ORIGINAL ARTICLE

Preliminary Evaluation of a Particle-enhanced Turbidimetric Immunoassay (PETIA) for the Determination of Serum Cystatin C-Like Immunoreactivity in Dogs

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Abstract: **Serum cystatin C often is used in humans as a rapid and more sensitive marker than serum creatinine for glomerular filtration rate. The purpose of the present study was to evaluate whether cystatin C–like immunoreactivity (CLI) could be measured reliably in canine serum and to investigate whether dogs with clinical renal insufficiency had higher CLI levels than did clinically healthy dogs and dogs with nonrenal diseases. A commercially available particle-enhanced turbidimetric immunoassay (PETIA) for human serum cystatin C was used to measure canine serum CLI in a linear and proportional manner, with a mean recovery of 104% ± 7.5% and coefficients of variation of 1.7 to 9.6%. The assay was then applied to serum samples from 17 clinically healthy dogs, 12 dogs with nonrenal diseases, and 8 dogs with renal insufficiency. Serum CLI was significantly higher in dogs with renal insufficiency (median serum CLI = 5.01 mg/L) than in clinically healthy dogs and dogs with nonrenal diseases (median serum CLI = 1.06 mg/L and 1.62 mg/L, respectively). Thus, canine serum CLI could be reliably measured using a commercially available PETIA designed for human serum cystatin C, and dogs with clinical renal insufficiency had, as expected, significantly higher serum CLI levels. (*Vet Clin Pathol.* 2001;30:86-90)**

Key Words: **Cystatin C, dog, immunoassay, renal insufficiency, test validation**

Cystatin C is a small low-molecular-mass cysteine protease inhibitor (molecular mass approximately 13 kd) consisting of a nonglycosylated polypeptide chain containing 120 amino acid residues. In humans, it is produced at a constant rate in all nucleated cells.[1,2] Because of its low molecular mass and its positive charge at normal pH, it is freely filtered by the glomerulus, without any significant tubular secretion, and is almost completely reabsorbed, catabolized, and broken down in the cells of the proximal convoluted tubule in the rat.[3] The constant production rate of cystatin C in all tissues, its elimination via the glomerular filter, and its minor dependence or even nondependence on many extrinsic factors, including sex, age, diet, inflammation, and neoplasia, make serum cystatin C an attractive endogenous biochemical marker of glomerular filtration rate (GFR).[4-10] Although recent studies have suggested that serum cystatin C may be influenced by nonrenal diseases such as inflammation and neoplasia,[9,10] studies in humans have shown that serum cystatin C is better than serum creatinine as a marker for GFR, especially in individuals with small to moderate decreases in GFR.[4-10]

To our knowledge, no one has reported on the determination of cystatin C in canine serum samples. However, rabbit anti-human cystatin C antibodies (Dako A/S, Glostrup, Denmark) have been shown to react with a 13-kd protein in canine cerebrospinal fluid.[11] We examined whether a commercial particle-enhanced turbidimetric immunoassay (PETIA), based on the use of rabbit anti-human cystatin C antibodies to measure cystatin C in human serum samples, could be used to reliably measure cystatin C–like immunoreactivity (CLI) in canine serum samples. Further, we investigated whether serum CLI levels among dogs with clinical renal insufficiency were higher than serum CLI levels in healthy dogs and dogs with nonrenal diseases, thereby indicating that canine serum CLI could be an attractive candidate for future investigation as a marker for estimation of GFR.

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Material and Methods

Particle-enhanced turbidimetric immunoassay (PETIA)

The Cystatin C PET Kit (Dako) designed for the determination of human serum cystatin C was used for the heterologous determination of cystatin C in canine serum samples. The principle of the analysis is the binding of cystatin C to rabbit anti-human cystatin C antibody coupled to polystyrene particles. The resulting change in turbidity is used as a measure of the cystatin C concentration in the samples. The analysis was performed with an automated analyzer (Cobas Fara II, Hoffmann-La Roche, Ltd, Basel, Switzerland) according to the manufacturer's description. The lowest detectable concentration was 0.4 mg/L, and cystatin C levels of <0.4 mg/L were reported as 0.4 mg/L. According to the manufacturer, analytical imprecision is low (coefficients of variation of 1.3% and 3.2%) when the assay is applied to human serum samples with 4.24 mg/L and 0.97 mg/L, respectively. In healthy humans, serum cystatin C levels are expected to be <2.5 mg/L,[6,12] and rheumatoid factors, hemoglobin, bilirubin, and triglycerides do not interfere in the assay.[7] Detailed investigation of the rabbit anti-human cystatin C antibodies against canine cystatin C was not conducted, but rabbit anti-human cystatin C antibodies from the same manufacturer (Dako) have been shown to react to a 13-kd protein in canine cerebrospinal fluid.[11]

