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ORIGINAL RESEARCH

Validation of a commercially available automated canine-specific immunoturbidimetric method for measuring canine C-reactive protein

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Key Words
Acute phase protein, CRP, method comparison, reference interval, stability

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Background: Measurement of C-reactive protein (CRP) is used for diagnosing and monitoring systemic inflammatory disease in canine patients. An automated human immunoturbidimetric assay has been validated for measuring canine CRP, but cross-reactivity with canine CRP is unpredictable.
Objective: The purpose of the study was to validate a new automated canine-specific immunoturbidimetric CRP method (Gentian cCRP).
Methods: Studies of imprecision, accuracy, prozone effect, interference, limit of quantification, and stability under different storage conditions were performed. The new method was compared with a human CRP assay previously validated for canine CRP determination. Samples from 40 healthy dogs were analyzed to establish a reference interval.
Results: Total imprecision was < 2.4% for 4 tested serum pools analyzed twice daily over 10 days. The method was linear under dilution, and no prozone effect was detected at a concentration of 1200 mg/L. Recovery after spiking serum with purified canine CRP at 2 different concentrations was 123% and 116%, respectively. No interference from hemoglobin or triglycerides (10 g/L) was detected. CRP was stable for 14 days at 4°C and 22°C. In the method comparison study, there was good agreement between the validated human CRP assay and the new canine-specific assay. Healthy dogs had CRP concentrations that were less than the limit of quantification of the Gentian cCRP method (6.8 mg/L).
Conclusions: The new canine-specific immunoturbidimetric CRP assay is a reliable and rapid method for measuring canine CRP, suitable for clinical use due to the option for an automated assay.

Introduction

The concentration of major acute phase proteins can increase several hundred fold in the blood during systemic inflammatory disease, as a result of altered protein synthesis in the liver.[1,2] C-reactive protein (CRP) is a major acute phase protein in dogs, and is a valuable diagnostic test in this species, used for detecting systemic inflammation, and for monitoring disease progression and response to treatment.[3–7] A validated canine-specific ELISA for measuring CRP is available[8], but the method has high inter-assay variation, and ELISAs are not optimal for routine clinical use because they do not allow random-access analysis and are time consuming to perform. A validation study of a canine-specific immunoturbidimetric assay was published previously, but the assay is not commercially available.[9] A more recently available human automated immunoturbidimetric assay permitted rapid random-access CRP results in clinical samples from dogs at a low cost.[10] The method was first validated using human CRP as calibrator material, and thus the reported results were in human equivalents of

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CRP.[10,11] Although later purified canine CRP became available for calibration[12], the polyclonal antibody is specific for human CRP, and the cross-reactivity with dog CRP remains unpredictable[13], requiring careful validation of every new batch prior to measurement of canine samples.[12,14] Therefore, a canine-specific method would reduce the risk of batches with antibodies that have no or insufficient cross-reactivity with canine CRP. A new immunoturbidimetric canine CRP assay based on chicken anti-canine CRP antibodies has recently been developed. The aim of this study was to validate the new method by investigating imprecision, accuracy, prozone effect, limit of quantification, and interference from hemolysis and lipemia, and to perform a method comparison to the human immunoturbidimetric assay previously validated and widely applied with canine samples.[10] In addition, a reference interval (RI) for canine CRP with the new method was to be established, and the stability of CRP under different, clinically relevant storage conditions was evaluated.

Materials and Methods

Animals and samples

Specimens used in the method validation study were obtained for diagnostic purposes, and submitted to the Clinical Chemistry Laboratory, University Animal Hospital (UDS) at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, for routine analysis. Serum was prepared by centrifugation (2000*g*, 5 min) after clot formation. Serum pools were prepared by mixing samples from 2 to 3 dogs with similar CRP concentrations. Samples were stored for a maximum of 3 months in cryotubes (Sarstedt AG & Co, Nümbrecht, Germany) at −20°C until analysis. Samples that were to be analyzed on different occasions were frozen in aliquots to avoid repeated freeze-thaw cycles, except the study where freeze-thawing effects were investigated. Samples were thoroughly mixed prior to analysis.

Reference interval

For establishing a RI, samples from 40 healthy dogs were collected between May 2012 and April 2013. The animals were blood donors or dogs owned by staff at UDS, and sampling was performed after written consent from the owners. The project was approved by the local ethical committee (Uppsala Animal Ethics Committee, C413/12). Serum was prepared as described above and stored in cryotubes at −80°C for a maximum of 11 months until analysis. Inclusion criteria were owner confirmation that the dog was healthy at the time of sampling and without signs of illness 2 months prior to and during the 2 weeks following sampling, and that laboratory test results were within the normal range. A routine biochemistry profile was performed on all dogs (Abbott Archtect c4000, Abbott Park, IL, USA). A full automated hematology profile (Advia 2120; Siemens Healthcare Diagnostics, Deerfield, IL, USA) including manual WBC differential count was performed in 23 dogs; the remaining 17 dogs had only a manual PCV value reported. Twenty-three of the dogs were clinically examined by a veterinarian prior to sampling, and in these animals, normal clinical examination was added as inclusion criterion.

