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Mukesh Kumar Srivastava1
Department of Medicine,
DUVASU, Mathura

Anil Ahuja
Department of Medicine,
RAJUVAS, Bikaner

RD Velhankar
Department of Medicine,
Bombay Veterinary College,
Mumbai

PN Panigrahi1
Department of Medicine,
RAJUVAS, Bikaner

AP Singh
Department of Medicine,
RAJUVAS, Bikaner

Ankita Sharma
Department of Medicine,
RAJUVAS, Bikaner

Subhash Kacchawaha
Department of Medicine,
RAJUVAS, Bikaner

Correspondence
Mukesh Kumar Srivastava1
Department of Medicine,
DUVASU, Mathura

Performance analysis of endothelin-1 for diagnosis of dilated cardiomyopathy in dogs

Mukesh Kumar Srivastava1, Anil Ahuja, RD Velhankar, PN Panigrahi1, AP Singh, Ankita Sharma and Subhash Kacchawaha

Abstract
The present study aimed to evaluate performance analysis of canine specific ET-1 sandwich enzyme-linked immunosorbent assay for the diagnosis and therapeutic evaluation of naturally occurring dilated cardiomyopathy in dogs. The estimation of endothelin-1 was performed by the commercially available canine specific quantitative sandwich ELISA kit. Recorded level of Endothelin 1 (mean ± S.E.) in healthy dog was 133.33 ± 15.26 ng/dl, while in dilated cardiomyopathy affected dogs it was 243.82 ± 18.89 ng/dl. The range in healthy dogs was 48.0 - 534.34 ng/dl with undetectable level in 2.5 % dogs, while in DCM affected dogs it's range was 4.23 - 487.49 ng/dl. Further analysis at three cut off point of i.e. 170, 180 and 190 ng/dl showed 93 %, 86 % and 72 % sensitivity and 45 %, 58 % and 63 % specificity, respectively. Cohen's Kappa value showed fair agreement (0.348, 0.276 and 0.388 at cutoff level of) with gold standard test at these three cut off points, while area under curve analysis showed fair diagnostic potential (0.77 and 0.72) at 170, 180 ng/dl concentration and poor (0.67) at 190 ng/dl concentration of this biomarker. Range of endothelin level seen in both groups showed huge overlapping between groups, which indicated poor discriminatory power of this biomarker for diagnosis of dilated cardiomyopathy. Although overall poor, but among three cut off levels analyzed for test performance, cut off level of 180 ng/dl has shown moderate sensitivity, specificity, false negative rate, fall-out rate and fair agreement.

Keywords: endothelin, dilated cardiomyopathy, test performance, therapeutic evaluation, enzyme-linked immunosorbent assay

1. Introduction
Heart failure is a clinical syndrome distinguished by signs and symptoms of interstitial and intravascular volume over-load and/ or manifestations of deficient tissue perfusion [1] secondary to inability of the heart to cover systemic metabolic needs. Hemodynamic state of dog with heart failure involves several neurohormonal systems, such as the adrenergic system, the renin-angiotensin-aldosterone system (RAAS), and endothelin [2], most of them are vasoconstrictor with positive chronotropic and inotropic stimuli and aimed for maintaining adequate cardiac output and organ perfusion [3]. These system have adverse effects on the cardiovascular system and contribute significantly to the process of cardiovascular remodeling and, thereby, morbidity and mortality.

Currently diagnosis of overt DCM involves either detection of ventricular arrhythmias on ECG or 24-hour ambulatory ECG recording, detection of left ventricular systolic dysfunction on echocardiographic examination, or both [4]. Most of these diagnostic tests are relatively expensive and typically require referral to a specialty practice with equipment and trained personnel capable of accurate assessment. Because of the high incidence of overt DCM with increasing age, yearly screening is recommended and incurs cumulative financial cost and owner commitment over the course of the dog's lifespan. Blood-based testing represents a relatively inexpensive, easily performed, and widely available screening method that could aid in counseling owners regarding the likely value of further examination, such as echocardiography [5]. In the available literature, B-type natriuretic peptide (BNP), pro-atrial natriuretic peptide (pro-ANP), and endothelin-1 (ET-1) have gained much interest and have been shown to be significantly increased in CHF due to DCM [6]. One of the several factors involved in hemodynamic state of dog is endothelin (ET), which is an autocrine and paracrine factor with vasoconstrictive, mitogenic, and inotropic activities in vascular and cardiac muscles. Its elevation has been reported in chronic heart failure and its production may be

conditioned by activation of other neurohumoral factors that are stimulated by the disease [7].

