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SALIVARY BIOMARKERS IN CANINE CHRONIC KIDNEY DISEASE: A DIAGNOSTIC STUDY

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ABSTRACT
Background: Chronic kidney disease (CKD) is a common and clinically significant disease in dogs characterized by progressive and irreversible loss of renal function. Diagnosis of CKD is mainly done on the basis of biomarkers such as blood urea nitrogen (BUN) and serum creatinine (sCr). Non-invasive diagnostic techniques such as saliva analysis offer a promising alternative to serum-based diagnostics providing an easy-to-collect, stress-free biological sample for clinical evaluations. The aim of this study was to evaluate and correlate the levels of salivary and serum creatinine and urea in dogs diagnosed with chronic kidney disease (CKD) along with diagnostic assessment of saliva.
Method: The study was conducted on dogs presented with clinical signs suggestive of CKD at the VCC, College of Veterinary Science, Assam Veterinary and Fishery University, Khanapara, Guwahati, Assam, India. Serum and salivary samples of confirmed 38 CKD cases and 10 healthy control dogs were collected and analysed for creatinine and urea levels. T- test, Pearson's correlation test and Receiver Operating Characteristic (ROC) analysis was used to compare, correlate and check the diagnostic ability of saliva respectively.
Result: The most consistent clinical finding observed was significantly higher concentrations of creatinine and urea in both serum and saliva samples from CKD affected dogs when compared to healthy dogs. A strong positive correlation was observed between the salivary and serum concentrations of creatinine and urea. The ROC analysis showed the cutoff value for salivary creatinine was determined to be 0.89 mg/dL with a sensitivity of 89.47% and a specificity of 100%. The cutoff value for salivary urea was 18.50 mg/dL, with a sensitivity of 97.37% and a specificity of 90%. These findings suggest that salivary biomarkers are nearly as reliable as serum biomarkers in reflecting kidney function and detecting CKD.
Conclusion: These findings suggest that salivary biomarkers are equally reliable as serum biomarkers in reflecting kidney function and can be utilised for diagnosis of CKD in dogs.

KEYWORDS: *Chronic kidney disease, saliva, biomarkers, creatinine, urea and dog*.

INTRODUCTION

Chronic kidney disease (CKD) is defined as the presence of structural and/or functional abnormality in one or both the kidneys with the abnormality persisting for a period of more than 3 months. CKD is often progressive and results in irreversible loss of renal function and structure (Bartges, 2012). With kidneys being involved in homoeostasis CKD thus affects not only the excretory system but also several other organ systems in the dog's body as well. Furthermore, CKD disrupts metabolic acid-base balance and the production of erythropoietin and vitamin D, contributing to systemic complications. Secondary manifestations include weight loss, muscle wasting, hyporexia, vomiting, halitosis, ulcerative stomatitis, and gastroenteritis (Bartges, 2012). Despite the clinical significance of CKD in dogs' early diagnosis remains a challenge for veterinarians. There is a direct correlation between the severity of illness and the associated cost of treatment suggesting early diagnosis is of paramount importance, particularly in developing countries where healthcare resources are constrained. The clinical progression of CKD in dogs typically involves a prolonged asymptomatic phase, followed by the gradual onset of clinical manifestations, ultimately culminating in severe renal dysfunction and death in the absence of appropriate intervention (Zuniga *et al.* 2016). Currently, in dogs, the diagnosis of CKD is made on the levels of blood urea nitrogen (BUN) and serum creatinine (sCr). Both markers though widely used have limitations with regard to detection of renal disease at an early stage (Hokamp and Nabity, 2016).
Venipuncture has been the standard invasive method for obtaining blood samples in dog that requires physical restraint or chemical sedation, can induce iatrogenic stress and pain, carry risks of hematoma formation and haemolysis and consequently limit the feasibility of frequent monitoring and may compromise animal welfare (Yoshizawa *et al*., 2013). Non-invasive diagnostic techniques represent a viable alternate to conventional methods having advantages of sample collection being less painful and anxiety inducing to the patients, samples being easier to collect and store without intervention requirements by trained professionals and little cold chain involvement. Amongst all non-invasive techniques, saliva sampling is emerging as the most popular diagnostic tool for detecting and monitoring various non-infectious disorders through the assessment of specific biomarkers (Javaid *et al.,* 2016). Therefore, to investigate the utility of saliva as a diagnostic material, the present study was designed to evaluate and correlate the diagnostic potential of salivary

biomarkers, specifically creatinine and urea, for CKD in dogs.

