Development of a canine blood C-reactive protein-measuring device using a flow-type immunosensor

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Abstract
This study aimed to construct a measurement system with the same performance as a measurement system using an automated analyzer and immunoturbidimetric reagents (comparative method) using a flow-type immunosensor (FIS) based on the fluorescence-linked immunosorbent assay technology. In the FIS constructed in this study, all control samples were within the indicated values. The coefficient of variation of repeatability and intermediate precision were less than 2.4% and less than 4.4%, respectively. The lower limit of quantification in this measurement system was 3.9 mg/L, and linearity was confirmed for quantification values, ranging from 3.9 to 465 mg/L. Canine plasma samples (N = 39) were used to measure C-reactive protein (CRP) levels using the comparative method (x) and FIS (y). The regression equation between the measurements was $y = 1.035x - 0.002$, with a correlation coefficient of 0.9809, indicating a significantly high correlation. Although the Brandt–Altman analysis suggested the possibility of a proportional systematic error between the two measurements, 38 of the 39 canine plasma samples measured fell within the acceptable range of error, indicating that the measurements are highly consistent. These results suggest that the analytical accuracy of the FIS constructed in this study and the quantitative value of canine CRP are equivalent to those of measurement systems using automated analyzers and immunoturbidimetric reagents.

Keywords Fluorescence-linked immunosorbent assay · Flow immunosensor · Acute phase proteins · C-reactive protein · Clinical test

Introduction
When infection or tissue damage occurs in the body, inflammatory cytokines, such as interleukin (IL)-1 and IL-6, are released from cells, such as monocytes and neutrophils. Substances that dramatically increase or decrease in blood concentration in response to these cytokines are called acute phase reactants and are clinically used as markers of acute inflammation in medicine and veterinary medicine [1, 2]. C-reactive protein (CRP) is a serum protein with a molecular weight of approximately 100 kDa that undergoes a sedimentation reaction with C polysaccharide extracted from the cell wall of Streptococcus pneumoniae, discovered by Tillet et al. in 1930, and is produced in the liver in response to inflammatory cytokines [3]. Blood CRP is frequently used in practice as an inflammation marker in humans and dogs [4–8]. Blood CRP in healthy dogs is significantly low (less than 6.8 mg/L) [9], and CRP concentrations increase rapidly in the acute phase of inflammation and have been reported to be $165 \pm 82$ mg/L in systemic inflammation and sepsis [10]. It then rapidly decreases once the inflammation resolves. Therefore, a limit of quantification (LOQ) of approximately 5 mg/L is likely to be required for the canine blood CRP measurement system, and the range of quantification should be roughly from 5 to 300 mg/L.

Antigen–antibody reactions are commonly used to measure CRP by immunoturbidimetry [9],
immunochromatography [11, 12], enzyme-linked immuno-sorbent assay [12], and immunofluorescence [13]. Among them, the measurement system combining an automated biochemical analyzer and immunoturbidimetric reagents has excellent analytical performance and has been introduced to university hospitals and analytical companies [9]. Desktop analyzers, which are widely used in small- and medium-sized hospitals, utilize a variety of measurement principles and use special reagents for each device. Although they are easy to administer and operate and have sufficient clinical performance, there is no correlation in measurement values among the various measurement methods, and the accuracy of analysis differs among them [14]. Hence, the quality of CRP test data currently differs from facility to facility. In addition to the lack of interoperability of laboratory data from a clinical perspective, this is also an obstacle to data accumulation and statistical analysis from a research perspective. Therefore, even testing equipment that can be introduced into small- and medium-sized hospitals must have the same analytical accuracy as the automated analyzers and measurement systems using immunoturbidimetric reagents introduced in flagship hospitals and specialized laboratories and still show correlative measurement values. Therefore, we used a flow immunosensor (FIS) based on the fluorescence-linked immunoabsorbent assay (FLISA). FIS is a type of analytical method that uses antigen–antibody reaction, and is generally based on the KinExA® (Kinetic Exclusion Assay, Sapidyne Inc. Boise, ID, USA) principle [15, 16]. The KinExA method is a highly sensitive method, but is expensive and time-consuming to develop, because it requires the solidification of a large amount of CRP or a portion of the constituent peptides. The sandwich FLISA method is a low-cost and quick method to construct an assay system by solid-phasing a commercial anti-dog CRP antibody. Since the purpose of this study was to detect CRP at the mg/L level, which is relatively high in volume, we adopted this method based on the expectation that FLISA would be sufficient in terms of sensitivity. However, FIS based on FLISA has not yet been applied to the clinical laboratory field, and this study is a novel attempt. This study aimed to develop a measurement system for canine CRP that is highly accurate and easy to manage and operate. For this purpose, we aimed to construct a measurement system using an FIS device based on FLISA.

