

Use of a time-resolved immunofluorometric assay for determination of canine C-reactive protein concentrations in whole blood

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Objective—To develop and validate a time-resolved immunofluorometric assay (TR-IFMA) for measurement of C-reactive protein (CRP) in canine whole blood.

Animals—12 healthy dogs and 35 dogs with inflammatory processes.

Procedure—CRP was isolated from acute-phase serum by affinity chromatography and used as a standard for calibration. Analytic and functional limit of detection and intra-assay and interassay precision were calculated. Accuracy was evaluated by recovery assays and by comparison with results of a commercial ELISA. Correlation between CRP concentrations in whole blood and corresponding plasma fractions was tested by use of TR-IFMA. Stability of blood samples at 4°C was assessed during a 1-month period, and effects of anticoagulants were evaluated. Measurements of CRP in blood samples from 12 healthy dogs were compared with those of 35 dogs with inflammatory diseases.

Results—Analytic and functional limits of detection were 0.53 and 3.26 µg/mL, respectively. Intra-assay and interassay coefficients of variation varied between 2.1% to 8.9% and 8.0% to 12.3%, respectively. Mean recoveries of added CRP were 104% and 114%. Measurements of CRP by use of TR-IFMA and ELISA were highly correlated ($R^2 = 0.97$). Measurements of CRP in whole blood and in corresponding plasma fractions by use of TR-IFMA were also highly correlated ($R^2 = 0.97$). Neither storage nor use of anticoagulants disturbed measurement of CRP concentrations in whole blood. Concentrations of CRP in whole blood of dogs with inflammation were significantly higher than in healthy dogs.

Conclusions and Clinical Relevance—Determination of CRP concentrations in whole blood may provide a diagnostic test for inflammation in dogs. (*Am J Vet Res* 2005;66:62–66)

C-reactive protein (CRP) is a major acute-phase protein (APP) in dogs,¹ with a pronounced and rapid increase in response to infection and tissue

injury. Its blood concentration, usually < 5 mg/L,^{2,3} can increase > 100 times within 24 hours after damage, making serum canine CRP analysis useful for evaluating acute inflammation in clinical and experimental situations.^{4–12}

In time-resolved fluorometry-based immunoassays, antigens or antibodies are labeled with complexes of lanthanide (III) ions (eg, europium, terbium, and samarium) and the specific antigen-antibody binding reaction is observed by measuring the long-decay fluorescence emitted from labels.¹³ Application of this technology offers the following advantages: high sensitivity, accuracy, stability of reagents, lack of radiation, low background, and wide test range.¹⁴

Whole blood cannot be used in most types of immunoassays because of the background signal and interference from samples. The use of time-resolved fluorescence has successfully solved this background problem, but the use of typical lanthanide labels has been compromised because ions may dissociate from chelates in the presence of other chelating compounds such as EDTA.

This problem has been solved in the study reported here by use of an intrinsically fluorescent and stable lanthanide chelate as a label, the europium chelate of 4-[2-(4-isothiocyanatophenyl)ethynyl]-2,6-bis{[N,N-bis(carboxymethyl)-amino]methyl}pyridine. This chelate has already enabled the use of whole blood as well as EDTA-treated plasma in the measurement of several human^{15,16} and animal¹⁷ analytes including human CRP. The measurement of human CRP concentrations in whole blood is highly correlated with its measurement in serum and plasma.^{16,18} However, to our knowledge, no studies exist regarding the use of whole blood for CRP measurements of dogs. The purpose of the study reported here was to develop a time-resolved immunofluorometric assay (TR-IFMA) for the measurement of canine CRP in whole blood.

Material and Methods

Animals and blood samples—Following the criterion of Solter et al,¹⁹ dogs were placed into 2 groups on the basis of disease status (ie, dogs with or without inflammation). In the first group, blood samples from 31 dogs that were admitted to the Veterinary Hospital of Murcia University with various inflammatory diseases were used, with the prerequisite of client consent. Additionally, blood samples from 4 dogs with experimentally induced inflammation caused by SC injection of 5 mL of 5% (wt/vol) casein in water⁴ were included in the study. This procedure followed an animal protocol approved by the Murcia University Ethical Committee. In the second group, blood samples were obtained from 12 healthy dogs that were supplied by the Animal Center of University of Murcia.

