

Investigation of a Commercial ELISA for the Detection of Canine Procalcitonin

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Background: Rapid identification of sepsis enables prompt administration of antibiotics and is essential to improve patient survival. Procalcitonin (PCT) is a biomarker used to diagnose sepsis in people. Commercial assays to measure canine PCT peptide have not been validated.

Objective: To investigate the validity of a commercially available enzyme-linked immunosorbent assay (ELISA) marketed for the measurement of canine PCT.

Animals: Three dogs with sepsis, 1 healthy dog, 1 dog with thyroid carcinoma.

Methods: Experimental study. The ELISA's ability to detect recombinant and native canine PCT was investigated and intra-assay and interassay coefficients of variability were calculated. Assay validation including mass spectrometry of the kit standard solution was performed.

Results: The ELISA did not consistently detect recombinant canine PCT. Thyroid lysate yielded a positive ELISA signal. Intra-assay variability ranged from 18.9 to 77.4%, while interassay variability ranged from 56.1 to 79.5%. Mass spectrometry of the standard solution provided with the evaluated ELISA kit did not indicate presence of PCT.

Conclusions and Clinical Importance: The results of this investigation do not support the use of this ELISA for the detection of PCT in dogs.

Key words: Biomarker; Diagnostic test; Dog; Sepsis; Validation.

Severe bacterial infections can result in marked morbidity and death in veterinary patients, with 50–70% of dogs with sepsis succumbing to their disease.¹ Early diagnosis of infection is essential for the appropriate management of sepsis, as it allows rapid administration of antibiotics resulting in improved outcomes.² A rapid diagnostic marker for infection would assist veterinarians in determining whether antibiotic therapy is indicated, thereby facilitating early initiation and discriminate use of antibiotics.

Procalcitonin (PCT) is a 14 kDa prohormone identified as a biomarker for sepsis in people. Normally, PCT is produced only in the thyroid C-cells as a precursor to calcitonin; however, during sepsis this peptide is ubiquitously expressed.³ Although the biologic role of PCT is poorly understood, studies in people suggest a potential role in modulating inflam-

Abbreviations:

CV	coefficient of variation
LCMS/MS	liquid chromatography-tandem mass spectrometry
OD	optical density
PCT	procalcitonin
rcPCT	recombinant canine PCT
SIRS	systemic inflammatory response syndrome

mation.⁴ Advantages of PCT as a serum biomarker for sepsis include absence in healthy individuals, rapid induction with the onset of sepsis, and a moderately long half-life, making daily monitoring more practical compared to other biomarkers such as tumor necrosis factor- α and interleukin-6.⁴ In people, marked elevations in serum PCT are used to distinguish patients with severe bacterial, fungal, or parasitic infections from those with severe nonseptic SIRS, to guide and shorten the course of antimicrobial treatment, and to prognosticate outcome.⁴ As such, PCT is becoming part of the standard of care to rapidly diagnose sepsis, minimize morbidity and mortality, and decrease unnecessary antibiotic use resulting in fewer adverse effects, lower health care costs, and decreased antimicrobial resistance. An assay for PCT might have similar value in dogs as in people.

Although PCT mRNA expression from nonthyroidal tissue has been shown in dogs with sepsis and SIRS, very little is known about serum PCT concentration in dogs attributable to the lack of a validated assay.^{5,6} Sequence differences with the human peptide⁷ could preclude cross-reactivity of canine PCT with assays for human PCT. Hence, an existing ELISA for human PCT did not differentiate samples from dogs with septic from nonseptic SIRS.⁵ A commercially available canine PCT ELISA^a incorporating a polyclonal antibody to PCT has been marketed for the

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quantification of this peptide in research; however, this kit has not been validated. The purpose of this study was to validate a commercial canine PCT ELISA with the future goal to apply the assay to investigate the kinetics of PCT in dogs. Because a gold standard for the quantification of canine PCT does not exist, alternate techniques were required to validate this ELISA. We hypothesized that the kit would allow for accurate and precise determination of canine PCT with intra-assay and interassay coefficients of variation (CV) of less than 10%.