Materials and methods

Principle of canine CRP measurement using a FIS

A schematic diagram of the prototype FIS-based CRP measurement system developed in this study is shown in Fig. 1a. Figure 1b shows a schematic diagram of the measurement principle. Fluorescent-labeled anti-canine CRP antibody prepared at a certain concentration is reacted with canine CRP to form an antigen–antibody complex, which is used as the measurement sample. The measurement cell is filled with a solid-phase on which an anti-canine CRP antibody is immobilized. When the measurement sample is passed through the cell, the antigen–antibody complex binds to the solid-phase antibody and forms a sandwich of canine CRP. A laser beam that excites the fluorescent dye is irradiated into the cell, and fluorescence proportional to the amount of antigen–antibody complex bound to the solid phase (i.e., the amount of canine CRP) is obtained. The concentration of the unknown sample is determined using a fluorescence intensity calibration curve prepared using standards. After measurement, the antigen–antibody complexes bound to the solid phase are removed by passing regenerated buffer, and the cell can be used repeatedly.

Reagents used for the FIS system

Anti-canine CRP monoclonal antibody from mouse (Catalog No. MCRP-40ALY-14H5, Immunology Consultants Laboratory, Inc.) was used to prepare fluorescent-labeled antibody. Fluorescent labeling of antibodies was performed using the Alexa Fluor™ 647 Protein Labeling Kit (Invitrogen, MA, USA). The solid-phase antibody was an anti-canine CRP monoclonal antibody derived from mouse (Catalog No. MCRP-40ALY-14D3, Immunology Consultants Laboratory, Inc.). Micro-polystyrene beads (φ100 μm) used as solid phase were purchased from AICA Industries (Aichi, Japan). The measuring cell was made of acrylic resin (Nalux, Osaka, Japan) with an outer diameter of 5 mm, a total length of 40 mm (including cap), an inner diameter of 2 mm, and a bead-filled portion with a length of 11 mm. The cell was filled with approximately 30,000 antibody-coated beads (fill rate; approximately 100%). Block Ace (Yukijirushi Megmilk, Tokyo, Japan) was used as a blocking agent for solid-phase antibodies. Methanol and Dulbecco’s phosphate-buffered saline (D-PBS) (−) were purchased as special grade reagents from Nacalai Tesque (Kyoto, Japan). Reagents used for measurement and wash buffers and regeneration solution were also special grade reagents purchased from Nacalai Tesque. Pure water used in the experiments was supplied by Integral 3 (Merck Millipore, MA, USA).