Assay characteristics of the PETIA method

The variation between replicates was determined as the coefficient of variation (CV) from a pooled variance estimate of the difference between duplicate determinations at low (<1 mg/L), medium (1-2 mg/L), and high (>2 mg/L) CLI levels from 11, 23, and 24 observations, respectively. Inaccuracy was investigated by evaluating the linearity under dilution. Specifically, duplicate determinations of CLI were made on serum samples with high, medium, and low CLI levels, respectively, diluted 75%, 50%, 25%, and 12.5% using physiologic saline (0.9% NaCl).

Animals

Thirty-seven dogs were included in the study. Ten dogs were clinically healthy Beagle dogs from a research colony, and the remaining 27 dogs were client-owned dogs presented at the Small Animal Hospital, Royal Veterinary and Agricultural University, for various diagnostic, therapeutic, or prophylactic measures. All dogs were subjected to clinical examination, and for all dogs the following blood components were measured: RBC sedimentation rate, total and differential leukocyte count, RBC count, hemoglobin concentration, hematocrit, platelet count, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities, and concentrations of urea, creatinine, fructosamine, cholesterol, total bile acids, albumin, total protein, glucose, calcium, inorganic phosphate, sodium, and potassium. Hematologic analyses were performed on K_3–EDTA stabilized whole blood using an Abbott CELL-DYN 3500 automated hematologic analyzer (Abbott Laboratories, Abbott Park, Ill, USA) , except for RBC sedimentation rate and differential leukocyte count, which were performed manually. All biochemical analyses were performed on serum samples using an automated spectrophotometric analyzer (Cobas Fara II, Hoffmann-La Roche), except for calcium, which was analyzed using an atomic absorption spectrophotometer (Perkin-Elmer 5000, Perkin-Elmer Corp. Analytic Instruments, Norwalk, Conn, USA). All analyses were based on methods routinely employed in our laboratory and were subjected to daily internal quality control and quarterly external quality control. Only test results originating from accepted analytical runs were included. Urine was collected from all 37 dogs by means of a urinary catheter and was subjected to urinalysis. Urine specific gravity was estimated using a refractometer. Semiquantitative determinations of urine glucose, protein, hemoglobin, bilirubin, ketone bodies, and urobilinogen concentrations were performed using Multistix 10SG (Bayer Austria, Vienna, Austria). Urinary sediment, both unstained and stained (Hemacolor, Merck KgaA, Darmstadt, Germany), was subjected to microscopy (×100 and ×500). Additional diagnostic testing on each dog was conducted at the discretion of the individual clinician (eg, ultrasonography, radiography, endocrine testing, and biopsy of the liver, kidney, or other organs).

Based on the examinations and the final clinical diagnoses, the 37 dogs were retrospectively assigned to 3 groups: clinically healthy dogs, dogs with nonrenal diseases, and dogs with renal insufficiency. Clinically healthy dogs had unremarkable findings on clinical and clinical pathologic examination. Dogs with nonrenal diseases had serum levels of urea and creatinine not exceeding the reference intervals for our laboratory, and a final clinical diagnosis other than renal insuffi-

Table 1. Analytical imprecision of cystatin C–like immunoreactivity measurements in canine serum samples.

Mean (mg/L)	SD (mg/L)	No. of Samples	Coefficient of Variation (%)
0.66	0.0636	11	9.6
1.39	0.0825	23	5.9
4.80	0.0795	24	1.7

Table 2. Analytical inaccuracy of cystatin C–like immunoreactivity measurements in canine serum samples assessed by dilution of serum samples.