Endothelin-1 is one of the most potent vasoconstrictors, was first discovered in 1988 from supernatant of cultured porcine aortic endothelial cells [8]. The formation of ET-1 is dependent on the cleavage of a circulating 39 amino acid propeptide ''big ET-1''by membrane-bound glycoprotein metallopeptidases referred to as endothelin-converting enzymes. Out of three isoforms, ET-1, is a 21-amino acid peptide, having molecular weight of 2,492, free carboxyl and amino termini and has two intra-molecular disulfide bonds. ET-1 is the predominant isoform expressed in vascular endothelium and acts as a locally active paracrine factor and probably also as a circulating hormone in the regulation of arterial and venous tone [9]. Plasma concentrations of endothelin-1 are elevated in patients with moderate to severe chronic heart failure (CHF) and correlate with the symptomatic and haemodynamic severity of CHF [10]. ET-1 also is a potent growth factor for several other cell types, including cardiomyocytes and endothelial cells. Moreover, ET-1 has direct effects on contractile function, protein synthesis, and electrophysiological events in cardiac myocytes. The usefulness of ET and BNP as bad prognosis indicators in dogs with DCM has been confirmed by Oyama *et al.* [11] and O'Sullivan *et al.* [12]. The purpose of this study was to evaluate canine specific ET-1 sandwich enzyme-linked immunosorbent assay (ELISA) for performance analysis of serum ET-1 for the diagnosis of naturally occurring DCM in dogs.

2. Materials and Methods

Screening of dogs in DCM group was based on the history of classical sign of DCM i.e. murmur, exercises intolerance, dyspnea, coughing, breathlessness, ascites and or cyanosis or hypo perfusion at rest, moribund, depression, prostration, however to be included, dogs must furthermore have presented with at least 3 of the following clinical signs, including at least 1 of cough, dyspnea, syncope, and at least 1 of decreased activity, decreased mobility, altered demeanor [2].

2.1 Screening criteria for dogs for "dilated cardiomyopathy (DCM) group"

1. No age, sex or breed prejudice.
2. History of classical sign of DCM i.e. murmur, exercises intolerance, dyspnea, coughing, breathlessness, ascites and or cyanosis or hypoperfusion at rest, moribund, depression, prostration [13]. Animal having any one of the above mentioned signs underwent electrocardiography and thoracic radiography for further evaluation.
3. Electrocardiographically cardiomegaly was considered as per suggestion of Tilley [14] and Martin [15].
4. Radiographic evidence of cardiomegaly, which included vertebral heart score more than 10.7 [16].
5. All the screened dogs underwent electrocardiography [15], thoracic radiography [16] and finally through echocardiography [17], 29 dogs were confirmed for dilated cardiomyopathy, which constituted DCM group.

2.2 Echocardiographic confirmation criteria of DCM in dogs

A major criterion for DCM positive dogs was presence of three or more than three findings mentioned below, but criteria no. 1 was mandatory [17].

1. Left ventricular dilation (especially in systole but also in diastole) either M mode right ventricular dimension in diastole (RVDd) (mm) more than 11.0 mm and left ventricular dimension in diastole(LVDd) of 36.0 mm and left ventricular dimension in systole (LVDs (mm) more than 25 mm.
2. Either M mode fractional shortening (25 %) (<20%) or left ventricular ejection fraction less than 40%.
3. M mode left atrium and aorta (LA: AO) ratio (more than 1.3).
4. Two dimensional mode sphericity index (>1.65).
5. E point septal separation (EPSS) (> 4 mm).

DCM group had 10 breeds representation (Labrador (11), German Shepherd (5), Non descript (4), Pomeranian (2), Golden Retriever (1), Doberman (2), Boxer (1), Great Dane (1), St. Bernard (1) and Neapolitan Mastiff (1)), with a male female sex ratio of 3.14 (male-22 and female- 7) , 28.68 ± 1.28 (Mean ± SE) kg body weight (range= 15-41 kg) and 6.72 ± 0.50 years age (range- 0.5 to 11.5). Healthy group consist of apparently healthy 40 dogs with 12 breeds representation (Labrador (10), German Shepherd (7), Non descript (6) Pomeranian (3) Golden Retriever (3), Doberman (2), Boxer (2), Great Dane (2), St. Bernard (2), Neapolitan Mastiff (1), Bull Mastiff (1) and Cocker Spaniel (1)), sex ratio of 2.33 (male- 28 and female- 12), body weight (Kg) (Mean ± SE) of 29.62 ± 1.48 (range=13-48) and average age (years) of 4.13 ± 0.14 (range = 2.5- 5.5). All the dogs of healthy of which underwent similar diagnostic test to confirm their healthy status.