Received January 23, 2004.

Accepted May 3, 2004.

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The authors thank Drs. Shizuo Yamamoto and Tomokazu Jinbo for technical assistance.

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Blood samples were collected from all dogs by venepuncture into EDTA tubes.^a Aliquots of the blood samples were centrifuged (3,000 × g, 10 minutes, room temperature [approx 25°C]), and the resulting plasma was stored at -20°C. Whole blood was stored at 4°C during the study. Hematocrits of blood samples were determined by use of an automated blood counter.^b

Purification of CRP—To prepare acute-phase serum, turpentine oil (0.5 mL/kg) was injected SC into a healthy dog.²⁰ The procedure had the approval of the Murcia University Ethical Committee. Three milliliters of the acute-phase serum was put through a column of agarose^c coupled with phosphorylethanolamine^d (PEAE-agarose) according to a previous report²¹ but with slight modifications. The column was equilibrated with 50mM Tris-HCl solution (pH, 8.0) that contained 150mM NaCl, 2mM CaCl₂, and 0.02% NaN₃. The CRP was eluted with 10mM Tris-HCl solution (pH, 8.0) that contained 150mM NaCl, 2mM EDTA, and 0.02% NaN₃.²⁰ Fractions of 0.8 mL were collected, and the protein content of each fraction was estimated by measurement of absorbance at 280 nm. Fractions with high protein concentrations were pooled and assessed for homogeneity by SDS-PAGE²² under reducing conditions in 10% polyacrylamide gels. The concentration of purified CRP was estimated by use of TR-IFMA with a standard curve constructed with pure CRP.^{23,e}

Labeling of antibodies with europium chelates—Anti-canine CRP goat polyclonal antibodies^c were labeled by incubating antibodies with a 100-fold molar excess of the europium chelate of 4-[2-(4-isothiocyanatophenyl)ethynyl]-2,6-bis{[N,N-bis (carboxymethyl)-amino]methyl}pyridine^f in 50mM carbonate buffer solution (pH, 9.8) overnight at 4°C. The free label was removed from labeled antibodies by use of gel-filtration chromatography,^g equilibrated, and run with Tris-HCl solution (pH, 7.5) containing 150mM NaCl and 0.02% NaN₃ (TSA) with a flow rate of 20 mL/h. Fractions containing the labeled antibodies were pooled and filtered through a 0.22-μm-diameter pore filter. Protein concentration of preparation was determined by absorbance at 280 nm.

Biotinylation of antibodies—Goat anti-canine CRP polyclonal antibodies (1 mg/mL) were added to 50mM carbonate buffer solution (pH, 9.8) that contained an 80-fold excess of biotin isothiocyanate^h dissolved in dimethylformamide. The reaction, kept at room temperature, was stopped after 4 hours by removing the excess of biotin isothiocyanate after passing the mixture through ready-made columnsⁱ with TSA. Finally, 0.1% bovine serum albumin was added to avoid aggregation.

Immunoassay method—Firstly, biotinylated anti-CRP antibodies (300 ng/well) were pipetted into streptavidin-coated microtitration wells^j and incubated for 45 minutes at room temperature. Strips were washed 4 times with 5mM Tris-HCl solution (pH, 7.75; ie, wash buffer solution) that contained 0.9% NaCl and 0.05% Tween 20. Next, 200 μL/well of whole blood; EDTA-treated plasma; or standards that were diluted (1:10,000) in 50mM Tris-HCl solution (pH, 7.75; ie, assay buffer solution) containing 0.9% NaCl and 0.05% NaN₃, 0.5% bovine serum albumin, 0.01% Tween 40, 0.05% bovine γ-globulin, 20μM diethylenetriaminepentaacetic acid, and an inert red dye (20 μg/mL) were added. The standard curve was constructed with pure CRP at a concentration of 200 μg/mL. Samples and standards were incubated for 45 minutes, and after a second wash, 200 μL of labeled anti-CRP antibodies was added to each well. Strips were incubated for 45 minutes and then washed again. Finally, enhancement solution^k (200 μL/well) that contained 50mM glycine-NaOH (pH, 10), 1.75M NaSCN, 5mM