The composition of D-PBS (−) (0.1 mol/L, pH 7.5) was as follows: sodium chloride, 107.3 mmol/L; disodium hydrogen phosphate, 8.3 mmol/L; potassium chloride, 3.4 mmol/L; and potassium dihydrogen phosphate, 1.5 mmol/L. The composition of the measurement buffer was as follows: dimethyl sulfoxide, 5 v/v%; disodium hydrogen phosphate dodecahydrate, 8.1 mmol/L; sodium chloride, 136.9 mmol/L; potassium dihydrogen phosphate, 1.5 mmol/L; potassium chloride, 2.7 mmol/L; and bovine serum albumin, 0.1 w/w%.
The composition of the wash buffer was as follows: disodium hydrogen phosphate dodecahydrate, 8.1 mmol/L; sodium chloride, 136.9 mmol/L; potassium dihydrogen phosphate, 1.5 mmol/L; potassium chloride, 2.7 mmol/L; and bovine serum albumin, 0.1 w/w%. The composition of the regeneration solution was as follows: dimethyl sulfoxide, 5 v/v%; and sodium hydroxide, 2.6 mmol/L. Gentian Canine CRP Calibrator Kit (Gentian AS, Moss, Norway) was used as a calibrator. Calibrator concentrations were 0.0, 10.0, 30.0, 78.0, 155.0, and 310.0 mg/L. The Gentian Canine CRP Control Kit was used to evaluate accuracy and precision; the manufacturer’s indicated values for control low and control high were 26–35 and 87–118 mg/L, respectively.

**Fluorescence labeling of anti-canine CRP antibody**

The Alexa Fluor™ 647 Protein Labeling Kit was used to prepare a fluorescent-labeled antibody stock solution (antibody concentration, 1.06 mg/mL) by fluorescently labeling a mouse-derived anti-canine CRP monoclonal antibody according to the manufacturer’s instructions. The antibody solution was stored under 4 °C until use. Before assay, it was diluted 5300-fold with an assay buffer to create a fluorescent-labeled antibody solution (antibody concentration, 0.2 μg/mL) and was used for the assay.

**Solidification of anti-canine CRP antibodies on beads**

Anti-dog CRP antibody was solidified on micro resin beads according to the method of Robert et al. [17]. A measurement cell was prepared by filling an empty flow cell cartridge with 0.1 g of antibody-coated beads and fixing it using a cotton filter and plastic tubing. The cell was sealed with a rubber stopper and stored at 4 °C until use.

**Analyzer based on the FIS used in this study**

An FIS instrument, DXS-610 (Seeds Tec, Ehime, Japan), was used for CRP measurement. The measurement conditions were as follows: excitation wavelength, 650 nm;
fluorescence measurement wavelength, 665 nm; cell temperature, 20–25 °C; and mobile phase flow rate, 0.36 mL/min. The volumes of liquid passed per sample were 0.2 mL of measurement sample, 0.36 mL of measurement buffer, 2.0 mL of washing buffer, and 0.36 mL of regeneration solution. Light Emitting Diodes were used as the excitation light source, and bandpass filters were used to illuminate a specific range of wavelengths. A photodiode was used for fluorescence detection, and a long-pass filter prevented stray light from entering. The light source and detector were placed in opposite positions across the measurement cell.

**Preparation of calibration curves**

For each concentration of calibrators (0.0, 10.0, 30.0, 78.0, 155.0, 310.0 mg/L), 2 μL of calibrator and 2 mL of fluorescence-labeled antibody solution (0.2 pg/mL concentration) were mixed in a micro tube and incubated at room temperature for 1 h. The sample was used as the measurement sample. The 0.2 mL of the measurement sample was introduced into the FIS, and the fluorescence intensity was measured. A calibration curve was created with canine CRP concentration and fluorescence intensity on the x- and y-axes, respectively.

**Assessing the accuracy and precision of the FIS system**

The Gentian Canine CRP Control Kit low (manufacturer’s indicated value, 26–35 mg/L) and high (87–118 mg/L) were used to evaluate the accuracy of readings and analytical precision. The procedure for preparation of the analytical samples is the same as for the calibrator assay. Ten consecutive measurements were performed to evaluate reproducibility. To evaluate intermediate precision, measurements were performed for 20 days. In this case, the column was replaced with a new one every day, and a calibration curve was prepared each time before measuring the control.

