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Assessment of serum cystatin C concentration in dogs by an enzyme-linked immunosorbent assay: An early and prognostic marker in renal failure

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Abstract
The study was conducted at the Department of Veterinary Medicine, Veterinary College Bangalore with objective to determine the efficacy of cystatin C as biomarker in early diagnosis of renal failure and to predict the progression of renal failure in dogs. Dogs aged above five years with history, clinical signs and laboratory findings suggestive of renal disease were taken up for the study. Based on the creatinine values, animals were randomly divided into 3 groups, Group-I (at risk group), Group-II (renal failure group) and Group-III (control). Serum cystatin C was measured by canine cystatin C ELISA kit. There was a significant difference ($P \leq 0.05$) in mean values between Group-II and Group-I as well as Group-II and Group-III while there was no significant difference ($P > 0.05$) in mean values between Group-I and Group-III (control). However, of the 10 animals of Group-I, 6 animals on day 0 and 8 animals on day 28 showed elevation in serum cystatin C levels compared to Group-III (control dogs). Therefore it was concluded that elevated serum cystatin C levels in individual animals in Group-I were indicative of early onset renal failure and serum cystatin C in all animals of Group-II correlated well with increasing creatinine values suggesting that serum cystatin C was a good index to predict the progression of renal failure.

Keywords: renal failure, serum cystatin C, ELISA, creatinine, early diagnosis

Introduction
Renal failure is one of the most common disorders in dogs a small animal practitioner may be presented with and estimation of the glomerular filteration rate is the most reliable measure of overall renal function, Despite several methods being available to measure the GFR, estimation of serum creatinine concentration is still utilized as a gold standard to measure the GFR and thereby overall kidney function as laid out by the International Renal Interest Society (IRIS). However serum creatinine concentration does not increase until the GFR is lowered by 75 per cent or more (Braun *et al.,* 2003) [2]. Hence it is imperative that a biomarker that measures GFR earlier be used in conjunction with serum creatinine values so as to detect the failing kidney function at a much earlier stage for timely therapeutic intervention. Serum cystain C is increasingly used in human medicine with this very objective.

Cystatin C (Cys-C), a cysteine protease with a low molecular weight (13 kDa), is produced at a constant rate by all nucleated cells and cleared by glomerular filtration and tubular epithelial cells reabsorbed nearly all (99%) of the filtered Cys-C. The reabsorbed Cys-C is catabolized by tubular epithelial cells, so it does not return to circulation. In addition, Cys-C is not secreted by renal tubules (Kaseda *et al.,* 2007) [9]. Because of these factors, serum Cys-C concentration is regarded as a GFR biomarker (Ghys *et al.,* 2014) [4]. Serum Cys-C concentrations predict a decrease in GFR in humans more accurately than serum Crt levels (Peralta *et al.,* 2011) [14]. As a result, serum Cys-C concentration is used as a marker in humans for the early detection of chronic kidney disease (Hari *et al.,* 2014) [5] and as a prognostic factor of renal functions and has shown considerable promise as an early biomarker of renal disease in dogs.

Further since all prior investigations of serum Cys-C concentration in dogs were retrospective, the prognostic significance of serum Cys-C concentration for renal prognosis is still unknown. Hence keeping in view the above facts, the present study was carried out with the objectives to monitor the progression of renal disease using Cys-C as biomarker and to determine its efficacy in the early diagnosis of renal disease.

Materials and Methods

The study was conducted in the Department of Veterinary Medicine, Veterinary College Bangalore, Karnataka Veterinary Animal and Fisheries Science University, Bidar (Karnataka). Dogs aged above five years with history, clinical signs and laboratory findings suggestive of renal disease were taken up for the study. Blood was collected from these animals and the serum creatinine values were determined. Based on the creatinine values, animals were randomly divided into 3 groups.

1. At risk group (Group I)

Ten animals with clinical signs suggestive of renal failure but with normal serum creatinine values of ≤1.4 mg/dl were selected to evaluate the possibility of occurrence of renal failure.

