ORIGINAL RESEARCH

Analytical validation of an ELISA for measurement of canine pancreas-specific lipase

Stacey P. Huth1, Roberta Relford1, Jörg M. Steiner2, Marilyn I. Strong-Townsend1, David A. Williams3

1IDEXX Laboratories Inc., One IDEXX Drive, Westbrook, ME, USA; 2Gastrointestinal Laboratory, Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA; and 3Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, Urbana, IL, USA

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Correspondence
Stacey P. Huth, IDEXX Laboratories Inc., One IDEXX Drive, Westbrook, ME 04096, USA
E-mail: stacey-huth@idexx.com
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Background: The diagnosis of canine pancreatitis is challenging. Clinical presentation often includes nonspecific clinical signs, such as vomiting, anorexia, and abdominal discomfort. Increased serum lipase activity can be indicative of pancreatitis; however, it can also be increased with other conditions. An immunoassay for measurement of canine pancreas-specific lipase in canine serum that would be suitable for commercial application and provide rapid results would be beneficial.

Objective: The goal of this study was to validate the Spec cPL assay, a commercially available ELISA for the quantitative measurement of canine pancreas-specific lipase.

Methods: Dynamic range, dilutional linearity, precision, interfering substances, assay stability, and reproducibility were investigated for analytical validation. The method was compared with the reference assay, canine pancreatic lipase immunoreactivity (cPLI), and included evaluation of a sample population of dogs and bias.

Results: Analytical validation showed a dynamic range of 36–954 \( \mu \)g/L; good precision (intra- and interassay coefficient of variation < 12%); absence of interference from lipid, hemoglobin, or bilirubin; 12-month kit stability; and good reproducibility. Method comparison showed a positive bias relative to the cPLI reference method; however, the bias can be accommodated by adjustment of decision limits. The upper limit of the reference interval for Spec cPL was determined to be 216 \( \mu \)g/L based on the upper 97.5th percentile of results from 93 clinically healthy, kennel-housed dogs.

Conclusions: Validation data demonstrated that the Spec cPL assay provides reproducible results for canine pancreas-specific lipase. A readily available assay for measurement of this enzyme allows broader clinical utilization of this analytical tool, generating timely results to aid in the diagnosis of canine pancreatitis.

Introduction

Pancreatitis is believed to be common in dogs1–3 but the diagnosis is considered to be challenging in some cases. Clinical signs often include vomiting, anorexia, and abdominal discomfort, but these clinical signs can be observed in a variety of other diseases, including hepatic disease, renal disease, hypoadrenocorticism, gastritis, and many others.4 A variety of changes are noted on CBCs and serum biochemical and are mostly related to systemic complications of pancreatitis, but are not specific and thus are not useful for arriving at the diagnosis of pancreatitis.4 Several methodologies have been used to diagnose canine pancreatitis, including imaging, eg, abdominal radiography, ultrasonography, and computed tomography, and measurement of serum analytes, eg, amylase and lipase activities, trypsin-like immunoreactivity, pancreatic lipase immunoreactivity, and trypsinogen activation peptide, and have been reviewed in detail elsewhere.5–7

Serum lipase activity has been used to diagnose pancreatitis in people and dogs for several decades.8–13 However, this activity lacks sensitivity and specificity in...
both canine and human patients with exocrine pancreatic disorders.

This is most likely due to the fact that many different cell types synthesize and secrete lipases and that catalytic activity-based assays cannot differentiate between lipases of different cellular origins. Recently, development and analytical validation of a radioimmunoassay and, subsequently, an ELISA for measurement of canine pancreatic lipase immunoreactivity (cPLI) in serum has been reported. Both techniques use small-scale manufacturing processes not suitable for commercialization and broad-based use. Using these assays, serum cPLI concentration is severely decreased or undetectable in serum from dogs with exocrine pancreatic insufficiency. Results suggest that the serum cPLI measured originates exclusively from exocrine pancreas and that these assays are thus specific for exocrine pancreatic function. Also, sensitivity of different minimally invasive diagnostic tests has been compared in a group of dogs with a diagnosis of pancreatitis. Sensitivity of serum trypsin-like immunoreactivity concentration was below 35% and that of serum lipase activity was < 55%. In contrast, sensitivity of serum cPLI concentration for pancreatitis was above 80%.

In another study, different diagnostic markers were compared in a group of 22 dogs with mild to moderate pancreatitis. Serum cPLI concentration had the highest sensitivity in this group of patients of 63.6%, compared with 36.4% for serum canine trypsin-like immunoreactivity concentration, 18.2% for serum amylase activity, and 13.6% for serum lipase activity, again suggesting that serum cPLI concentration is the most sensitive test for canine pancreatitis currently available.

