration as the response variable, interferent as a fixed effect, and individual sample as a random effect. If the interferent had a statistically significant effect, Dunnett's multiple comparison tests were performed to evaluate at which interferent concentration the serum fPLI concentrations would be different from the neat fPLI concentrations.

2.7 | Reference interval and decision threshold

One-hundred and seven surplus serum samples acquired from clinically healthy cats from past blood drives or research projects (2004-2019) with Institutional Animal Care and Use Committee approval and informed owner consent form were stored at −80°C until further analysis. Internal data suggest that feline pancreatic-specific lipase is stable at −80°C over a long period of time (ie, several years; personal communication JMS 2022). Cats were judged to be clinically healthy based on physical examination, owner questionnaire, complete blood count, and serum biochemistry profile. Age, sex, and breed were not available for this convenience set of samples. Results were used to determine the upper limit of the reference interval for the Spec fPL assay by calculating the upper 95th percentile. The median and range of the data were also calculated. The decision threshold (a theoretical cutoff) for diagnosing pancreatitis was determined with an expected specificity of 99%. Data were first log-transformed to better approximate normality, then the mean and SD were calculated. Then, a value was obtained by adding SD times $Z_{0.99}$ to the mean to yield an expected specificity of 99%. By exponentiating this value, a decision threshold was obtained that has the same units as the Spec fPL assay.

2.8 | Statistical analyses

All analyses were performed using R Statistical Software (v4.2.0; R Core Team 2022) or GraphPad Prism (v9.0.0, California, USA). Statistical significance was set as a P-value <0.05. Normality was assessed with quantile-quantile plots when appropriate.

3 | RESULTS

3.1 | Dilutional linearity

The O/E ratios for dilutional linearity ranged from 79.0% to 200.0% with a mean (±SD) of 115.6% (±27.1%) (Table 1, Figure 1). Higher O/E ratios were found when further diluting samples with an expected serum fPLI concentration below 10 µg/L (n = 11, mean ± SD: 138.7% ± 26.9%; Range: 112.6%-200.0%). Out of 11 such samples, seven samples had an O/E ratio larger than the targeted 120%. When excluding these 11 measurements that would not practically require

TABLE 1 Dilutional parallelism for the Spec fPL shown for 9 serum samples at dilutions of 1:1 (neat), 1:2, 1:4, and 1:8.

Dilution	Observed (µg/L)	Expected (µg/L)	O/E ratio (%)
Sample 1			
Neat	12.2		
1:2	6.7	6.1	109.8
1:4	4.2	3.1	137.7
1:8	3	1.5	200.0
Sample 2			
Neat	19.9		
1:2	10.0	10.0	100.5
1:4	5.8	5.0	116.6
1:8	3.8	2.5	152.8
Sample 3			
Neat	34.1		
1:2	15.3	17.1	89.7
1:4	8.4	8.5	98.5
1:8	4.8	4.3	112.6
Sample 4			
Neat	28.3		
1:2	14.2	14.2	100.4
1:4	7.6	7.1	107.4
1:8	5	3.5	141.3
Sample 5			
Neat	34.5		
1:2	16.6	17.3	96.2
1:4	8.8	8.6	102.0
1:8	4.9	4.3	113.6
Sample 6			
Neat	49.6		
1:2	19.6	24.8	79.0
1:4	11	12.4	88.7
1:8	6.8	6.2	109.7
Sample 7			
Neat	13.7		
1:2	8.0	6.9	116.8
1:4	4.5	3.4	131.4
1:8	3.0	1.7	175.2
Sample 8			
Neat	30.1		
1:2	14.3	15.1	95.0
1:4	8.1	7.5	107.6
1:8	4.5	3.8	119.6
Sample 9			
Neat	35.0		
1:2	15.6	17.5	89.1
1:4	9.2	8.8	105.1
1:8	5.6	4.4	128.0