Analysis of C-reactive protein

CRP concentration was determined using a canine-specific immunoturbidimetric method (Gentian cCRP lot 1212703; Gentian AS, Moss, Norway) on a fully automated, open-system clinical chemistry/immunoassay analyzer (Abbott Architect c4000, Abbott Park, IL, USA). The main reagent consists of polyclonal chicken anti-canine CRP antibodies resulting in increased turbidity upon reaction with canine CRP, measured spectrophotometrically. Calibration was performed once weekly with canine CRP (cCRP calibrator lot 1212406; Gentian AS). Samples with CRP concentrations of > 300 mg/L were diluted 1:5 with 0.9% NaCl by the instrument, and re-analyzed. Two canine control samples (cCRP low control lot 1212401 and cCRP high control lot 1212402; Gentian AS) were analyzed each day where experiments were performed.

Experimental design

Imprecision was determined by analyzing 4 serum pools in duplicates twice daily, with a minimum of 2 h between runs, for 10 days. The assay was recalibrated once during the imprecision study. Linearity under dilution was investigated by manually diluting a serum sample with an initial CRP concentration of 293 mg/L, with the aim of testing linearity in the range up to 300 mg/L where no autodilution of samples was performed. The sample was serially diluted to concentrations of 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, and 0.025 of the original concentration, using 0.9% NaCl as diluent. Samples were measured in duplicate in random order in a single run. To test linearity of samples with CRP concentrations exceeding 300 mg/L, a serum sample was spiked with purified canine CRP (Life Diagnostics, West Chester,

PA, USA) to a concentration of 1201 mg/L, and manually diluted to concentrations of 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.025 of the original concentration, using 0.9% NaCl as diluent. Samples were measured in duplicate in a random order in a single run, and samples > 300 mg/L were auto-diluted by the instrument.

The presence of a prozone effect, which may cause false low results due to antigen excess, was investigated by analyzing an undiluted spiked serum sample. In a recovery study, a serum sample with a CRP concentration of 23 mg/L was spiked with purified canine CRP (Life Diagnostics) to predicted concentrations of 52 mg/L and 100 mg/L. Samples in the recovery study were measured in triplicate in a single run. Limit of quantification (LoQ), the lowest amount of analyte in a sample that can be quantitatively determined with stated acceptable imprecision and trueness[15], was determined by preparing 3 samples with expected CRP concentrations of 2.9 mg/L, 4.9 mg/L, and 6.8 mg/L, by diluting a serum sample with CRP 36 mg/L (base pool) with 0.9% NaCl. Each sample was analyzed in 36 replicates, with 12 replicates daily on 3 different days. The base pool was analyzed in 12 replicates, with 3 replicates daily on 3 different days. A new calibration was performed each day of experiment in the LoQ study.

For interference studies, a hemolytic solution was prepared by an osmotic shock procedure following guidelines in CLSI EP7-A2.[16] EDTA-stabilized canine blood was centrifuged and the plasma discarded. Blood cells were washed 3 times by adding 0.9% NaCl, centrifuging the sample and then removing the supernatant. In the next step, cells were lysed by dilution with distilled water and freezing. After thawing, centrifugation, and discarding cell debris, a hemolytic solution with a hemoglobin concentration of 100 g/L was obtained. For testing the effect of lipemia, a commercial fat emulsion was purchased (Intralipid 200 g/L; Fresenius Kabi AB, Uppsala, Sweden). Two serum pools with CRP concentrations of 33 mg/L (low pool) and 115 mg/L (high pool) were prepared, and each pool was divided into 4 vials of equal volumes. The hemolytic solution was added to one vial to obtain a test pool with a final hemoglobin concentration of 10 g/L. An equal volume of saline was added to another vial, to obtain a control pool for the hemolytic test pool. The fat emulsion was added to one of the 2 remaining vials, to obtain a test pool with a final triglyceride concentration of 10 g/L. The same volume of distilled water was added to the forth vial, to obtain a control pool for the lipemic test pool. Samples were measured in triplicate in random order in a single run.

Storage stability was evaluated using 3 serum samples collected within 3 hours. Samples were analyzed in triplicate and then stored in aliquots at approximately 22°C and at 4°C, respectively. They were analyzed in triplicates on days one, 2, 3, 4, 5, 7, 10, and 14 (22°C) and 2, 4, 7, 10, and 14 (4°C), or until the deviation from baseline (day 0) was exceeding 10% at 2 subsequent test points. Three fresh serum pools with CRP concentrations 27 mg/L, 46 mg/L, and 113 mg/L were used for studying the stability of CRP during freezing and thawing. Samples were analyzed in triplicate when fresh, and after one, 2, 3, and 4 freeze-thaw cycles in −20°C. In each cycle, samples were frozen for 24 h, thawed, analyzed, and then immediately refrozen.

