Assessment of three automated assays for C-reactive protein determination in dogs

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Objective—To determine the characteristics of an automated canine C-reactive protein (CRP) assay and evaluate 2 human CRP assays for use in dogs.

Animals—56 client-owned dogs with pyometra and 11 healthy control dogs.

Procedures—Samples from 11 dogs with high (≥100 mg/L) or low (<10 mg/L) CRP concentrations (determined by use of a canine ELISA) were evaluated by use of the automated canine CRP assay. Intra- and interassay imprecision was determined (by use of those 2 plasma pools), and assay inaccuracy was assessed by use of logistic regression analysis of results obtained via ELISA and the automated canine CRP assay. Two automated human CRP assays were used to measure plasma CRP concentration in 10 dogs.

Results—By use of the ELISA, mean ± SD plasma CRP concentration was 96.1 ± 38.5 mg/L and 10.1 ± 23.2 mg/L in dogs with pyometra and control dogs, respectively. The automated canine assay had intra-assay coefficients of variation (CVs) of 7.8% and 7.9%, respectively, and interassay CVs of 11.1% and 13.1%, respectively. Results from the automated assay were highly correlated with results obtained via ELISA. The human assay results did not exceed 0.4 mg/L in any dog.

Conclusions and Clinical Relevance—The automated canine CRP assay had less interassay imprecision, compared with the ELISA. The 2 human CRP assays were not suitable for analysis of canine plasma samples. The automated canine CRP assay was more precise than the ELISA for serial evaluations of plasma CRP concentration in dogs. (Am J Vet Res 2007;68:1281–1286)

Assessment of serum or plasma CRP concentration has become an important prognostic tool for a variety of diseases in humans, and its clinical use is widespread.1–4 Nearly 2 decades ago, it was determined that dogs with various inflammatory disorders have higher concentrations of the canine analogue of this acute-phase protein than healthy dogs,5 and it is known that the circulating concentration of CRP often relates to the extent or activity of disease.5,7 In recent years, interest in determination of CRP concentration in dogs has escalated and results of studies have identified alterations in CRP concentration in dogs with uterine disease8; acute pancreatitis;9 and various infectious,10,11 hematologic,12 and neoplastic13–15 disorders. In the past, clinical use of CRP concentration assessments in dogs has been stifled by the lack of an automated assay. For many years, the only commercially available assay was an ELISA.16 Unfortunately, that canine CRP ELISA is associated with several limitations that make it impractical for clinical use. In general, performance of an ELISA is labor-intensive and time-consuming, thereby rendering the procedure cost-ineffective for analysis of a single sample. In addition, the canine CRP ELISA has some undesirable assay characteristics.15 Assay performance is often expressed in terms of imprecision and inaccuracy. Inaccuracy relates to the difference between a measurement determined by use of the assay and the true value (which often is difficult to obtain), whereas imprecision relates to the degree of variation (closeness) among serial measurements of the same material and is expressed as a CV (%). The canine CRP ELISA has high interassay imprecision (reflected by CVs as high as 29%), which makes the assay less desirable for clinical diagnostic use.15 Preferably, an imprecision CV should be <5%.16 However, a maximum CV of 12.2% has been suggested as the limit of an objective analytical performance standard for canine CRP concentration determinations on the basis of data regarding the biological variation of this acute-phase protein.17 Nevertheless, the accuracy of the ELISA is good; thus, the assay is a valuable tool for determinations of canine CRP concentrations under research circumstances in which all samples can be analyzed in 1 batch, thereby avoiding the issue of interassay imprecision.15

ABBREVIATIONS

CRP C-reactive protein
CV Coefficient of variation
CI Confidence interval
The use of human CRP assays for canine CRP concentration determinations has been considered of little value after reports indicated that there was insufficient cross-reactivity between the proteins for assay purposes. Although canine CRP and human CRP have structural similarities, the 2 protein types did not share common antigenicity. However, a commercially available turbidometric immunoassay for human CRP can reliably measure canine CRP concentration, with acceptable precision within and between runs.