Materials and Methods

Samples

Thyroid lysate acquired postmortem from a dog with a thyroid carcinoma and canine recombinant PCT (rcPCT) were tested with an ELISA for detection of native and rcPCT, respectively. Canine recombinant PCT was cloned by PCR-amplifying complementary DNA reverse transcribed from canine thyroid gland mRNA. Restriction enzyme^b sites *NheI* and *SacI* were integrated into the forward and reverse primer, respectively, for cloning PCT with a HIS tag into the pET100/D TOPO expression vector.^c One-Shot BL-21 star competent *E. coli* cells^d were transformed with the pET/PCT construct according to the manufacturer's instructions, grown in lysogeny broth containing ampicillin, and induced with isopropyl β -D-1-thiogalactopyranoside. The rcPCT was purified by histidine tag affinity for nickel resin, dialyzed against phosphate-buffered saline, and then centrifuged in a Vivaspin column.^e The recombinant protein was analyzed by polyacrylamide gel electrophoresis^f and Coomassie staining (data not shown), and quantified by a Bradford Lowry assay.^g The thyroid lysate was diluted with the sample diluent provided in the ELISA kit to yield neat, 1 : 2 and 1 : 4 dilutions.

Serum from 3 dogs obtained within 24 hours of a diagnosis of sepsis (2 dogs with septic peritonitis and 1 dog with sepsis secondary to penetrating wounds) was pooled to establish a "septic" sample. Sepsis was diagnosed if 2 of any of the following 4 criteria were present: body temperature <38 or $>39.2^{\circ}\text{C}$ (<100.6 or $>102.6^{\circ}\text{F}$), heart rate >120 beats per minute, respiratory rate >20 breaths per minute, white blood cell count <6 or $>16 \times 10^3$ cells/dL, or $>3\%$ band neutrophils.⁸ Serum from a healthy dog undergoing elective surgery (determined on the basis of results of a normal physical examination, complete blood count, and serum biochemistry profile) was used as a "nonseptic" sample. The serum collected was left over after routine biochemical evaluation in the hospital laboratory performed at the discretion of the attending veterinarian. All samples were stored at -80°C from the time of collection and thawed just before use.

PCT ELISA

The reagents were prepared and all incubations and washes were performed according to the manufacturer's instructions.^a The standard solution provided was diluted to yield 1,000, 500, 250, 125, 62.5, 31.2, and 15.6 pg/mL solutions, with a final blank well containing only the standard diluent (0 pg/mL PCT). The optical density (OD) of the samples was determined with a microplate reader set to a wavelength of 450 nm.^h The OD of the blank well was subtracted from each sample OD. Log transformation of the OD values from the standard dilutions was performed to plot the standard curve, which was subsequently

used to determine the PCT concentration in samples by means of the formula $10^{[(\log\text{OD} - \text{intercept})/\text{slope}]}$.

Detection of Canine Recombinant and Native PCT. Eight 1 : 4 dilutions of rcPCT, resulting in concentrations ranging from 22,938 to 1.4 pg/mL, as well as neat, 1 : 2, and 1 : 4 dilutions of thyroid lysate were assayed as positive controls.

Intra-Assay and Interassay Variation. Stored serum samples from dogs with and without sepsis were thawed, vortexed, and centrifuged before being assayed in 5 repeat wells of the same ELISA kit to determine the intra-assay variability. The same samples were then divided into 4 aliquots, which were stored at -20°C until analyzed in 4 additional different assays to determine the interassay variability.

Mass Spectrometry

The standard solution from 2 separate ELISA kits and an aliquot of rcPCT were analyzed by liquid chromatography-tandem mass spectrometry (LCMS/MS) to identify peptides in the samples.ⁱ

Database Searching. All LCMS/MS spectra were analyzed by MASCOT.^j MASCOT was set up to search in the National Centre for Biotechnology Information database (selected for *Canine lupus familiaris*, 24,867 entries) assuming the digestion enzyme trypsin. MASCOT was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 20 parts per million. Iodoacetamide derivation of cysteine was specified in MASCOT as a fixed modification. Pyroglutamate formation at the N-terminus, S-carbamoylmethylcysteine cyclization of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine, and acetylation of the N-terminus, were specified in MASCOT as variable modifications.ⁱ

Criteria for Protein Identification. Scaffold^k was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.⁹

Statistical Analysis

Intra-assay and interassay CVs were calculated by dividing the standard deviation (SD) by the mean of the 5 results obtained for each sample. All data are reported as mean \pm SD. Graphs were generated by commercial software.¹

Results

PCT ELISA

Detection of rcPCT and Native PCT. Analysis of dilutions of the standard solution supplied in the ELISA kit yielded a standard curve with $R^2 = 0.985$. Analysis of specific concentrations of rcPCT yielded random results with no apparent relationship to the amount of rcPCT (Fig 1A). Assessment of different dilutions of thyroid lysate yielded PCT concentrations of 15, 19, and 38 pg/mL, respectively (Fig 1B).