Na₂CO₃, 1M NaCl, 5% glycerol, and 20% 1-propanol was added and strips were incubated for 20 minutes. The enhanced fluorescence, proportional to the quantity of CRP in the sample, was measured in a time-resolved fluorometer.^l

Evaluation of the assay—The analytic limit of detection was evaluated by repeated analysis of the zero standard (assay buffer solution), whereas the functional limit of detection was determined by analyzing 8 blood samples from healthy dogs. Both limits of detection were expressed as the mean concentration resulting + 2 SD.¹⁷ The intra-assay precision was determined by calculating coefficients of variation (CV) of 4 blood samples with various CRP concentrations that were analyzed in triplicate. To assess the interassay precision, CV of CRP measurements were calculated for duplicates of the same blood samples on 3 different days during a single week.

Accuracy was assessed by investigating the recovery of CRP that was added to 3 blood samples at concentrations of 10 and 20 μg/mL (obtained from standards). Additionally, CRP concentrations in whole blood from 12 samples were measured by use of TR-IFMA and CRP concentrations in corresponding plasma fractions were measured by use of a commercially available ELISA kit for canine CRP.^m Results obtained with both methods were compared.

Correlation between CRP concentrations of 16 blood samples (8 from healthy dogs and 8 from dogs with high CRP concentrations as a result of various inflammatory processes) and CRP concentrations in corresponding plasma fractions was tested by the TR-IFMA.

Stability of CRP in whole blood—Six blood samples with various amounts of CRP were analyzed on days 1, 3, 7, 15, and 30 after sample collection. All blood samples were stored at 4°C between assays.

Anticoagulants—For this study, 4 healthy dogs were injected with casein to cause inflammation and increase the blood CRP concentrations. At 24 hours after casein injection, blood samples were obtained by venepuncture into tubes containing 1 of 3 anticoagulants (ie, EDTA, lithium heparin, and trisodium citrate). Tubes were stored at 4°C. Serum from blood samples of each dog was stored at -20°C.

Clinical validation—The EDTA-treated blood samples from dogs (n = 35) with various pathologic processes (14 with leishmaniosis, 5 with pyometra, 4 with experimentally induced inflammation caused by the injection of casein, 3 with acute renal failure, 2 with ehrlichiosis, 1 with metritis, 1 with hemangiosarcoma, 1 with chronic granulocytic leukemia, 1 with lymphoma, 1 with a subcutaneous abscess, 1 with microfilaremia, and 1 after an epilepsy episode) and from 12 healthy dogs were used to determine whether inflammation was detectable by use of TR-IFMA. Each dog was assessed by rigorous physical examination and laboratory tests that included hematologic evaluation^b and serum biochemical analysis.ⁿ

Statistical analysis—Simple linear regression analysis was used to compare CRP concentrations in whole blood determined by use of TR-IFMA with concentrations in corresponding plasma fractions determined by use of ELISA and also to compare CRP concentrations in whole blood with CRP concentrations in corresponding plasma fractions when both are measured by use of TR-IFMA. Paired Student *t* tests were performed for analysis of data from stability and anticoagulants studies. ANOVA was used to compare CRP levels in healthy and sick dogs. Data analysis was performed by use of a statistical software program.^o Values of *P* < 0.05 were considered significant.

Results

Isolation of CRP—Canine CRP was purified from acute-phase serum by PEAE-agarose affinity chro-

matography. The protein was eluted from the column with elution buffer solution in a single peak and detected by measuring the absorbance at 280 nm in the collected fractions. Results of SDS-PAGE under reducing conditions of fractions with high protein content revealed that the eluted sample consisted of 2 bands of 29 and 24 kd (Figure 1).

Evaluation of the assay—The analytic limit of detection was calculated as the mean of repeated analysis of the zero calibrator + 2 SD and resulted in a value of 0.53 µg/mL. The functional limit of detection was determined by analyzing blood samples from 8 healthy dogs and resulted in a value of 3.26 µg/mL. The highest imprecision was observed at the lowest CRP concentration. However, for medium and high CRP concentrations, the intra-assay precision varied between 2.1% and 8.9%, whereas the interassay precision ranged between 8.0% and 12.3% (Table 1).

