Comparative Analysis of C-reactive Protein Levels in the Saliva and Serum of Dogs with Various Diseases

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ABSTRACT

Objective: We performed this study to characterize the difference between inflammatory and non-inflammatory status in diseased dogs by measurement of salivary C-reactive protein (CRP). In addition, we assessed whether a correlation exists between CRP levels in saliva and those in serum.

Materials and Methods: CRP levels were measured in 32 client-owned dogs, which were then divided into inflammation and non-inflammation groups based on serum CRP level.

Results: The salivary CRP level was higher in the inflammation group than in the non-inflammation group ($p < 0.05$). Furthermore, there was a positive correlation between salivary and serum CRP levels ($R = 0.866$, $p < 0.001$).

Conclusion: These data suggest that canine salivary CRP measurements can effectively and non-invasively detect an inflammatory state in dogs.

INTRODUCTION

In dogs, C-reactive protein (CRP) is a major acute phase protein produced by non-specif-

Table 1 *Comparison of clinical characteristics and CRP levels of inflammation group and non-inflammation group*

	Inflammation group	Non-inflammation group
Clinical characteristics	n = 19	n= 13
Age (year, median)	9 (2-18)	11 (1-16)
Sex	3 M, 7 CM, , 3 SF	10 CM, 1 M, 1F, 1SF
CRP levels (mg/dl)		
Serum	7.53 (6.27-11.97)*	0.79 (0.65-0.96)
Saliva	0.1033 (0.0721-0.1502)*	0.0007 (0.0006-0.0080)

*Significant statistical difference between inflammation group and non-inflammation group indicates * (p < 0.001)*

ic tissue injury.[1] Its resting serum concentration is low. However, it could increase rapidly after exposure to inflammatory stimuli, then decrease after the resolution of inflammation.[2] Because of these characteristics, CRP comprises a sensitive and specific biomarker of systemic inflammation, useful for the diagnosis and monitoring of various inflammatory diseases.[3,4]

CRP levels are typically measured using serum collected by venipuncture, a painful and stress-inducing procedure that requires involvement of professional and skilled clinicians or technicians, as well as laboratory equipment. Saliva collection is non-invasive, low-stress, and pain-free. Therefore, it is an attractive alternative method for the evaluation of individual immune activity via CRP levels.5 Saliva is an easily available biological fluid that contains many local and systemic factors that enter the oral cavity.[6] In humans, saliva samples have been successfully used for detection of several biomarkers, such as cortisol and alpha-amylase.[7,8] In addition, saliva samples have been used in several studies of cortisol, catecholamine, and phenobarbital in veterinary medicine.[9-11]

The objectives of this study were to examine differences in salivary CRP levels between diseased dogs classified into inflammation or non-inflammation groups, without the use of chemical stimulants. Moreover, we assessed whether a correlation exists between CRP levels in serum and those in saliva.