Recently, an automated canine CRP assay was made commercially available by the same manufacturer as that of the canine CRP ELISA. The manufacturer reports the precision of the assay in control samples with 8.2 to 50.8 mg of CRP/L, with a positive relationship between precision and higher CRP concentration. The assay imprecision for lower CRP concentrations was moderate (interassay CV, 11%). However, to our knowledge, this assay has not yet been evaluated in clinical cases or in dogs with severely high CRP concentrations (> 50 mg/L). The purpose of the study reported here was to determine the imprecision and inaccuracy of the automated canine CRP assay. We hypothesized that in sick dogs with high CRP concentrations, the precision of canine CRP concentration determinations between runs would be improved by use of the automated assay, compared with that associated with the previously evaluated ELISA. In addition, the use of 2 newer automated human CRP assays for determination of CRP concentrations in dogs was evaluated.

Materials and Methods

Animals and samples—The study was approved by the Board for Ethical Use of Animals in Research, Djurforsoksetiska Namnden, Tierp, Sweden. Of the client-owned dogs that were evaluated at the Department of Small Animal Clinical Sciences, Swedish University of Agricultural Science, Uppsala, Sweden from March 2001 through May 2002, those with a presumptive diagnosis of pyometra for which treatment included ovariohysterectomy were entered in the study after informed owner consent was obtained. The presumptive diagnosis was made on the basis of signalment, history, clinical signs, laboratory data, and findings of diagnostic imaging (ie, radiographic or ultrasonographic detection of a large, fluid-filled uterus). For each of these study dogs, the diagnosis of pyometra was confirmed after surgery via histologic examination of the uterus.

Immediately after admission to the hospital, blood (4 mL) was collected into 2 tubes containing EDTA, which were placed on ice and transported within 30 minutes to the Department of Clinical Chemistry, Swedish University of Agricultural Science for separation of plasma. Immediately after separation, the plasma was stored at −70°C.

Three healthy adult sexually intact female dogs and 8 healthy spayed female dogs that had previously been treated for pyometra were included as control dogs after informed owner consent was obtained. A history questionnaire was completed by the owner of each control dog to ensure that the dog had been healthy for at least 2 months prior to inclusion in the study. Physical examinations were performed by 2 of the authors (AB and RH), and blood samples were collected by use of the same procedures as those applied to the dogs with pyometra. Only female dogs with a history of apparent health, no abnormal physical examination findings, and results of hematologic and serum biochemical analyses that were within reference limits were enrolled as control dogs.

Plasma samples from dogs with pyometra and control dogs were transported to Washington State University, Pullman, Wash, on dry ice (duration of transport, < 24 hours). After confirmation that the samples were still frozen after transport, they were stored at −70°C until analysis. All samples were thawed on the day that the ELISA and automated human CRP assays were performed. After removal of the required plasma volume for the ELISA and, from 10 samples, the required volume for the automated human CRP assays, the samples were refrozen and stored at −70°C until analysis via the automated canine CRP assay. None of the samples contained plasma of sufficient volume for ELISA repeated analyses. Thus, for interassay analysis, plasma samples were categorized and pooled on the basis of the ELISA results: samples that had a high concentration of CRP (> 100 mg/mL) and samples that had a low concentration of CRP (< 10 mg/mL) were selected to provide 2 pools. Each pool was divided into 7 aliquots, and 1 aliquot from each pool was analyzed immediately; the remaining 6 aliquots from each pool were refrozen for subsequent analysis of interassay CV. Only vials needed for each analytic run were thawed to minimize variation as a result of repetitive freeze-thaw cycles. The total time period for determination of interassay precision was 55 days.

Canine-specific CRP assays—C-reactive protein was determined by use of a commercially available canine sandwich ELISA. Single plasma samples were analyzed in 1 batch immediately after thawing.

The automated canine CRP assay evaluated in the study was a commercially available polyethylene glycol–enhanced immunoturbidimetric canine CRP assay, which was used with an automated chemistry analyzer. The lower limit of detection of the assay had been determined as 3.99 mg/L, whereas the functional sensitivity (corresponding to the lowest standard on calibration curve) was 6.25 mg/L.

For determination of intra-assay CV, plasma samples were thawed and analyzed immediately in duplicate during 1 session. The analysis, including calibration curve generation and quality-control assessment (through evaluation of assay control samples), was performed as recommended by the manufacturer. Briefly, canine plasma samples were mixed with the kit buffer and antisera containing a polyclonal anti-canine CRP antibody. After incubation at 37°C, agglutination with sample CRP was monitored via spectrophotometry at 340 nm. Samples with CRP concentrations > 100 mg/L (maximum calibration value) were diluted and reanalyzed according to the manufacturer’s instructions. The same batch of reagents was used for all tests.

