Original Research

Evaluation of NT-proBNP a Cardiac Biomarker for the Early Diagnosis of Cardiac Disease in Dogs

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Abstract

The present study investigates the diagnostic efficacy of NT-pro BNP (N Terminal pro B–type natriuretic peptide) a cardiac marker in evaluation of cardiac diseases in dogs. 356 dogs suspected of having cardiac diseases based on clinical signs were subjected to detailed physical examination, electrocardiography and radiography. Serum samples from dogs positive for cardiac diseases were evaluated for NT-pro BNP and triglycerides, cholesterol, CK and LDH were also estimated for comparative study. 36 dogs were found positive with a prevalence of 10.11%. The diseases include Dilated Cardiomyopathy, Myocardial Ischemia and Infarction, Hypertrophic Cardiomyopathy and Conduction Defects. Radiography revealed a Vertebral Heart Score of 9.44±0.16 in dogs with cardiac diseases. A highly significant increase in NT-pro BNP was noted in dogs with cardiac diseases as compared to normal healthy dogs. Values of triglycerides, cholesterol, creatine kinase (CK) and lactate dehydrogenase (LDH), though elevated in affected dogs, were within the normal range of the given species. In conclusion, NT-pro BNP can be used as a reliable diagnostic cardiac marker in the absence of sophisticated imaging techniques in veterinary practice.

Key words: Cardiac Disease, Cardiomyopathy, Dogs, NT-proBNP

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Introduction

Cardiac diseases are a potential cause of death in canines besides prolonged suffering and physiological disability (Egenvall *et al.*, 2006). According to the American Veterinary Medical Association, the estimated morbidity related to canine cardiac diseases has been found to be 1 in every 10 dogs (Dove, 2001). The

evaluation of cardiac disease in dogs can often be challenging as the patient history is often nonspecific. The presence or intensity of a heart murmur on physical examination is not always a reliable measure of disease severity and concurrent pulmonary disease can obscure the interpretation of thoracic radiographs. More recent advances in veterinary diagnostic imaging technology have enabled us to detect cardiac diseases much earlier and more accurately. However, the accuracy and reliability of this technique is vastly dependent on a specialist or highly trained cardiologist, to interpret the results and depict the state of a disease or give prognosis. They are relatively expensive and might not be readily available.

Owing to these, a considerable amount of research has been dedicated to the development of a more robust and easy method than diagnostic imaging technology. These researches have highlighted the importance of using cardiac biomarkers, as a reliable method for the early detection and evaluation of cardiac diseases. Several potential cardiac biomarkers have been identified and tested for their suitability in the diagnosis of heart failure in dogs. The natriuretic peptides (NP) have emerged as important marker in the early diagnosis and therapeutic monitoring of heart diseases. The principal NPs today are the atrial NP (ANP), the brain NP (BNP) and the c-type NP, which take part in cardiovascular and cardiorenal homeostasis. NT-proBNP has been proven to be a useful marker for diagnosis of heart disease in human medicine for more than a decade (Morita *et al*., 1993). Recent studies on canine NT-proBNP assay have been found useful in assessment of heart disease in dogs (De Francesco *et al*., 2007; Boswood *et al*., 2008). Research has shown that serum and plasma levels of NT-proBNP in dogs and cats are the only biomarkers that can diagnose and monitor congestive processes and indirectly, assess the myocardial function of small animals (Boswood, 2009). Although, the interest in NT-proBNP in veterinary medicine has grown in the past 10 years, its usage remains in some research contexts because proper methodologies have yet not been validated and standardized for clinical practice (Oyama, 2010; Freitas *et al*., 2013; Alves *et al*., 2015 and Ferreira *et al*., 2016). Reports of its use in clinical practice are sporadic (Wess *et al.*, 2011; Kumar *et al*., 2012; Sjostrand *et al*., 2014; Thirunavukkarasu, 2014; Fox *et al*., 2015; De Lima and Fereira, 2017; Svete *et al*., 2017). Therefore, the present study was conducted to evaluate the diagnostic efficiency of NT-proBNP as a cardiac biomarker in dogs with dilated cardiomyopathy and other cardiac disease in a clinical setup.