The Gentian cCRP method was compared with a Randox CRP test lot CP9742 (Randox Laboratories Ltd, Crumlin, UK), which is a human immunoturbidimetric CRP test that has been previously validated for measuring canine CRP.[10] Analyses were performed on an Abbot Architect (Abbott Architect c4000). The method was calibrated with canine CRP (Life Diagnostics). The intra- and inter- assay coefficients of variation for the Randox method at a concentration of approximately 30 mg/L were 1.9% and 4.2%, respectively. The range of linearity was 10–241 mg/L, and samples with CRP concentrations > 241 mg/L were autodiluted 1:3 with 0.9% NaCl. All samples were analyzed in duplicate by both methods immediately after each other.

Statistical methods and performance goals

Statistical analyses were performed using a statistical software for Microsoft Excel (Analyse-it Software Ltd, Leeds, UK). Quality goals were derived from previously reported data on biological variability in healthy dogs.[17] The allowable imprecision (CV_{max}) was set at 12%, allowable bias ($bias_{max}$) at 9.5%, and total allowable error (TE_a) at 29.6%.

Arithmetic means, standard deviations, and coefficient of variations (CV) were calculated using routine descriptive statistics. Imprecision was assessed following recommendations in NCCLS EP5-A2[18], using a nested ANOVA to obtain information about within run, between run and between day variation, and total imprecision. To evaluate linearity under dilution, an ordinary least square regression analysis was performed to test if the intercept was different from 0 and the slope different from one, with significance level $\alpha = 0.05$. Acceptable recovery after spiking a serum sample with purified canine CRP was set to 80–120%. Recovery was calculated by dividing the observed CRP result with the expected CRP result, after subtracting

the concentration of the original serum sample (23 mg/L) from both results. The expected CRP concentration was calculated based on the concentration provided from the manufacturer of the purified CRP (Life Diagnostics). In the LoQ study, the observed mean value and standard deviation (SD) of the 36 replicates of each low pool were determined. The expected concentrations of the low pools were calculated based on the known concentration of the base pool and dilution factors. Bias was the difference between observed and expected concentrations. The total error (TE) was calculated with the following formula:

$$TE = \text{bias} + 2SD$$

The LoQ was the concentration of the lowest sample that had a total error smaller than the allowable total error.

The maximum allowable change in CRP concentration caused by interfering substances was set to ± 10%. Number of replicates required for detecting interference effects with 95% confidence and power was determined according to the following formula:[16]

$$n = 2((z_{1-\alpha/2} + z_{1-\beta})s/d_{\max})^2$$

where $\alpha = 0.05$, $\beta = 0.05$, s = within run precision of the method, and $d_{\max}$ = maximum allowable difference caused by interference, expressed as 10% of the mean concentrations of the low pool and high pool, respectively. The calculated number of samples (n) was < 3. Triplicate measurements were used in the study.

Bias caused by interference was calculated as the difference in mg/L (d_{obs}) between the test pool containing an interferent and the control pool. The 95% confidence intervals (CI) for d_{obs} were calculated according to the following equation:[16]

$$d_{obs} \pm t_{0.975\ n-1} s \sqrt{\frac{(2)}{n}}$$

The acceptance limits, expressed in mg/L, were ± 0.1 × $CRP_{control\ pool}$. If the 95% CI of d_{obs} was within the acceptance limits, it was concluded with 95% confidence that an interfering effect of ≥ 10% was not present.

In the stability study, a deviation below ± 10% was considered acceptable.

Data from the method comparison study were analyzed as previously described.[19] Correlation was calculated and Passing–Bablok regression analysis performed to derive regression data.[20] Results were compared against the inherent imprecision of both methods, calculated as $\sqrt{((CV^2_{Gentian}/2) + (CV^2_{Randox}/2))}$. A Bland–Altman difference plot was created including limits representing the 0 ± 95% CI of the combined inherent imprecision, and the methods were considered identical within inherent imprecision if ≥ 95% of the observations were within the limits.

Reference interval

For determining a RI, samples from healthy dogs were thawed and analyzed in a single run.

The RI was to be calculated with the robust method including Box–Cox transformation, using the Reference Value Advisor.[21] A prerequisite for this approach was that the healthy dogs had CRP concentrations above the LoQ of the Gentian method; otherwise, an exact RI could not be established and results were reported as < LoQ.

Results

All experiments were performed during a 5-week period in 2013. The imprecision was lower than the allowable imprecision (12%) for all tested serum pools (Table 1). In the study of linearity under dilution, regression analysis revealed a small constant error when diluting the sample with CRP concentration 293 mg/L, but no proportional error. The intercept was 2.5 mg/L (95% CI 1.4–3.7 mg/L) and the slope was 1.00 (95% CI 0.99–1.01). The dilution of the spiked sample with CRP concentration 1201 mg/L revealed no constant or proportional error when analyzed with regression analysis; the intercept was not different from 0, and the slope not different from one (Figure 1). The sample with CRP concentration 1201 mg/L was correctly reported to be higher than the upper calibrator point when analyzed undiluted, which indicated that no analytically relevant prozone effect was present at this concentration. In the recovery study, the observed recoveries were 123% and 116% for the spiked samples with expected CRP concentrations of 52 mg/L and 100 mg/L, respectively. Limit of Quantification was determined at 6.8 mg/L,

Table 1. Imprecision: the coefficient of variation for within and between run, and between day imprecision and total coefficient of variation (CV) for 4 canine serum pools analyzed in duplicate twice daily for 10 days with a new automated canine-specific immunoturbidimetric method to measure C-reactive protein (CRP).