Note: Spec fPL refers to the assay used.
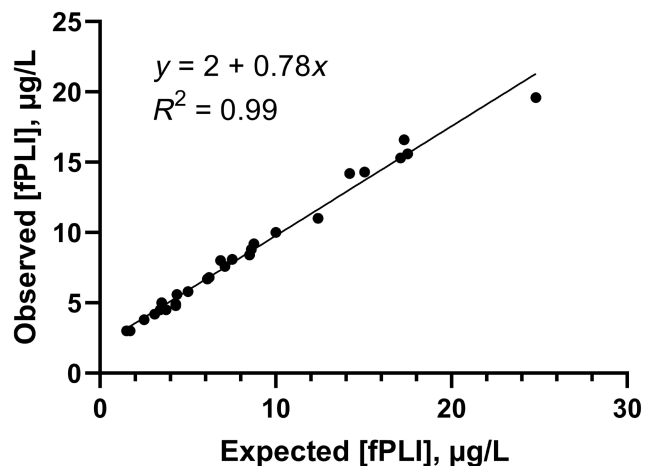
Abbreviation: O/E, observed/expected.

FIGURE 1 Spec fPL dilutional linearity. Observed and expected results for dilution series (1:2, 1:4, 1:8) for nine serum samples demonstrate the reliability of the Spec fPL assay. Spec fPL refers to the assay used.

dilution for measurement, O/E ratios for dilutional linearity ranged from 79.0% to 116.8% with a mean ($\pm$SD) of 100.7% ($\pm$10.4%).

3.2 | Accuracy

The O/E ratios for spiking recovery obtained from a set of 36 samples that were made from combinations of nine individual samples ranged from 76.0% to 112.0% (mean$\pm$SD, 93.7%$\pm$8.4%; Table 2, Figure 2). Out of 36 samples, 34 (94.4%) were within the targeted O/E ratio of 80% to 120%. Two of the prepared samples that had an O/E ratio outside the targeted range had an O/E ratio of 76.0% and 79.7%, with an expected concentration of 18.6 and 5.9 (μg/L), respectively.

3.3 | Precision

The mean %CV of serum samples with low ($\leq$3.5μg/L), medium (3.6-5.3μg/L), and high concentration ($\geq$5.4μg/L) on the Spec fPL assay was 5.6% (range: 4.1%-7.5%) for intra-assay and 6.7% (range: 5.7%-8.3%) for inter-assay. Both the lowest and highest %CVs were observed on serum samples with high concentration ($\geq$5.4μg/L) on the Spec fPL assay. Intra-assay and inter-assay variabilities were both acceptable based on the two sets of nine different surplus serum samples tested (Table 3).

3.4 | Interference study

The O/E ratios mostly fell within the targeted 80%-120% with the addition of lipids (Table 4, Figure S1A,B), hemoglobin (Table 5, Figure S1C,D), or bilirubin (Table 6, Figure S1E,F) to various serum specimens, but the percent change from neat fPLI concentration

was higher than the targeted 20% for more samples with high-range Spec fPL concentrations that had been spiked with the very high concentrations of bilirubin (Table 6). Hemoglobin (P=0.02) and bilirubin (P=0.01) were statistically significant as a fixed effect but not lipid (P=0.51). Dunnett's multiple comparison tests revealed that samples with a hemoglobin concentration of 250mg/dL had an fPLI concentration significantly different from the neat fPLI concentration (adjusted P=0.02), but not different from the neat fPLI concentration at lower (125mg/dL) or higher (375, 500mg/dL) hemoglobin concentrations. Samples with a bilirubin concentration of 15 or 22.5mg/dL had an fPLI concentration statistically significantly different from the neat fPLI concentration (adjusted P=0.04 and 0.03, respectively).

3.5 | Reference interval and decision threshold

The median Spec fPL concentration in serum from 107 clinically healthy cats was 2.1μg/L (range: 1.4-$\geq$200.0 [detection limit] μg/L; Figure 3). The upper limit of the reference interval for Spec fPL assay was determined to be 4.4μg/L based on the upper 95th percentile of the serum Spec fPL concentrations in these 107 cats tested. The decision threshold calculated as described was 8.8μg/L. Based on the decision threshold, two clinically healthy cats (Spec fPL concentration: 11.3 and $\geq$200μg/L) would have a result consistent with pancreatitis, yielding an actual specificity of 98% for this cohort of cats.