**Evaluation of quantifiable range**

In general, the limit of quantitation in clinical testing is defined as the coefficient of variation (CV%) of repeated measurements within 10% or 20%. In the present study, the limit of quantitation was evaluated following this rule. The results of three measurements of low-concentration dilutions of CRP standard solutions were used to calculate CV%. To confirm the accuracy of measurement in low-concentration samples, a “precision profile diagram of quantitative values” was prepared, plotting the CV% at each CRP concentration. The upper LOQ was evaluated by the linearity of the quantification value when dilutions of high-concentration samples were measured. Specifically, CRP solutions of ten concentrations between 0 and 465 mg/L were measured in triplicate at each concentration, and deviations from the theoretical values were evaluated using the F statistic from the ratio of the residual variance to the quasi-error variance by analysis of variance. Linearity was judged to be nonlinear at a significance level of $p < 0.001$. Validation support software (version 3.5, Japanese Society for Clinical Chemistry) was used for the statistical analyses.

**Reagents and equipment for comparative assays**

An automated biochemical analyzer and an immunoturbidimetric reagent assay system were used as the comparative method for this study. The reagent was the Particle-Enhanced Turbidimetric Immunoassay Gentian Canine CRP Reagent Kit (Gentian Diagnostics, Moss, Norway). Previous studies have reported that this reagent is reliable and suitable for clinical assays [9]. The analyzer was a Model 3100 automated biochemical analyzer (Hitachi High-Tech, Tokyo, Japan), and the measurement parameters were those recommended by the manufacturer. Calibrators and controls included with each product were used, and control measurements fell within the manufacturer’s reference range. The calibrators and controls are the same products used for FIS calibration and precision control.

**Canine plasma samples**

Dog plasma was from residual samples ($N = 39$) at the Animal Clinical Laboratory Center (Doubutsu Kensa Inc., Kanagawa, Japan). Specimens were plasma with heparin Li as anticoagulant and were transported from the veterinary clinic to the laboratory at 4 °C. Heparin Li is the most common anticoagulant in pet animal clinical practice, and the majority of blood biochemistry, including CRP, uses this plasma. CRP was measured at this laboratory using an automated biochemical analyzer and an immunoturbidimetric reagent assay system. The samples were then frozen and stored at $−80$ °C until FIS analysis at another laboratory. Beforehand, there was no change in CRP measurement values due to freezing and storage at $−80$ °C. According to previous studies, up to four freeze-thaws do not affect canine CRP levels [9]. In this study, blood was not collected from the animals, and the test residual plasma was used when the individuals could not be identified. Therefore, the study was outside the scope of ethical considerations by the Ethics Committee on Clinical Research of Okayama University of Science (Review No. 2020-0007). In this study, the cell was replaced approximately every ten samples for measurement.

**Statistical analyses**

Scatterplots were prepared using the x- and y-axes for the comparative and FIS measurements, respectively. To
evaluate the concordance between the CRP measurements of the two methods, a Brandt–Altman plot was used with the $x$- and $y$-axes representing the mean of the reference control and FIS measurements and the difference between the two measurements (reference control—FIS), respectively.

The relationship between the comparative method and FIS measurements was evaluated by standard principal axis regression analysis using the Validation Support/Excel version 3.5 program for validation calculation (Japanese Society for Clinical Chemistry, Special Committee on Quality Control). The slope of the regression equation and the 95% confidence interval of the intercept were calculated using the bootstrap method.

**Results**

**Calibration curve of canine CRP analyzed by FIS**

A calibration curve was prepared from the fluorescence intensity obtained from the calibrator measurement and is shown in Fig. 2.

**Measured values of control samples**

The acceptable range for control low was 26–35 mg/L, and the measured value in FIS was 31.5 ± 0.09 mg/L ($N=12$, average ± standard deviation (SD)]. The acceptable range for control high was 87–118 mg/L, and the measured value in FIS was 96.2 ± 0.50 mg/L ($N=12$). Both control measurements fell within the acceptable range, confirming that the canine CRP measurement system by FIS constructed in this study was accurately calibrated.