Regression	Y Intercept (95% Confidence Interval)	Slope (95% Confidence Interval)	*P* (LOF)*	R^2
Linear	0.08 (–0.02, 0.18)	0.99 (0.97, 1.03)	.07	.994
Logarithmic	0.01 (–0.01, 0.04)	0.99 (0.95, 1.05)	.11	.983

*Probability of lack of fit to the model, ie, that the data follow a straight line.

ciency. Dogs with renal insufficiency were azotemic and had clinical and pathologic findings consistent with renal insufficiency (eg, polyuria, polydipsia, and proteinuria) that were not explained by a nonrenal diagnosis. Renal histopathology results were available for some dogs with renal insufficiency.

Serum samples from 20 unclassified dogs were used to investigate the characteristics of the PETIA method. All serum samples subjected to the PETIA method were aliquotted into small plastic vials and stored at –55°C until analysis, for a maximum of 5 weeks.

Statistical analysis

Arithmetic means, SDs, CVs, 95% confidence intervals, and median values were calculated using routine statistical procedures. Linear regression with and without the variance stabilizing logarithmic transformation plus lack of fit (LOF) test were performed.[13-15] Comparison between groups was made using the Kruskal-Wallis test and Dunn's multiple comparison test with *P* values of < .05 considered indicative of significant differences.

Results

Characterization of the PETIA

Analytical imprecision is summarized in Table 1. The CV ranged from 1.7% to 9.6% at 3 CLI levels, with the highest CV in serum samples with low CLI levels. Inaccuracy of the PETIA was investigated by determination of recovery of CLI in diluted canine serum samples (Table 2). The mean recovery was 104.2% ± 7.5%. Analysis of dilutions of canine serum samples resulted in a linear regression equation in which (X,Y) = (degree of dilution, CLI relative to the concentration in the undiluted samples), and whose intercept and slope did not differ from zero and 1, respectively (Table 2). The LOF test revealed that the dilution curve was linear ($P = .07$). In the corresponding logarithmic regression analysis, the slope was not different from 1, thereby indicating parallelism, and the logarithmic model also followed a straight line (LOF, $P = .11$). Hence, the PETIA method measured canine serum CLI in a linear and proportional manner.

Comparison of serum CLI levels in healthy and diseased dogs

Based on laboratory and clinical diagnoses, 17 dogs were classified as clinically healthy, 12 dogs had nonrenal diseases, and 8 dogs had renal insufficiency (Figure 1, Table 3). In 4 of the 8 dogs in the latter group, lesions consistent with renal insufficiency were detected on histopathologic examination of kidney tissue obtained either by kidney biopsy or at necropsy. Histologic diagnoses included membranous glomerulonephritis, glomerulosclerosis, and interstitial fibrosis. The median serum CLI levels were 1.06 mg/L, 1.62 mg/L, and 5.01 mg/L for clinically healthy dogs, dogs with nonrenal diseases, and dogs with renal insufficiency, respectively (Figure 1). The median serum CLI level in dogs with clinical renal insufficiency was significantly higher than that in clinically healthy dogs and dogs with nonrenal diseases ($P < .05$). There was no difference in the median serum CLI level among clinically healthy dogs and dogs with nonrenal diseases ($P < .05$). One dog with nonrenal disease had Cushing's disease and had a high serum CLI concentration (6.25 mg/L).

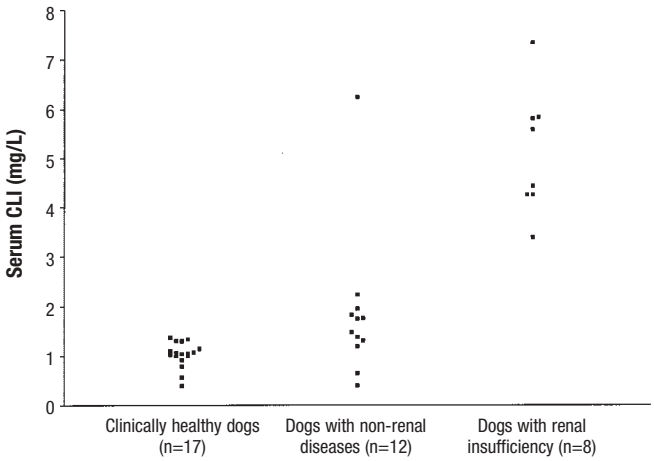

Figure 1. Serum cystatin C–like immunoreactivity (CLI) levels in 3 groups of dogs.

Table 3. Clinical characteristics and serum urea and creatinine concentrations for 37 dogs in which serum cystatin C–like immunoreactivity (CLI) was measured.