2. Renal failure group (Group II)

Ten animals with clinical signs of renal failure and serum creatinine values of >1.4 mg/dl were selected and were monitored to evaluate the progression renal failure with specific or supportive therapy.

3. Healthy control group (Group III)

Ten apparently healthy animals with serum creatinine values of ≤1.4 mg/dl were selected as control group and were monitored.

Blood was collected on day 0 and day 28 for Group-I and Group-III, and on days 0, 7, 14 and 28 for Group-II. Blood was collected by venipuncture of saphenous or cephalic vein into 2 ml tubes with and without EDTA for hematology and biochemistry (serum). Samples in EDTA for hematology were analyzed using automatic blood analyzer manufactured by BC 2800 Mindray Hematology Analyzer and serum after separation was analyzed for creatinine using semi biochemical analyzer by Micro Lab Biochemical Analyzer RX 50 using serum creatinine kit (Erba diagnostics Germany). The remaining serum was stored at -80 °C until used for measurement of serum cystatin C using ELISA.

Serum cystatin C was measured using canine Cystatin C ELISA Kit (Serial No. E0148 Ca) supplied by Bioassay Technology Laboratory (BT Lab), a brand of Shanghai Korain Biotech Co Ltd, Shangai (China) a commercially available sandwich ELISA according to methods and instructions provided with the assay (Fig.1). The kit contained a plate precoated with an antibody against canine cystatin C from the tested sample, as well as an additional site-specific biotinylated anti-canine cystatin C monoclonal antibody and reference standards and reagents. A standard curve for cystatin C was created using 6 dilutions (ranging from 0.05 mg/L to 15 mg/L) of canine cystatin C reference standard (included in kit), which were evaluated on the same plate as the clinical samples. Reference standards were diluted 1: 100 using the included proprietary sample diluents and were added to standard wells. After that samples were added to all wells with the secondary biotinylated antibody and streptavidin-HRP reagent was added and incubated for 1 hour at 37 °C. After this incubation, wells were washed with wash buffer for 5 times. Then substrate solution A and substrate solution B were added and incubated for 10 minutes at room temperature in the dark for color development. At 10 minutes, a dilute stop solution was added, and the optical density of the solution in the well was measured at 450 nm using a plate reader (Fig.1). The concentrations of the experimental samples were calculated from a standard curve using curve fitting software (Fig. 2).

