Development and validation of an enzyme-linked immunosorbent assay for feline trypsin-like immunoreactivity

Jörg M. Steiner, vetmed, Dr med vet; David A. Williams, MA, VetMB, PhD; Erik M. Moeller; Tonatiuh Melgarejo, DVM, PhD

Objective—To develop and validate an ELISA for quantitative analysis of feline trypsin-like immunoreactivity (fTLI).

Sample Population—Purified feline cationic trypsin (fCT) and rabbit anti-fCT antiserum; blood samples from 63 healthy cats.

Procedures—A sandwich capture ELISA was developed, using anti-fCT antiserum purified by affinity chromatography that underwent biotinylation. Purified fCT was used for standards. The assay was validated by determination of sensitivity, working range, linearity, accuracy, precision, and reproducibility. A reference range was established by assaying serum samples from the 63 healthy cats.

Results—Sensitivity was 1.23 µg/L; working range was 2 to 567 µg/L. Ratios of observed versus expected results for 4 samples tested at various dilutions ranged from 90.0 to 120.7%. Ratios of observed versus expected results for 5 samples spiked with various concentrations of fCT ranged from 82.0 to 101.8%. Intra- and inter-assay coefficients of variability ranged from 9.9 to 11.1% and from 10.2 to 21.7%, respectively. The reference range for serum fTLI measured with this ELISA was 12 to 82 µg/L.

Conclusions and Clinical Relevance—Results suggest that an ELISA can be used to measure serum fTLI in cats. The ELISA was sufficiently sensitive, linear, accurate, precise, and reproducible for clinical use. (*Am J Vet Res* 2000;61:620–623)

Evaluation of exocrine pancreatic function by use of serum or fecal markers has been the goal of gastroenterologists for more than a century. Initially, catalytic assays were used to test serum for tryptic activity.[1-3] Not surprisingly, these assays were found to be unreliable, and it was subsequently shown that under physiologic conditions, trypsin circulates in the blood stream as its inactive zymogen, trypsinogen.[4] The development of **radioimmunoassays (RIA)** for the measurement of **trypsin-like immunoreactivity (TLI)** in humans and dogs marked a major breakthrough in noninvasive pancreatic function testing.[5] Recently, a radioimmunoassay for determination of serum **feline trypsin-like immunoreactivity (fTLI)** has been described.[6] Since its introduction, this assay has been used clinically for diagnosis of exocrine pancreatic disorders in cats.[7,8] However, even though this assay has been shown to be sensitive, linear, accurate, precise, and reproducible, a few concerns remain. First, tracers for the assay have to be produced in the laboratory by radio-iodination of pure feline cationic trypsin every few weeks.[6,9] Although this procedure is relatively safe, frequent iodination of proteins still raises important regulatory and operator safety issues. Second, the RIA has a reported working range of 2 to 50 µg/L. This range encompasses values for healthy cats (range, 17.1 to 48.6 µg/L) and cats with exocrine pancreatic insufficiency (typically, ≤ 8 µg/L). However, the upper limit of 50 µg/L does not allow direct measurement of extremely high serum fTLI concentrations. Analysis of routine 1:4 dilutions of serum samples does allow quantification of these high fTLI concentrations, but evaluation of diluted samples alone does not allow accurate determination of low serum fTLI concentrations. Therefore, the working range of the currently available RIA necessitates routine evaluation of each serum sample twice—once undiluted and once in a 1:4 dilution—which is time consuming. To overcome these limitations of the RIA, the purpose of the study reported here was to develop and validate an ELISA for quantitative analysis of serum fTLI.

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From the Gastrointestinal Laboratory, Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4474.
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Materials and Methods

Feline cationic trypsin (fCT) was purified from feline pancreatic tissue by means of sulfuric acid extraction, ammonium sulfate fractionation, gel filtration, and affinity chromatography, as described.[10] Freshly purified fCT was irreversibly inhibited by incubation with **Nα-*p*-tosyl-L-lysine chloro-methyl ketone (TLCK)** in methanol and then dialyzed against 1 m*M* HCl. The TLCK-fCT was then adjusted to a concentration of 1 mg/ml and immediately frozen and stored at –80 C.

