Alteration in Serum Cystatin-C and SDMA as biomarkers of early renal dysfunction associated with Canine Monocytic Ehrlichiosis (CME)

Anubhav Singh
DUVASU: Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan

Mukesh Kumar Srivastava
DUVASU: Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan

Kapil Kumar Gupta (DR.KAPIL09@GMAIL.COM)
Indian Veterinary Research Institute https://orcid.org/0000-0003-3086-7767

Research Article

Keywords: Cystatin-C, serum symmetric dimethylarginine, Ehrlichiosis, ELISA, PCR

Posted Date: June 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-591312/v1

Abstract

Present study was carried out on 30 dogs, positive for ehrlichiosis in nested PCR. Important clinical symptoms exhibited by the canine monocytic ehrlichiosis (CME) positive dogs were mucosal pallor, epistaxis, ascites, ecchymotic and petechial hemorrhages, corneal opacity, icterus and vomiting. All positive dogs were divided into two group (each containing 15 dogs) for the purpose of providing different therapeutic intervention. Six healthy dogs irrespective of age sex or breed were taken as control for comparison of parameters. Whole blood was collected and serum was separated by using standard protocol and serum cystatin C and symmetric dimethylarginine (SDMA) were estimated by using commercial ELISA kit. There was significant increase in serum cystatin C and serum symmetric dimethylarginine (SDMA) in both the treatment groups of dogs in comparison with control. However, there was non-significant decrease in serum cystatin C and SDMA on day 14 post-treatment in both the treatment groups. Total 17 (56.67%) dogs were found to have higher serum cystatin C out of which 10 (33.33%) dogs were also having elevated serum creatinine while remaining 7 (23.33%) dogs were having normal creatinine values. Elevated serum level of SDMA was found in15 (50%) dogs of which 10 (33.33%) were also having concurrent higher creatinine while 5 (16.67%) dogs were have normal creatinine value in spite of having elevated SDMA level. Results indicate the early rise of serum cystatin C and SDMA during renal damage which may considered as better biomarkers for early screening of renal impairment associated with canine monocytic ehrlichiosis (CME).

1. Introduction

Renal failure may be one of the severe irreparable complication in Ehrlichiosis, therefore, needs early diagnosis. In case of acute *E. canis* infection a possible glomerulopathy with minimal changes and without histologic evidence of glomerular disease could be seen (Codner et al., 1992). In veterinary clinical practice, serum urea and creatinine concentrations are widely used as endogenous markers for evaluating renal function in dogs because they are easy and inexpensive to perform (Miyagawa et al., 2009). On the other hand, serum Creatinine (Cr) usually only becomes elevated once more than 75% of nephron are lost, in addition creatnine could create a false sense of security in early stages of renal disease (Srivastava et al., 2011). Over several years serum levels of cystatin- C (Cys-C) have been considered as a significant marker for renal function (Antognoni et al., 2005). Cystatin C (Cys-C) is a small, non-glycosylated, 13 kDa, 120 amino acid polypeptides constantly produced by most nucleated cells within the body and is released during phagocytosis and inflammation. Its production in body is a stable process that is not influenced by renal conditions, dietetic factors or increased protein catabolism. Moreover, it does not change with age or muscle mass like creatinine does. Its biochemical characteristics allow free filtration in the renal glomerulus, and subsequent metabolism and reabsorption by the proximal tubule. For these reasons, serum Cys-C has been suggested to be an endogenous marker of glomerular filtration rate (Antognoni et al., 2005; Villa et al., 2005). Hence serum Cys-C levels can be considered as another renal marker with superior reliability compared to creatinine. Symmetric dimethylarginine (SDMA) was first identified in 1970 by Kakimoto and Akazawa as a urine metabolite

and was suspected to have a functional role in protein synthesis (Kakimoto et al., 1970). It is now known that SDMA and a similar protein, asymmetric dimethylarginine (ADMA) are derived from post-translational modification (methylation) of arginine residues within almost every cell. After proteolysis, or protein breakdown, these protein residues are released into the circulation. SDMA is eliminated from the body primarily via renal excretion (> 90% via glomerular filtration without tubular reabsorption or secretion), but some small percentage of its elimination may be associated with non-renal enzymatic cleavage and subsequent degradation Serum concentrations of SDMA are increased in human patients with chronic kidney disease (CKD) and it has been shown that serum SDMA concentrations are inversely correlated with glomerular filtration rate (GFR) (Kielstein et al., 2006). SDMA is not only a molecule of importance for measurement and evaluation of renal excretory function in CKD, but perhaps increased levels of SDMA can be linked to inflammation and progression of CKD. Keeping in view the importance of detection of ehrlichiosis induced renal dysfunction the present study was conducted to evaluate the clinical significance of canine specific Serum cystatin-C and Symmetric dimethylarginine (SDMA) for the early diagnosis of renal dysfunction associated with canine monocytic ehrlichiosis (CME).