Group	Breed	Sex	Age (years)	Urea (mmol/L)*	Creatinine (µmol/L)†	CLI (mg/L)
Clinically healthy	Beagle	Male	1	5.65	83	1.105
	Beagle	Male	4	4.77	91	1.005
	Beagle	Male	6	2.12	72	1.045
	Beagle	Male	7	4.19	55	1.345
	Beagle	Male	2	5.70	66	0.920
	Beagle	Male	7	2.78	67	1.070
	Beagle	Female	5	2.97	65	1.055
	Beagle	Female	5	3.34	69	1.080
	Beagle	Female	4	3.29	71	1.375
	Beagle	Female	6	3.90	79	1.150
	American Bulldog	Male	1	7.79	48	1.315
	American Bulldog	Female	2	9.03	90	1.305
	Dachshund	Female	9	7.54	94	0.400
	Rottweiler	Female	2	7.73	82	1.050
	Mixed breed	Male	2	7.64	106	0.790
	Mixed breed	Female	8	4.18	55	0.570
	Mixed breed	Female	8	6.50	81	1.030
Nonrenal diseases						
Discoid lupus	Mixed breed	Male	9	3.68	64	1.380
Idiopathic polydipsia	Golden Retriever	Male	10	12.60	58	0.650
Chronic atopic dermatitis	Gammel Dansk Hoensehund	Male	3	3.92	73	1.315
Cystitis	Mixed breed	Male	13	9.13	78	1.765
Diabetes insipidus	Dachshund	Female	13	5.18	68	1.955
Acromegaly	German Shepherd Dog	Female	9	2.92	56	2.240
Polyarthritis	Border Collie	Female	0.5	1.77	54	1.200
Cushing's disease	Rottweiler	Female	6	2.69	47	6.250
Cholangioadenocarcinoma	Scottish Terrier	Female	9	3.33	44	1.755
Atrophic gastritis	Labrador Retriever	Female	12	4.81	60	0.400
Polyarthritis	Collie	Female	8	4.30	80	1.830
Systemic lupus erythematosis	Mixed breed	Female	8	9.98	112	1.485
Renal insufficiency						
	Lhasa Apso‡	Male	8	51.40	650	4.255
	Labrador Retriever	Male	0.5	61.82	498	4.255
	Vizsla	Male	1	49.31	394	5.585
	Miniature Schnauzer	Female	0.5	67.21	395	5.810
	Boxer	Female	2	29.51	330	4.430
	Cairn Terrier‡	Female	5	92.00	860	7.350
	Mixed breed‡	Female	9	42.12	400	5.840
	Mixed breed‡	Female	1	33.98	203	3.390

*Reference range for serum urea, 3.30-13.50 mmol/L.
†Reference range for serum creatinine, 40-130 µmol/L.
‡Histopathologic findings consistent with chronic renal failure.

Discussion

Serum cystatin C is being used more often in humans as a rapid and sensitive marker for GFR, and it may be a better marker than serum creatinine for GFR estimation, especially when GFR is only moderately reduced.[4-10] Serum cystatin C is much less affected than serum creatinine by nonrenal factors such as sex, age, muscle mass, inflammation, and neoplasia.[4-10] If cystatin C production and metabolism are similar in dogs and humans, the present findings indicate that serum cystatin C may be an attractive candidate for estimating GFR in dogs.

The heterologous serum cystatin C PETIA employed in this study measured canine serum CLI in a linear and proportional manner, with a mean recovery of 104% ± 7.5% and characterized by CVs ranging from 1.7% to 9.6%. The CVs obtained when using canine serum samples were slightly higher than but still compared favorably to those reported by the manufacturer for human serum samples (CV of 1.3% when cystatin C = 4.24 mg/L; CV of 3.2% when cystatin C = 0.97 mg/L). The serum CLI level in dogs with renal insufficiency was, as expected, higher than that in clinically healthy dogs. Thus, the assay measured canine serum CLI in a reliable manner and adequately reflected the clinically recognized impairment in renal function.