MATERIALS AND METHODS

The study was conducted over six months at the Department of Veterinary Medicine, College of veterinary Science, Assam Veterinary and Fishery University, Khanapara, Assam. Dogs irrespective of age, sex and breed presented at Veterinary Clinical Complex (VCC) from different parts of Assam and neighbouring states of North Eastern region with history (inappetence, polyuria/polydipsia, weight loss, lethargy) and clinical signs (vomition, oral ulcer, pale mucous membrane) suggestive of kidney disease were screened and subjected to further detailed physical examination, haematology, serum biochemical profile and ultrasonography. Samples of saliva were also collected from these dogs. Dogs with confirmed CKD were selected for the study. Apparently healthy dogs registered for vaccination or routine annual health checkup were also selected as control and serum as well as saliva was collected for evaluation of renal biomarkers (creatinine and urea).

Collection of blood and saliva samples

Blood samples were collected from dogs into clot activator coated vacutainer for biochemical analysis. The blood was allowed to clot at room temperature. The tubes were centrifuged at 3,000 rpm for 15 minutes to separate the serum and the sample was analysed immediately. Unstimulated whole saliva was collected from the dogs using commercially available unflavoured disposable oral care sponge swab for tooth cleaning in children. The swabs were placed under the tongue and in the cheek pouches of the dogs for 30 seconds. The swabs were then squeezed into a labelled micro centrifuged tube and centrifuged at 3000 rpm for 5 min so that debris can settle down and the clear extract was collected using a micro pipette and stored at -20ºC until further analysis.

Estimation of Biochemical Parameters

Serum and saliva samples were used for determination of creatinine and urea concentration, at 37 ºC in a Semi- Automated Clinical Chemistry Analyser (Bene SpheraTM C61) by using commercially available *in vitro* diagnostic kits.

Estimation of creatinine (mg/dl) in serum and saliva

Creatinine concentration in serum and saliva was determined using the modified Jaffe's method with a commercially available diagnostic kit (Asritha Diatech India Pvt. Ltd., Hyderabad), as described by Newman (1999). The reaction mixture containing sample or standard and working reagent was incubated at room temperature for approx. 30 sec. The absorbance was measured at 505 nm at two time intervals, initially (A1) and after 60 seconds (A2) against the reagent blank. The change in absorbance (ΔA) was calculated for both standard and test samples. The creatinine concentration in the sample was calculated using the following formula:

$$\text{Creatinine (mg/dl)} = \frac{\Delta A_{\text{Test}}}{\Delta A_{\text{Standard}}} \times \text{Concentration of Standard}$$

where $\Delta A = (A2 - A1)$.

Estimation of urea (mg/dl) in serum and saliva

Urea concentration in serum and saliva was estimated by a UV enzymatic method using a commercial diagnostic kit (Asritha Diatech India Pvt. Ltd., Hyderabad) following the method by Thomas (1998). The reaction mixture containing sample or standard and working reagent was incubated at room temperature for approx. 30 sec. The absorbance was measured at 340 nm at two time intervals, initially (A1) and after 60 seconds (A2) against the reagent blank. The change in absorbance (ΔA) was calculated for both standard and test samples. The creatinine concentration in the sample was calculated using the following formula:

$$\text{Urea (mg/dl)} = \frac{\Delta A_{\text{Test}}}{\Delta A_{\text{Standard}}} \times \text{Concentration of Standard}$$

where $\Delta A = (A1 - A2)$.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel and GraphPad Prism 9. Student T- test was carried out to compare the values between serum and salivary concentrations of creatinine and urea of CKD affected dogs and apparently healthy dogs. Correlation between serum and salivary biomarkers were carried out using Pearson's correlation. Receiver operating characteristic (ROC) curves were plotted for the estimation of area under the curve (AUC) value, cut off value, specificity and sensitivity of the biomarkers.

RESULT AND DISCUSSION

Serum and salivary creatinine and urea values of dogs

Data presented in Table 1 summarizes the concentration of creatinine and urea in serum as well as saliva from apparently healthy dogs and dogs with CKD. A significant increase (≤0.001) in the mean value of serum creatinine (sCr) in dogs suffering from CKD (6.27±0.53 mg/dl) was recorded as compared to mean value of sCr of apparently healthy dogs (1.127±0.04 mg/dl). These findings were similar with Cowgill *et al.* (1998) and Mrudula *et al*. (2005) in dogs. The increase in sCr concentrations is a result of progression of kidney disease and decline of GFR (Dibartola *et al*., 1983). A significant increase (≤0.001) was recorded in the mean value of serum urea in dogs suffering from CKD (122.79±11.59 mg/dl) when compared to the mean value of serum urea of apparently healthy dogs (37.45±3.99 mg/dl). These findings are in agreement with Bradea *et al*., (2013) and Rusenov *et al*., (2014). The increase in urea levels in serum in CKD dogs might be due to retention of nitrogenous substances that are normally excreted by healthy kidneys (Cowgill *et al*., 1998 and Polzin, 2010).