Mean CRP Concentration mg/L	$CV_{within\ run}$%	$CV_{between\ run}$%	$CV_{between\ day}$%	CV_{total}%
26.5	1.7	0.2	1.6	2.4
141	0.5	0.3	1.1	1.3
227	0.5	0.0	1.9	2.0
370	1.2	0.0	0.8	1.5

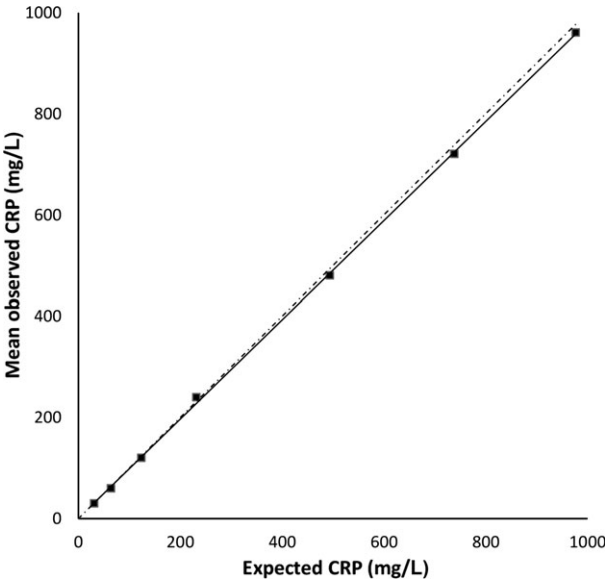

Figure 1. Linearity under dilution of a spiked canine serum sample with a C-reactive protein (CRP) concentration of 1201 mg/L, manually diluted to concentrations of 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.025 of the original concentration, and measured with a new automated canine-specific immunoturbidimetric CRP method. The solid line represents regression line (95% confidence interval), intercept −0.9 mg/L (−10.5–8.7 mg/L), and slope 1.02 (1.00–1.04). Broken line represents $Y = X$.

Table 2. Limit of quantification (LoQ) determined in three canine serum pools diluted to low C-reactive protein (CRP) concentrations. Samples were analyzed 36 times on 3 different days with a new automated canine-specific immunoturbidimetric CRP method. The total error (TE) was calculated (TE = bias + 2 standard deviations [SD]). LoQ was 6.8 mg/L, as TE exceeded the set quality goal (29.6%) for lower concentrations.

Expected CRP Concentration (mg/L)	Mean Observed CRP Concentration (mg/L)	SD (mg/L)	TE (%)	TE < TEa
6.8	7.1	0.39	16	Yes
4.9	5.9	0.35	38	No
2.9	4.8	0.39	90	No

TEa indicates allowable total error.

because the TE exceeded the set quality goal for lower concentrations (Table 2). No interference was detected for hemoglobin or triglycerides at a concentration of 10 g/L (Table 3). CRP was acceptably stable during storage for 14 days at approximately 22°C and 4°C (Figure 2). In the freeze-thaw experiment, the CRP concentrations observed after one, 2, 3, and 4 freeze-thaw cycles were 97–102% of the initial concentrations.

The method comparison study was performed over a period of 23 days. Thirty-eight fresh and 11 frozen samples, in total 49 specimens, were included. Three samples had CRP concentrations below LoQ for the Gentian method (6.8 mg/L), and with the Randox method, these samples had CRP concentrations < 10 mg/L. For the remaining 46 samples, the Passing–Bablok regression analysis revealed small constant and proportional errors with intercept 7.3 mg/L (95% CI 5.1–11.7 mg/L) and slope 0.92 (95% CI 0.88–0.95) (Figure 3). The correlation coefficient (r) was .995. For samples that were not diluted for any of the methods (n = 33), there was no constant or proportional error; the intercept was 2.2 mg/L (95% CI −1.2–5.8 mg/L), and the slope was 0.98 (95% CI 0.95–1.01). Thirteen samples with high CRP concentrations were autodiluted with either the Randox or both methods. Due to the low number of diluted samples, no regression analysis was performed for this subgroup of samples, but there appeared to be mainly a constant error, with Randox measuring approximately 11% higher values than the Gentian method. The Randox and Gentian methods were not identical within the inherent imprecision of both methods, because > 5% of the observations were outside the limits representing the 0 ± 95% CI of the combined inherent imprecision (Figure 4).