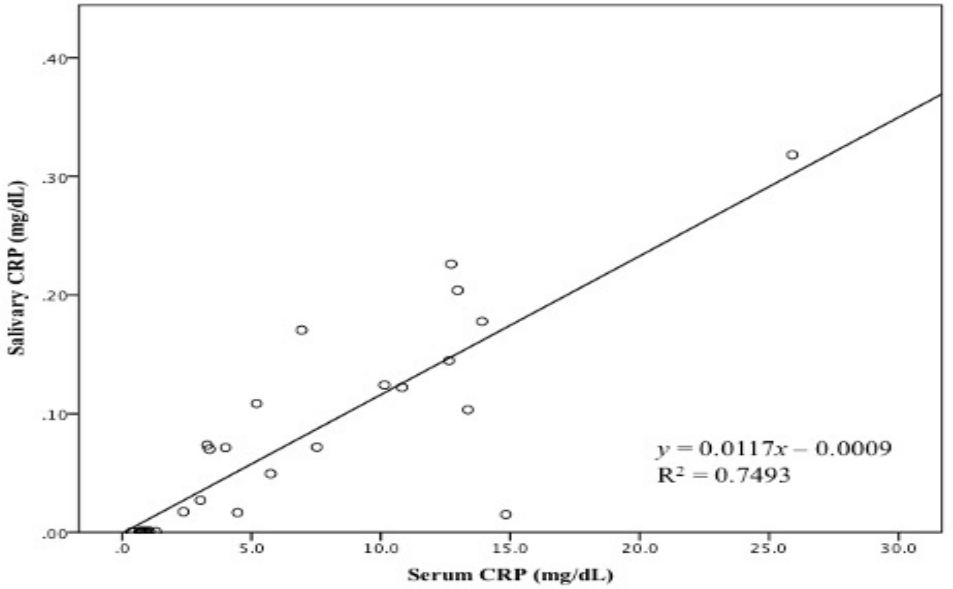
MATERIALS AND METHODS

Animals and Sample Collection

Thirty-two client-owned dogs with various diseases (Table 1) presented to the Veterinary Medical Teaching Hospital at Chungnam National University during the period from May 2017 to September 2017. All dogs underwent a complete physical examination, blood count (Advia2120; Siemens Healthcare Diagnostics, Deerfield, IL, USA), CRP measurement, and serum biochemistry profile (Mindray BS-300; Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China). Dogs were classified into inflammation or non-inflammation groups according to the laboratory results: dogs with serum CRP values higher than the reference range of 0–2 mg/dl were included in the inflammation group, whereas dogs with lower serum CRP values were included in the non-inflammation group. Both serum and saliva specimens were collected from each dog at a single time point. Before collection of saliva samples, dogs were fasted for 6–12 hr. No acid stimulants were used to increase salivary secretion. Dogs with apparent periodontal disease on physical examination or bleeding gums were excluded from the study.

Saliva was obtained using a cotton pad and a 1.5-ml tube (Greiner Bio-One, Kremsmünster, Austria). The cotton pad was placed in the oral cavity in contact with the buccal mucosa, or dogs were allowed to chew the cotton pad for 1–2 min. The pad was then placed in the tube and centrifuged for 15 min at 3,000 x g. Immediately after

Figure 1. *Correlation between C-reactive protein levels in serum and saliva.*



centrifugation, salivary samples were stored in Eppendorf tubes and frozen at -70 °C until biochemical analysis. Blood samples were obtained from each dog by venipuncture and collected in tubes containing a coagulation activator. Blood was allowed to clot at room temperature and centrifuged at 3,000 x g at room temperature for 10 min. Serum samples were stored at -70°C until biochemical analysis. All saliva and serum samples were frozen in aliquots, and only vials needed for each assay were thawed to prevent any potential variation as a result of repetitive freeze-thaw cycles.

Measurement of CRP Concentration in Both Saliva and Plasma

CRP levels in both serum and saliva samples were measured using a commercial canine CRP ELISA kit (Abcam, Cambridge, MA, USA). Dilutions of serum and saliva samples used in analyses were 1:1000, 1:10, and undiluted. All thawed salivary samples were centrifuged at 1,500 x g for 15 min at 4 °C to remove cellular debris and minimize the turbidity of the saliva, which could negatively impact the accuracy of analysis.[12] Supernatants were transferred into fresh Eppendorf tubes and appropriately labeled. A total of 64 samples, consisting of 32 serum and 32 saliva samples, were assayed in duplicate. For ELISA analysis, all samples were thawed and mixed thoroughly; ELISA was then performed in accordance with the manufacturer's instructions. Absorbance was detected at a wavelength of 450 nm on a microplate reader (Biotek Instruments Inc., Winooski, VT, USA).

Statistical Analysis

Statistical analyses were performed using IBM SPSS software (version 24, IBM Corp., Ehningen, Germany). CRP levels were expressed as median values (25th and 75th percentiles). Pearson's product-moment correlation and simple regression analysis were used to compare CRP levels in saliva and serum. Differences in salivary CRP were analyzed using independent-samples t-tests. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The 32 client-owned diseased dogs consisted of 15 different breeds, including Maltese (n = 9), Shih Tzu (n = 4), Schnauzer (n = 3), Yorkshire terrier (n = 2), Welsh Corgis (n = 2), Pomeranian (n = 2), Beagle (n = 2), Sapsaree (n = 1), Poodle (n = 1), German

Shepherd (n = 1), French Bulldog (n = 1), Cocker Spaniel (n = 1), Chihuahua (n = 1), Cane Corso (n = 1), and Australian Shepherd (n = 1). Based on a general health screening and laboratory evaluation, the dogs were diagnosed with various diseases.