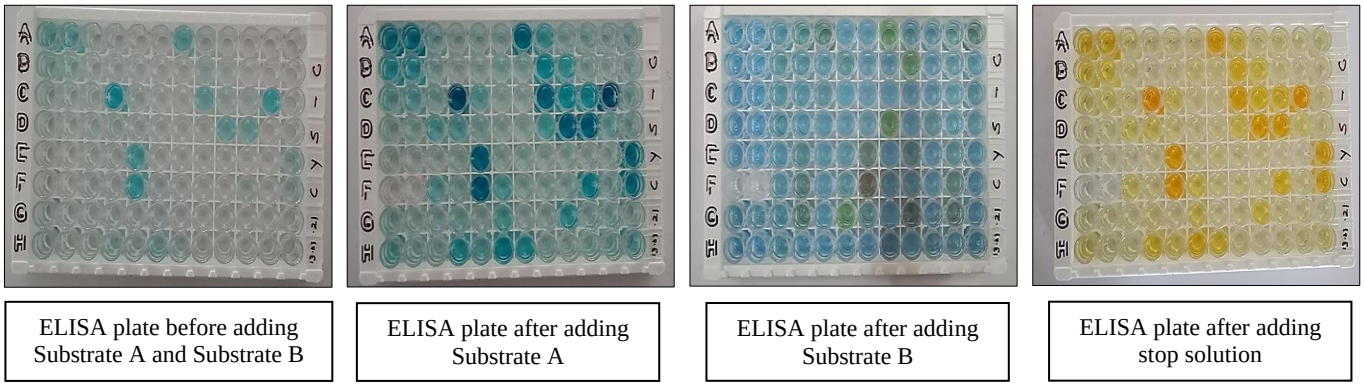

Fig 1: Procedure of sandwich ELISA for serum cystatin C estimation

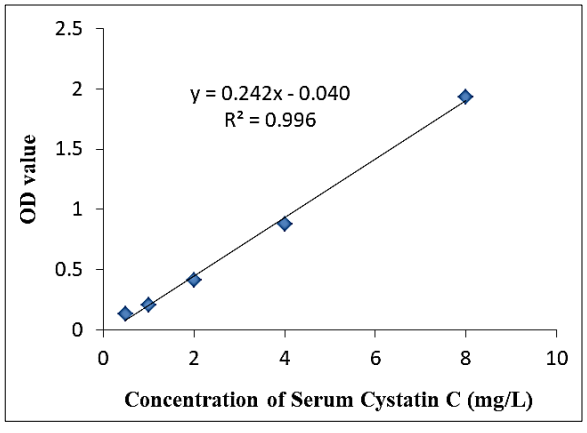

Fig 2: Standard curve for serum cystatin C at 95% confidence level

Statistical analysis

Data are presented as mean ± standard error. One way ANOVA was performed of collected data with IBM SPSS software (New York, USA) and GraphPad Prism 8.4 software (GraphPad Software, CA, USA).

Results and Discussion

Dogs of Group-I (at risk group) and Group-II (renal failure) exhibited clinical signs suggestive of renal failure such as vomition, anorexia, hemorrhagic diarrhoea, paleness of mucous membrane, dullness, depression and restlessness, whereas Group-III animals were apparently normal with no clinical signs and were used as control.

The creatinine values were studied to evaluate the renal damage and values ≤ 1.4mg/dl was considered normal. The

normal levels of creatinine in blood are indicators of ability of kidney to eliminate nitrogenous waste products successfully. According to the classification of IRIS (2017) [6] creatinine values of ≤1.4 mg/dl is considered as stage I renal failure. The creatinine values of Group I, II and III are depicted in Table.1.Animals in Group I had normal creatinine values though clinical signs were suggestive of renal failure. Therefore some of animals in Group-I with normal creatinine levels (≤1.4 mg/dl) could be in stage I renal failure. Creatinine inversely correlates with GFR and is a better indicator of renal function but creatinine is insensitive to detect early renal insufficiency because the values increase above the reference range only when the damage to the kidneys is more than 60-75 per cent (Lefebvre, 2011) [11].

Table 1: Mean ± SE of serum creatinine values in Group I, II and III dogs

Groups	Serum creatinine	
	Day 0	Day 28
Group I	1.20 ± 0.06ax	1.18 ± 0.05ax
Group II	5.30 ± 0.87ay	4.32 ± 1.12ay
Group III	1.10 ± 0.06ax	1.03 ± 0.04ax

Note: [a, b, c, d] Mean values in a row with different superscripts differ significantly ($P \leq 0.05$)
[w, x, y, z] Mean values in a column with different superscripts differ significantly ($P \leq 0.05$)

In the present study, serum cystatin C was measured by sandwich ELISA technique by using canine serum cystatin C ELISA kit. The mean ± SE of serum cystatin C value in Group-III (control) was 1.14 ± 0.12 mg/L on day 0 and 1.17 ± 0.32 mg/L on day 28. The mean ± SE of serum cystain C value in dogs in Group-II (renal failure) in the present study was 4.38 ± 1.28 mg/L, 4.26 ± 1.38 mg/L, 3.55 ± 1.51 mg/L, 3.56 ± 1.45 mg/L on days 0,7,14 and 28 and ranged between 1.17 to 13.26 mg/L (Table. 2, 3 and 4 and Fig. 3). There was a significant difference ($P \leq 0.05$) in the mean between Group-III (control group) and Group-II and this is in agreement with findings of Kavitha (2010) [10], Murty *et al.* (2013) [13], Chacar *et al.* (2016) [3] and Subapriya *et al.* (2020) [16] where in all of these workers reported low levels of serum cystatin C in healthy control group as compared to much higher values in animals with renal failure. Further all the animals in Group-II had higher than reference levels of serum cystatin C which correlated with increased serum creatinine values in all the animals, thus proving that serum cystatin C has good correlation with serum creatinine in renal failure, and hence was found to be a useful indicator for diagnosis as well as progression of renal failure.