2.3 Inclusion criteria for animals to be included in "Healthy control group"

3 Apparently healthy owned dogs of any age, breed or sex with regular deworming and vaccination history followed by through clinical examination.

4 No any arrhythmia or cardiac enlargement on electrocardiography.

5 No any evidence of cardiomegaly or any other disease radiographically.

6 Healthy range of complete blood count, liver function tests, kidney function tests and serum electrolytes (Na^+, K^+ and Cl^-) values for dogs.

7 No any evidence of cardiovascular abnormality on echocardiography.

Blood samples from the dogs of both group were allowed to clot for two hours at room temperature then centrifuged for 20 minutes at 3000 rpm. Supernatant was collected carefully and stored in three aliquot at -70ºC to avoid repeated freeze/thaw cycles and perform batch analysis. The estimation of endothelin-1 was performed by the commercially available canine specific quantitative sandwich ELISA kit (My Bio Source, Cat.No: MBS028059), which is intended to analyze endothelin concentrations in canine serum, plasma and other body fluids. Whole procedure was followed as per manufacturer recommendation.

2.4 Assay procedure

All reagents and samples were prepared and brought to room temperature (18°C- 25°C) naturally for 30min before starting assay procedure. All standards and samples were added in duplicate to the plate. Standard wells, sample wells and blank (control) wells were set and 50 µl standard added to each standard well, 50 µl sample was added to each sample well and 50 µl sample diluent was added to each blank/control well. Horse radish peroxidise (HRP) conjugate reagent (100 µl) was added to each well, covered with an adhesive strip

and incubated for 60 minutes at 37°C. Automated washing was done four times using wash buffer (1×). For this purpose washer was adjusted to aspirate as much liquid as possible and set to fill volume at 350 µl/well/wash. After final wash, plates were inverted and blot dried by hitting plate onto absorbent paper or paper towels until no moisture appears. Chromogen solution A (50 µl) and chromogen solution B (50µl) was added to each well successively. Gently mixed and then protected from light and incubated for 15 minutes at 37°C. Stop solution (50 µl) was added to each well. If the colour in the wells is green or the colour change does not appear uniform, gently tapping of the plate was done to ensure thorough mixing. Optical Density (OD) was read at 450 nm using a Microelisa strip plate reader within 15 minutes.

2.5 Statistical analysis

Professional curve fitting software was used to make a standard curve and concentrations of the samples were calculated. Echocardiography as gold standard techniques for confirmatory diagnosis [17, 18] was used for evaluating the test performance of the endothelin. Receiver operating characteristics (ROC) analysis was used for above purpose [19]. The validity of dichotomous of diagnostic tests in the form of two outcome categories i.e. positive test (+) and negative test (–) was compared with the gold standard test to determine by sensitivity and specificity. Area under the ROC curve was considered as an effective measure of inherent validity of a diagnostic test. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. An area of 1 represents a perfect test; an area of 0.5 represents a worthless test. A rough guide for classifying the accuracy of a diagnostic test is the traditional academic point system: 0.90-1 = excellent, 0.80-0.90 = good, 0.70-0.80 = fair, 0 .60-0.70 = poor and 0.50-0.60 = fail.

For analysis of agreement between gold standard and endothelin Kappa Statistic was calculated, which is is based on the difference between how much agreement is actually present ("observed" agreement) compared to how much agreement would be expected to be present by chance alone ("expected" agreement). Interpretation of Kappa was done as describe below [20].

Poor	Slight	Fair	Moderate	Substantial	Almost perfect
0.0	0.01–0.20	0.21– 0.40	0.41–0.60	0.61–0.80	0.81–0.99

3. Results

The present study demonstrated a significant increase of plasma ET-1 immunoreactivity in dogs DCM with or without CHF compared with normal dogs. Recorded level of Endothelin 1 (mean± S.E.) in healthy dog was 133.33 ± 15.26 ng/dl, while in DCM affected dogs it was 243.82 ± 18.89 ng/dl (tab-1). The range in healthy dogs was 48.0 - 534.34 ng/dl with undetectable in 2.5 % (1 out of 40) dogs, while in DCM affected dogs it's range was 4.23 - 487.49 ng/dl. Results of significance tests showed significant difference between healthy and DCM affected dogs. Further analysis was performed at three cut off point of i.e. 170, 180 and 190 ng/dl, which showed 93 %, 86 % and 72 % sensitivity and 45 %, 58 % and 63 % specificity at these levels, respectively. False negative rate at these cutoff levels were 7, 14 and 28, while false positive rate were 55, 43 and 38. Cohen's Kappa value showed fair agreement (0.348, 0.276 and 0.388) at these three cut off points, while area under curve analysis showed fair diagnostic value (0.77 and 0.72) at 170, 180 ng/dl concentration and poor (0.67) at 190 ng/dl concentration of this biomarker. False negative rate were 7, 14 and 28%, while fall-out rates were 55, 43 and 38 % at these concentrations of endothelin. The result of conventional treatment in DCM affected dogs showed non-significant but reduced level in DCM affected pre treatment dogs (223.27 ± 12.17 ng/dl) in comparison to post treatment (198.53 ± 18.89 ng/dl)

