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Multi-centered investigation of a point-of-care NT-proBNP ELISA assay to detect moderate to severe occult (pre-clinical) feline heart disease in cats referred for cardiac evaluation

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KEYWORDS
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Abstract *Objective:* To prospectively evaluate the diagnostic accuracy of a point-of-care (POC) N-terminal pro-B-type natriuretic peptide (NT-proBNP) ELISA to assess the likelihood of moderate to severe occult heart disease (OcHD) in a clinical population of cats suspected to have heart disease.
Animals: One hundred and forty-six asymptomatic client-owned cats with a heart murmur, gallop rhythm, arrhythmia, or cardiomegaly.
Methods: Physical examination, blood pressure measurement and echocardiography were performed prospectively. Point-of-care ELISA was visually assessed as either positive or negative by a reader blinded to the echocardiographic results.
Results: Forty-three healthy cats, 50 mild OcHD, 31 moderate OcHD, 6 severe OcHD, and 16 cats equivocal for OcHD were examined. Cats with OcHD included 65 with hypertrophic cardiomyopathy, 6 with restrictive or unclassified cardiomyopathy, 1 with arrhythmogenic right ventricular cardiomyopathy, and 15 with non-cardiomyopathic forms of heart disease. Point-of-care ELISA differentiated cats with moderate or severe OcHD with sensitivity/specificity of 83.8%/82.6% and overall accuracy of 82.9%. Positive POC ELISA increased likelihood of moderate or severe OcHD by a factor of 4.8 vs. those that tested negative. Point-of-care ELISA differentiated cats with moderate or severe cardiomyopathic OcHD with sensitivity/specificity of 88.6%/81.3% and overall accuracy of 83.2%.
Conclusion: In a select sample of cats referred for cardiac evaluation, positive POC NT-proBNP ELISA increases likelihood of moderate to severe OcHD while negative POC NT-proBNP ELISA result excludes moderate to severe OcHD.

Abbreviations

ARVC	arrhythmogenic right ventricular cardiomyopathy
AV	atrioventricular
HCM	hypertrophic cardiomyopathy
IQR	interquartile range
IVSd	interventricular septum thickness in diastole
LA:Ao	left atrium to aorta ratio
LVIDd	left ventricular internal diameter in diastole
LVIDs	left ventricular internal diameter in systole
LVPWd	left ventricular free wall thickness in diastole
NT-proBNP	N-terminal pro-B-type natriuretic peptide
NPV	negative predictive value
OcHD	occult heart disease
POC	point-of-care
PPV	positive predictive value
R/UCM	restrictive/unclassified cardiomyopathy

Introduction

Diagnosis of occult (preclinical) feline heart disease (OcHD) is challenging. Echocardiography is useful for non-invasive evaluation of cardiac structure and function, but is costly, technically challenging and might not be readily available. The sensitivity of the medical history, physical examination, thoracic radiography, and serum biochemistries for detection of OcHD is relatively low. Moreover, many heart murmurs in cats are benign in origin.[1] One study found that only 53% of cats with heart murmurs had echocardiographic evidence of heart disease.[2] Thus, a simple, widely-available, and cost-effective means to predict the presence of OcHD is attractive.

The physiology of B-type natriuretic peptide and its N-terminal pro-BNP precursor (NT-proBNP) in cats has been reviewed.[3] Previous studies revealed that quantitative NT-proBNP concentration discriminates occult cardiomyopathy from healthy cats with relatively high sensitivity and specificity and is best at detecting cats with more advanced severity of disease vs. those with only mild structural changes.[4–9] Collectively, these studies indicated that NT-proBNP assay detected occult cardiomyopathy with a sensitivity between 86 and 100% and specificity between 89 and 100%. Samples from these studies were submitted to a central reference laboratory where a plate ELISA assay was performed. Return of assay results can take up to 72 h.

Point-of-care (POC) assays enable testing and return of results at the time of patient

examination. Bidirectional flow devices[g] are one form of POC testing and use a colorimetric ELISA. Test results are based on the color of the patient sample spot compared to a reference spot. The relative color density of the spots can be assessed visually or with an automated POC ELISA reader that electronically measures and compares the optical densities of the two spots. We sought to prospectively evaluate the diagnostic accuracy of a new POC feline NT-proBNP ELISA to identify moderate or severe OcHD within a selected study population of cats referred to secondary or tertiary referral hospitals.