Cystatin C is a cysteine-proteinase inhibitor that is widely distributed in biological fluids. It has a low molecular mass and is formed from 120 amino acid residues in a single polypeptide chain. The physiological and the biochemical role of cystatin C in diagnosis of renal disorders has been described by Abharhamson *et al.* (1990) [1] where it was stated that cystatin-C is constantly produced by nucleated cells and is filtered by the glomeruli, with approximately 99 per cent of filtered cystatin-C reabsorbed by tubular epithelial cells. However, the tubular epithelial cells catabolise the reabsorbed cystatin-C, so it does not return into circulation. For these reasons, serum cystatin-C concentration is considered to be a biomarker of the GFR. This has been corroborated by Ghys *et al.* (2014) [4].

Table 2: Mean ± SE values of serum cystatin C in Group I, II and III dogs

Groups	Serum cystatin C	
	Day 0	Day 28
Group I	1.31 ± 0.35ax	1.41 ± 0.17ax
Group II	4.38 ± 1.28ay	3.56 ± 1.45ay
Group III	1.14 ± 0.12ax	1.17 ± 0.32ax

Note: [a, b, c, d] Mean values in a row with different superscripts differ significantly ($P \leq 0.05$)
[w, x, y, z] Mean values in a column with different superscripts differ significantly ($P \leq 0.05$)

In addition, cystatin-C is not secreted by renal tubules (Kaseda *et al.*, 2007) [9]. According to Peralta *et al.* (2011) [14] decrease in GFR is predicted more accurately by serum cystatin-C concentrations than by serum creatinine levels. Hence serum cystatin-C concentration is used as a marker in humans for the early detection of chronic kidney disease and as a prognostic factor of renal functions (Hari *et al.*, 2014) [5]. Miyagawa *et al.* (2009) [12] stated that increased serum cystatin-C concentrations indicate a decrease in GFR in dogs.

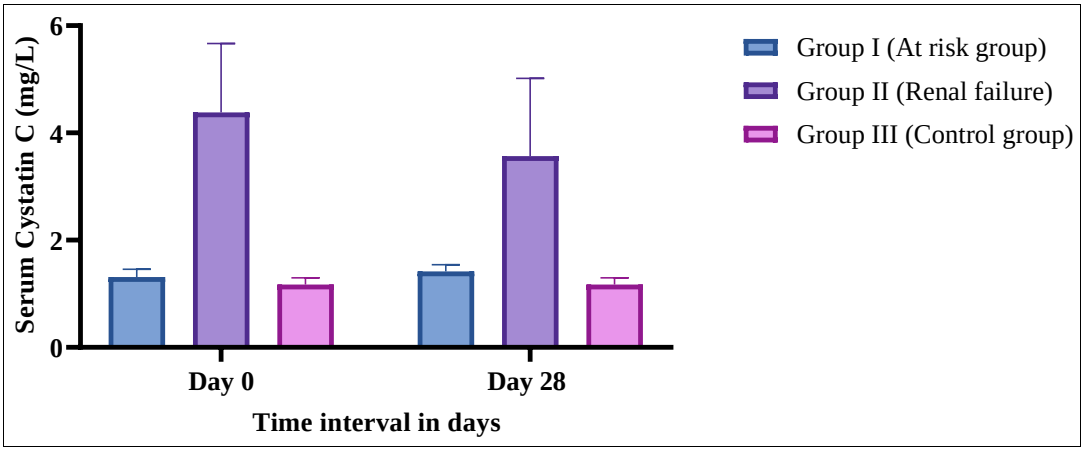

Fig 3: Mean ± SE values of serum cystatin C in Group I, Group-II and Group-III dogs

In humans, the use of serum cystatin-C concentrations has improved the detection and prediction of outcome in chronic kidney disease (Shlipak *et al.*, 2013) [15]. Similarly in the present study serum cystatin C concentration provided a good indication of future risk of progression of renal disease and correlated well with serum creatinine.