The goal of this study was to develop and analytically validate an immunoassay for measurement of canine pancreas-specific lipase in dog serum that would be suitable for commercial application and provide rapid results. In this study, the new assay, Spec cPL, was validated through a collaborative effort between IDEXX Laboratories and the Gastrointestinal Laboratory at Texas A&M University (GI Lab). The GI Lab’s cPL ELISA was used as a gold standard reference assay and utilizes 2 purified polyclonal antibodies and a streptavidin detection system. Spec cPL was developed using dual monoclonal antibodies with a direct enzyme-labeled detection reagent. The intent of this study was to conduct a technical correlation between the 2 platforms.

**Materials and Methods**

**Canine pancreatic lipase (cPL) antigen**

Native cPL was obtained by purification from canine pancreata and was used for monoclonal antibody development. A recombinant antigen (rcPL) was later developed and utilized for all remaining work pertaining to Spec cPL. Briefly, the gene for cPL was cloned from mRNA that had been purified from canine pancreata. The full-length gene sequence and nucleotides for a 6-His tag were inserted into a baculovirus expression vector for protein expression in insect cells. The secreted protein was purified from culture supernatant using nickel affinity chromatography. Following buffer exchange to phosphate-buffered saline (PBS), the purified protein was stored, frozen at −80°C. With optimal expression and purification, enzymatically active antigen yields ranged from 2 to 4 mg/L of culture supernatant.

**Production of monoclonal antibodies for Spec cPL**

Purified, native cPL was used to immunize Balb/C mice (Charles River Laboratories, Wilmington, MA, USA) following NIH-equivalent guidelines for the care and use of mice. Fusion (using myeloma cell line FO) generated 2 positive clones, 4G11 and 7E11, that expressed mouse IgG anti-cPL activity. Both of these hybridomas resulted in successful ascites production. Cell lines secreting these antibodies were deposited with the American Type Culture Collection (ATCC), Manassas, VA, on March 30, 2005. Strain designations are CPL 7E11 clone 2/A5 and CPL 4G11/14D, bearing ATCC Patent Deposit numbers PTA-6653 and PTA 6652, respectively.

**Anti-cPL horseradish peroxidase (HRP) conjugate for Spec cPL**

Monoclonal anti-cPL 4G11 was covalently coupled to HRP (Roche Molecular Biochemicals, Mannheim, Germany) by following a published method. Briefly, HRP was activated with sulfo-NHS-SS-biotin by following a published method. Next, disulfides on the antibody were reduced to thiol groups using 2-mercaptoethanol amino hydrochloride (MEA; Sigma, St. Louis, MO, USA). After removing excess MEA by passing solution over a desalting column (HiTrap; GE Healthcare Biosciences, Pittsburgh, PA, USA), reduced antibody was combined with HRP-SMCC at a molar ratio of 1:4 (antibody:HRP). The resulting conjugate was stored frozen at −70°C until use.

**Microtiter plate for Spec cPL**

High-binding 96-well microtiter plates (Thermo Electron, Waltham, MA, USA) were coated overnight with 5 μg/mL monoclonal anti-cPL 7E11 in phosphate buffered saline (PBS) at room temperature. Following incubation at room temperature for 1 hour, plates were washed and blocked with 1% bovine serum albumin in PBS for 1 hour at room temperature. Following washing, plates were incubated with canine serum samples for 1 hour at room temperature, followed by washing and incubation with the monoclonal anti-cPL 7E11 antibody at 1 μg/mL for 1 hour at room temperature. Following washing, plates were incubated with anti-cPL horseradish peroxidase (HRP) conjugate diluted 1:1000 in PBS at room temperature for 1 hour. Following washing, plates were incubated with tetramethylbenzidine (TMB) substrate (Pierce Chemical Company, Rockford, IL, USA) at room temperature for 15 minutes. Following a second wash, TMB substrate was stopped with 1 M sulfuric acid. Plates were then read at 450 nm in a microtiter plate reader.
buffer, pH 7.4. After washing off unbound protein, plates were overcoated with bovine serum albumin (BSA, Sigma), and dried under vacuum for subsequent storage at 4°C until use.