Human automated CRP assays—Two human CRP assays were evaluated: a human CRP latex assay (designated assay A) that was analyzed on a high-volume
chemical autoanalyzer* and a high-sensitivity human CRP assay† (designated assay B). Assay A was a turbidimetric immunoassay that used latex particles coated with monoclonal murine anti-human CRP antibody for agglutination with sample CRP. Plasma samples were thawed and analyzed without dilution within 2 hours. The analyzer was in clinical use at a regional human hospital and quality-control, calibration, and control analysis was performed according to routine procedures as recommended by the manufacturer.

Assay B was an immunometric chemiluminescent assay. The reagent in assay B contained murine anti-human CRP monoclonal antibody and alkaline phosphatase conjugated to rabbit polyclonal anti-human CRP antibody. Analysis of plasma samples was performed without sample dilution and after samples were diluted (1:100) with the sample diluent (included in the component set for the assay) according to the procedure manual. The samples were thawed and analyzed within 1 hour. Calibration and standard curves were performed on the analyzer, according to the procedure manual instructions, immediately prior to sample testing.

Assay validation and statistical analysis—Arithmetic means and intra- and interassay CVs were calculated by use of routine descriptive statistical procedures. Intra-assay variation between replicates was calculated as the CV from a pooled variance estimate of the difference between the duplicate determinations. The interassay CV was determined from the mean and SD of 7 replicate determinations from 1 pool of high–CRP-concentration plasma samples and from 1 pool of low–CRP-concentration plasma samples. The relationship between results obtained via the ELISA and the automated canine CRP assay was analyzed via linear regression. Differences between groups of continuous variables were evaluated by use of a Student t test. A value of P < 0.05 was considered significant.

Results

Canine CRP ELISA—Plasma samples from 56 dogs with pyometra and 11 control dogs were analyzed by use of the ELISA. Mean ± SD plasma CRP concentration among dogs with pyometra was 96.1 ± 38.5 mg/L (range, 0.1 to 167.4 mg/L). Among control dogs, mean plasma CRP concentration was 10.1 ± 23.2 mg/L (range, 0 to 77.7 mg/L). The difference between these mean values was significant (P < 0.001).

Automated canine CRP assay—Plasma samples from 11 dogs were analyzed in duplicate for determination of intra-assay precision. On the basis of results of the ELISA, 6 samples with CRP concentrations > 100 mg/L and 5 samples with CRP concentrations < 10 mg/L were selected for analysis. Among the dogs with high–CRP-concentration plasma samples, all 6 had pyometra; among the dogs with low–CRP-concentration plasma samples, all 5 were control dogs. Mean CRP concentrations in individual plasma samples were calculated (Table 1). The intra- and interassay CVs for the automated assay were also determined; for purposes of these determinations, plasma samples with high and low CRP concentrations were defined as > 100 mg/L and < 25 mg/L, respectively (Table 2).

The relationship between plasma CRP concentrations obtained via the canine CRP ELISA (CRPELISA) and plasma CRP concentrations obtained via the automated turbidimetric canine CRP assay (CRPAUTOMATED) was investigated (Figure 1). The linear regression analysis yielded an estimated intercept and slope of 11.7 (95% CI, −25.2 to 48.6) and 1.3 (95% CI, 0.9 to 1.7), respectively. A proportional bias of CRP concentration determined via the automated assay was thus detected as follows:

\[
\text{CRP}_{\text{AUTOMATED}} = 1.3 \times \text{CRP}_{\text{ELISA}} + 11.7
\]

The correlation between CRPELISA and CRPAUTOMATED was 0.93.

Human automated assays—Assay A detected CRP concentrations ranging from 0.1 to 0.4 mg/L in 6 of the 10 plasma samples that were evaluated. The assay did not detect CRP in the remaining 4 samples. Three of the 4 samples in which CRP was not detected by assay A were from control dogs for which the ELISA revealed CRP concentrations of < 1 mg/L. However, the sample

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<th>Table 1—Plasma concentrations (mg/L) of CRP determined in plasma samples obtained from 11 dogs* via a canine-specific ELISA (mean values) and via duplicate assessments performed by use of an automated canine CRP turbidimetric assay (mean ± SD values).</th>
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<td><strong>Assay</strong></td>
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<td><strong>Dog</strong></td>
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<td><strong>High CRP concentration</strong></td>
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*Pooled variance estimate of the differences between duplicate determinations from 6 dogs with high plasma CRP concentrations and 5 dogs with low plasma CRP concentrations.
from the fourth dog (a dog with pyometra) had a CRP concentration of 114 mg/L as determined by use of the ELISA. The CRP concentrations determined by use of assay A were 0.1 mg/L in 1 sample for which the ELISA revealed a concentration of 94 mg/L; 0.2 mg/L in 4 samples for which the ELISA revealed concentrations ranging from 1 to 94 mg/L; and 0.4 mg/L in 1 sample for which the ELISA revealed a concentration of 133 mg/L. C-reactive protein was not detected by use of assay B in any of the 10 plasma samples evaluated.