The median serum CLI value was higher, although not significantly so, in dogs with nonrenal diseases (1.62 mg/L) than in clinically healthy dogs (1.06 mg/L). The reason for this tendency was not investigated in the present study, but serum CLI may depend to some degree on the breed of the dog (the majority of the clinically healthy dogs were Beagles). Some of the dogs classified as having nonrenal diseases, eg, the dog with Cushing's disease, may have had an actual reduction in GFR, which was not detected by the methods employed in the present study. Also, GFR in the dog may be influenced by several nonrenal factors such as hydration status;[16] alterations in the fluid balance and extracellular volume in some of the dogs with nonrenal diseases may have affected serum CLI concentration without a corresponding change in GFR.[17] Serum CLI in dogs may be affected by inflammation and neoplasia, as has been reported recently in humans.[9,10] The possibility that the stability of CLI was compromised during freezing seems unlikely because human cystatin C is stable for at least 6 months when serum samples are stored at −80°C.[18]

Because serum CLI can be measured reliably in dogs and because dogs with renal insufficiency have higher serum CLI levels, serum CLI could be used as a marker for GFR in dogs. Additional studies should be performed on the relationship between canine serum CLI concentration and GFR measured by other methods, and on serum CLI variations in dogs with nonrenal diseases. ◊

References

1. Grubb A, Löfberg H. Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. *Proc Natl Acad Sci USA.* 1982;79:3024-3027.
2. Abrahamson M, Olafsson I, Palsdottir A, et al. Structure and expression of the human cystatin C gene. *Biochem J.* 1990;268: 287-294.
3. Tenstad O, Roald AB, Grubb A, Aukland K. Renal handling of radiolabelled human cystatin C in the rat. *Scand J Clin Lab Invest.* 1996;56:409-414.
4. Simonsen O, Grubb A, Thysell H. The blood serum concentration of cystatin C (γ-trace) as a measure of the glomerular filtration rate. *Scand J Clin Lab Invest.* 1985;45:97-101.
5. Grubb A, Simonsen O, Sturfelt G, et al. Serum concentration of cystatin C, factor D and β_2-microglobulin as a measure of glomerular filtration rate. *Acta Med Scand.* 1985;218:499-503.
6. Grubb A. Diagnostic value of analysis of cystatin C and protein HC in biological fluids. *Clin Nephrol.* 1992;38(suppl 1):S20-S27.
7. Kyhse-Andersen J, Schmidt C, Nordin G, et al. Serum cystatin C, determined by a rapid, automated particle-enhanced turbidimetric method, is a better marker than serum creatinine for glomerular filtration rate. *Clin Chem.* 1994;40:1921-1926.
8. Newman DJ, Thakkar H, Edwards RG, et al. Serum cystatin C measured by automated immunoassay: a more sensitive marker of changes in GFR than serum creatinine. *Kidney Int.* 1995; 47:312-318.
9. Randers E, Erlandsen EJ. Serum cystatin C as an endogenous marker of renal function—a review. *Clin Chem Lab Med.* 1999; 37:389-395.
10. Kos J, Stabuc B, Cimerman N, Brunner N. Serum cystatin C, a new marker of glomerular filtration rate, is increased during malignant progression. *Clin Chem.* 1998;44:2556-2557.
11. Uchida K, Kuroki K, Yoshino T, et al. Immunohistochemical study of constituents other than beta-protein in canine senile plaques and cerebral amyloid angiopathy. *Acta Neuropathol (Berl).* 1997;93:277-284.
12. Finney H, Newman DJ, Price CP. Adult reference ranges for serum cystatin C, creatinine and predicted creatinine clearance. *Ann Clin Biochem.* 2000;37:49-59.
13. Box GEP, Hunter WG, Hunter JS. *Statistics for Experimenters.* New York: Wiley & Sons; 1978.
14. Weisberg S. *Applied Linear Regression.* New York: Wiley & Sons; 1985.
15. Høier R, Jensen AL. Evaluation of an enzyme linked immunosorbent assay (ELISA) for determination of insulin in dogs. *J Vet Med Series A.* 1993;40:26-32.
16. Tabaru H, Finco DR, Brown SA, Cooper T. Influence of hydration state on renal functions of dogs. *Am J Vet Res.* 1993;54:1758-1764.
17. Peters AM. Expressing glomerular filtration rate in terms of extracellular fluid volume. *Nephrol Dial Transplant.* 1992;7:205-210.
18. Erlandsen EJ, Randers E, Kristensen JH. Evaluation of the Dade Behring N Latex Cystatin C assay on the Dade Behring Nephelometer II System. *Scand J Clin Lab Invest.* 1999;59:1-8.