Reference interval

The 40 dogs included in the RI study had a median age of 5 years (range 0.5–11 years) and included 19 intact males, one castrated male, and 20 intact females. The dogs were of 12 different breeds including Golden Retrievers (n = 7), Labrador Retrievers (n = 7), Giant Schnauzers (n = 5), Flat-Coated Retrievers (n = 4), German Shepherds (n = 4), Border Terriers (n = 3), mixed breed dogs (n = 3), Norfolk Terriers (n = 2), German Pointer (n = 1), Rottweiler (n = 1), Landseer (n = 1), and Dalmatian (n = 1). All reference dogs except one had CRP concentrations lower than the LoQ of 6.8 mg/L; therefore, the RI could not be calculated with the robust method. One male Dalmatian had a CRP concentration of 16 mg/L. This dog did not undergo a clinical examination prior to sampling. A serum sample removed from the same dog 6 months earlier and stored at −80°C had a CRP concentration < 6.8 mg/L.

Discussion

Two important components of a method validation study are imprecision and accuracy. Imprecision for the Gentian cCRP method was low with a total coefficient of variation of < 2.4% for all tested samples, compared with the maximal allowable imprecision of 12%.[17] In this study, data on biological variability in

Table 3. Interference: the observed difference in C-reactive protein (CRP) concentration between a canine test pool with interfering substance and control pool, when measured with a new automated canine-specific immunoturbidimetric CRP method. The maximum allowable change in CRP concentration caused by interference was set to ± 10%.

Interferent	$CRP_{control\ pool}$ (mg/L)	$CRP_{test\ pool}$ (mg/L)	d_{obs} [95% CI] (mg/L)	Acceptance Limits* of Interference (mg/L)
Hemoglobin 10 g/L	29.0	29.3	0.33 [−1.28; 1.95]	[−2.9; 2.9]
	111.5	111.5	−0.03 [−2.39; 2.32]	[−11.2; 11.2]
Triglycerides 10 g/L	30.3	31.3	1.00 [−0.62; 2.62]	[−3.0; 3.0]
	119.5	119.8	0.27 [−2.08; 2.62]	[−12.0; 12.0]

d_{obs} (95 CI) indicates observed difference (95% confidence interval).
*Acceptance limits = $\pm 0.1 \times CRP_{control\ pool}$.

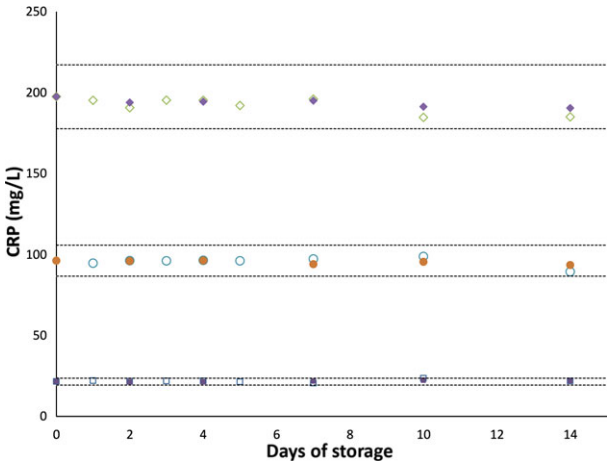

Figure 2. Stability of C-reactive protein (CRP) at approximately 22°C (open symbols) and 4°C (filled symbols) during 14 days of canine sample storage, and measured by a new automated canine-specific immunoturbidimetric CRP method. The CRP concentrations for the low, medium, and high specimens were 22 mg/L, 96 mg/L, and 197 mg/L, respectively. Dashed lines represent acceptable deviation (± 10%).

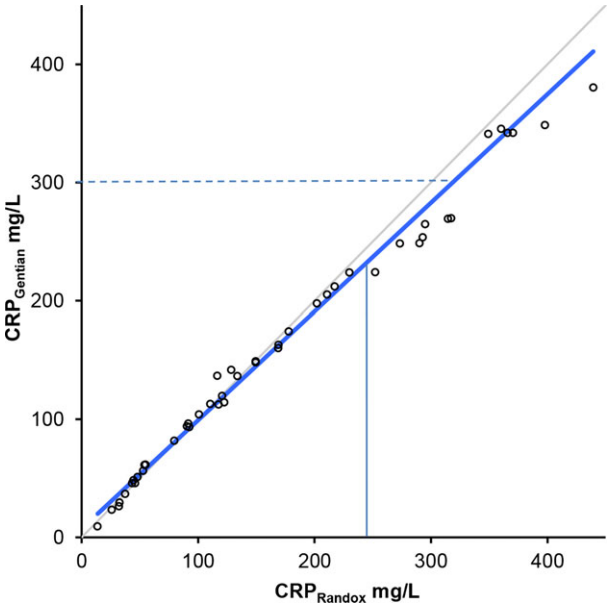

Figure 3. Passing–Bablok regression analysis for C-reactive protein (CRP) concentrations in canine sera measured with a new automated canine-specific immunoturbidimetric CRP method, and a human CRP assay previously validated in dogs (Randox) (n = 46). The solid blue line represents the line of best fit from regression analysis $CRP_{Gentian} = 0.92 \times CRP_{Randox} + 7.3$, and the gray line represents line of agreement $Y = X$. The correlation coefficient (r) was .995. The vertical line represents Randox CRP concentration 241 mg/L; samples with higher concentrations were autodiluted 1:3 by the Randox method. The dashed horizontal line represents Gentian CRP concentration 300 mg/L; samples with higher concentrations were autodiluted 1:5 by the Gentian method.