4 | DISCUSSION

The Spec fPL assay is a sandwich ELISA that uses dual monoclonal antibodies specific for feline pancreatic lipase. This study aimed to analytically validate this assay as well as to re-evaluate the reference interval and propose a clinically useful decision threshold for the diagnosis of pancreatitis in cats. Dilutional linearity results demonstrated the reliability of Spec fPL measurement is narrower than the reportable dynamic range of 0.5-50.0μg/L, based on the O/E ratios, for which a ratio of 80%-120% is generally seen as acceptable.[26] However, the narrower reliable range would not affect the clinical use since serum samples with a Spec fPL concentration less than 10μg/L typically would not need to be diluted, and samples with a Spec fPL concentration larger than 10μg/L that were diluted showed good reliability with an O/E ratio well within the targeted range. The O/E ratios for spiking recovery ranged between 76.0% and 112.0%, with only 2/36 measurements outside the range of 80%-120%; this is aligned with the guideline from FDA for acceptable accuracy.[26] The dilutional linearity results demonstrate the validity of using this method to assess accuracy. The coefficients of variation for both intra-assay and inter-assay variability for samples across different ranges of Spec fPL concentration were all below 9%, which is below the generally targeted 20%, suggesting good precision.[26]

The quality of serum samples obtained from cats suspected of pancreatitis might be impacted by lipemia, hemolysis, or icterus.

TABLE 2 Spiking recovery for the Spec fPL assay projected from undiluted measurement of 6 dilutional linearity samples with three additional samples with an fPLI concentration <10 μg/L.

Spiking	fPLI concentrations mixed (μg/L)		Observed (μg/L)	Expected (μg/L)	O/E ratio (%)
Sample 1&2	1.8	3.3	2.2	2.6	86.3
Sample 1&3	1.8	8.5	4.3	5.2	83.5
Sample 1&4	1.8	12.2	6.1	7.0	87.1
Sample 1&5	1.8	19.9	10.1	10.9	93.1
Sample 1&6	1.8	34.1	15.0	18.0	83.6
Sample 1&7	1.8	28.3	14.3	15.1	95.0
Sample 1&8	1.8	34.5	13.8	18.2	76.0
Sample 1&9	1.8	49.6	22.8	25.7	88.7
Sample 2&3	3.3	8.5	4.7	5.9	79.7
Sample 2&4	3.3	12.2	7.1	7.8	91.6
Sample 2&5	3.3	19.9	10.8	11.6	93.1
Sample 2&6	3.3	34.1	18.5	18.7	98.9
Sample 2&7	3.3	28.3	15.7	15.8	99.4
Sample 2&8	3.3	34.5	16.1	18.9	85.2
Sample 2&9	3.3	49.6	23.8	26.5	90.0
Sample 3&4	8.5	12.2	9.3	10.4	89.9
Sample 3&5	8.5	19.9	13.7	14.2	96.5
Sample 3&6	8.5	34.1	18.9	21.3	88.7
Sample 3&7	8.5	28.3	18.4	18.4	100.0
Sample 3&8	8.5	34.5	18.9	21.5	87.9
Sample 3&9	8.5	49.6	26.5	29.1	91.2
Sample 4&5	12.2	19.9	13.8	16.1	86.0
Sample 4&6	12.2	34.1	21.3	23.2	92.0
Sample 4&7	12.2	28.3	21.5	20.3	106.2
Sample 4&8	12.2	34.5	20.9	23.4	89.5
Sample 4&9	12.2	49.6	29.6	30.9	95.8
Sample 5&6	19.9	34.1	27.4	27.0	101.5
Sample 5&7	19.9	28.3	27.0	24.1	112.0
Sample 5&8	19.9	34.5	27.2	27.2	100.0
Sample 5&9	19.9	49.6	34.0	34.8	97.8
Sample 6&7	34.1	28.3	34.1	31.2	109.3
Sample 6&8	34.1	34.5	36.6	34.3	106.7
Sample 6&9	34.1	49.6	37.7	41.9	90.1
Sample 7&8	28.3	34.5	33.6	31.4	107.0
Sample 7&9	28.3	49.6	39.7	39.0	101.9
Sample 8&9	34.5	49.6	38.8	42.1	92.3

Note: Spec fPL refers to the assay used.

Abbreviations: fPLI, feline pancreatic lipase immunoreactivity; O/E, observed/expected.