Anti-fCT antiserum was raised in New Zealand White rabbits by 4 SC injections, at 4 week intervals, of 0.5 mg of fCT in 0.5 ml of **phosphate-buffered saline solution (PBSS**; pH 7.2) thoroughly emulsified with 0.5 ml of complete Freund adjuvant for the initial injection and 0.5 ml of Freund incomplete adjuvant for all following injections.[6] Serum was collected 10 days after each injection and tested for anti-fCT activity by use of a radial immunodiffusion assay; activity was quantitated by evaluating binding to radioactively labeled TLCK-fCT.[6]

Anti-fCT antibodies were purified from antiserum by means of affinity chromatography. Briefly, approximately 0.5 mg of TLCK-fCT was coupled to a 1 ml NHS-activated Sepharose column,[a] following the manufacturer's instructions. Coupling efficiency was calculated according to the manufacturer's recommendations. For lipoprotein precipitation, 35 ml of rabbit antiserum was thawed and 35 ml of 1*M* $CaCl_2$ and 1,400 µl of 10% dextran sulfate solution were added. The mixture was mixed by gentle inversion of the centrifuge tube for

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15 minutes. Then, the mixture was centrifuged at 10,000 × g for 10 minutes at 4 C, and the precipitate was discarded. A gel filtration column[b] was used for buffer change to 75 m*M* Tris hydrochloride, 150 m*M* NaCl, pH 8.0 (buffer A), following manufacturer's instructions. The material was collected and applied to the affinity column mounted on a **fast performance liquid chromatography (FPLC)** purification system.[c] Absorbance of the effluent was measured at a wavelength of 280 nm. After the column was washed with buffer A until absorbance had returned to baseline, the mobile phase was changed to 100 m*M* glycine, 500 m*M* NaCl, pH 2.5 (buffer B), and 1 ml fractions of the effluent were collected into test tubes containing 300 µl of 1*M* Tris hydrochloride, pH 8.0. The purified antibodies were concentrated,[d] the buffer was changed to PBSS, pH 7.2,[e] and the concentration was adjusted to 1 mg/ml. Aliquots (200 µl) were frozen at –20 C.

For biotinylation, 5,800 µg of the purified monospecific polyclonal antibody in PBSS, pH 7.2, was injected into a dialysis cassette,[f] and a 20-fold molar excess of biotin[g] (770 nmol or 430 µg) was added. After incubation for 30 minutes at room temperature (approx 20 C), the material was dialyzed 3 times against 800 ml of PBSS, pH 7.2, for 1 hour at 4 C. Biotinylation efficiency was determined by use of a 2-(4'-hydroxyazobenzene) benzoic acid avidin assay kit.[h] This procedure was repeated until a biotinylation coefficient of 3.44 was reached. The biotinylated antibodies were adjusted to a concentration of 1 mg/ml and frozen in aliquots of 100 µl.