healthy dogs were used to set the maximal allowable imprecision and TE. There are different ways of establishing analytic quality specifications[22,23], and optimally, decisions are taking into account "the effect of analytical performance on clinical outcomes in specific clinical settings."[23] However, such studies are seldom available for veterinary tests. The next best way to establish quality specifications is either to use data on biological variation, which is available for CRP in dogs[17,24,25], or to analyze clinicians' opinions about the highest acceptable error of a test that can be accepted without negatively affecting the clinical decisions.[23] To our knowledge, the latter approach has not yet been reported for interpretation of canine CRP. Hence, data on biological variability were used in this study to determine allowable imprecision and allowable total error.[17] The quality goals in the recovery, interference, and stability studies were set arbitrarily with the intention that clinical decisions are not affected as long as the goals are fulfilled. Considering the marked difference in CRP concentration between healthy dogs and dogs with systemic inflammatory disease, it can be questioned how useful it is to base quality specifications for CRP on biological variability data.[26] For future studies, preference may be given to the establishment of quality goals based on clinicians' assessment to what degree different CRP test results affect patient care.

True bias could not be assessed in this study due to the lack of a gold standard method or available standard material for accurate measurement of canine

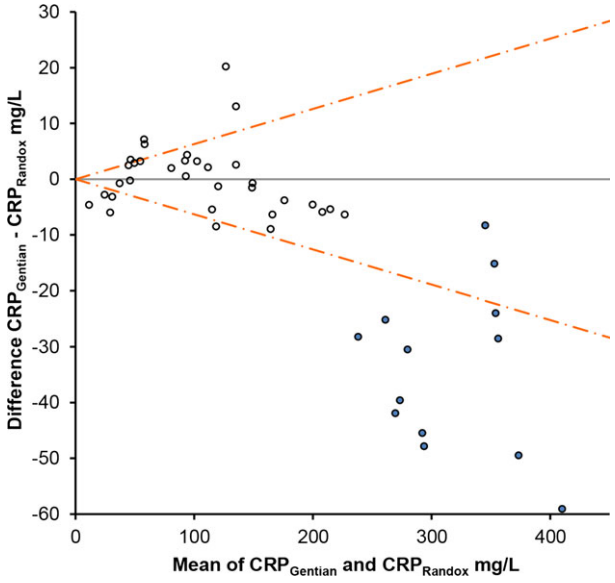

Figure 4. Bland–Altman difference plot for C-reactive protein (CRP) concentrations in canine sera measured with a new automated canine-specific immunoturbidimetric CRP method and a human CRP assay previously validated in dogs (Randox) (n = 46). The methods were not identical within inherent imprecision, as 21 observations (46%) were not within the dotted lines representing 0 ± 1.96 × inherent imprecision of both methods (3.2%). Open symbols represent samples that were analyzed undiluted, whereas filled symbols were autodiluted by the instrument. Autodilution was performed for samples > 241 mg/L by the Randox method (dilution 1:3), and for samples > 300 mg/L by the Gentian method (dilution 1:5), and the difference between the methods was most pronounced for samples that were autodiluted.

CRP. In the spike and recovery study, where purified CRP was added to a serum sample, the recovery was slightly higher than the preset quality goals at one of the 2 tested concentrations. However, if laboratory test results are interpreted with guidance from RIs and/or clinical decision limits established at the individual laboratory, as recommended earlier[27], bias should not affect interpretation negatively. Accuracy was indirectly assessed by linearity studies and was found to be acceptable. There was a small constant error in one of the 2 dilution studies; if linear, the intercept was expected at 0 mg/L; in this study, it was at 2.5 mg/L (95% CI 1.4–3.7 mg/L). The low absolute value and narrow CI close to zero implicates that this finding was of minor importance. It can be compared with the results from the linearity study previously performed with the Randox method, where the intercept was 6.3 mg/L (95% CI −3.4–15.9 mg/L).[10] Bias could not be determined in the method comparison study either, as the comparative method was not a gold standard method. A human immunoturbidometric assay was used for comparison, but because it was calibrated with purified canine CRP, the results were reported in canine CRP and not human equivalents of CRP. Agreement was found between the 2 methods based on results from regression analysis, and although the difference between them was larger than could be explained by inherent imprecision of the methods, the result indicates that both methods yielded similar results for canine CRP. The biggest discrepancy between the methods was found in samples that were autodiluted in one or both assays. This could be due to a dilution error, but the cause for the discrepancy was not investigated further.