Spec cPL assay protocol

Quantitative measurement of cPL was performed using 96-well microtiter plates coated with monoclonal anti-cPL 7E11 as described in the previous section. Samples were mixed 1:3 with anti-cPL HRP conjugate (monoclonal 4G11). A volume of 40 µL of mixture was added to each microtiter well and incubated for 60 minutes at 24 – 26°C. Contents of the microtiter wells were aspirated, washed with PBS solution (pH 7.4) containing 0.05% Tween 20 (Sigma), and incubated for 5 minutes at 18 – 24°C with substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB; SeraCare, Milford, MA, USA). The reaction was stopped using a 1% SDS solution. Optical density (OD) values were determined spectrophotometrically at 650 nm.

With each set of samples, a 5-member calibration curve was included. cPL calibrators were prepared using recombinant cPL antigen in PBS, pH 7.4, containing 1% BSA, 0.05% Tween 20, and a preservative. Calibrator concentrations were 0, 100, 200, 500, and 1000 µg/L. The average OD produced for duplicate calibrators was used in all calculations. A second order polynomial best fit line was fit through all calibrator points to generate a regression formula. Concentrations of cPL for samples were calculated in micrograms per liter by solving the quadratic formula using Microsoft Excel.

Gold standard reference method: cPLI protocol

The protocol for the gold standard reference assay, cPLI, has been described in detail previously, but briefly the assay uses a rabbit-derived polyclonal antibody on both sides of a sandwich ELISA. Plates were coated with affinity purified polyclonal antibody and blocked with a milk-free blocking solution (Superblock in PBS; Pierce Chemical Company). Unknown samples were prepared in a 1:200 dilution of PBS, pH 7.2, containing 1% BSA and 0.05% Tween 20. Standards were prepared by a 1:2 serial dilution of a cPL stock solution. Microtiter wells were each loaded with 100 µL of either unknown sample or standard and incubated for 1 hour at 37°C. Following a PBS wash, wells were incubated with secondary antibody solution containing biotinylated rabbit anti-cPL antibody and incubated at 37°C for 1 hour. Next, HRP-labeled streptavidin solution (100 µL per well) was added and incubated for 1 hour at 37°C. After PBS wash, TMB substrate (100 µL per well, ImmunoPure Turbo TMB; Pierce Chemical Company) was added and developed for 12 minutes. Finally, a combination of 4 M acetic acid and 0.5 M sulfuric acid was used to stop the reaction. Plates were read at 450 nm. Standard curves were calculated using a 4-parameter fit with an algorithm based on the Levenberg–Marquardt method (SOFTMAX PRO; Molecular Devices, Sunnyvale, CA, USA).

Dynamic range and comparison of antigen sources

An evaluation of the dynamic range for the Spec cPL assay was conducted. Sets of calibrators were prepared using both native and recombinant cPL antigens. Antigens were assigned concentrations in units of micrograms per liter based on enzymatically active protein. Calibrators were prepared at final concentrations spanning the range of 0 – 1000 µg/L cPL. Calibration curves for both antigen sources were plotted for comparison.

Precision

Precision of the Spec cPL assay was determined by assaying each of 3 canine serum samples obtained from IDEXX Laboratories (Columbus, OH, USA) in 12 replicates across each of 5 plates over multiple days. SD and coefficient of variation (CV) were calculated for 12 replicates within a plate (intra-assay) and for individual replicates across 5 plates (interassay).

Dilutional linearity

To test linearity of the Spec cPL assay, 3 canine serum samples (IDEXX Laboratories) containing cPL concentrations > 700 µg/L (determined using the assay under development) were diluted using canine serum containing < 30 µg/L cPL. The dilution series consisted of 1:1.5, 1:2, 1:3, 1:4, and 1:8. cPL concentrations of each sample dilution were calculated as described in the section on the Spec cPL assay protocol. Spec cPL-reported values were plotted against expected values. Results from all 3 sample dilution series were combined in a single graph and the slope of the linear best fit line was calculated.

Interference study

To determine if increased concentrations of commonly occurring sample matrix components would interfere with accuracy of the Spec cPL assay, the effects of lipid, hemoglobin, or bilirubin added to serum samples (IDEXX Laboratories) with low- and mid-range Spec cPL concentrations were evaluated. Lipid (up to 7.0 OD units at 660 nm; Intralipid, Baxter Healthcare,
Deerfield, IL, USA), hemoglobin (up to 520 mg/dL; bovine, Sigma), and bilirubin (up to 20 mg/dL; ditaurobilirubin, Promega, Madison, WI, USA) were added in incremental concentrations into each of 2 canine serum samples (IDEXX Laboratories) with low- (<30 μg/L) and mid-range (200–400 μg/L) Spec cPL concentrations. Each serum sample was also tested in the absence of interfering substances. For those samples, PBS was added in a volume equivalent to the volume of added interferent to compensate for dilutional effects. Spec cPL concentrations were calculated as described in the section on the Spec cPL assay protocol.