Cardiological and neurological diseases were most common in the non-inflammation group. Post-operative inflammation within 4 days and acute pancreatitis at the time of diagnosis were the most common conditions in the inflammation group. The clinical characteristics of all dogs are shown in Table 1. Salivary CRP levels in the dogs were 0.0004-0.3182 mg/dl and serum CRP levels were 0.3772-25.90 mg/dl. Salivary CRP levels were significantly higher in the inflammation group than in the non-inflammation group ($p < 0.001$) (Table 1). Furthermore, there was a positive correlation between serum and salivary CRP levels in all dogs ($R = 0.866$, $p < 0.001$; Figure 1).

DISCUSSION

The results of this study indicate that salivary CRP is an accurate method for identifying inflammation in diseased dogs. Saliva can be collected without the use of invasive methods, and is advantageous for pediatric patients or those with a specific clinical pathologic state (e.g., anemia or hemostatic disease), physical sensitivity (e.g., to needles), and geographic handicaps (e.g., those who reside far from the hospital).[6,13]

In this study, salivary CRP was able to serve as an indicator of inflammatory status and showed a moderate-to-strong association ($R = 0.866$ and $R2 = 0.749$) with serum CRP level. The quantification of salivary CRP and its correlation with serum have been assessed in human,[5] porcine,[14] and canine13 studies. Although correlation in dogs ($R2 = 0.69$) was higher than that in humans ($R2 = 0.49$) or pigs ($R2 = 0.52$), a moderate association was detected in all groups.

We observed a difference of salivary and serum CRP levels which do not meet the regression equation. The low ratio of saliva CRP level to serum CRP level could reduce the precision of salivary CRP measures, particularly at low serum CRP concentrations in a non-inflammation state.[15] Although the mechanisms by which CRP is transported from serum to saliva remain unclear,[5] CRP could potentially enter in the saliva in its high-molecular weight form and its 2 glycosylated subunits could increase its lipid-insolubility,[15,16] preventing CRP from entering saliva. For large molecules such as proteins and charged steroids, the primary route of entry into the oral cavity is through plasma exudates of systemic origin from gingival crevicular fluid (GCF),[17] as well as from minor abrasions in the mouth, instead of diffusion or ultrafiltration.[9,18] Whole saliva used in this study comprised oral fluids from the major and minor salivary gland, as well as fluids of non-salivary origin, including GCF, serum transudate from the mucosa and sites of inflammation, epithelial and immune cells, food debris, and many microbes.[18,19] Poor oral health in diseased dogs might have caused minor bleeding or GCF overflow from micro-injuries, which could have increased salivary CRP in the absence of elevated serum CRP. Furthermore, steroid hormones are known to be metabolized by oral bacteria and epithelial cells in the salivary gland during transcellular movement.[20] Although this mechanism is not known in salivary CRP, this modification and subsequent metabolism could considerably modify or reduce detection in saliva.

Our investigation has different experimental settings and collection methods compared to previously published studies. The composition of whole saliva can be rapidly altered by flow rate, the degree of stimulation in various glands, and the time of day.21 Our protocol controlled for animal factors by fasting, acid stimulant exclusion, and collection time, as well as by the exclusion of dogs with periodontitis and other oral diseases.

In addition, we collected samples before treatment, which may have increased the association with inflammation. A previous study in humans reported that mechanical stimulation, but not acid stimulation,

is the most viable option for acquisition of saliva without changes in CRP levels.[21] Based on these results, we used mechanical stimulation for saliva collection in our study. Further studies in veterinary medicine are needed to confirm the optimal collection protocol.

There were some limitations in this study. First, we included a relatively small number of dogs. Most diseased dogs who presented to the clinic showed signs of dehydration and dry mucosa in the oral cavity. Therefore, we were unable to obtain a sufficient volume of saliva for analysis. Second, our study used sample pretreatment (centrifugation) to remove cellular debris. Processing methods can adversely affect the concentration of CRP in humans.[21] Therefore, it is necessary to verify whether the same adverse effect is present in veterinary medicine samples.

CONCLUSION

Taken together, the results of this study suggest that salivary CRP measurements in dogs can provide a straightforward, adaptable, and non-invasive method for detection of inflammation. This method could be used as a tool to monitor health and assess inflammation in diseased animals. To use saliva for assessment of inflammation in clinical practice, further studies should focus on creating reference ranges in different species, as well as the stability of saliva samples in various storage conditions.

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CONFLICT OF INTERESTS

The author should declare any conflict of interests.

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