An attempt was made to evaluate the salivary concentration of creatinine and urea as potential markers for CKD in dogs. The study revealed a significant increase (≤0.001) in the mean value of salivary creatinine in dogs suffering from CKD (1.96±0.17 mg/dl) when compared to mean value of apparently healthy dogs (0.59±0.07 mg/dl). Similar findings were observed in humans (Venkatapathy *et al.* 2014 and Yajamanam *et al.* 2016) and in dogs (Raja *et al.* 2017 and Tvarijonaviciute *et al.* 2018) affected with kidney disease. Similar to creatinine, salivary urea concentration was also significantly increased in dogs suffering from CKD (63.43±6.29 mg/dl) when compared to apparently healthy dogs (12.04±1.44 mg/dl). Similar findings were reported by Raja *et al.* (2017) and Tvarijonaviciute *et al.* (2018) in dogs and Xia *et al.* (2012), Renda (2017) and Aravind and Manokaran (2018) in humans. The reason for this could be that the concentration of serum creatinine and urea builds up and a gradient is created, which causes diffusion of creatinine and urea to saliva as well. This process is aided by the fact that an alteration in permeability is seen in the salivary glands of dogs afflicted by CKD (Nakahari *et al*., 1996; Guyton and Hall, 2006 and Zhang *et al*., 2016). Also, low level creatinine in saliva is seen in comparison to serum which has also been reported by Venkatapathy *et al*., (2014), Yajamanam *et al*., (2016) and Pham (2017). This could be due to the fact that creatinine is a large molecule with high molecular weight of 113 Da and molecular radius of 0.3nm maintained at constant plasma levels by kidneys and exhibit low lipid solubility which can limit the diffusion of creatinine across the cells and intercellular junctions of the salivary glands (Chiou *et al*., 1977). Saliva acts as an alternative route of excretion by the body when renal function is in compromised state, because of anatomic and physiologic similarities of its secretive units to the renal tubules. The primary salivary secretion which contains electrolytes, water, mucus and enzymes, flows out into collecting ducts to be managed: reabsorbing sodium and secreting phosphate and potassium (Savica *et al*., 2007).

Table 1: Mean ± S.E. of serum and salivary creatinine and urea in apparently healthy dogs and dogs with CKD

Parameter	Apparently healthy dogs (n=10)	CKD dogs (n=38)	P-value
Serum creatinine (mg/dl)	1.127±0.04 (0.86–1.28)	6.27±0.53 (2.30–12.50)	≤0.001***
Salivary creatinine (mg/dl)	0.59±0.07 (0.10–0.78)	1.96±0.17 (0.40–3.80)	≤0.001***
Serum urea (mg/dl)	37.45±3.99 (18.80–55.00)	122.79±11.59 (54.10–332.30)	≤0.001***
Salivary urea (mg/dl)	12.04±1.44 (6.51–22.00)	63.43±6.29 (21.00-200.40)	≤0.001***

Values within bracket indicates range

Table 2: Pearson correlation analysis of serum and salivary creatinine and urea in dogs with CKD (n=38)

	Serum	Saliva	r	P-value
Creatinine	6.27±0.53	1.96±0.17	0.77	≤0.001***
Urea	122.79±11.59	63.43±6.29	0.81	≤0.001***