Assay stability
Spec cPL assay stability at the intended storage condition of 2–7°C was evaluated by testing kit components at regular intervals over a period of 12 months. Calibrator OD values were collected in duplicate throughout the study. Multiple lots of Spec cPL were evaluated in this manner for stability at 2–7°C.

Healthy canine population
Ninety-three serum samples were acquired from adult, male and female, medium-sized dogs (nonpurebred hounds) housed and cared for at Covance (Princeton, NJ, USA) following guidelines of the Institutional Animal Care and Use Committee. The dogs had no signs of vomiting or diarrhea and were fed a standardized laboratory diet. Each sample was tested on multiple lots of the Spec cPL assay. Results were used to determine the upper limit of the reference interval for Spec cPL by calculating the upper 97.5th percentile. Median, mean, SD, and range of the data were also calculated.

Agreement with the GI Lab cPLI ELISA
To evaluate agreement between IDEXX Spec cPL results and results obtained from the GI Lab cPLI ELISA, 58 canine serum samples (IDEXX Laboratories) were tested with both assays. These samples consisted of canine serum samples that spanned the dynamic range of the assay. At IDEXX Laboratories, samples were tested on a single lot of Spec cPL assay across 2 days. At the GI Lab, samples were tested on multiple coated plate lots across multiple days. Each sample was tested once with each assay. Calculated Spec cPL dose values were plotted against GI Lab cPLI dose values. A 45° line of equality was included in the plot to assess bias. Subsequent analysis of data were done using the Bland–Altman approach to assess any bias observed between the 2 methods.30

Spec cPL reproducibility
Forty-three random canine serum samples were obtained from IDEXX Reference Laboratories and were used for Spec cPL reproducibility studies. Each sample was tested on duplicate runs on each of 3 manufactured lots of Spec cPL across multiple days. For each lot, results from duplicate runs were plotted against each other to demonstrate within lot run-to-run reproducibility. Additionally, results between lots were plotted to demonstrate lot-to-lot reproducibility.

Results

Analytical validation
Dynamic range and comparison of antigen source
Evaluation of Spec cPL dynamic range showed an OD response of approximately 0–1.4 absorbance units across the range of 0–1000 μg/L for both recombinant and native cPL antigens (Figure 1).

Dilutional linearity
The dilutional linearity study demonstrated that when reported cPL values were compared with expected cPL values of 3 sample-dilution series, cPL measurement was reliable between 36 and 954 μg/L (Figure 2).

Precision
Intraplate and interplate variability were comparable for all 3 samples tested (Table 1).

Figure 1. Dynamic range and comparison of antigen source. Both recombinant and native canine pancreas lipase (cPL) antigens give similar responses when assayed using the Spec cPL ELISA. cPL is the analyte being measured (canine pancreas-specific lipase); Spec cPL refers to the test method used for the comparison. OD, optical density.

Table 1. Spec cPL assay precision values. Values are expressed as mean and SD.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Intraplate SD</th>
<th>Interplate SD</th>
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<tbody>
<tr>
<td>1</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td>2</td>
<td>0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Interference study

The diagnostic interpretation was unchanged with addition of lipids (at 7.0 OD units [\(>1600\) mg/dL]), 520 mg/dL hemoglobin, or 20 mg/dL bilirubin in specimens with low- and mid-range analyte concentrations (Table 2).

Assay stability

The stability study showed no statistically significant trends in calibrator OD values over 12 months at \(2\pm0.7\)\(^\circ\)C (Figure 3). Additional lots tested (data not shown) demonstrated similar performance over 12 months.

Spec cPL reproducibility

Results from duplicate runs within each of 3 manufactured lots of Spec cPL correlated with each other with intraclass correlation coefficients ICC\(^{31}\) ranging from 0.968 to 0.999 and a SD of measurement error ranging from 8 to 52, indicating <3% total variation due to the measurement system and good within-lot reproducibility (Figure 4A). Results from multiple manufactured lots of Spec cPL correlated with each other with ICC > 0.970 with a SD range of 27–47, indicating <3% total variation due to the measurement system and good lot-to-lot reproducibility (Figure 4B).