Intra-Assay Variability. To determine the precision of the ELISA, 5 replicates of 2 different serum samples were analyzed in the same ELISA. Replicate analysis

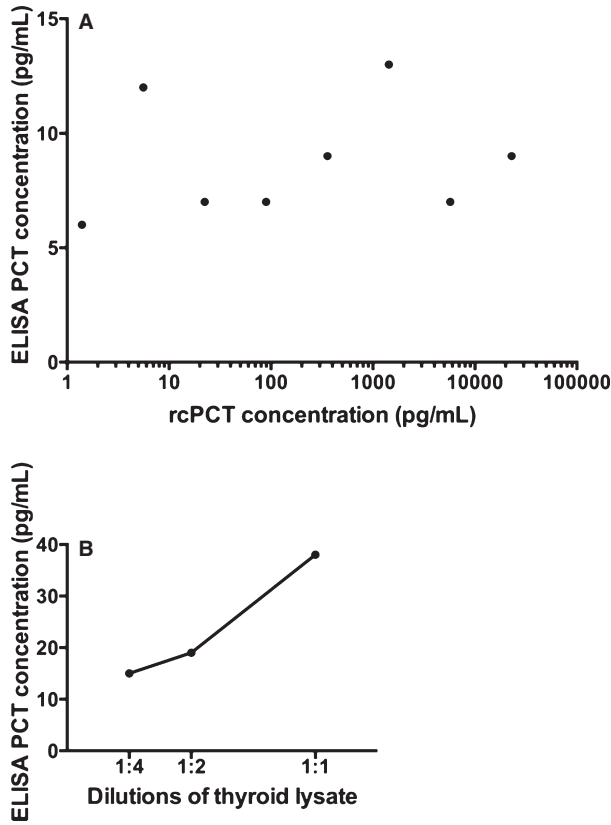


Fig 1. (A) Specific concentrations of rcPCT detected by a commercial PCT ELISA. (B) PCT concentration in different dilutions of thyroid lysate as measured by commercial ELISA.

of a serum sample from a dog without sepsis yielded PCT concentrations ranging from 0 to 213 pg/mL with a mean \pm SD of 99 ± 77 pg/mL and a CV of 77.4%. Replicate analysis of a pooled serum sample from dogs with sepsis yielded PCT concentrations ranging from 218 to 380 pg/mL with a mean \pm SD of 305 ± 58 pg/mL and a CV of 18.9% (Fig 2).

Interassay Variability. To investigate repeatability of the ELISA across assays performed on different days, serum samples from dogs with and without sepsis were

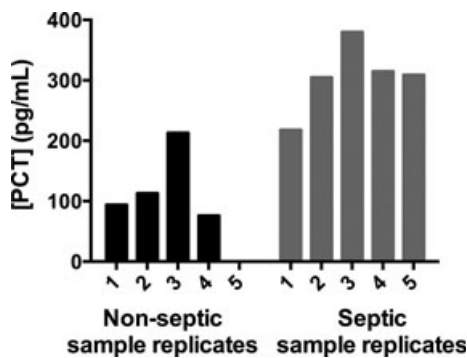


Fig 2. PCT as measured 5 times by commercial ELISA in single samples from a dog with and a dog without sepsis. Replicate 5 of the nonseptic sample had a concentration of 0 pg/mL.

analyzed on 5 different days. Standard curves prepared for each assay had R^2 values >0.96 . The sample from a dog without sepsis yielded PCT concentrations ranging from 28 to 120 pg/mL with a mean \pm SD of 68 ± 38 pg/mL and a CV of 56.1%. The sample from dogs with sepsis yielded PCT concentrations ranging from 6 to 309 pg/mL with a mean \pm SD of 160 ± 127 pg/mL and a CV of 79.5% (Fig 3).

Mass Spectrometry

To verify that the rcPCT preparation indeed contained PCT, an aliquot was analyzed by LCMS/MS. Results indicated that with $>95\%$ probability canine PCT and canine albumin were contained in the preparation. To investigate whether the standard provided in the commercial ELISA contained PCT, 2 samples of the standard solution submitted from two separate ELISA kits were also analyzed by MS/MS. Spectra for PCT were not detected in either of the 2 samples, but both contained spectra for 2 or more peptides yielding a probability of 100% for canine albumin.

Discussion

The assay investigated in this study is marketed for the detection of both native and rcPCT in serum, tissue homogenates, or other biological fluids.^a Based on the investigation presented here, this commercially available canine PCT ELISA could not be validated and data generated do not support use of this assay to measure PCT in dogs.