Materials and Methods

The present study was conducted in Teaching Veterinary Clinical Complex (TVCC), Department of Veterinary Medicine and Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022, Assam, India. The period of study was from August, 2017 to May, 2018. Dogs registered at Medicine Unit, TVCC were considered for the present study. Six apparently healthy adult dogs registered for routine annual health checkup were selected as control group for obtaining normal data for comparison of parameters under study. A total of 356 dogs suspected to be affected with cardiac disease and showing signs of lethargy, weakness, anorexia, dyspnea, exercise intolerance, chronic

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coughing, ascites, pedal edema, cyanosis of tongue or mucous membrane and syncope, or their combination were considered for further screening. These dogs were subjected to thoracic radiography and electrocardiograms for detection of cardiomyopathy and cardiac diseases other than cardiomyopathy. Those dogs suffering from gastro-intestinal disorders, excretory system disorders, nervine disorders and muscular injury, endocrine disorders, skin and reproductive system disorders and acute infections of bacterial, viral, rickettsial and protozoal origin were excluded from the study.

For electrocardiography a standard ECG recorder (Cardiart 6208-BPL machine) with a built-in thermal printer was used to record the electrocardiogram of the dogs. Briefly, the dogs were laid on a rubber matted table on its right lateral recumbency and allowed to relax. The electrodes with alligator tooth clips were attached to the skin of the dog after generous application of ECG gel for proper conduction as per standard procedure described by Tilley, 1992. No pharmacological preparation was used prior to recording ECG. Paper size used was 80mm x 20m and paper speed was kept at 25mm/sec. However, for dogs with faster heart rate, paper speed of 50 mm/sec was used. Sensitivity was kept at 1cm=1mV.

For radiography, Type-ME 0610M (Tech-60, KV-100) X-ray machine (Siemen Healthcare Pvt. Ltd., Mumbai 400079) was used. Measurement of the radiographic image of the dilated heart was taken with a built-in electronic caliper and expressed in millimeters (mm). Both lateral (right lateral recumbency) and dorso-ventral views of thoracic cavity were taken. Vertebral heart score/scale was calculated as per Buchanan and Buechler (1995).

For estimation of NT proBNP assay a commercially available kit (Canine N-Terminal pro-brain natriuretic peptide (NT-proBNP) ELISA Kit from Arsh Biotech) was employed. The assay was performed by strictly following the manufacturers' protocol. Briefly, the given standard solution was serially dilute using standard diluents to acquire a concentration of 60 pg/ml, 30 pg/ml, 15 pg/ml, 7.5 pg/ml and 3.75 pg/ml. 50µl of each diluted standard solution were added to the 96 well microplate in duplicate. 10 µl of serum sample and 40 µl of standard diluent were added to the 96 well microplate in duplicate. Two wells were left as blank. The plate was covered with a plate cover and incubated for 45 minutes at 37˚C. Each well was aspirated and washed using wash buffer, repeating the process four times for a total of five washes. The washing buffer is aspirated or removed by decantation after the final wash. The plate is inverted and blotted against clean paper towels. Except the blank well, to each well was added 50µl HRP-Conjugate detection antibody. The plate was covered with a new adhesive strip and incubated for 30 minutes at 37˚C. All the wells were subjected to washing/aspiration for 5 times again. 50µ chromogen solution A and 50µl chromogen solution B was added to each well. The solutions were gently mixed and incubated for 15 minutes at 37˚C. This step required the plate to be protected from light.50µl of stop solution was added to each well. The color in the wells changed from blue to yellow. The Optical Density (O.D.) was read at 450nm using an ELISA plate reader within 15 minutes of colour development. The duplicate readings for

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each standard, control and sample were added and average was calculated. The average zero standard optical density (O.D.) was subtracted from it. A standard curve was created by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve fit (cvxpt32).

Traditional markers of cardiac disease including triglycerides, cholesterol, CK and LDH were also estimated for comparative study. Standard procedures were followed for estimation. Triglycerides (mg/dL) was estimated by GPO-POD (Glycerol phosphate dehydrogenase – peroxidase) method and cholesterol was estimated by Trinder CHOD-POD End Point method using standard kits following manufacturers protocol. The AST, triglyceride, cholesterol, CK and LDH diagnostic kits were purchased from DIATEK bearing catalogue numbers OT02, TG02, CH02, CK01 and LDH01.