Table 3: Mean ± SE values of serum creatinine and serum cystatin C in Group-II on different days

	Day 0	Day 7	Day 14	Day 28
Serum creatinine (mg/dl)	5.32 ± 0.87	5.0 ± 1.01	4.85 ± 1.34	4.32 ± 1.12
Serum cystatin C (mg/L)	4.38 ± 1.28	4.26 ± 1.38	3.55 ± 1.51	3.56 ± 1.45

The mean ± SE of serum cystatin C value in Group-I was 1.31 ± 0.35 mg/L and 1.41 ± 0.17 mg/L on day 0 and 28, respectively (Table. 2 and Fig. 3). There was no significant difference (P>0.05) in mean between Group-I and Group-III (control). However, of the 10 animals of Group-I, 6 animals on day 0 and 8 animals on day 28 showed elevation in serum cystatin C levels compared to Group-III (control dogs) (Table. 4). According to IRIS (2019) [7], dogs and cats with Stage 1 CKD have normal or near-normal GFR and serum creatinine levels. This means that many of the clinical signs that are classically associated with renal failure maybe absent. Thus, many patients with stage 1 disease will be asymptomatic. Further it can be observed that serum creatinine values of these animals (Group-I) were within the normal reference range although the animals exhibited clinical signs suggestive of renal failure. Hence taking into account the increased serum cystatin C levels in Group-II (renal failure) animals that correlated with increased serum creatinine levels, it can be concluded that 8 animals in Group-I could be in stage I of renal failure probably progressing to stage II. Further the mean ± SE of serum cystatin C levels in Group-I was elevated as compared to Group-III (control) which was though not statistically significant substantiates that the Group-I animals could be progressing towards renal failure. This is in agreement with earlier workers Kavitha (2010) [10] and Iwasa *et al*. (2018) [8]. Wu *et al*. (2010) [17] reported that human patients with high serum cystatin-C concentrations carry a high risk of subsequent mortality, even in patients with a normal estimated GFR. Similarly in the present study, dogs of Group-I exhibited high serum cystatin C value along with normal creatinine values which could be a risk factor and an early predictor of renal disease and subsequent mortality.

Table 4: Values of serum cystatin C (mg/L) of the individual dogs in Group-I and II

Animal	Group I		Group II			
	Day 0	Day 28	Day 0	Day 7	Day 14	Day 28
1	0.79669	1.24174	1.28512	0.99587	1.04959	1.02479
2	1.49587	1.73554	1.39669	1.1157	1.17355	1.09917
3	1.30682	1.31818	1.17851	1.23554	1.23719	3.81405
4	1.01653	1.00207	1.26198	1.1405	1.18595	0.89256
5	1.86653	1.74587	1.20413	0.8719	1.28926	1.41322
6	0.81818	1.29628	12.4914	14.1983	*	*
7	1.83884	1.31198	12.3514	13.3298	*	*
8	1.98146	1.85331	6.59504	6.14876	3.71901	3.19008
9	0.67273	0.71612	13.2686	12.686	5.71901	5.09917
10	1.33843	1.96281	9.50826	10.9917	13.2562	12.9959
mean ± SE	1.31 ± 0.35	1.41 ± 0.17	4.38 ± 1.28	4.26 ± 1.38	3.55 ± 1.51	3.56 ± 1.45

Note: Mean ± SE value of serum cystatin C of Group-III was 1.14 ± 0.12 on day 0 and 1.17 ± 0.02 on day 28. *Dead animals

Conclusion

It was therefore concluded following the present study that elevated serum cystatin C levels in dogs with normal creatinine levels could be an indicator of early onset of renal failure. Further, it was concluded that serum cystain C was a good index to predict the progression of renal failure and hence a promising endogenous GFR marker, which would be particularly useful to overcome the shortfalls associated with sCr assessment.

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