Assay accuracy was evaluated by measuring the

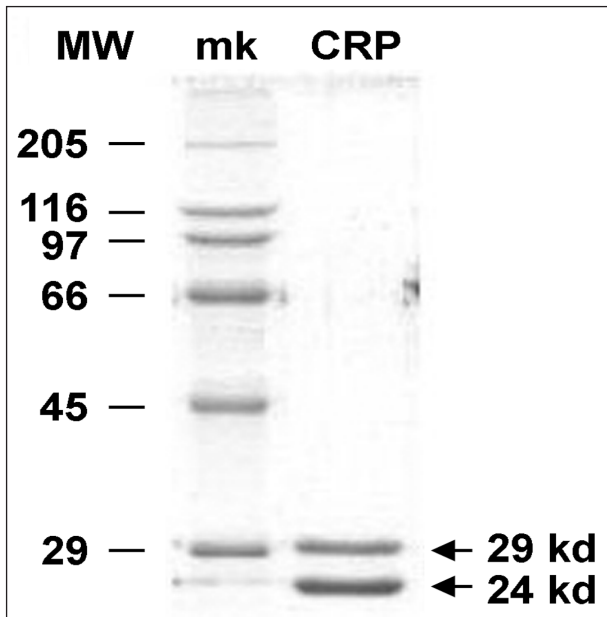


Figure 1—SDS-PAGE of purified C-reactive protein (CRP) from canine acute-phase serum. Electrophoresis in 10% polyacrylamide gels was performed under denaturing and reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250, and molecular masses (MW) were assigned by use of myosin (205 kd), β-galactosidase (116 kd), phosphorylase B (97 kd), bovine serum albumin (66 kd), ovalbumin (45 kd), and carbonic anhydrase (29 kd) as molecular mass markers (mk).

Table 1—Intra-assay and interassay precision of the time-resolved immunofluorometric assay (TR-IFMA) for determination of C-reactive protein (CRP) concentrations in canine whole blood.

Sample	Mean CRP (µg/mL)	CV (%)	
		Intra-assay (n = 3)	Interassay (6)
1	1.39	17.2	NA
2	13.63	8.9	12.3
3	49.45	2.1	9.3
4	56.45	4.6	8.0

CV = Coefficients of variation. NA = Not available.

recovery CRP that was added to 3 blood samples at concentrations of 10 and 20 µg/mL (Table 2). Mean recoveries for CRP concentrations of 10 and 20 µg/mL were 104% and 114%, respectively. Measurements obtained from 12 blood samples by use of TR-IFMA were highly correlated with those obtained by measuring corresponding plasma fractions by use of an ELISA ($R^2 = 0.97$; $y = 1.08x - 1.39$).

By use of TR-IFMA, CRP concentrations in whole blood from 16 EDTA-treated blood samples (8 from healthy dogs and 8 from dogs with pathologic conditions) and those for corresponding plasma fractions also had a good correlation ($R^2 = 0.97$; $y = 0.86x - 1.03$). The CRP concentration in whole blood was slightly lower than in plasma fractions, although no significant ($P = 0.649$) difference in concentrations was observed between whole blood and plasma. The Hcts fluctuated between 39% and 50% in healthy dogs and between 29% and 50% in the 8 dogs with various inflammatory diseases, with 3 dogs having Hcts below the reference range of 37% to 55% for our laboratory. When CRP values in whole blood were corrected for Hct following the recommendation of Urdal et al,¹⁸ the correlation did not improve further (results not shown).

Stability—Results for measurements of CRP in whole blood from 6 samples on different days during a single month were determined (Figure 2). No significant change was found in CRP concentrations in whole blood when measured on different days.