Statistics

The data generated was analyzed statistically following standard statistical methods (Snedecor and Cochran, 1994). Data are shown as Mean±Standard error. Statistical significance was defined at $P<0.05$. One-way ANOVA with F test was done and in case of significant F, pair wise comparison was done by Tukey Honest Significant Difference test. The software's used were IBM SPSS Statistics Version20 and JMP 10 of fast SAS 9.3.

Results and Discussion

In the present study, out of 356 dogs screened for cardiac diseases based on history and clinical findings, 36 dogs were found to be suffering from various forms of cardiac diseases with a prevalence of 10.11 per cent. Out of these 36, 10 dogs (27.78%) had dilated cardiomyopathy, Myocardial ischemia and infarction (02, 5.67%), Hypertrophic cardiomyopathy (01, 2.78%) and Conduction defects (23, 63.89%). Several variations ranging from 9.8 per cent to 58.2 per cent were reported in the prevalence of cardiac diseases (24.2% by Vollmar, 2000; 9.8% by Castro *et al.*, 2009; 58.2% by Wess *et al.*, 2010; Jeyaraja *et al*., 2015). This variation in the prevalence of cardiac diseases may be attributed to the differences in the criteria adopted during screening and diagnosis of the cases for the study.

Electrocardiographic changes observed in dogs with cardiac disease were mild increase in the heart rate along with cardiac arrhythmias. The most common among them were Sinus tachycardia, Atrial fibrillation and Flutter followed by Sinus arrhythmia with Wandering Pacemaker (WPM), Sinus arrhythmia without WPM, Low voltage complex, Sick sinus syndrome, Atrioventricular junctional premature complex and Third-degree heart block. Similar observations were also noted by Satish *et al*. (2011), Noszczyk *et al.* (2010), Martin *et al*. (2010), Singh *et al*. (2004) and Pereira *et al.* (2004) in various cardiac conditions. The arrhythmias observed could be due to heredity or acquired damage to the conduction system, atrial and ventricular affections, extra cardiac causes like previous therapy with digoxin or its toxicity and alteration

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in concentrations of minerals like Calcium and Potassium (Tilley, 1992). Anaemia could also result in tachyarrhythmias (Bolton, 1975) and atrial fibrillations with atrial enlargement are often associated with chronic valvular insufficiency and cardiomyopathy (Edward and Tilley, 1985).

In the present study, radiography revealed the Vertebral Heart Scores (VHS). Mean value of VHS in dogs with cardiac diseases was found to be 9.44±0.16, which was comparatively higher than control group with a mean VHS score of 8.15±0.08. Dogs with cardiac diseases often have a higher VHS value. Statistically, the score did not reveal any significant alteration, however the study also documented other changes with respect to the positioning, shape and size of the heart, typical of cardiac disease. Similar findings were reported by other workers on the subject, like Kibar and Alkan (2005), Marin *et al.* (2007), Gugjoo *et al.* (2012), Maria (2012) and Jan *et al.* (2018). The alterations in VHS and other criteria however should be studied only with breed considerations as the anatomy varies with breeds (Sleeper and Buchanan, 1999). Studies explain the cardiac chamber dilatation as an outcome of myocarditis, toxicities and genetic influences. The cardiomegaly may also be assumed to be the resultant of hypertrophy of cardiac musculature which may be symmetrical or apical in nature (Jubb *et al.*, 2007).

Data presented in Table 1 shows the Mean±SE values of cardiac biomarkers evaluated in the present study. NT-proBNP was highly elevated (P<0.01) in both the groups with cardiac diseases as compared to the normal dogs under study. The traditional markers of cardiac diseases like triglycerides, cholesterol, CK, LDH and AST showed a statistically significant increase in comparison to the values of normal dogs however the increase was not as remarkable as those of NT-proBNP.