The absence of a prozone effect should be documented prior to introducing a new immunologic test for measuring CRP, as false low results can result in incorrect clinical decisions with consequent suboptimal patient care, for example, when repeated CRP measurements are used to monitor treatment effect. In this study, no prozone effect was found for a spiked serum sample with a CRP concentration of approximately 1200 mg/L, which, to our knowledge, is a higher concentration than has been reported in canine patients with inflammatory disease in the literature, and higher than any patient sample so far admitted to the authors' laboratories (unpublished data).

Limit of quantification, the lowest measurable concentration of an analyte within preset quality criteria, was 6.8 mg/L for the Gentian cCRP assay. The optimal clinical cut-off value for canine CRP has been established by analyzing samples from dogs with various types of diseases with the Randox method and set to 16.8 mg/L.[28] Therefore, a LoQ of 6.8 mg/L should be sufficient for a CRP test intended for clinical use. In human medicine, high-sensitivity CRP assays are commonly used to evaluate risk of cardiovascular disease[29], and research projects have evaluated CRP concentrations in the low range in dogs with heart failure and obesity.[30,31] For this type of research where small differences at low CRP concentrations are expected, the Gentian cCRP assay is unsuitable. The limit of blank was not determined in this study as it was not of clinical importance, but it should be evaluated if a high-sensitivity CRP assay is to be developed.

No interference in hemolytic or lipemic specimens was detected with the Gentian cCRP method. This was also true for a time-resolved immunofluorometric assay[32], whereas interference from hemolysis and lipemia was documented in a canine CRP ELISA test[33], and hemolytic specimens yielded false low results with a canine-specific immunoturbidimetric method.[9] The Randox method at UDS in Uppsala, used as comparative method in the study, is slightly affected by hemolysis, and markedly affected by lipemia

(A.H., unpublished data). Lack of interference from hemolysis and lipemia is an advantage in a clinical setting, because hemolytic and/or lipemic samples are common in canine patients predetermined for CRP analysis. The tested levels of hemolysis and lipemia (10 g/L) in this study are macroscopically obvious, and the concentration of interferents was similar to what has been previously tested for canine CRP.[32,33] Increased bilirubin concentrations have been shown to interfere with CRP tests[33], but were not tested in this study. Future studies should address this further.

As previously shown, canine CRP is stable at −10°C for 3 months[34], and our study demonstrated good stability for 14 days both at room temperature and at approximately 4°C. Up to 4 freeze-thaw cycles had no effect on results.

For establishing RIs, it is recommended to use 120 animals; numbers as low as 40 individuals may be used, but negative effects on the accuracy and precision of the RI have to be anticipated.[27] However, for a major acute phase protein like CRP, a clinical cut-off value higher than the RI is preferred[28], and it was estimated that inclusion of only 40 animals would be sufficient in this study. Our study showed that in 40 healthy dogs, CRP concentrations were < 6.8 mg/L with the Gentian cCRP method. An exact RI could not be established because the concentrations were lower than the method's LoQ. One presumed healthy dog had an increased CRP concentration of 16 mg/L. From this dog, another specimen obtained 6 months earlier was < 6.8 mg/L as in the other healthy dogs. A possible explanation for the increased CRP at one of the occasions is that the dog had a subclinical inflammation.

In conclusion, the new Gentian cCRP method is a reliable method for measuring canine CRP, and as it is an automated assay with short turnaround time, mountable on random-access machinery, the method is suitable for clinical use. Healthy dogs are expected to have CRP concentrations less than the LoQ of the method (6.8 mg/L), and serum may be stored up to 14 days refrigerated or at room temperature prior to analysis.

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References

1. Ceron JJ, Eckersall PD, Martynez-Subiela S. Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Vet Clin Pathol*. 2005;34:85–99.
2. Eckersall PD, Bell R. Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. *Vet J*. 2010;185:23–27.
3. Yamamoto S, Shida T, Santsuka H, et al. Changes in serum C-reactive protein levens in dogs with various disorders and surgical traumas. *Vet Res Commun*. 1993;17:85–93.
4. Nakamura M, Takahashi M, Ohno K, et al. C-reactive protein concentration in dogs with various diseases. *J Vet Med Sci*. 2008;70:127–131.
5. Lowrie M, Penderis J, Eckersall PD, McLaughlin M, Mellor D, Anderson TJ. The role of acute phase proteins in diagnosis and management of steroid-responsive meningitis arteritis in dogs. *Vet J*. 2009;182:125–130.
6. Gebhardt C, Hirschberger J, Rau S, et al. Use of C-reactive protein to predict outcome in dogs with systemic inflammatory response syndrome or sepsis: original study. *J Vet Emerg Crit Care*. 2009;19:450–458.
7. Dabrowski R, Kostro K, Lisiecka U, Szczubial M, Krakowski L. Usefulness of C-reactive protein, serum amyloid A component, and haptoglobin determinations in bitches with pyometra for monitoring early post-ovariohysterectomy complications. *Theriogenology*. 2009;72:471–476.
8. Kjelgaard-Hansen M, Kristensen AT, Jensen AL. Evaluation of a commercially available enzyme-linked immunosorbent assay (ELISA) for the determination of C-reactive protein in canine serum. *J Vet Med A Physiol Pathol Clin Med*. 2003;50:164–168.
9. Eckersall PD, Conner JG, Harvie J. An immunoturbidimetric assay for canine C-reactive protein. *Vet Res Commun*. 1991;15:17–24.
10. Kjelgaard-Hansen M, Jensen AL, Kristensen AT. Evaluation of a commercially available human C-reactive protein (CRP) turbidometric immunoassay for determination of canine serum CRP concentration. *Vet Clin Pathol*. 2003;32:81–87.
11. Klenner S, Bauer N, Moritz A. Evaluation of three automated human immunoturbidimetric assays for the detection of C-reactive protein in dogs. *J Vet Diagn Invest*. 2010;22:544–552.
12. Kjelgaard-Hansen M. Comments on measurement of C-reactive protein in dogs. *Vet Clin Pathol*. 2010;39:402–403.