Therefore, the effect of sample quality on Spec fPL concentrations was evaluated by adding lipid, hemoglobin, or bilirubin to various samples at various serum fPLI concentrations. The interference study demonstrated that most samples had an acceptable O/E ratio (targeted O/E ratios between 80% and 120%), suggesting that Spec fPL concentrations were unaffected by these potentially interfering substances. While most O/E ratios were within the targeted range, samples with a spiked hemoglobin concentration of 250 mg/dL

or with a spiked bilirubin concentration of 15 or 22.5 mg/dL were found to have a serum fPLI concentration statistically different from the neat fPLI concentration (Figure S1) Hemoglobin (Figure S1C,D) appeared to have a dose-dependent effect on the Spec fPL assay, causing an underestimation of the serum fPLI concentrations that were not great enough to shift most the O/E ratios outside the targeted range. One possible explanation for the statistical difference between the fPLI concentrations of samples with 250 mg/dL of

hemoglobin added and neat samples was the difference in variability between the two sample sets. Samples with less or more than 250 mg/dL added showed a similar variability, while the samples with 250 mg/dL added, likely by chance, showed a much lower variability (Figure S1D). Bilirubin (Figure S1E,F) also appeared to cause an underestimation, but it does not appear to be dose-dependent after exceeding certain bilirubin concentrations. The effect of bilirubin interference on serum fPLI concentration also seems to be dependent on the neat serum fPLI concentration. Taken together, higher serum fPLI concentrations decreased slightly as higher concentrations of bilirubin were added, resulting in an O/E ratio at the lower range of the targeted range. However, this decrease did not change the clinical interpretation of the evaluated samples, even when the levels of bilirubin would be consistent with severe icterus.
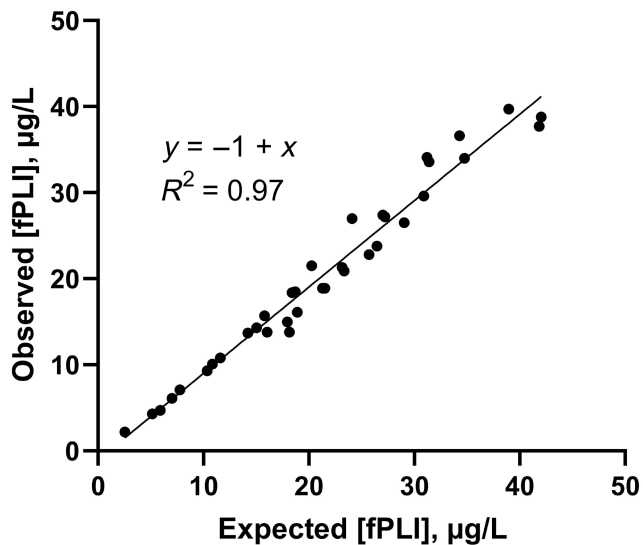

$$y = -1 + x$$
$$R^2 = 0.97$$

FIGURE 2 Spec fPL spiking recovery. Observed and expected results for the undiluted measurement of six samples from dilutional linearity experiments with three additional samples with an fPLI concentration <10 μg/L. Spec fPL refers to the assay used.

The results from this re-evaluation of the Spec fPL reference interval and proposed decision threshold for diagnosing pancreatitis in cats gave us a reference interval of ≤4.4 μg/L, an equivocal range of 4.5-8.7 μg/L, and a range consistent with pancreatitis of ≥8.8 μg/L. A high expected specificity for excluding clinically healthy cats was chosen because, although no single test is sufficient in diagnosing feline pancreatitis alone, in practice, it might be one of the few or the only diagnostic test used for diagnosing feline pancreatitis. The revised reference interval and decision threshold are slightly higher than those currently used.[8] The previous study that established 5.4 μg/L as a decision threshold included disease groups with different levels of confidence in diagnosing pancreatitis (ie, "definitely not," "probably not," "possibly not," "possibly," "probably," and "definitely") and a group of healthy cats (n=41). The reference interval was determined from the central 95th percentile of results from healthy cats, and the cutoff at 5.4 μg/L had a sensitivity of 79% for differentiating cats classified as "definitely" plus "probably" pancreatitis and a specificity of 82% for differentiating cats as "probably not" plus "definitely not" pancreatitis.[8] The current study used variability observed in clinically healthy cats to establish a theoretical decision threshold that would have higher specificity for excluding any clinically healthy cat. The higher decision threshold obtained in this study could possibly exclude some cats with pancreatic pathology with slightly elevated serum fPLI concentration (between 5.4 and 8.7 μg/L) from the "definitely" plus "probably" pancreatitis groups in the previous study, which could suggest that these cats might have had milder forms of pancreatitis, which the assay may not be sensitive enough to detect,[6] or a reduction in acinar cell mass in advanced stages of pancreatitis due to pancreatic atrophy and fibrosis.[19] If these cats had other abnormalities found on other diagnostics consistent with but not specific for pancreatitis, they could possibly be attributed to other comorbidities if present. Whether raising the upper limit for the reference interval and decision threshold for diagnosing pancreatitis could avoid overinterpreting age-related changes or reactive pancreatic changes requires further examination.