**Assessment of analytical accuracy**

For repeatability, the CV% of ten measurements of control low and high were 1.8% and 2.4%, respectively. For intermediate precision, the CV% for 20-day measurements of control low and high were 3.6% and 4.4%, respectively.

**Evaluation of measurable range**

A precision profile diagram of canine CPR quantification values in the low-concentration range is shown in Fig. 3. The CV% of the measured value was within 10% for CRP solutions above 3.9 mg/L and within 20% for CRP solutions above 3.9 mg/L. Therefore, the LOQ of this measurement system was 3.9 mg/L.

Canine CRP solutions ranging from 0 to 465 mg/L were measured and plotted on a scatterplot with theoretical values on the $x$-axis and measured values on the $y$-axis, as shown in Fig. 4. The linearity was evaluated in the range of 3.9 to 465 mg/L, excluding diluted standard solutions below the limit of quantitation, and the $F$ statistic from the ratio of the residual variance to the net error variance yielded a significance probability of 0.9611. Therefore, there was linearity in the quantitation values at least up to 465 mg/L.

Based on these results, the LOQ of the canine CRP measurement system according to the FIS constructed in this study was 3.9 mg/L, and the upper LOQ was 465 mg/L.

**Comparison of measurements between the comparative method and FIS**

The distribution range of the measured values for canine plasma samples ($N=39$) using the automated analyzer and measurement system using immunoturbidimetric reagents was 5.9–151.4 mg/L. The distribution range of FIS measurements was 5.3–160.7 mg/L. A scatterplot of measurements made by the automated analyzer and measurement system.
using the immunoturbidimetric reagent on the x-axis and measurements made by the FIS on the y-axis is shown in Fig. 5a. The regression equation for the relationship between the two was $y = 1.035x - 0.002$, with a Pearson’s correlation coefficient of 0.9809. The 95% confidence interval for the slope calculated by the bootstrap method was 0.953–1.112, and the 95% confidence interval for the intercept was $-0.24$ to 0.323. The Brandt–Altman plots of the comparative method and FIS measurements are shown in Fig. 5b. The difference between the two methods on the y-axis tended to increase as the mean value of the two methods on the x-axis increased, indicating that a proportional systematic error exists between the two measurements. Thirty-eight of the 39 samples fell within the acceptable range of error (mean ± 1.96 SD).

**Discussion**

In this study, we aimed to construct a measurement system using FIS based on KinExA that has the same analytical accuracy as a measurement system using an automated analyzer and immunoturbidimetric reagents and that still shows correlated measurement values. Both the accuracy and reproducibility (repeatability and intermediate precision) of the canine CRP measurement system constructed in this study were satisfactory. According to a previous study, the CV% of repeatability and intermediate precision of the comparison methods using samples with CRP concentrations ranging from 26.5 to 370 mg/L were less than 1.7% and 1.9%, respectively [9]. Since the FIS system developed in this study uses a hand method for mixing samples and reaction reagents, it is hypothesized that the repeatability was more likely to vary than that of automated analyzers. In this study, flow cells were replaced on each measurement day; thus, it is hypothesized that individual cell differences were reflected in the intermediate precision variation.