Materials and methods

Study methods were approved by Institutional Animal Care and Use Committees at sites where it was required. Informed owner consent was obtained at all participating sites. Cats were prospectively recruited at Michigan Veterinary Specialists (Southfield, MI), The Animal Medical Center (New York, NY) or the veterinary teaching hospitals of the University of Pennsylvania (Philadelphia, PA), Texas A&M University (College Station, TX), Tufts University (North Grafton, MA) or the University of Wisconsin (Madison, WI) from 2011 to 2012. Inclusion was based on referral for evaluation of a heart murmur, gallop rhythm, arrhythmia, or cardiomegaly. Exclusion criteria included cats $<$1 year of age, presence of known congenital heart disease, current or historical diagnosis of congestive heart failure, systemic hypertension (systolic blood pressure $>$ 180 mmHg), untreated or uncontrolled hyperthyroidism (total T4 $>$ 4.0 μg/dL), current or historical history of renal disease (serum creatinine $>$ 2.8 mg/dL), fluid therapy within the previous 72 h, current treatment with diuretics, or concurrent systemic disease judged as severe by the attending clinician.

Cats underwent routine physical examination and non-invasive blood pressure measurement by Doppler[h] method using an unspecified thoracic or pelvic limb and inflatable cuff. Timing and environment during measurement was left to the discretion of the clinician. Electrocardiographic examination was performed at the discretion of the clinician. Approximately 3 mLs of venous blood was drawn, divided between an EDTA tube or plain red top tube, centrifuged, and the resulting plasma or serum was banked at −80 °C for batch analysis. Point-of-care ELISAs were performed by a trained individual (MCM) blinded to the echocardiography results. Three drops of plasma (n = 111) or serum (n = 35) were mixed with 4 drops of assay conjugate in a sample tube and the contents poured into the POC ELISA sample well. The conjugate-diluted sample flowed across the device until it reached an indicator window. At this time, the operator activated the device, initiating the wash and color development steps of the ELISA process. After a 10-min incubation time, the relative color densities of the patient and reference spots were evaluated by both visual and automated[i] inspection. The assay result was based on the color of the sample spot compared to the positive reference spot. Visual assay results were recorded as either positive (abnormal) when the color of sample spot was equal to or darker than the reference spot, or negative (normal) when the color of sample spot was lighter than reference spot. The automated POC ELISA reader utilizes an LED light source and digital photoreceptor to measure and compare the optical densities of the sample and reference spots.

Remaining plasma and serum samples were stored frozen at −20 °C to −80 °C prior to batch analysis at a central reference laboratory. Plasma NT-proBNP concentration was quantitatively determined by a new second-generation feline NT-proBNP ELISA plate assay, which utilizes the same second-generation anti-NT-proBNP antibodies as the POC ELISA device. Range of detection for the ELISA plate assay is between 24 and 1500 pmol/L and concentrations ≥100 pmol/L are considered abnormal.[5,6]

All cats received routine two-dimensional, M-mode, and color Doppler echocardiograms that were performed by either a board-certified cardiologist or a cardiology resident-in-training under the direct supervision of a board-certified cardiologist. Personnel performing and overseeing echocardiographic exams were blinded to the POC ELISA results. Criteria for diagnosis of various forms of cardiomyopathy were based on a previous study[5] with the attending cardiologist responsible for the final classification of disease presence and severity. Left atrial to aortic ratios (LA:Ao) and the ventricular septal and left ventricular wall thicknesses were measured from 2D right short-axis views and the average of 3 measurements was calculated. Cats with OcHD were classified by the examining cardiologist as having either cardiomyopathic forms of OcHD, including hypertrophic cardiomyopathy (HCM), restrictive/unclassified cardiomyopathy (R/UCM), or arrhythmogenic right ventricular cardiomyopathy (ARVC),

[g] Ultrasonic Doppler Flow Detector, Parks Medical, Aloha, OR.
[h] SNAP™, IDEXX Laboratories Inc., Westbrook, ME.
[i] SNAPshot DX™ Optical Analyzer, IDEXX Laboratories Inc., Westbook, ME.

or as having non-cardiomyopathic forms of OcHD (eg., valvular disease, arrhythmias).

Cats with OcHD were further classified by the cardiologist performing the exam as having mild, moderate or severe disease. The cardiologist was blinded to the POC ELISA status and based their assessment on the entirety of the clinical and echocardiographic findings. Cats whose echocardiographic findings did not permit a definitive diagnosis as to the presence of absence of OcHD, were assigned an equivocal diagnosis. For the purposes of the pre-specified primary objective, cats were analyzed in two groups, those classified as normal, equivocal, or with mild OcHD (Group A) and those with moderate or severe OcHD (Group B).