4. Discussion

Dilated cardiomyopathy associated circulatory dysfunction causes activation of a number of neurohormonal systems, including the endothelin (ET) system, which is only beginning to be utilized in veterinary clinical medicine, but measurement of these circulating neurohormones provides potential utility in the diagnosis, staging and assessment of prognosis in cardiac disease. In addition to its potent vasocontractile (both venous and arterial) and vascular remodeling effects, ET-1 has potent positive inotropic and chronotropic effects [21] for raising blood pressure [22]. Up-regulation of endothelin pathways may be beneficial in providing short-term inotropic support for the failing myocardium in which beta-adrenergic responsiveness frequently is attenuated [23].

The present study demonstrated a significant increase of plasma ET-1 immunoreactivity in dogs DCM with or without CHF compared with normal dogs. Similar results were previously recorded in experimentally induced CHF [24] and clinical studies [25] with human commercial ELISA for measuring ET-1 immunoreactivity in dogs. Recorded level of Endothelin 1 (mean± S.E.) in healthy dog was 133.33 ± 15.26 ng/dl, while in DCM affected dogs it was 243.82 ± 18.89 ng/dl. The range in healthy dogs was 48.0 - 534.34 ng/dl with undetectable in 2.5 % (1 out of 40) dogs, while in DCM affected dogs it's range was 4.23 - 487.49 ng/dl. Results of significance tests showed significant difference between healthy and DCM affected dogs, which was in agreement with the previous study of Prosek *et al.* [25], who reported significant difference between healthy and dogs with DCM with CHF, however they did not reported significant difference between healthy dogs and dogs with heart disease but without CHF. Vollmar *et al.* [26] found no significant difference among ET-1 concentrations when comparing normal dogs with dogs with naturally occurring CHF, which may be due to the use of different assays and sample preservation techniques and effect of therapy used in their study. In our study, we included dogs with DCM with or without CHF, which might be a factor for different results from previous studies. Fukumoto *et al.* [27] reported that dogs with cardiopulmonary disease have significantly higher level (4.6 ± 4.6 pmol/l) of ET-1 than healthy control dogs. Previously Tessier-Vetzel *et al.* [28] reported significantly higher plasma concentrations of ET-1 in dogs with spontaneous cardiac and respiratory diseases (5·3 ± 0·3 and 5·3 ± 0·6 pg/ml, respectively) than in healthy dogs, but the mean level of ET-1 of our study in both the groups was much higher than study of Tessier-Vetzel *et al.* [28]. Suggested reason for this variation is use of different machines and use of canine specific ELISA kits in our study. There are several pieces of evidence suggesting that the endothelins are increased during course of congestive heart failure in dogs [12, 29]. Increment may range from two to ten times in CHF compared to healthy animals, regardless of the underlying etiology, which reported to correlate well with compromise hemodynamic and functional class of heart failure [30]. Range

of ET level seen in both groups of present study clearly showed huge overlapping between groups, which indicated poor discriminatory power of this biomarker for diagnosis of DCM, which was previously pointed out by Schober [30], but O'Sullivan *et al.* [12] suggested to add this biomarker in the list of neurohormonal biomarkers in dogs with DCM and CHF as increasing big ET-1 concentrations over time can be useful predictors of poor prognosis. Certain factors present in or associated with heart failure could induce endothelin overproduction. Prosek *et al.* [25] reported 62.5 % dogs with DCM without CHF have geometric mean within the range of geometric mean of healthy dogs, while very few DCM with CHF dogs have concentration borderline to the healthy dogs.