In this study, different dilutions of rcPCT were not detected in a dose-dependent or consistent fashion. Possible reasons might be that the recombinant version of PCT was not in its native form and contained a polyhistidine tag, which is required for purification but could have interfered with immunodetection. Therefore, thyroid lysate, which should contain native PCT, was also assayed. A neat preparation of this lysate yielded a putative PCT concentration of 38 pg/mL. This result might reflect the actual concentration of PCT in canine thyroid tissue or another protein. Based

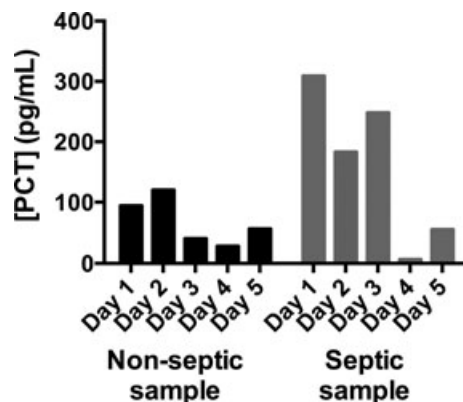


Fig 3. PCT as measured on 5 different days by commercial ELISA in the same samples from dogs with and without sepsis.

on results of MS/MS analysis, the standard within the ELISA kit did not contain PCT; therefore, it is more likely that another protein such as albumin was detected in the thyroid lysates. Analysis of dilutions of a standard containing albumin in an ELISA incorporating an antibody to albumin might yield a standard curve with an acceptable slope, however, of an irrelevant substance.

Typically accepted intra-assay and interassay CVs for ELISAs are less than 10%.¹⁰ According to the certificate of analysis supplied by the manufacturer for the commercial assay, the intra-assay CV is <4.5% and interassay CV is <7.4%.^a However, the intra-assay and interassay CVs determined in this study were much higher, and of a magnitude typically considered unacceptable for ELISA technology.¹⁰ Hence, the ELISA assessed here yielded neither accurate nor precise results.

A limitation of this study was that a suitable gold standard for measuring canine PCT was not available. Hence, the ELISA under investigation could not be compared to a known assay. However, absence of PCT in the standard solution as investigated with a highly sensitive method such as MS/MS precluded further attempts at validating this assay. Substances yielding an optical signal in thyroid lysate and serum samples from septic and nonseptic dogs may consist of albumin or other proteins that bind nonspecifically to wells in the kit. Ideally, validation of an ELISA would entail assessment of precision and accuracy over multiple analyte concentrations within an assay, between different assays and between different laboratories. Within the time frame of this study, additional kits for the detection of canine PCT became available.^m To the authors' knowledge, these kits are marketed for research purposes only and have not undergone industrial or governmental review. As a result, information regarding their components may be proprietary. Whether these newer kits are more suitable for the detection of canine PCT remains unknown. Nevertheless, the results of this investigation do not support the use of this ELISA for the detection of PCT in dogs.

Footnotes

^a Canine procalcitonin, PCT ELISA kit, EIAab Science Co Ltd, Wuhan, China

^b *NheI* and *SacI* restriction endonucleases, New England BioLabs, Ipswich, MA

^c Topo TA Kit, Invitrogen, Grand Island, NY

^d One-Shot BL-21 star competent *E. coli*, Invitrogen

^e Sartorius Vivaspin sample concentrators, GE Life Sciences, Piscataway, NJ

^f NuPage 4–12% Bis-Tris gels, Invitrogen

^g Pierce BCA protein assay, Thermo Scientific, Rockford, IL

^h PowerWave XS2 with Gen5.0 version 1.11 software, BioTek Instruments Inc, Winooski, VT

ⁱ Mass Spectrometry Facility, Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Canada

^j MASCOT version 2.3.02, Matrix Science, London, UK

^k Scaffold version 4.0.3, Proteome Software Inc, Portland, OR

^l GraphPad Prism version 6.0a for Mac OS X, GraphPad Software, La Jolla, CA

^m <http://www.amsbio.com/productpage.aspx?code=AMS.E08P0208&cty=CANADA&cur=USD>; http://www.cusabio.com/ELISA_Kit-95491/#.UP1luY7_RUQ; <http://www.biotangusa.com/bt/canine-dog-procalcitonin-pct-elisa-kit-96t.html>; http://www.mybiosource.com/datasheet.php?products_id=943719; <http://www.novateinbio.com/en/dog-canine-elisa-kit/59626-dog-canine-procalcitonin-pct-elisa-kit.html> (January 21, 2013)

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Conflict of Interest: Authors disclose no conflict of interest.

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