2. Material And Method

2.1. Screening of dogs for study

Screening of dogs, suspected for *Ehrlichia* was done on the basis of observation of classical signs of *Ehrlichia* in dogs. Based on results of previous studies, clinical signs considered for screening are mucosal pallor, high fever, lymphadenomegaly, epistaxis, splenomegaly, hyphema, depression, ocular abnormalities, vomiting, haematuria, melena, haematemesis, hind limb /or scrotal edema, bleeding diathesis, icterus, central nervous system signs, ascites and weight loss (Fig. 1–4). Dogs with above mentioned 2–3 classical signs of ehrlichiosis listed above will be included in screening procedure. Dogs' positive for ehrlichiosis with a concurrent disease and dog without proper history of vaccination and deworming were excluded from screening procedure. A total of thirty-five (35) dogs were screened in the present investigation, which were presented to Veterinary Clinical Complex, DUVASU, Mathura between August 2018 to April 2019.

2.2. Confirmation of screened dogs for ehrlichiosis

Screened dogs further underwent a series of diagnostic procedure to confirm the disease. Out of 35 screened samples, 30 samples were found positive for ehrlichiosis on the basis of Nested PCR. The primary confirmation of canine monocytic ehrlichiosis was done by detection of morulae in monocytes in the blood smear which was taken from ear tip and stained by Leishman stain. Dogs having symptoms of CME but negative for blood smear examination; were further confirmed by polymerase chain reaction test. The thirty (30) dogs confirmed for ehrlichiosis were used for further clinical studies. Specific primer pairs were used for *E. canis* gene (16 S rRNA). Details of primers and thermal cycling profile used for nested PCR analysis are given in Table 1. Amplification reactions were carried out in Thermocycler (BioRad, USA). PCR results were analysed using agarose gel electrophoresis under UV light (Fig. 5). All

positive dogs were grouped into two group (G_1 and G_2) with identical number of dogs in each group (n = 15) for the purpose of evaluating different therapeutic protocol.

Table 1

details of primer and thermal cycling profile for nested PCR analysis of *Ehrlichia canis*

Species	***Ehrlichia canis***	
Primer sequence	ECAN5: 5'- CAATTATTTATAGCCTCTGGCTCTGGCTATAGGA-3' HE3: 5'- TATAGGTACCGTCATTATCTTCCCTAT- 3'	
Thermal cycling profile	Initial denaturation- 94°C for 3 min	
	First step	**Second step**
	Denaturation (3 cycles)- 94°C for 1 min Annealing- 55°C for 2 min Extension- 72°C for 1.5 min	Denaturation (37 cycles)- 94°C for 1 min Annealing- 55°C for 2 min Extension- 72°C for 1.5 min Final extension- 72°C for 8 min

2.3. Determination of Canine Cystatin C and canine symmetric dimethylarginine (SDMA)

These parameters were analyzed by using commercial ELISA kit (Canine Cystatin C ELISA kit, catalog no.- E0148Ca, Bioassay Technology Laboratory) as per the manufacturer's protocol and results were summarized in tabular form for easy understanding.

2.4. Statistical analysis

The parameters were expressed as mean ± S.E. and Data were analysed by using one-way (ANOVA) Analysis of Variance followed by the Post-Hoc Tukey HSD test using Statistical Package for the Social Sciences (SPSS 20.0). The level of statistical significance for all comparisons was established at ($P < 0.05$).