Anticoagulants—Measurements of CRP were performed on serum and heparin-, citrate-, and EDTA-treated blood samples from 4 dogs with high blood concentrations of CRP as a result of casein injection

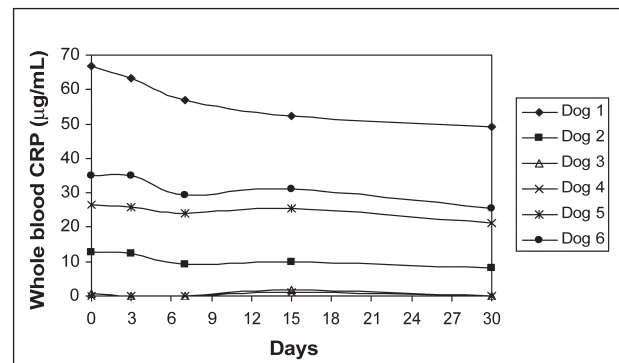


Figure 2—Stability of canine CRP measurements in 6 blood samples during a 1-month period by use of a time-resolved immunofluorometric assay.

Table 2—Recovery of various CRP concentrations after the addition of pure CRP to blood samples.

Sample	Recovery (%)	
	Addition of CRP 10 µg/mL	Addition of CRP 20 µg/mL
1	106.1	130.8
2	112.3	112.8
3	93.6	100.3
Mean	104	114.7

Table 3—Concentrations of CRP measured from blood samples containing different anticoagulants by use of TR-IFMA.

Dog	CRP (μg/mL)			
	Serum	EDTA-treated blood	Heparin-treated blood	Citrate-treated blood
1	75.7	74.4	70.5	68.8
2	55.6	49.9	49.2	44.9
3	42.3	42.1	40.6	39.0
4	35.0	38.1	34.0	29.7

Table 4—Concentrations of CRP in whole blood of 12 healthy dogs and 35 dogs with various inflammatory processes.

No. of dogs	Condition	CRP (μg/mL)	
		Median	Range
12	Healthy	1.08	0–2.78
14	Leishmaniasis	28.11	6.19–66.98
5	Pyometra	26.21	15.97–59.0
4	Inflammation by casein	40.35	25.57–60.40
3	Acute renal failure	65.52	41.14–98.63
2	Ehrlichiosis	66.42	56.54–76.30
1	Postpartum metritis	9.62	NA
1	Hemangiosarcoma	58.25	NA
1	Chronic granulocytic leukemia	76.25	NA
1	Lymphoma	64.28	NA
1	Subcutaneous abscess	39.42	NA
1	Microfilaremia	63.99	NA
1	Epilepsy episode	56.49	NA

NA = Not available.

(Table 3). In general, serum yielded the highest CRP concentrations, whereas citrate-treated blood samples had the lowest whole blood CRP concentrations; however, the differences were not significant.

Clinical validation—The CRP concentration was measured with no correction for variations in Hcts of blood samples from 35 dogs with various inflammatory processes, which ranged from 6.19 to 98.63 μg/mL (Table 4). The highest CRP concentration in whole blood of 12 healthy dogs (not having inflammation) was 2.82 μg/mL. The CRP concentrations in whole blood of dogs with inflammation were significantly higher than those observed in healthy dogs ($P < 0.001$).

The Hcts of dogs with inflammation fluctuated between 18.1% and 55% with 13 values below the reference range. When CRP concentrations were corrected for Hct, the CRP concentration range changed to 6.19–65.04 μg/mL.

Discussion

Investigation during the last decade indicates that the quantification of APPs in plasma or serum can provide valuable diagnostic information in the detection, prognosis, and monitoring of disease.²⁴ Indeed, in the future, any health check regime that omits the APP response may be less than optimal.²⁵

In dogs, blood concentrations of CRP increase in many infections⁹ and inflammatory conditions such as surgical trauma,⁵ *Ehrlichia canis* infection,⁶ gastric mucosal injury,⁷ or leishmaniasis,¹⁰ and APP assays could be used as rapid tests for inflammation providing better

sensitivity, compared with other inflammatory markers such as leukocytosis and neutrophilia.¹⁹ In a recent study,⁹ combined determination of blood concentrations of CRP and haptoglobin had the greatest value in differentiating between alternative pathologic states.