**Discussion**

The present study focused on evaluation of the assay imprecision of an automated turbidometric canine CRP assay that was recently made commercially available. Results of our evaluations indicated that intra-assay CVs in canine plasma samples with high and low CRP concentrations were 7.8% and 7.9%, respectively. These CV values are higher than those reported by the manufacturer (reported CV range, 1.54% to 5.78%) but similar to the imprecision associated with the canine CRP ELISA (intra-assay CV range, 6.9% to 10.1%) and that associated with an automated human CRP assay (intra-assay CV range, 5.2% to 10.8%). The latter assay is a commercially available turbidimetric immunoassay that has been validated for use in dogs. That human assay has also been extensively investigated with respect to quality control and has excellent long-term analytic performance for canine CRP concentration determinations. However, it is manufactured in Europe and may, at present, be less readily available in the United States, compared with its availability in Europe. The newer commercially available automated canine CRP assay can be used with either of 2 automated analyzers, potentially making its use more widespread. The manufacturer of the automated canine assay, as well as the human automated assay, identified less imprecision among samples with higher CRP concentrations, which contrasts with the results of the present study indicating that the CVs at both high and low CRP concentrations were similar. However, our data set contained 1 sample with a high CRP concentration and a substantial difference between duplicate values. The CV of the duplicate plasma CRP determinations for this dog was 5.0% as high as the CV for any of the other dogs. Among the dogs with high plasma CRP concentrations, the intra-assay CV would be 8.3% if this dog were excluded. We could not detect any assay errors to explain the large difference in duplicate determinations in this single sample; thus, we included the dog in the reported data. Further investigation of greater numbers of samples would be necessary to determine whether the intra-assay variation is actually less than the results of the present study have indicated.

The imprecision between runs of an assay used for clinical diagnostic purposes is of particular interest. In the automated canine CRP assay evaluated in the present study, the interassay imprecision was reflected by interassay CVs of 13% determined from the pool of plasma samples with low CRP concentration (mean concentration, 37 ± 4.8 mg/L) and 11% determined from the pool of plasma samples with high CRP concentration (mean concentration, 159 ± 17.6 mg/L). The interassay CVs reported by the manufacturer range from 3.8% to 6.6%, with the highest variation among samples with low CRP concentrations (mean concentration, 9 ± 0.9 mg/L); in samples with the highest CRP concentration (51 ± 2.8 mg/L), interassay CV was 5.5%. Other assays used for canine CRP determinations have interassay CVs of 4.8% to 13.3%; 8.0% to 12.3%; and 3.0% to 10.2%. The canine CRP ELISA used in the present study has been associated with interassay CVs of 7.5% to 29.0%, with increasing imprecision evident with increasing CRP concentrations.

In our study, the data were based on 7 analyses of the same plasma pools. The second through seventh replication was performed after 1 additional freeze-thaw cycle, compared with the first analysis. The high- and low-CRP-concentration pool values obtained from the first replication differed by only 0% and 10%, respectively, from the mean value in each group. This result is similar to findings in assessments of human plasma CRP concentrations, which are unaffected by as many as 7 additional freeze-thaw cycles. It appears from the interassay CV values in the present study that the new automated turbidimetric assay is comparable to other assays and that imprecision between runs of the automated assay is indeed improved, compared with that of the ELISA, especially in plasma samples with high concentrations of CRP.

Accuracy of the new automated canine CRP assay was only indirectly investigated in our study through comparison with the canine CRP ELISA. The accuracy, evaluated by linearity under dilution, of the ELISA has been previously evaluated and is considered good. In the present study, there was good correlation (correlation, 0.93; R², 0.86) between the results obtained via ELISA and the automated turbidimetric assay; this finding was similar to the correlation reported by the manufacturer (correlation, 0.91; R², 0.84). These results are also highly similar to data obtained for the human turbidometric assay that was recently validated for use in dogs, in which logistic regression comparison of
the canine ELISA and the human turbidometric assay revealed a slope of 0.79 with correlation of 0.91 and $R^2$ of 0.84. 