3. Results

3.1. Serum Cystatin C alteration

The Mean ± SE values of serum cystatin C of healthy dogs at day 0 and 14 were 0.88 ± 0.41 and 0.87 ± 0.04, respectively. The Mean ± SE values of serum cystatin C at day 0 and day 14 of Group 1 were 2.29 ± 0.57 and 1.95 ± 0.44, while the values of this parameter at day 0 and 14 in dogs of Group 2 were 3.18 ± 0.75 and 3.03 ± 0.71, respectively. Test of significance showed that serum cystatin C level of both the treatment group were significantly higher than that of control group on the day of presentation. However, there was non-significant decrease in serum cystatin C on day 14 post-treatment in both the treatment groups. (Table 2)

Table 2
Serum cystatin-C alterations in various groups of dogs suffering from CME

Group	Serum Cystatin C (mg/l)	
	Day 0	Day 14
Healthy	$0.88^{a} \pm 0.41$	$0.87^{a} \pm 0.04$
G1	2.29ab ± 0.57	1.95ab ± 0.44
G2	3.18b ± 0.75	3.03b ± 0.71

*Mean with different superscript (a, b) in columns are differing significantly in between the groups, otherwise non-significant

3.2. Serum symmetric dimethylarginine (SDMA) alteration

Values of serum SDMA have been summarized in the table 16. The Mean ± SE values of serum SDMA of healthy dogs at day 0 and 14 were 21.69 ± 4.29 and 16.99 ± 3.72 respectively. The Mean ± SE values of serum SDMA at day 0 and day 14 of Group 1 were 16.45 ± 3.17 and 11.59 ± 2.18, while the values of this parameter at day 0 and 14 in dogs of Group 2 were 21.69 ± 4.29 and 16.99 ± 3.72, respectively. Test of significance showed that serum SDMA level of both the treatment group were significantly higher than that of control group on the day of presentation. However, there was non-significant decrease in serum SDMA on day 14 posttreatment in both the treatment groups. (Table 3)

Table 3
Serum SDMA alterations in various groups of dogs suffering from CME

Group	SDMA (µg/dl)	
	Day 0	Day 14
Healthy	$7.82^{a} \pm 0.29$	$7.81^{a} \pm 0.29$
G1	$16.45^{ab} \pm 3.17$	$11.59^{ab} \pm 2.18$
G2	$21.69^{b} \pm 4.29$	$16.99^{b} \pm 3.72$

*Mean with different superscript (a, b) in columns are differing significantly in between the groups, otherwise non-significant.

4. Discussion

In the present investigation serum cystatin C level of both the treatment group were significantly higher than that of control group on the day of presentation (Day 0). However, there was non-significant decrease in serum cystatin C on day 14 post treatment in both the treatment groups. In the current study the serum cystatin C was standardised from the healthy control group by ELISA assay and its normal upper limit was calculated and set as ≤ 1.2 mg/l (Mean ± 2 Standard deviation) which includes 95 % of the cystatin C data from control group and is therefore having 95 % of the confidence level. In this study a total of 30 CME positive dogs were included, among which 17 dogs were having elevated level of serum cystatin C (> 1.2 mg/l). Out of these 17 dogs, 10 dogs which were having elevated cystatin C and elevated serum creatinine. But the remaining 7 dogs were having elevated serum cystatin C with normal creatinine values, indicating the beginning of renal dysfunction. Comparison of serum creatinine, SDMA and serum cystatin C as a biomarker for early renal dysfunction has been summarized in Table 4. The present findings of increase in serum cystatin C in kidney dysfunction are in agreement with the previous findings (Iwasa et al., 2019; Wu et al., 2010; Miyagawa et al., 2009). This increase in serum Cyst C might be due to decrease in GFR in renal dysfunction. A study conducted by Wehner et al., 2008 in 60 dogs with early renal impairment revealed that cystatin C concentrations will probably increase before serum creatinine concentrations and may be regarded as a suitable biochemical marker for diagnosis of renal diseases not only in human sector but also in livestock sector but the validation in respect with analytical, biological and clinical testing is still needed for further clinical application. In present study total 15 dogs were identified having elevated serum level of SDMA and out of these 15 dogs, five dogs have normal creatinine while 10 dogs have elevated creatinine suggesting the early rise of SDMA during renal damage. Significant increase in serum SDMA may attribute to early renal dysfunction which is in agreement with the findings of Dahlem et al., 2017; Hall et al., 2016; Nabity et al., 2015; Hall et al., 2014. Significantly higher SDMA concentrations could be explained by a decreased GFR caused by renal hypoperfusion and/or intrinsic renal damage (Dahlem et al., 2017). Moreover, in kidney diseases, SDMA seems to increase earlier that creatinine (Nabity et al., 2015; Hall et al., 2016) and is not influenced by dog's muscle mass or inflammatory states. Serum cystatin C and serum SDMA and has higher sensitivity than serum creatinine, making it a better screening test for early kidney disease and therefore it could detect renal impairment earlier than serum creatinine and therefore earlier treatment intervention extends the renal survival period even in asymptomatic dogs that have increased SDMA or serum cystatin C.