TABLE 3 Intra-assay and inter-assay variability of Spec fPL assay.

	Intra-assay n = 10				Inter-assay n = 10		
	Mean fPLI (μg/L)	SD	%CV		Mean fPLI (μg/L)	SD	%CV
Sample 1	1.6	0.1	4.3	Sample 1*	2.4	0.2	6.8
Sample 2	2.9	0.1	4.2	Sample 2*	4.4	0.3	6.0
Sample 3	7.4	0.5	6.4	Sample 3*	7.9	0.4	5.1
Sample 4	11.8	0.7	6.1	Sample 4*	11.5	0.8	7.1
Sample 5	21.0	1.6	7.5	Sample 5*	24.6	1.9	7.8
Sample 6	40.0	1.8	4.4	Sample 6*	29.2	2.4	8.3
Sample 7	31.0	1.9	6.2	Sample 7*	31.2	2.2	7.2
Sample 8	36.5	1.5	4.1	Sample 8*	41.1	2.4	5.9
Sample 9	48.5	3.4	7.0	Sample 9*	56.7	3.4	6.0

Note: Spec fPL refers to the assay used. *denotes samples used were different between intra-assay and inter-assay variability.
Abbreviations: CV, coefficient of variation; fPLI, feline pancreatic lipase immunoreactivity; n, the number of replicates.

TABLE 4 Effect of lipid on Spec fPL concentrations.

Lipid added (mg/dL)	0 (neat)	300	600	900	1200	Mean O/E ratio ± SD (%)
Low-concentration fPL (≤3.5 µg/L) serum sample, µg/L (% change from neat concentration)						
	1.8	1.8 (+0%)	1.9 (+5.6%)	1.8 (+0%)	1.7 (−5.6%)	100 ± 3.9
Mid-concentration fPL (3.6-5.3 µg/L) serum sample, µg/L (% change from neat concentration)						
	3.8	3.9 (+2.6%)	3.8 (+0%)	3.8 (+0%)	3.3 (−13%)	97.9 ± 6.3
	4.1	4.6 (+12.2%)	4.3 (+4.9%)	4.4 (+7.3%)	4.5 (+9.8%)	106.8 ± 4.7
	4.2	4.3 (+2.4%)	4.2 (+0%)	4.3 (+2.4%)	4.3 (+2.4%)	101.4 ± 1.3
High-concentration fPL (≥5.4 µg/L) serum sample, µg/L (% change from neat concentration)						
	5.5	5.2 (−5.5%)	5.4 (−1.8%)	5.5 (+0%)	5.4 (−1.8%)	98.2 ± 2.2
	12.1	10.4 (−14.0%)	10.3 (−14.9%)	9.4 (−22.3%)	9.1 (−24.8%)	84.8 ± 9.7
	25.3	23.4 (−7.5%)	31.2 (+23.3%)	31.2 (+23.3%)	31 (+22.5%)	112.3 ± 14.9
	26.9	25.3 (−5.9%)	26.1 (−3.0%)	25.4 (−5.6%)	24.7 (−8.2%)	95.5 ± 3.1
	42.1	39.3 (−6.7%)	39.1 (−7.1%)	38.3 (−9.0%)	38.4 (−8.8%)	93.7 ± 3.7
Mean O/E ratio ± SD (%)		97.5 ± 7.7	100.8 ± 10.5	99.6 ± 12.3	96.9 ± 13.6	98.7 ± 10.9

Note: Spec fPL refers to the assay used.