A sandwich ELISA was developed. First, wells in 96-well flat bottom ELISA plates[i] were coated with 200 ng of affinity purified anti-fCT antibodies in 100 µl of carbonate-bicarbonate buffer, pH 9.4/well.[e] Plates were incubated for 1 hour at 37 C and washed 2 times with 200 µl of PBSS, pH 7.2,[e] with 0.5% polyoxyethylenesorbitan monolaurate (Tween)/well and 1 time with 200 µl of PBSS, pH 7.2/well. Remaining binding sites were blocked with 200 µl of a milk-free blocking solution[j]/well. Plates were incubated for 1 hour at 37 C and washed 3 times in the same way. Coated plates were stored in a refrigerator for up to 2 weeks. Phosphate-buffered saline solution, pH 7.2, with 5% **bovine serum albumin (BSA)** was used as the negative control solution. Standard solutions of TLCK-fCT were prepared by serial dilution in PBSS, pH 7.2, with 5% BSA and frozen in aliquots of 250 µl at –20 C. Standard solutions were thawed immediately prior to loading on the plates. A volume of 100 µl of negative control solution or standard solution/well was used. All samples (control samples and test samples) were prepared in a 1:10 dilution with PBSS, pH 7.2, with 5% BSA, and wells were loaded with 100 µl of each diluted sample/well. Plates were incubated for 1 hour at 37 C and washed 3 times as described. Antibody solution containing 10 ng of the biotinylated anti-fCT antibodies in 100 µl of buffer C (0.05 *M* monobasic and dibasic sodium phosphate mixed to pH 7.5 with 0.2 g/L NaN_3 and 5 g/L BSA) was then added to each well. Plates were again incubated for 1 hour at 37 C and washed 3 times, and 100 µl of horseradish peroxidase-labeled streptavidin[k] solution (1 µg/ml) in buffer C was added to each well. Plates were incubated for another hour at 37 C and washed 3 times, and color was developed by adding 100 µl of a 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate solution[l] to each well. The reaction was stopped after 20 minutes by adding 100 µl of 2 N sulfuric acid/well, and absorbance was read at a wavelength of 450 nm.[m] Standard curves were calculated by use of a 4-parameter equation:

$$y = ([A - D]/[1 + (x/C)^B]) + D,$$

where D is the y-value corresponding to the asymptote at high values of x, A is the y-value corresponding to the asymptote at low values of x, C is the x-value corresponding to the midpoint between A and D, and B describes how rapidly the curve makes its transition from the asymptotes in the center of the curve. All 4 parameters were calculated by use of an algorithm based on the Levenberg-Marquardt curve fitting method.[m]

The ELISA was validated by determining assay sensitivity, working range, dilutional parallelism, spiking recovery, intra-assay variability, and interassay variability. Assay sensitivity was determined by loading 10 sets of negative control samples as unknown samples and determining mean plus 3 time the SD on the standard curve. The lower limit of the working range was defined as the next higher integer greater than the assay sensitivity. The upper limit of the working range was defined as the apparent value of an absorbance that equaled mean maximum absorbance minus 3 times the SD of the maximum absorbance. The maximum absorbance of the assay was measured by assaying 10 duplicate wells containing 250 µg/L TLCK-fCT. Four samples with serum fTLI ranging from 23.4 to 136.6 µg/L were used to determine dilutional parallelism; 1:10, 1:20, 1:40, and 1:80 dilutions of each sample were assayed. Spiking recovery was determined by adding 7.81, 15.63, 31.25, and 62.5 µg/L TLCK-fCTto each of 5 serum samples with fTLI ranging from 9.0 to 141.8 µg/L. Intra-assay variability was determined by evaluating 5 serum samples multiple times during the same assay run. Interassay variability was determined by evaluating 5 serum samples during multiple consecutive assay runs.

A reference range for the assay was determined by evaluating serum samples from 63 clinically normal cats. Food was withheld from all cats for at least 12 hours prior to collection of blood samples. The reference range was calculated as the mean ± 2 SD.

Results

After approximately 0.5 mg of TLCK-fCT was coupled to an NHS-activated column, coupling efficiency was calculated to be 100%. Several buffers for application and elution of anti-fCT antiserum were tried before optimal conditions were found. Optimal yield was obtained when the antiserum was applied with buffer A, and the antibodies were eluted with buffer B. The 35 ml of rabbit anti-fCT antiserum yielded a total of 11.6 mg of pure polyclonal antibodies. Half of this material was frozen at –20 C, and the other half was biotinylated until it reached a biotinylation coefficient of 3.44 mol of biotin/mol of antibody. The biotinylated polyclonal anti-fCT antibody was collected and frozen at –20 C.