r: Pearson correlation coefficient, ***statistically highly significant

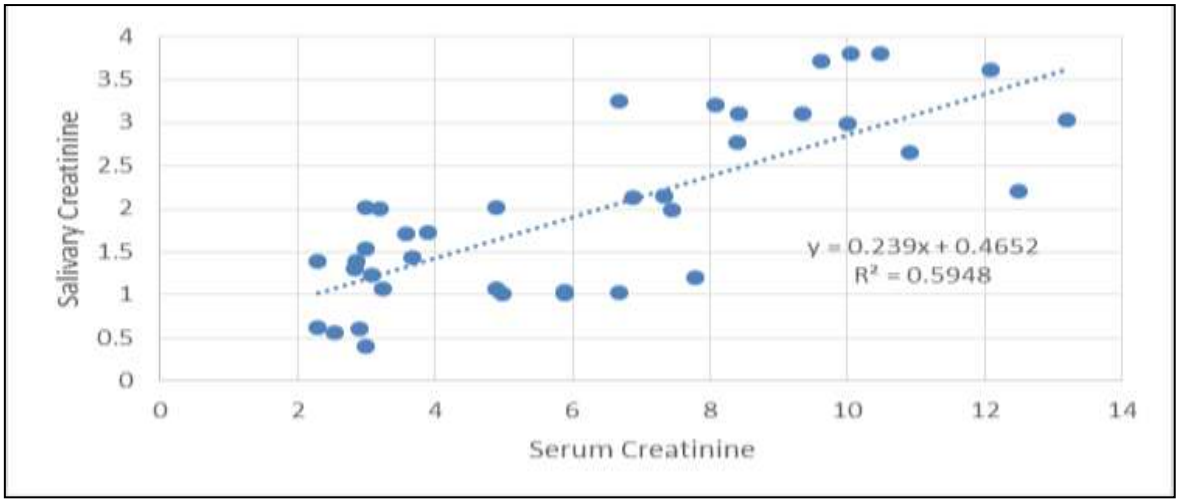

Fig. 1 (A) Scatter Plot showing correlation between serum and salivary creatinine

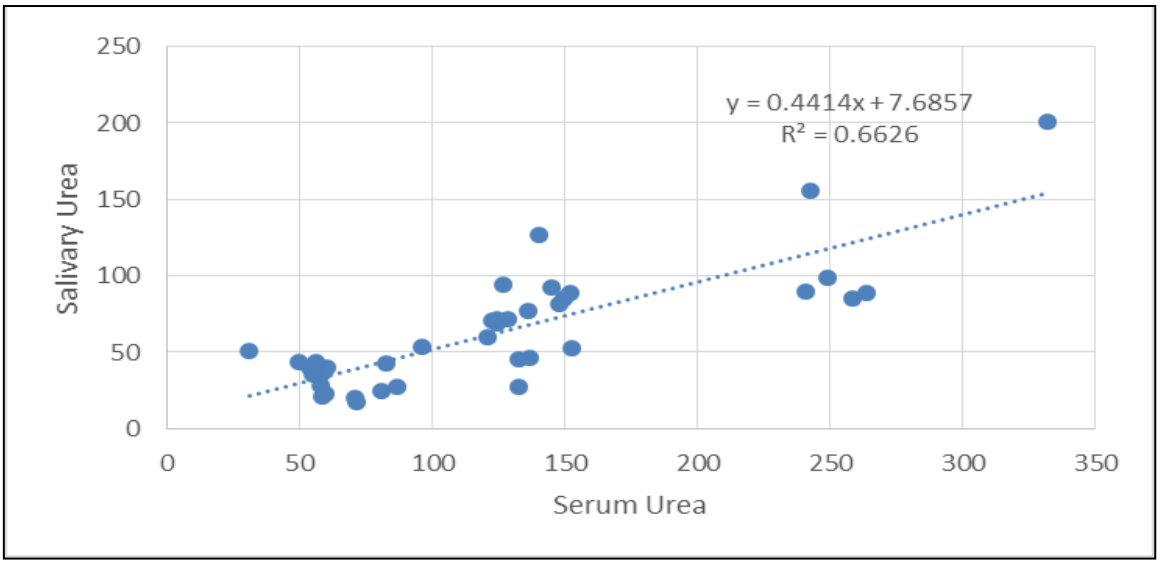

Fig. 1 (B) Scatter Plot showing correlation between serum and salivary urea

Table 3: AUC value of salivary creatinine and urea

PARAMETERS	AUC	SE	95% CI
Salivary creatinine	0.92	0.03	0.85 to 1.00
Salivary urea	0.98	0.01	0.96 to 1.00

Table 4: Sensitivity and Specificity of salivary and serum creatinine and urea with cut off value in dogs

PARAMETERS	CUT OFF VALUE (mg/dl)	SENSITIVITY (%)	SPECIFICITY (%)
Salivary creatinine	0.89	89.47	100
Salivary urea	18.50	97.37	90