Abbreviations: fPLI, feline pancreatic lipase immunoreactivity; O/E, observed/expected.

TABLE 5 Effect of hemoglobin on Spec fPL concentrations.

Hb added (mg/dL)	0 (neat)	125	250	375	500	Mean O/E ratio ± SD (%)
Low-concentration fPL (≤3.5 µg/L) serum sample, µg/L (% change from neat concentration)						
	2.7	2.6 (−3.7%)	2.6 (−3.7%)	2.8 (+3.7%)	2.7 (+0%)	99.3 ± 3.1
Mid-concentration fPL (3.6-5.3 µg/L) serum sample, µg/L (% change from neat concentration)						
	3.6	3.5 (−2.7%)	3.5 (−2.7%)	3.6 (+0%)	3.7 (+2.7%)	99.4 ± 2.3
High-concentration fPL (≥5.4 µg/L) serum sample, µg/L (% change from neat concentration)						
	9	8.6 (−4.4%)	8.5 (−5.6%)	8.2 (−8.9%)	8.3 (−7.8%)	94.7 ± 3.5
	12	13.1 (+9.2%)	11.1 (−7.5%)	11.4 (−5%)	10.9 (−9.2%)	97.5 ± 7.4
	24.2	21.1 (−12.8%)	22.5 (−7.0%)	22.5 (−7.0%)	20.9 (−13.6%)	91.9 ± 5.5
	27.1	27.2 (+0.4%)	26.1 (−3.7%)	26.2 (−3.3%)	24.2 (−10.7%)	96.5 ± 4.4
	34.3	31.6 (−7.9%)	32.4 (−5.5%)	30.8 (−10.2%)	31 (−9.6%)	93.4 ± 4.1
	43.9	40.4 (−8.0%)	41.2 (−6.2%)	39 (−11.2%)	34.7 (−21.0%)	90.8 ± 7.7
	48.9	50.2 (+2.7%)	47.9 (−2.0%)	49.2 (+0.6%)	46.1 (−5.7%)	99.1 ± 3.2
Mean O/E ratio ± SD (%)		97.0 ± 6.5	*95.1 ± 1.9	95.4 ± 5.2	91.7 ± 7.0	94.8 ± 5.6

Note: Spec fPL refers to the assay used; *indicates the fPLI concentrations of the group were statistically different from the neat fPLI concentrations using the Dunnett's multiple comparison tests.

Abbreviations: fPLI, feline pancreatic lipase immunoreactivity; Hb, hemoglobin; O/E, observed/expected.

While the exact ages for the clinically healthy control cats enrolled in this study were not available, clinically healthy cats of various ages were included in the research projects that served as the source of the serum samples in this study, which should thus mirror the animal population for which the reference interval will be used.[27] Age and its exact effect on the pancreas are still unclear. Since chronic pancreatitis in cats often presents with milder signs and is the most prevalent histological form of pancreatitis in cats, with 60% of pancreases showing consistent histological changes,[1] it has been suggested that chronic inflammation of the pancreas is common in cats and possible age-related changes in some cats that are associated with mild inflammation.[25] The concern of overinterpreting possible age-related pancreatic changes could stem from the difficulty of associating the clinical presentation with pancreatitis, especially in senior cats having concurrent diseases with overlapping clinical signs. However, how age would affect the Spec fPL concentration and whether life-stage specific reference intervals are needed remains to be evaluated. A limited number of publications looking at age-related pancreatic changes in cats showed that age could be associated with pancreatic duct width,[28,29] pancreatic duct width/

TABLE 6   Effect of bilirubin on Spec fPL concentrations.