The lower limit of canine CRP quantification in this measurement system was 3.9 mg/L, and linearity was confirmed for quantification values in the range of 3.9 to 465 mg/L. Blood CRP levels have been reported to be less than 6.8 mg/L in healthy dogs [9] and 165 ± 82 mg/L in systemic inflammation and sepsis [10]. Therefore, the quantitative range of the canine CRP measurement system by FIS developed in this study is applicable to the clinically required canine CRP concentration.
In the evaluation of the correlation between the comparative assay and FIS using canine plasma samples (N = 39), the regression equation between the assay system (x) and FIS (y) measurements using the automated analyzer and immunoassay reagent was \( y = 1.035x - 0.002 \), with a correlation coefficient (r) of 0.9809, indicating a significantly high correlation. Brand–Altman analysis suggested that a proportional systematic error may exist between both measurements. However, 38 of the 39 canine plasma samples measured fell within the acceptable range of error, and the agreement between the measurements can be evaluated as high. Therefore, the quantitative values in the FIS constructed in this study are equivalent to those of the measurement system using an automated analyzer and immunoturbidimetric reagents. The automated biochemical analyzers installed in university hospitals and laboratories are large and expensive, because they are equipped with high-precision optics system, refrigerated reagent storage for more than 20 types of reaction reagents for multiparameter analysis, autosamplers for mounting multiple specimens, and automatic sampling mechanisms for specimens and reagents. In addition, the operation of the analyzer requires operators who are familiar with reagent and instrument management and precision control. Therefore, it is difficult to introduce automated biochemical analyzers and measurement systems using immunoturbidimetric reagents in small- and medium-scale animal care facilities. If the system is specialized for CRP measurement, even the immunoturbidimetric method has the potential to achieve smaller size, lower cost, and simpler operation. However, we are developing this method with a view of constructing a measurement system with a higher sensitivity than that of the turbidimetric method. In human clinical testing, highly sensitive CRP assays (capable of measuring up to around 0.2 mg/L) have become popular, because low concentrations of CRP are risk markers for cardiovascular diseases, such as atherosclerosis. [18, 19]. Although high-sensitivity CRP measurement is not common in canine clinical practice, some reports suggest its usefulness; hence, we considered it desirable to establish a measurement system that can achieve high sensitivity in the future [20]. Because of the superior sensitivity of fluorescence analysis compared to that of immunoturbidimetry, we developed an FIS based on the FLISA principle.

The FIS-based CRP measurement system developed this time enables measurement simply by mixing standard solution or sample plasma with fluorescent-labeled antibody solution and introducing it into the device. Moreover, analysis is automated so that testing can be performed without requiring specialized skills. Using biological substances other than CRP as measurement targets by replacing antibodies immobilized in cells in the FIS is possible. This study confirmed the basic performance of the FIS CRP measurement system. For future practical use, it is necessary to shorten the measurement time, evaluate the effects of blood anticoagulants (ethylenediamine tetra acetic acid Na or K, sodium citrate), assess the effects of interfering substances (hemoglobin, triglycerides, bilirubin) on the optical system, and confirm correlation with a comparative method using more canine plasma samples.

Conclusion

The canine CRP measurement system in FIS showed sufficient basic performance and good correlation with the measurement system using an automated analyzer and immunoturbidimetric reagents. Although it is necessary to evaluate the influence of interfering substances and to conduct sufficient evaluation on actual specimens for clinical application in the future, canine CRP measurement is possible with FIS based on the FLISA principle. Measurement of clinical marker substances using FIS is expected to make a significant contribution to future medical care at small- and medium-sized medical institutions and veterinary clinics.

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N.A.

Author contributions Conceptualization: NF; methodology: NT and AH; formal analysis and investigation: TK, and NK; writing—original draft preparation: AH and TK; writing—review and editing: NT and NF; funding acquisition: HT; resources: NK; and supervision: HT and NF.

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Data availability The data that support the findings of this study are available from the corresponding author, Noboru Fujitani, upon reasonable request.

Declarations

Conflict of interest This study was funded by Seeds Tec Co., Ltd. Hideki Toita runs Seeds Tec Co., Ltd. Nobutoshi Kanaki runs Doubutsu Kensa Inc. Kubota Tomoko and Norio Tateishi are employees of Seeds Tec Co., Ltd. Noboru Fujitani received research funding from Seeds Tec Co., Ltd.

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