Reproducible standard curves were achieved with the ELISA (**Fig 1**). Mean absorbance of 10 sets of negative control samples was 0.0003, with an SD of

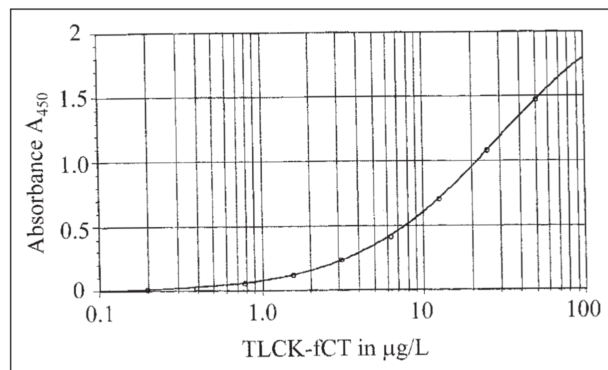

Figure 1—Standard curve of absorbance at 450 nm versus concentration of feline cationic trypsin (incubated with Nα-*p*-tosyl-L-lysine chloro-methyl ketone; TLCK-fCT) obtained with an ELISA developed to measure serum trypsin-like immunoreactivity in cats. The curve was created with standard software ($y = ([-0.005 - 2.319]/[1 + (x/28.134)^{0.982}]) + 2.319$; $R^2 = 1$).

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Table 1—Evaluation of dilutional parallelism of an ELISA developed to measure serum feline trypsin-like immunoreactivity (fTLI) in cats

Sample No.	Dilution	Observed (µg/L)	Expected (µg/L)	Ratio (%)*
1				
	1:10	23.4	NA	NA
	1:20	12.6	11.7	107.7
	1:40	6.1	5.9	103.4
	1:80	3.5	2.9	120.7
2				
	1:10	61.0	NA	NA
	1:20	32.8	30.5	107.5
	1:40	15.6	15.3	102.0
	1:80	8.2	7.6	107.9
3				
	1:10	79.9	NA	NA
	1:20	36.9	40.0	92.3
	1:40	18.1	20.0	90.5
	1:80	9.0	10.0	90.0
4				
	1:10	136.6	NA	NA
	1:20	76.7	68.3	112.3
	1:40	40.7	34.2	119.0
	1:80	19.6	17.1	114.6

All samples were evaluated in duplicates.
*Ratio of observed versus expected value.
NA = Not applicable.

Table 2—Evaluation of spiking recovery for an ELISA developed to measure serum fTLI in cats

Sample No.	Spiking concentration (µg/L)	Observed (µg/L)	Expected (µg/L)	Ratio (%)*
1				
	0	5.0	NA	NA
	7.8	11.9	12.8	93.0
	15.6	17.9	20.6	86.9
	31.3	29.8	36.3	82.1
	62.5	57.1	67.5	84.6
2				
	0	21.9	NA	NA
	7.8	28.8	29.7	97.0
	15.6	33.3	37.5	88.8
	31.3	46.7	53.2	87.8
	62.5	69.5	84.4	82.3
3				
	0	60.6	NA	NA
	7.8	65.0	68.4	95.0
	15.6	73.0	76.2	95.8
	31.3	88.0	91.9	95.8
	62.5	119.3	123.1	96.9
4				
	0	69.8	NA	NA
	7.8	75.0	77.6	96.6
	15.6	87.0	85.4	101.9
	31.3	97.6	101.1	96.5
	62.5	131.6	132.3	99.5
5				
	0	137.9	NA	NA
	7.8	143.9	145.7	98.8
	15.6	148.5	153.5	96.7
	31.3	153.0	169.2	90.4
	62.5	183.4	200.4	91.5

Spiking recovery was determined by adding 7.8, 15.6, 31.3, and 62.5 µg/L of feline cationic trypsin incubated with Nα-*p*-tosyl-L-lysine chloro-methyl ketone to each of 5 serum samples.
See Table 1 for key.