Method comparison

Healthy canine population

Median Spec cPL concentration in serum from 93 clinically healthy dogs was 39.4 mg/L (mean 53.1 mg/L, SD 50.4 mg/L, range 4.0–275.0 mg/L). The upper limit of the reference interval for Spec cPL was determined to be 216 mg/L based on the upper 97.5th percentile of the 93 dogs tested.

Agreement with GI Lab cPLI ELISA

The comparison between Spec cPL and cPLI ELISA illustrates a bias exists, with higher concentrations for Spec cPL relative to cPLI ELISA (Figure 5). The data were plotted by difference against the mean to illustrate the magnitude of difference observed between the 2 assays (Figure 6).

**Table 1.** Intra-assay and interassay precision of Spec cPL assay.

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay n = 12</th>
<th>Interassay n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean cPL (mg/L)</td>
<td>SD %CV</td>
</tr>
<tr>
<td>Sample 1</td>
<td>77</td>
<td>6 7.8</td>
</tr>
<tr>
<td>Sample 2</td>
<td>177</td>
<td>16 9.0</td>
</tr>
<tr>
<td>Sample 3</td>
<td>704</td>
<td>79 11.2</td>
</tr>
</tbody>
</table>

\(n\), the number of replicates; CV, coefficient of variation. Spec cPL refers to the test method.
Discussion

The Spec cPL assay, an ELISA utilizing dual monoclonal antibodies specific for canine pancreatic lipase, was developed and analytically validated relative to the Texas A&M GI Lab cPLI ELISA. Dilutional linearity results demonstrated reliability of cPL measurement over the reportable dynamic range of 30–1000 μg/L for the Spec cPL assay, and precision testing indicated that interassay and intra-assay variability were comparable for samples tested over this range with CVs within the range of what is generally accepted for ELISA technology. Additionally, variability presented for Spec cPL was comparable to that of cPLI ELISA. Serum samples obtained from animals suspected of pancreatitis may be hemolyzed or have increased bilirubin or lipid owing to biochemical alterations related to an animal’s disease status. The effect of sample quality on Spec cPL results was evaluated by adding lipid, hemoglobin, and bilirubin to normal canine samples. These commonly occurring serum components were added at various levels considered to be abnormal, and the added lipid, hemoglobin, or bilirubin did not affect the Spec cPL results. Further, assay protocol requires a 1:3 sample dilution with conjugate before addition to the plate, and this dilution provides additional protection from interference due to poor sample quality. The shelf-life of the Spec cPL assay kit components was good over 12 months of refrigerated storage with no significant loss of signal. As calibrators are included on every test plate, Spec cPL
calculated dose values remain constant throughout the 12-month shelf-life of the assay. Reproducibility is critical to quality for quantitative assays and data generated in this study indicated that Spec cPL results are reliable.

Comparison of the Spec cPL assay with the reference cPLI ELISA resulted in a positive bias of approximately 50%. Based on the evaluation of healthy dogs in this study, Spec cPL assay results < 200 μg/L are considered normal. In comparison, results < 102 μg/L are considered normal using the GI Lab cPLI ELISA. Although both assays measure the same analyte, cPL, immunoassays cannot be considered to be truly analytical32,33 as they do not actually quantify the number of molecules present in the sample, but rather measure an immunologic response32 that is greatly dependent on type of antibody used and a variety of reaction conditions. Thus, it is expected that 2 immunoassays measuring serum concentration of the same analyte using a different protocol and different antibody pairs will provide different numerical results.33 However, the assays have general agreement, and independent reference intervals and cut-off values must be established for each assay. Multiple factors may contribute to the systematic shift demonstrated in the Bland–Altman plot across the dynamic range of the assay, including fundamental design differences between the Spec cPL and the cPLI reference system. The reference cPLI ELISA was developed using 2 polyclonal antibodies, a streptavidin detection system, and a 4-parameter calibration curve fit. Conversely, the Spec cPL assay was developed using dual monoclonal antibodies with direct detection and a second order polynomial calibration curve fit. Regardless of the origin of the shift, actual interpretation of patient results across the dynamic range remains unchanged. Results within the reference interval for cPLI ELISA were also within the reference interval for Spec cPL. The same was observed for results above the upper limit of the reference interval.

In summary, the Spec cPL assay is an ELISA available for the quantitative measurement of canine pancreas-specific lipase concentration in serum samples. Validation data demonstrate that the Spec cPL assay provides reproducible results for canine pancreas-specific lipase. Several studies have been reported that suggest the original cPLI assay is clinically useful,23–25,34 but further studies evaluating clinical utility of this new assay will be needed and are currently in progress.

Acknowledgment

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References