Statistical analysis

Commercial software was used for statistical analysis.[j,k,l] Data is expressed as median and interquartile range (IQR). Summary statistics at baseline were compiled and the two groups were compared using Mann—Whitney tests. Bonferroni corrections for multiple comparisons were performed within the groups of echocardiographic and signalment variables. The clinical utility of POC ELISA to differentiate Group B from Group A was assessed by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratios of the POC ELISA assay. These variables were calculated with and without inclusion of cats with non-cardiomyopathic forms of OcHD. Statistical significance was defined as $P < 0.05$.

Results

One hundred and sixty-two cats were initially examined. Sixteen cats were excluded from further analysis based on findings of systemic hypertension ($n = 3$), hyperthyroidism ($n = 1$), creatinine >2.8 mg/dL ($n = 2$), congenital heart disease ($n = 2$), or blood samples not of sufficient quantity to run all the assays ($n = 8$). The remaining 146 cats from 6 different referral institutions were used for analysis (Tufts, 39; Michigan Veterinary Specialists, 34; Penn, 31; Texas A&M University, 18; University of Wisconsin, 13; Animal Medical Center, 11). The study included 97 males and 49 females. Median age [IQR] was 8 [5—12] years. Breeds studied were 120 domestic cats, 8 Maine Coon, 6 Persian, 4 Siamese, 3 Sphynx, 1 Bengal, 1 Ragdoll, 1 Devon Rex, 1 Abyssinian, and 1 Exotic short hair. Ten cats were found to have arrhythmias during examination. Arrhythmias included ventricular premature complexes (4/10; 40%), supraventricular premature complexes or tachycardia (3/10; 30%), 2nd or 3rd degree heart block (2/10, 20%); and both supraventricular and ventricular ectopy (1/10; 10%). Entry criteria were based on the reason for referral (cats could fulfill more than one inclusion criteria) and included heart murmur (125/146; 85.6%), arrhythmia (25/146; 17.1%), gallop (18/146; 12.3%), cardiomegaly (6/146; 4.1%), left mean electrical axis shift in the frontal plane (3/146; 2.1%), and previous history of rear limb weakness (1/146; 0.68%).

Eighty-seven of 146 (59.6%) cats were diagnosed as OcHD, 43/146 (29.5%) as normal and 16/146 (11.0%) as equivocal. Cats with OcHD included 50/87 (57.5%) with mild disease, 31/87 (35.6%) with moderate disease, and 6/87 (6.9%) with severe disease. Thus, Group A consisted of 109 cats and Group B (moderate to severe OcHD) consisted of 37 cats, resulting in an overall prevalence of cats with moderate to severe OcHD of 25.3% (37/146 cats). Specific diagnoses in cats with OcHD included 65/87 (74.7%) with HCM, 6/87 (6.9%) with R/UCM, 1/87 (1.1%) with ARVC, and 15/87 (17.2%) with other diagnoses, including 3 cats with isolated basilar septal hypertrophy, 3 cats with mitral regurgitation, 2 cats with 3rd degree atrioventricular (AV) block, and 1 cat with each of the following diagnoses: tricuspid regurgitation, atrial premature complexes, isolated systolic anterior motion of the mitral valve, isolated mild papillary muscle hypertrophy, aortic insufficiency, interatrial cyst, and isolated dynamic mid-left ventricular obstruction.

There were no significant differences in age, body weight, heart rate, systolic blood pressure, left ventricular dimension at end-diastole (LVIDd) or end-systole (LVIDs), BUN, creatinine, or T4 between Groups A and B (Table 1; Fig. 1). In contrast, there was a significant difference in thickness of the interventricular septum (IVSd) and left ventricular posterior wall (LVPWd) in diastole, LA:Ao, and aortic blood flow velocity between Groups A and B (Table 1; Fig. 1).

The median ELISA plate NT-proBNP concentration between Group A and B was significantly different (Group A: 48 [27—88] pmol/L vs. Group B: 522 [223—1014] pmol/L; $P < 0.0001$; Fig. 2). Results were similar if only cats with cardiomyopathic forms of OcHD (HCM, R/UCM, or ARVC) were included in the analysis (Group A: 48, [27—89] pmol/L, $n = 96$ vs. Group B: 546, [268—1276] pmol/L, $n = 35$; $P < 0.0001$).