Bilirubin added (mg/dL)	0 (neat)	7.5	15	22.5	Mean O/E ratio ± SD (%)
Low-concentration fPL (≤3.5 μg/L) serum sample, μg/L (% change from neat concentration)					
	2.4	2.3 (−4.2%)	2.4 (+0%)	2.4 (+0%)	99.0 ± 2.1
Mid-concentration fPL (3.6-5.3 μg/L) serum sample, μg/L (% change from neat concentration)					
	3.6	3.3 (−8.3%)	3.3 (−8.3%)	3.2 (−11.1%)	93.1 ± 4.8
High-concentration fPL (≥5.4 μg/L) serum sample, μg/L (% change from neat concentration)					
	8.6	7.9 (−8.1%)	7.4 (−14.0)	7 (−18.6%)	89.8 ± 8.0
	12.5	11.8 (−5.6%)	10.6 (−15.2%)	10.1 (−19.2%)	90.0 ± 8.8
	24.9	19.4 (−22.1%)	18.1 (−27.3%)	17.8 (−28.5%)	80.5 ± 13.3
	27.1	27.7 (+2.2%)	25 (−7.7%)	24.7 (−8.9%)	96.4 ± 5.5
	39.8	33.7 (−15.3%)	31.4 (−21.1%)	29.3 (−26.4%)	84.3 ± 11.4
	43.5	41.1 (−5.5%)	38.1 (−12.4%)	35.5 (−18.4%)	90.9 ± 8.0
	46.9	37.9 (−19.2%)	34.7 (−26.0%)	33.5 (−28.6%)	81.6 ± 12.9
Mean O/E ratio ± SD (%)		90.4 ± 7.8	*85.3 ± 8.9	*82.3 ± 9.7	86.0 ± 9.2

Note: Spec fPL refers to the assay used; *indicates the fPLI concentrations of the group were statistically different from the neat fPLI concentration using Dunnett's multiple comparison tests.

Abbreviations: fPLI, feline pancreatic lipase immunoreactivity; O/E, observed/expected.
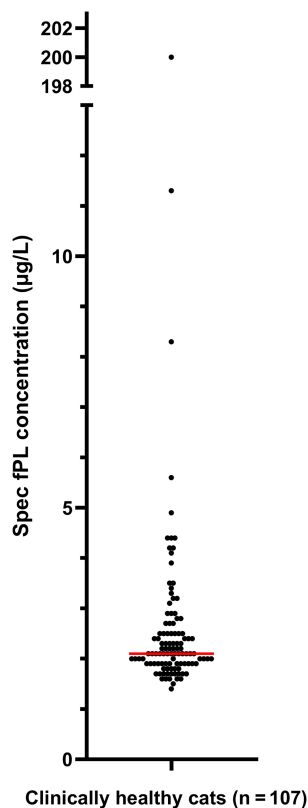

FIGURE 3   Spec fPL concentrations in 107 clinically healthy cats. The red line denotes the median of the Spec fPL concentrations.

pancreatic thickness ratio,[29] but not pancreatic echogenicity[28] or pancreatic width.[28] In human medicine, age has been associated with decreased perfusion, fibrosis, and atrophy, which might have an impact on exocrine pancreatic function.[30] None of these observed age-related changes seem to directly affect the amount of pancreatic lipase being released into the circulation, which, however, does increase with acinar cell damage during pancreatitis.[22] Moreover, as the occurrence of chronic pancreatitis has been demonstrated to be correlated with age in a histopathological study,[1] this could alternatively suggest a potential natural history of pancreatitis in cats that may or may not be perpetuated by similar risk factors and etiologies over a long time and could partially explain why higher serum fPLI concentration might be observed in senior cats. The often mild or asymptomatic presentation of pancreatitis in these cats could reflect the feline nature of hiding discomfort. The incidence of pancreatitis may therefore be underestimated. That said, the relationship between aging and its related pancreatic changes in cats requires further elucidation. To our knowledge, there are currently no publications documenting a clear definition of reactive pancreatic changes in veterinary or human medicine.

One limitation of this study is the lack of age information; thus, the relationship between age and Spec fPL concentrations was not evaluated. For future studies, it will be important to examine the sensitivity of Spec fPL in cats with proven pancreatitis with the proposed decision threshold for diagnosing pancreatitis. It will also be important to assess the specificity of the assay in cats with non-pancreatic illnesses.

5 | CONCLUSIONS

The Spec fPL assay is sufficiently linear, accurate, and precise for the measurement of fPLI concentrations in serum samples from cats and is unaffected by commonly encountered concentrations of potentially interfering substances. Several studies have reported that Spec

fPL assay is clinically useful.[8,9,11–13] The results of this study suggest that the use of a decision threshold of 8.8 μg/L would have high diagnostic specificity for excluding clinically healthy cats. Further studies evaluating the clinical utility of Spec fPL with the revised reference interval and decision threshold as a cutoff for diagnosing feline pancreatitis are warranted.

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