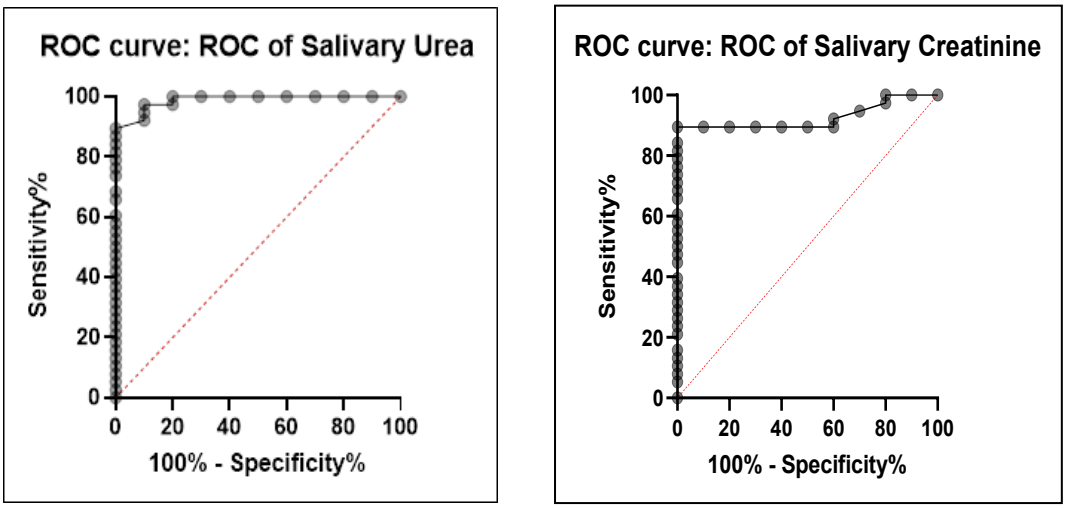

Fig. 2: ROC curve and AUC of salivary urea (A) and serum urea (B)

Pearson's correlation analysis of serum and salivary creatinine and urea in dogs

To check the relationship between serum and salivary creatinine and whether changes in serum creatinine are reflected in saliva, a Pearson's correlation analysis was performed in case study groups. The results of correlation of salivary to serum creatinine and urea in dogs suffering from CKD are depicted in Table 2 and Fig. 1 (A and B) shows the scattered diagram. A significantly high positive correlation (<0.001) was found between serum and salivary creatinine and serum and salivary urea in CKD cases with correlation coefficient r=0.77 and r=0.81 respectively. These findings are in accordance with Raja *et al.* (2017) and Tvarijonaviciute *et al.* (2018) who also found correlation of these analytes between serum and saliva of dogs suffering from CKD.

Area under the curve (AUC) value cut off value (mg/dl), sensitivity and specificity of creatinine and urea in saliva

To assess the diagnostic potential of salivary creatinine and urea, ROC curve analysis was performed. The ROC curve of salivary creatinine showed area under the curve=0.92, SE=0.03 and 95 % confidence interval of 0.85 to 1.00. Cut off point of salivary creatinine obtained was 0.89 mg/dl with sensitivity=89.47%, specificity=100%. Similar findings were reported by Xia *et al.* (2012), Bader *et al*. (2015), Lasisi *et al.* (2016), Zuniga *et al*. (2016) and Padwal *et al*. (2022) in humans

suffering from CKD. The ROC curve of salivary urea showed area under the curve= 0.98, SE= 0.01 and 95 % confidence interval of 0.96 to 1.00. Cut off point of salivary urea obtained was 18.50 mg/dl with sensitivity=97.37%, specificity=90%. Similar findings were obtained by other workers in humans with kidney disease (Xia *et al*., 2012; Bader *et al.,* 2015, Lasisi *et al*., 2016, Zuniga *et al.* (2016) and Padwal *et al*., 2022). In humans suffering from CKD, the levels of urea, creatinine and uric acid between the saliva and serum was closely related and the salivary concentration of urea, creatinine and uric acid could reflect the renal damage, monitor kidney function of the CKD patients and help diagnose middle to late-stage CKD patients (Xia *et al*., 2012 and Zuniga *et al.* 2016). In the present study, the areas under the ROC curves for salivary urea and creatinine suggest that these markers can be used as additional diagnostic parameters in dogs suffering with CKD. The area under the ROC curve with standard error and 95% confidence interval for salivary creatinine and urea are shown in Table 3 and respective cut-off values with their highest sensitivity and specificity in Table 4 with receiver operating characteristics curve in Fig. 2 (A and B).

CONCLUSION

From this study it can be conclude that that urea and creatinine can be measured in canine saliva with commercially available spectrophotometric assays. In addition, these analytes show higher values in saliva of dogs with chronic kidney disease compared with healthy dogs and were found to be almost nearly equally sensitive and specific when compared to serum analytes in reflecting kidney disease. Therefore, salivary creatinine and urea levels may serve as alternatives to serum values for assessing kidney function. Additionally, saliva collection, being non-invasive, could be used instead of blood sampling for the diagnosis and monitoring of CKD affected dogs.

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Informed consent

All animals' procedures for the experiments were approved by the Institutional Animal Ethics Committee.

Conflict of interest

The authors declare that they have no conflict of interest.

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