0.0017. Extrapolation from the standard curve indicated that an absorbance of 0.0003 plus 3 times the SD of 0.0017 was equivalent to an fTLI of 0.123 µg/L. Because samples were diluted 1:10 prior to assay, this indicated that assay sensitivity was 1.23 µg/L for serum samples, and the lower limit of the working range was

Table 3—Intra-assay variability of an ELISA developed to measure serum fTLI in cats

Sample No.	Mean (µg/L)	SD (µg/L)	CV (%)
1	6.9	0.726	10.5
2	26.8	2.66	9.9
3	74.2	8.11	10.9
4	79.6	8.86	11.1
5	172.5	17.86	10.4

Five serum samples were assayed 10 times during the same assay run; all samples were evaluated in duplicates.
SD = Standard deviation. CV = Coefficient of variation (CV = SD/mean).

Table 4—Interassay variability of an ELISA developed to measure serum fTLI in cats

Sample No.	Mean (µg/L)	SD (µg/L)	CV (%)
1	20.2	2.59	12.8
2	23.4	2.38	10.2
3	54.4	6.60	12.1
4	73.0	10.55	14.5
5	142.3	30.87	21.7

Five serum samples were assayed 10 times during consecutive assay runs; all samples were evaluated in duplicates.
SD = Standard deviation. CV = Coefficient of variation (CV = SD/mean).

2 µg/L. Mean absorbance of 10 duplicate wells containing 250 µg of TLCK-fCT/L was 2.917 with an SD of 0.122. Extrapolation from the standard curve indicated that an absorbance of 2.917 minus 3 times the SD of 0.122 was equivalent to an fTLI of 56.7 µg/L. Because samples were diluted 1:10 prior to assay, this indicated that the upper limit of the working range was 567 µg/L. Linearity of the assay was determined by evaluating dilutional parallelism. Ratios of observed versus expected results for the 4 samples tested ranged from 90.0 to 120.7% (**Table 1**). Accuracy of the assay was tested by determining spiking recovery. Ratios of observed versus expected results ranged from 82.0 to 101.8% (**Table 2**). Intra- and interassay variability ranged from 9.9 to 11.1% (**Table 3**) and from 10.2 to 21.7% (**Table 4**), respectively. Mean and SD serum fTLI for the 63 healthy cats were 46.9 µg/L and 17.5 µg/L, respectively. Thus, the reference range for serum fTLI measured with this ELISA was 12 to 82 µg/L.

Discussion

Results suggest that the ELISA described in the present study should be acceptable for measuring serum fTLI in cats. The sensitivity of this ELISA was 1.23 µg/L, which compares favorably with the sensitivity of 1.9 µg/L reported for the RIA.[6] Given the range of serum fTLI in healthy cats, a lower limit of the working range of 2.0 µg/L for this ELISA appears to be adequate for detecting subnormal serum fTLI concentrations. The upper limit of the working range (567 µg/l) was far greater than that reported for the RIA (50 µg/l) and should allow measurement of high serum fTLI without the need for assaying additional dilutions of the serum sample.

Evaluation of dilutional parallelism of this ELISA indicated ratios of observed to expected values of 90 to 120.7%, indicating acceptable linearity for clinical use. Evaluation of spiking recovery yield-

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ed observed to expected values of 82.1 to 101.9%, demonstrating acceptable accuracy. Coefficients of variation for intra-assay variability were acceptable, implying that the assay is precise. Coefficients of variation for interassay variability were also acceptable, indicating that the assay is reproducible. One could argue that an interassay variability of 21.7% for 1 sample was not acceptable. However, mean serum fTLI for this sample (142.3 µg/L) was almost twice the upper limit of the reference range. Thus, the high coefficient of variation would be clinically acceptable, because clinical interpretation of the result would not be changed. If, in the future, it is determined that a severe, rather than only a moderate, increase in serum fTLI is associated with a different clinical diagnosis or prognosis, the relative lack of reproducibility in the upper range of the assay should be addressed.