To our knowledge, our study is the first report of an assay for a canine APP that enables the use of whole blood. The possibility of directly using a fresh whole blood sample saves time and also minimizes the possibility of sample mix-up and other technical errors because of the simplified logistic involved.¹⁵ It also enables simpler and cheaper instrumentation (centrifuges are not needed). Furthermore, it makes the work of clinicians easier, bearing in mind that they may also choose to store samples and measure CRP concentration several days after blood collection without compromising the validity of results.

Another advantage of the method described in our study is that samples with various anticoagulants (EDTA, heparin, and citrate) and hemolytic samples can be used, which is in contrast to what has been reported^{3,6,9-12} with the ELISA methods, the most common technique used for measuring canine CRP so far.

Analyte concentrations are lower in whole blood than in serum or plasma because of the volume taken up by RBCs.¹⁷ For example, the CRP concentration in whole blood of humans is lower than in serum.¹⁶ Results of 1 study¹⁸ indicate that high Hcts slightly underestimate human CRP concentrations, whereas low Hcts overestimate CRP concentration; in instances of high or low Hcts, results may be adjusted by use of the following equation:

$$CRP_{0.40} = [(1 - 0.40)/(1 - Hct)] \times CRP \text{ (measured)}.$$

However, results of the TR-IFMA used in our study are hardly disturbed by the presence of RBCs as indicated by the good correlation between CRP concentration in whole blood and in corresponding plasma fractions obtained by use of the TR-IFMA ($y = 0.86x - 1.03$; $R^2 = 0.97$) or ELISA ($y = 1.08x - 1.39$; $R^2 = 0.97$). The stable europium chelate used as a label in the assay enables the measurement of the time-resolved fluorescence that is virtually free from the background signal derived from the sample components and plastics. Furthermore, the dilution factor used in our study for the canine blood samples is higher than that used for assays of human blood samples (1:10,000 vs 1:40¹⁸ or 1:400¹⁶); this is likely to have a positive contribution in reducing any blood-derived assay interference from blood samples. When corrections were made for Hcts, neither the correlation between CRP concentration in whole blood and plasma nor the assay capacity to differentiate blood values between healthy and sick dogs improved. Therefore, we postulate that rendering individual Hcts may not be necessary when determining CRP measurements from blood samples of dogs.

Our findings do indicate that dogs with inflammation can be distinguished from healthy dogs by quantifying the CRP concentrations in whole blood; and thus, CRP concentration in whole blood could be included as a complementary indicator for the diagnosis of inflammation in dogs. The TR-IFMA described in our study constitutes a sensitive, precise, and accurate

method for the measurement of canine CRP concentrations in whole blood. Use of the TR-IFMA may provide a user-friendly alternative to the ELISA and an efficient tool for the analysis of CRP concentrations in canine whole blood.

- a. Vacutest Plast, K3 EDTA (0.2 mg/mL), Arzergrande, Italy.
- b. Vet ABC, ABX Diagnostics, Montpellier, France.
- c. Epoxy-activated agarose (cross-linked 4% beaded agarose), Sigma-Aldrich, Spain.
- d. O-phosphorylethanolamine, Sigma-Aldrich, Spain.
- e. Courtesy of Dr. Shizuo Yamamoto, Azabu University, Japan.
- f. Innotrak Diagnostics, Turku, Finland.
- g. Superdex 200 HR column, Amersham Biosciences, Uppsala, Sweden.
- h. Innotrak Diagnostics, Turku, Finland.
- i. NAP-10 and NAP-5 columns, Amersham Biosciences, Uppsala, Sweden.
- j. Innotrak Diagnostics, Turku, Finland.
- k. DELFIA enhancement solution, PerkinElmer Lifesciences, Wallac Oy, Turku, Finland.
- l. VICTOR 1420 multilabel counter, PerkinElmer Lifesciences, Wallac Oy, Turku, Finland.
- m. Tridelta phase range canine CRP kit, Tridelta Development Ltd, Bray, Ireland.
- n. Cobas Mira Plus, ABX Diagnostics, Montpellier, France.
- o. SPSS software, version 10.0, SPSS Inc, Chicago, Ill.
- p. Eckersall PD. C-reactive protein and haptoglobin, markers of infection, inflammation, hyperadrenocorticism and stress in dogs (abstr). *Vet Clin Pathol* 2003;32:154.

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