In general, plasma CRP concentrations derived by use of the automated canine CRP assay used in the present study were higher than the CRP concentrations derived by use of the ELISA (reflecting by the y-intercepts of 11.7), despite the fact that the automated assay was applied to samples that underwent 1 additional freeze-thaw cycle after completion of the ELISA.

In the study of this report, we used plasma obtained from blood collected into tubes containing EDTA, whereas sera or plasma samples prepared from blood anticoagulated with lithium and heparin are the sample types recommended by the manufacturer. However, in previous studies performed by some of the authors to investigate plasma CRP concentrations in dogs with inflammatory and noninflammatory uterine disease, plasma prepared from blood mixed with EDTA was successfully evaluated by use of the ELISA. The decision to use plasma in those studies was made after discussions at the time with the representative for the manufacturer, who indicated that analysis of plasma from EDTA-treated blood would be equally as feasible as analysis of serum. In humans, CRP concentrations derived from serum samples and plasma samples from EDTA-treated blood have excellent agreement. In our study, the correlation between results obtained via ELISA and via the automated turbidometric assay for plasma samples separated from EDTA-treated blood was similar to results obtained for serum samples. Although this finding does not indicate the potential assay inaccuracy attributable to the use of plasma samples obtained from EDTA-treated blood, it at least indicates that the relationship between assays (automated vs ELISA) for evaluation of those samples is similar to the relationship between assays for evaluation for serum samples. Given that our study primarily investigated assay imprecision and the relationship between assays, in contrast to inaccuracy, we found the use of plasma samples obtained from EDTA-treated blood acceptable. Also, the plasma CRP concentrations determined in the present study were similar to those previously reported for dogs with pyometra and healthy control dogs.

The study of this report involved samples that were stored for a relatively long time (4 years) between collection and analysis. The mean CRP concentration among dogs with pyometra was slightly < 100 mg/L; this suggests that the CRP molecule is relatively stable, which is consistent with results from other studies. Human CRP is stable in plasma samples obtained from EDTA-treated blood that have been stored at −70 °C for 8 to 11 years. Refrgerated human and canine plasma samples have stable CRP concentrations after 3 weeks and 30 days, respectively. Indeed, human CRP remains stable after storage for a period of 3 weeks at room temperature (approx 20 °C). Another study of canine CRP revealed that this protein is stable at −10 °C for at least 3 months.

Neither of the human automated assays performed well in the present study. Unlike the results obtained by use of the human turbidometric immunoassay evaluated by Kjelgaard-Hansen et al, the values of CRP concentration determined by use of the human turbidometric assay by the authors corresponded poorly to the concentrations determined by use of canine CRP ELISA. It cannot be ruled out that the turbidometric assay detected other proteins that cross-reacted with the monoclonal anti-human CRP antibody. The human high-sensitivity CRP assay evaluated in the present study did not detect canine CRP in plasma at all. These results can be added to previous nonsuccessful attempts to use human assays for assessment of CRP concentrations in dogs. To the authors’ knowledge, there is only 1 human automated assay that cross-reacts with and detects canine CRP.

In our study, the commercially available automated canine CRP assay evaluated had superior assay characteristics, compared with the canine-specific CRP ELISA. The intra-assay variation (assessed from duplicate plasma samples [anticoagulated with EDTA] of 11 dogs) was < 8% with CRP concentrations ranging from 8 to 229 mg/L. The interassay variation was 13% and 11% in pooled plasma samples with mean CRP concentrations of 37 and 159 mg/L, respectively, which is lower than the ELISA. It has been suggested that the ELISA can be useful for diagnostic purposes, despite high interassay imprecision, because of the great magnitude of increases in circulating CRP concentration associated with inflammatory responses in dogs. Use of the automated assay is also less labor-intensive than use of the ELISA, making it even more promising for clinical use. For the automated canine CRP assay, both intra- and interassay imprecision were within the limits of the objective analytic performance standard for imprecision (maximum CV, 12.2%) derived from data on biological variation of circulating concentrations of CRP in dogs. The 2 human CRP assays evaluated in the present study do not appear to be suitable for detection of canine CRP in plasma.

References

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