Table 1: Mean±SE values of Cardiac biomarkers in dogs with Dilated cardiomyopathy and cardiac diseases other than DCM

Parameter	Healthy Control (n=6)	Dogs with DCM (n=10)	Dogs with cardiac diseases other than DCM(n=26)
NT-proBNP (pg/mL)	10.37^{a}±1.35	418.53**^{c}±16.13	275.70**^{b}±11.90
	(8.24-12.50)	(379.21-457.85)	(249.00-302.40)
Triglyceride (mg/dL)	40.33^{a}±2.46	66.9*^{c}±1.29	56.46*^{b}±1.77
	(32.00-48.00)	(47.00-59.00)	(47.00-86.00)
Cholesterol (mg/dL)	116.00^{a}±2.37	174.1*^{b}±1.98	168.35*^{b}±4.93
	(110.00-125.00)	(159.00-181.00)	(130.00-203.00)
CK (U/L)	29.00^{a}±2.07	107.3*^{c}±9.54	73.5*^{b}±0.97
	(23.00-36.00)	(82.00-93.00)	(57.00-85.00)
LDH (U/L)	52.83^{a}±5.76	151.6*^{b}±21.39	172.27*^{b}±3.47
	(132.00-145.00)	(181.00-201.00)	(132.00-286.00)
AST (U/L)	30.83^{a}±2.44	68.69*^{b}±5.21	41.80^{a}±1.52
	(24.00-37.00)	(49.10-63.00)	(24.10-56.00)

*Superscripts to be compared row-wise, significant at *P<0.05; **P<0.01; Values in bracket indicates range*

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N Terminal proBNP fragments (NT-pro BNP) and other natriuretic peptides are considered the most important of the various cardiac biomarkers till date. Their full-fledged clinical use is still under consideration in veterinary practice. Circulating B-type natriuretic peptide (BNP) levels are increased, primarily in response to increased myocardial wall stress (Boswood *et al*., 2008). The increased concentrations of BNP (Brain natriuretic peptide) are also linked to various causes including myocardial infarction, cardiac hypertrophy and heart failure (Morita *et al*., 1993).

BNP is one of the cardiac markers of heart failure. It correlates with symptoms of heart failure and may indicate left ventricular (LV) volume and pressure overload in the presence of shunt (Nir and Naseer, 2005). In the present study the mean±SE values of NT-proBNP showed significant rise ($P<0.01$) in dogs with DCM (418.53±16.13 pg/mL) in comparison to healthy control (10.37±1.35 pg/mL). The increase seen was comparatively less in dogs with cardiac diseases other than DCM (275.70±11.90 pg/mL), however it was significant compared to the healthy control. This indicates the utility of NT-proBNP as an indicator of cardiac diseases including DCM. Similar findings were reported by Zoair *et al.* (2014), DeLima and Ferreira (2017), Oyama (2010), Petric and Tomsic (2008), Boswood *et al.* (2008), Noszczyk-Nowak (2011) in various cardiac conditions. Goetze *et al.* (2004) and Pruszcyk (2005) explain the increase of NT-proBNP production in response to myocyte stress. However, the cut off values of NT-proBNP vary from study to study. Freitas *et al.* (2013), Alves *et al.* (2015) and Ferreira *et al.* (2016) have stated that NT-proBNP is until now, used only in some research contexts and no proper methodologies have been validated and standardized. Similarly, trigyceride also followed the same pattern like NT-proBNP and was found to be 40.33±2.46 mg/dL in healthy control and showed rise in dogs with DCM (66.9±1.29 mg/dL) and dogs with cardiac diseases other than DCM (56.46±1.77mg/dL) at $P<0.05$. Similar findings were reported by Freeman *et al.* (1996) and Petra *et al.* (2017). The mean values though showed increase from that of healthy control, however remained within the standard normal range for the given species corroborating to the findings of Sesh *et al.* (2013). This rise in the mean values of triglyceride in cardiac disease affected dogs including DCM may be considered because of the 13.88% cases reporting tachyarrhythmia as seen under the light of the studies conducted by Gupta *et al.* (2007) wherein some clinico-pathological changes in cardiac arrhythmia in dogs was observed.