Table 4
Comparison study of serum creatinine, SDMA and serum cystatin C as a marker for early renal dysfunction

Total No. of CME positive Dogs	Dogs with elevated serum Creatinine (Normal upper limit: ≤ 1.4 mg/dl)	Dogs with elevated SDMA (Normal upper limit: ≤ 10.09 µg/dl)		Dogs with elevated Cystatin C (Normal upper limit: ≤ 1.2 mg/l)	
30	10 (33.33%)	15 (50 %)		17 (56.67%)	
		Elevated SDMA and elevated creatinine	Elevated SDMA with normal creatinine	Elevated Cystatin C and elevated creatinine	Elevated Cystatin C with normal creatinine
		10 (33.33 %)	5 (16.67 %)	10 (33.33 %)	7 (23.33 %)

Declarations

Acknowledgment

The authors are thankful to Dean and Vice Chancellor DUVASU, for providing necessary facilities required for conducting present study.

Author contribution

Mukesh Kumar Srivastava designed the present study; Anubhav Singh performed various experiments; Kapil Kumar gupta and Mukesh Kumar Srivastava analyzed the data and Kapil Kumar Gupta wrote and corrected the manuscript. All authors have carefully read and approved the submitted manuscript.

Funding

This study was funded by department of Veterinary Medicine, DUVASU, Mathura.

Availability of data and material

All data and materials are available

Conflict of interest

The authors declare that they have no conflict of interest.

Code availability

Software application complies with field standard

Ethical approval

Approval was obtained from the Institutional Animal Ethics Committee (IAEC) of U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU) Mathura, India (approval no IAEC/19/12).

Consent to participate

Not applicable

Consent for publication

Authors are responsible for correctness of the statements provided in the manuscript. See also authorship principles. The editor-in-chief reserves the right to reject submissions that do not meet the guidelines described in this section.

References

Codner, E.C., Caceci, T., Saunders, G.K., Smith, C.A., Robertson, J.L., Martin, R.A. and Troy, G.C., 1992. Investigation of glomerular lesions in dogs with acute experimentally induced Ehrlichia canis infection. American Journal of Veterinary Research, 53, 2286- 2291.

Miyagawa, Y., Takemura, N. and Hirose, H., 2009. Evaluation of the measurement of serum cystatin C by an enzyme linked immunosorbent assay for humans as a marker of the glomerular filtration rate in dogs. Journal of Veterinary Medical Science, 71, 1169-1176.

Srivastava, M., Gaikwad, R.J., Samad, A., Sharma, B. and Srivastava, A., 2011. Relationship of Serum Creatinine and Glomerular Filtration Rate by 99mTc-DTPA Scintigraphy in Dogs with Renal Failure. Asian Journal of Animal Sciences, 5, 381-386.

Antognoni, M.T., Siepi, D., Porciello, F. and Fruganti, G., 2005. Use of serum cystatin C determination as a marker of renal function in the dog. Veterinary Research Communications, 29 (2), 265–267.

Villa, P., Jiménez, M., Soriano, M.C., Manzanares, J. and Casasnovas, P., 2005. Serum cystatin C concentration as a marker of acute renal dysfunction in critically ill patients. Critical Care, 9, 139–143.

Kakimoto, Y. and Akazawa, S., 1970. Isolation and identification of N-G, N-G- and N-G, N'-G dimethylarginine, N-epsilon-mono-, di-, and trimethyllysine, and glucosylgalactosyl and galactosyl-delta-hydroxylysine for human urine. Journal of Biological Chemistry, 245, 5751-5758.

Kielstein, J.T., Salpeter, S.R., Bode-Boger, S.M., Cooke, J.P. and Fliser, D., 2006. Symmetric dimethylarginine (SDMA) as endogenous marker of renal function, a metaanalysis. Nephrol Dial Transplant, 21, 2446-2451.

Iwasa, N., Takashima, S., Iwasa, T., Iwasa, K., Suzuki, T., Kumazawa, R. and Nishii, N., 2019. Evaluation of monitoring methods in asymptomatic dogs with high serum cystatin C concentrations. Journal of Veterinary Medical Science, 19, 289.

Wu, C., Lin, J., Caffrey, J.L., Chang, M., Hwang, J. and Lin, Y., 2010. Cystatin C and long-term mortality among subjects with normal creatinine-based estimated glomerular filtration rates. Journal of the American College of Cardiology, 56, 1930–1936.