Thus, we conclude that this ELISA is sensitive, linear, accurate, precise, and reproducible, but that reproducibility for samples with fTLI in the upper range of the assay is lower. In examining these results, it should be remembered that validation parameters were examined over an unusually wide range. Most assays are only validated over a narrow range, most commonly only using samples with results within the reference range, and may therefore have better values for validation parameters.

The reference range for serum fTLI measured with this assay was 12 to 82 µg/L. This reference range compares favorably with the control range reported for serum fTLI measured with the RIA (17.1 to 48.6 µg/L; calculated as the range from the lowest to highest serum fTLI for 21 healthy cats[6]). The difference may reflect the smaller number of cats used to establish the control range for the RIA.

[a]HiTrap NHS-activated Sepharose column, Amersham Pharmacia Biotech, Piscataway, NJ.

[b]PD-10, Sephadex G-25 M column, Amersham Pharmacia Biotech, Piscataway, NJ.

[c]ÄKTA purifier, Amersham Pharmacia Biotech, Piscataway, NJ.

[d]Centricon-30 concentrator, Amicon, Beverly, Mass.

[e]BupHTM dry blend buffers, Pierce Chemical Co, Rockford, Ill.

[f]Slide-A-Lyzer 10K dialysis cassette, Pierce Chemical Co, Rockford, Ill.

[g]EZ-Link Sulfo-NHS-LC biotin, Pierce Chemical Co, Rockford, Ill.

[h]ImmunoPure HABA assay kit, Pierce Chemical Co, Rockford, Ill.

[i]Flat-bottom, high binding EIA/RIA strip plate-8, Corning Costar, Cambridge Mass.

[j]Superblock in PBS, Pierce Chemical Co, Rockford, Ill.

[k]ImmunoPure Streptavidin HRP-Conjugated, Pierce Chemical Co, Rockford, Ill.

[l]1-Step Turbo TMB-ELISA, Pierce Chemical Co, Rockford, Ill.

[m]UV MAX ELISA plate reader and SOFTMAX PRO analysis software package, Molecular Devices, Sunnyvale, Calif.

References

1. Anson ML. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J Gen Physiol* 1938;22:79–89.
2. Wiggins HS. Simple method for estimating trypsin. *Gut* 1967;8:415–416.
3. Rinderknecht H, Silverman P, Geokas MC, et al. Determination of trypsin and chymotrypsin with remazol brilliant blue-hide. *Clin Chim Acta* 1970;28:239–246.
4. Borgström A, Ohlsson K. Studies on the turnover of endogenous cathodal trypsinogen in man. *Eur J Clin Invest* 1978;8:379–382.
5. Williams DA, Batt RM. Diagnosis of canine exocrine pancreatic insufficiency by the assay of serum trypsin-like immunoreactivity. *J Small Anim Pract* 1983;24:583–588.
6. Steiner JM, Medinger TL, Williams DA. Development and validation of a radioimmunoassay for feline trypsin-like immunoreactivity. *Am J Vet Res* 1996;57:1417–1420.
7. Steiner JM, Williams DA. Feline pancreatitis. *Compend Contin Educ Pract Vet* 1997;19:590–603.
8. Steiner JM, Williams DA. Feline exocrine pancreatic disorders: insufficiency, neoplasia, and uncommon conditions. *Compend Contin Educ Pract Vet* 1997;19:836–849.
9. Hunter WM, Greenwood FC. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature* 1962;194:495–496.
10. Steiner JM, Medinger TL, Williams DA. Purification and partial characterization of feline trypsin. *Comp Biochem Physiol* 1997;116B:87–93.

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