Cholesterol increased from 116±2.37mg/dL in control group to 168.35±4.93 mg/dL in dogs with other cardiac diseases other than DCM and 174.1±1.98 mg/dL in dogs with DCM. Although the increase was significant ($P<0.05$) compared to the control group, it was not so among the two diseased groups. Similar observations were also noted by other workers (Freeman *et al.* (1996), Devi (2008). This contradicts the observation of Sesh *et al.* (2013) who reported a decrease in total cholesterol (simultaneously with an increase in low density cholesterol). However, in both the studies, the values remain within the standard normal level of the parameter indicating the cases of DCM to be of per acute nature. The increase may be

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associated with liver dysfunction and congestive heart failure is considered as one of the causes of acute liver failure as low cardiac output results in reduced hepatic blood flow (Saner *et al.*, 2009). Liver dysfunction causes a decreased esterification of serum cholesterol (Jagannath and Ananda, 2001). Gupta *et al.* (2007) also reported lipemia in cases of tachyarrhythmia.

CK (CK) showed significant rise in both dogs with DCM (107.3±9.54 U/L) and dogs with other cardiac diseases (73.5±0.97 U/L) compared to the healthy control group (29.00±2.07 U/L) at P<0.05. Similar findings were reported in studies conducted by Gupta *et al.* (2007), Kumar (2012), Jan *et al.* (2018), Sesh *et al.* (2013) and O' Brien (1997) in DCM cases. Increase in CK values in tachyarrhythmia was also noted (Hamm, 1994 and Ladenson, 2007) due to hyper activity of the cardiac muscles in arrhythmias. Cases with atrial fibrillation (AF) also showed a higher level of CK which is in accordance with findings of Premlatha *et al.* (1997) indicating that DCM leads to AF. LDH in control group was seen to be at 52.83±5.76 U/L which showed significant (P<0.05) rise in both the groups with DCM (151.6±21.39 U/L) and any other cardiac disease (172.27±3.47 U/L). However, it did not vary significantly when the diseased groups were compared to each other. Identical pattern of alteration were also reported in DCM by Kumar *et al.* (2012), Petra *et al.* (2017) and Jan *et al.* (2018) and in other cardiac disease (based on findings of arrhythmia in electrocardiograms) by Hamm (1994), Gupta *et al.* (2007), and Devi (2008). However, it was reported by Rajkumar and Kamran (2013) that LDH does not act as a sensitive marker for cardiac cell degeneration and that its elevation in serum is seen even in cases of muscle injury in other body parts also. However, in the current study, it may be noted that cases with muscle injury due to trauma or incision were excluded from study. Hence, in present cases of cardiac ailments, the additional serum LDH may be assumed to be generated from myocyte injury alone.

AST values showed significant (P<0.05) increase in dogs with DCM (68.69±5.21 U/L) compared to healthy dogs (30.83±2.44 U/L) and dogs with other cardiac diseases (41.80±1.52 U/L). Similar observations were reported by Gupta *et al*. (2007) and Sesh *et al.* (2013) in cardiomyopathy in dogs. In cases with cardiac arrhythmia, Benjamin (2001) reported that AST was found to be increased in myocardial infarction and congestive heart failure indicating specificity in diagnosing cardiac diseases while Hamm (1994) reported that increased activity of cardiomyocytes in arrhythmias results in increased AST values in serum. In the present study, the number of cases showing arrhythmias and myocardial infarction were high in the present study in affected groups and hence may be used to explain the increased mean of AST levels. However, AST needs to be considered as segregation criteria for DCM in correlation with other cardio-specific parameters as AST level is also elevated in serum in case of damage of other muscles besides cardiac myofibers.

Conclusion

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In the present study it was observed that cardiac biomarkers like N-terminal pro-brain like natriuretic peptides were found to be equally effective or superior in diagnosis of dogs suffering from cardiac disease in comparison to both electrocardiography and radiography. Therefore, in the absences of advanced imaging techniques like echocardiography which is a gold standard for the diagnosis of cardiac diseases, using newer generation cardiac biomarkers like N-terminal pro-brain like natriuretic peptides for diagnosis of cardiac diseases in dogs will be beneficial for the practicing clinicians.

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