Wehner, A., Hartmann, K. and Hirschberger, J., 2008. Utility of serum cystatin C as a clinical measure of renal function in dogs. Journal of the American Animal Hospital Association, 44, 131-138.

Dahlem, D.P., Neiger, R., Schweighauser, A., Francey, T., Yerramilli, M., Obare, E. and Steinbach, S.M.L., 2017. Plasma symmetric dimethylarginine concentration in dogs with acute kidney injury and chronic kidney disease. Journal of Veterinary Internal Medicine, 31, 799–804.

Hall, J.A., Yerramilli, M. and Obare, E., 2016. Serum concentrations of symmetric dimethylarginine and creatinine in dogs with naturally occurring chronic kidney disease. Journal of Veterinary Internal Medicine, 30, 794–802.

Nabity, M.B., Lees, G.E., Boggess, M.M., Yerramilli, M., Obare, E., Rakitin, A., Aguiar, J. and Relford, R., 2015. Symmetric dimethylarginine assay validation, stability, and evaluation as a marker for the early detection of chronic kidney disease in dogs. Journal of Veterinary Internal Medicine ,29, 1036– 1044.

Hall, J.A,, Yerramilli, M., Obare, E., Yerramilli, M., Jewell, D.E (2014). Comparison of Serum Concentrations of Symmetric Dimthylarginine and creatinine as kidney function biomarkers in cats with chronic kidney disease. Journal of Veterinary Internal Medicine, 28, 1676-1683.

Graphs

Graphs 1-3 are in the Supplemental Files section.

Figures

Figure 1

Pale mucus membrane

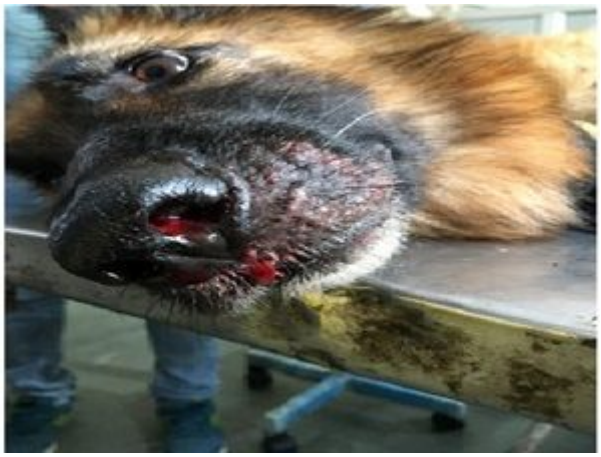

Figure 2

Epistaxis in GSD

Figure 3

hyphema in GSD

Figure 4

Limb edema in Rottweiler

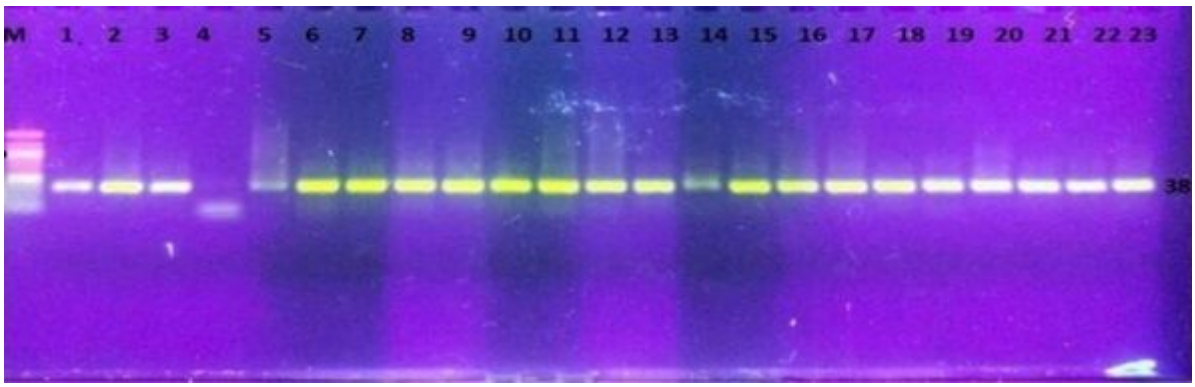

Figure 5

Result of nested PCR (M= Marker of 100 bp, Lane 1-23= Sample, primer: 460-540 bp)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphs.pdf