Further analysis was performed at three cut off point of i.e. 170, 180 and 190 ng/dl, which showed 93 %, 86 % and 72 % sensitivity and 45 %, 58 % and 63 % specificity at these levels, respectively. Cohen's Kappa value showed fair agreement (0.348, 0.276 and 0.388) at these three cut off points, while area under curve analysis showed fair diagnostic value (0.77 and 0.72) at 170, 180 ng/dl concentration and poor (0.67) at 190 ng/dl concentration of this biomarker. False negative rate were 7, 14 and 28%, while fall-out rates were 55, 43 and 38 % at these concentrations of endothelin. This peptide have a paracrine activity in the early stages of the disease, thus little quantity of is released during disease progression. Failure to identify increased plasma ET-1 concentrations in dogs with heart disease but no evidence of CHF should not be overemphasized because activation of local tissue systems is possible. A reported study of tachycardia-induced heart failure in dogs was characterized by an early activation of the cardiac and renal tissue endothelin systems, and this activation occurred before any changes in plasma and urinary ET-1 and could help explain the findings of our study. Although vascular endothelial cells are the major source of endothelin, it is also produced by a wide variety of cell types including renal tubular endothelium, glomerular mesangium, cardiac myocytes [30] glia, the pituitary, macrophages, mast cells, etc. [31] and leukocytes [32]. Several vasoactive substances stimulate production of ET-1, including angiotensin II, vasopressin, bradykinin, thrombin, and norepinephrine [33]. Cardiotrophin-1, tumor necrosis factor-alpha, interleukin-1, transforming growth factor-beta, lipoproteins, insulin, endotoxin, hypoxia, and low (5 dynes/cm2) shear stress, exercise, high levels of LDL cholesterol, diabetes mellitus, obesity, age also have been reported to involve in production of ET-1 [34, 35]. It has also been reported to be increased in acute renal failure [36], hypertension [37]. These different sources and releasing factors might be responsible for false positive results of our study.

Because endothelin is a potent stimulus for atrial natriuretic peptide secretion, it has been proposed that atrial natriuretic peptide opposes the actions of endothelin [38]. In the trial studies, it has been shown that short term oral endothelin receptor blockade with bosentan (a mixed endothelin receptor antagonist) resulted in improved hemodynamics without neurohormonal stimulation in patients who were symptomatic despite triple drug chronic heart failure therapy including an ACE inhibitor, diuretic, and digoxin [39]. Plasma ET-1 concentrations are high dogs with experimentally induced CHF [40] and treatment with ET-receptor antagonists has been shown to improve hemodynamic performance in such dogs [41]. Moreover, a normal endothelin concentration at a single time point does not exclude the possibility of future development of DCM, therefore, rescreening over the course of the dog's life is recommended. Conventional treatment through enalapril maleate @ 0.5 mg/kg PO q12h. furosemide @ 1–2 mg/kg b.wt PO/IM q12h initial dose, increased in nonresponsive cases up to 6-8 mg/kg b.wt depending on severity of pulmonary congestion/ pulmonary edema, L-carnitine @ 1 g PO q8h for less than 25 kg b.wt and 2 g PO q8h for 25–40 kg b.wt, spironolactone @ 2 mg/kg b.wt orally b.i.d. and digoxin @ 0.01 mg/kg to 0.02 mg/kg b.wt b.i.d. for 1 day, then 0.005 to 0.01 mg/kg b.wt for maintenance showed non-significant reduction in level of endothelin. Although non-significant but reduced trend of this biomarkers in post treatment group of dogs was indicative for halt of further damage and reduction of volume overload in DCM dogs after conventional treatment. Relevant data for comparison of therapeutic efficacy of this regimen was not available in referred literature.

Based on the result of present study, use of this biomarker for DCM diagnosis in dogs is not recommended due to its poor discriminatory power; however it may be use for cardiotoxicity studies and evaluation of efficacy of different drugs for improving volume overload secondary to DCM. Although many previous studies shown good correlation of this biomarker with the symptomatic and haemodynamic severity of CHF. Different sources in the body and releasing factors for this biomarker might be responsible for limitation of use in DCM affected dogs. Also there is need of establishment of own range of biomarkers as each assay have its own range. Comparisons of biomarker concentration though different machine and kits is not possible and limit the use of data of any study.

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Tab 1: Test performance of Endothelin 1 in healthy and DCM affected dogs

S. No.	Parameters	Cut off point of 170 ng/dl	Cut off point of 180 ng/dl	Cut off point of 190 ng/dl)
1.	Sensitivity (true positive rate)	93	86	72
2.	Specificity (true negative rate)	45	58	63
3.	False negative rate	7	14	28
4.	False positive rate (Fall-out)	55	43	38
5.	AUC	0.77 (fair)	0.72 (fair)	0.67 (poor)
6.	Cohen's Kappa value	0.348 (fair)	0.276 (fair)	0.388 (fair)
Effect of therapy on Endothelin 1 level after 45 days of therapy				
S. No.	**Biomarker**	**Pre-treatment**	**Post-treatment**	
1.	Endothelin 1	223.27 ± 12.17	198.53 ± 18.89 ^{NS}	

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