13. Yamamoto S, Miyaji S, Abe N, Otabe K, Furukawa E, Naiki M. Canine C-reactive protein (CRP) does not share common antigenicity with human CRP. *Vet Res Commun*. 1993;17:259–266.
14. Cerón JJ, Martinez-Subiela S, Ohno K, Caldin M. A seven-point plan for acute phase protein interpretation in companion animals. *Vet J*. 2008;177:6–7.
15. National Committee for Clinical Laboratory Standards. *Protocols for determination of limits of detection and limits of quantification; approved guideline*. EP17-A. Wayne, PA: National Committee for Clinical Laboratory Standards; 2004.
16. Clinical and Laboratory Standards Institute. *Interference testing in clinical chemistry; approved guideline*, EP7-A2. 2nd ed. Wayne, PA: Clinical and laboratory standards institute; 2005.
17. Kjelgaard-Hansen M, Mikkelsen LF, Kristensen AT, Jensen AL. Study on biological variability of five acute-phase reactants in dogs. *Comp Clin Pathol*. 2003;12:69–74.
18. National Committee for Clinical Laboratory Standards. *Evaluation of precision performance of quantitative measurement methods; approved guidelines*, EP5-A2. 2nd ed. Wayne, PA: National Committee for Clinical Laboratory Standards; 2004.
19. Jensen AL, Kjelgaard-Hansen M. Method comparison in the clinical laboratory. *Vet Clin Pathol*. 2006;35: 276–286.
20. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry. Part I. *J Clin Chem Clin Biochem*. 1983;21:709–720.
21. Geffré A, Concordet D, Braun JP, Trumel C. Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. *Vet Clin Pathol*. 2011;40:107–112.
22. Montori VM, Guyatt GH. What is evidence-based medicine and why should it be practiced? *Respir Care*. 2001;46:1201–1214.
23. Kenny D, Fraser CG, Petersen P, Kallner A. Consensus agreement. *Scand J Clinl Lab Invest*. 1999;59:585.
24. Carney PC, Ruaux CG, Suchodolski JS, Steiner JM. Biological variability of C-reactive protein and specific canine pancreatic lipase immunoreactivity in apparently healthy dogs. *J Vet Intern Med*. 2011;25:825–830.
25. Martínez-Subiela S, Tecles F, Cerón JJ. Critical differences of acute phase proteins in canine serum samples. *Vet J*. 2003;166:233–237.
26. Antonsen S. The estimation of biological and preanalytical variations of inflammation markers. *Scand J Clin Lab Invest Suppl*. 1994;54:55–60.
27. Friedrichs KR, Harr KE, Freeman KP, et al. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet Clin Pathol*. 2012;41:441–453.
28. Christensen MB, Langhorn R, Goddard A, et al. Comparison of serum amyloid A and C-reactive protein as diagnostic markers of systemic inflammation in dogs. *Can Vet J*. 2014;55:161–168.
29. Wilson AM, Ryan MC, Boyle AJ. The novel role of C-reactive protein in cardiovascular disease: risk marker or pathogen. *Int J Cardiol*. 2006;106:291–297.
30. Cunningham SM, Rush JE, Freeman LM. Systemic inflammation and endothelial dysfunction in dogs with congestive heart failure. *J Vet Intern Med*. 2012;26: 547–557.
31. Tvarijonaviciute A, Martinez S, Gutierrez A, Ceron JJ, Tecles F. Serum acute phase proteins concentrations in dogs during experimentally short-term induced overweight. A preliminary study. *Res Vet Sci*. 2011;90:31–34.
32. Parra MD, Cerón JJ. Effects of haemolysis, lipaemia and bilirubinaemia in canine C-reactive protein and haptoglobin determination by time-resolved fluorometry: short communication. *Acta Vet Hung*. 2007;55:295–299.
33. Martínez-Subiela S, Cerón JJ. Effects of hemolysis, lipemia, hyperbilirrubinemia, and anticoagulants in canine C-reactive protein, serum amyloid A, and ceruloplasmin assays. *Can Vet J*. 2005;46:625–629.
34. Riley RF, Zontine W. Further observations on the properties of dog C-reactive protein and the C-reactive protein response in the dog. *J Lab Clin Med*. 1972;80: 698–703.