[j] Prism 4.0, Graph Pad Software, La Jolla, CA.
[k] MedCalc 11.3.6.0, MedCalc Software, Ostend, Belgium.
[l] STATA 12.1, StataCorp, College Station, TX.

Table 1 Baseline signalment, echocardiographic, and clinical laboratory values obtained in 146 cats screened for occult heart disease.

	Age (yrs)	BW (kg)	HR (bpm)	SBP (mmHg)	IVSd (cm)	LVPWd (cm)	LVIDd (cm)	LVIDs (cm)	LA:Ao	AoV (m/s)	BUN (mg/dL)	Cr (mg/dL)	T4 (mcg/dL)
Normal ($n = 43$)	9 (6–13)	4.8 (4.1–6.0)	200 (168–210)	126 (119–138) (42)	0.46 (0.41–0.52)	0.46 (0.41–0.49)	1.44 (1.32–1.54)	0.70 (0.57–0.84)	1.25 (1.19–1.38)	0.91 (0.81–1.12) (34)	25 (21–30) (42)	1.3 (1.2–1.5) (41)	1.8 (1.6–2.2) (42)
Equivocal ($n = 16$)	11 (7–13.5)	4.7 (4.0–5.8)	181 (170–200)	130 (119–140)	0.50 (0.46–0.58)	0.49 (0.43–0.52)	1.50 (1.32–1.56)	0.66 (0.54–0.74)	1.28 (1.18–1.37)	1.04 (0.80–1.42)	25 (22–31)	1.4 (1.1–1.5)	1.9 (1.6–2.0)
Mild ($n = 50$)	6 (4–10)	5.1 (4.4–6.1)	200 (178–216)	137 (126–152)	0.55 (0.48–0.60)	0.51 (0.45–0.60)	1.43 (1.28–1.53)	0.65 (0.59–0.82)	1.31 (1.20–1.45)	1.15 (0.94–1.77) (49)	27 (23–30) (48)	1.5 (1.4–1.8) (48)	2.1 (1.6–2.6) (48)
Group A ($n = 109$)	**8 (5–12)**	**5.0 (4.2–6.1)**	**200 (170–210)**	**130 (120–150)**	**0.50 (0.45–0.57)**	**0.48 (0.43–0.52)**	**1.47 (1.30–1.53)**	**0.69 (0.58–0.82)**	**1.28 (1.19–1.40)**	**1.03 (0.85–1.38) (99)**	**26 (22–30) (106)**	**1.4 (1.2–1.6) (105)**	**1.9 (1.6–2.3) (106)**
Moderate ($n = 31$)	8 (4–11)	5.5 (4.5–6.5)	200 (172–200)	132 (119–150) (30)	0.63 (0.51–0.79)	0.63 (0.56–0.73)	1.50 (1.29–1.74)	0.77 (0.69–0.86)	1.56 (1.30–1.75)	2.22 (1.16–4.24) (29)	27 (24–32) (30)	1.5 (1.3–1.7) (30)	1.9 (1.8–2.1) (30)
Severe ($n = 6$)	12.5 (9–14)	4.4 (3.7–4.9)	180 (172–200)	107 (103–140)	0.60 (0.41–0.66)	0.49 (0.44–0.68)	1.43 (1.30–2.00)	0.68 (0.57–1.28)	1.82 (1.47–2.18)	1.87 (0.81–3.98)	26 (23–40)	1.6 (1.2–1.9)	1.7 (1.3–2.2)
Group B ($n = 37$)	**8 (4–12)**	**5.4 (4.5–6.3)**	**192 (172–200)**	**128 (110–149)**	**0.60 (0.51–0.76)**	**0.63 (0.54–0.70)**	**1.49 (1.30–1.74)**	**0.76 (0.67–0.86)**	**1.56 (1.34–1.76)**	**2.22 (1.06–4.24) (35)**	**27 (24–33) (36)**	**1.5 (1.3–1.7) (35)**	**1.9 (1.7–2.1) (36)**
P-value (A vs. B)	*0.864*	*0.388*	*0.316*	*0.446*	*<0.0001*	*<0.0001*	*0.202*	*0.020*	*<0.0001*	*0.0001*	*0.222*	*0.091*	*0.716*

Values are presented as median and interquartile range. For groups with missing data, the number of cats is displayed. Group A consisted of the combined groups of normal cats and cats with equivocal and mild occult heart disease. Group B consisted of the combined group of cats with moderate or severe occult heart disease. *P*-values that are underlined met criteria for statistical significance after adjustment for multiple comparisons.

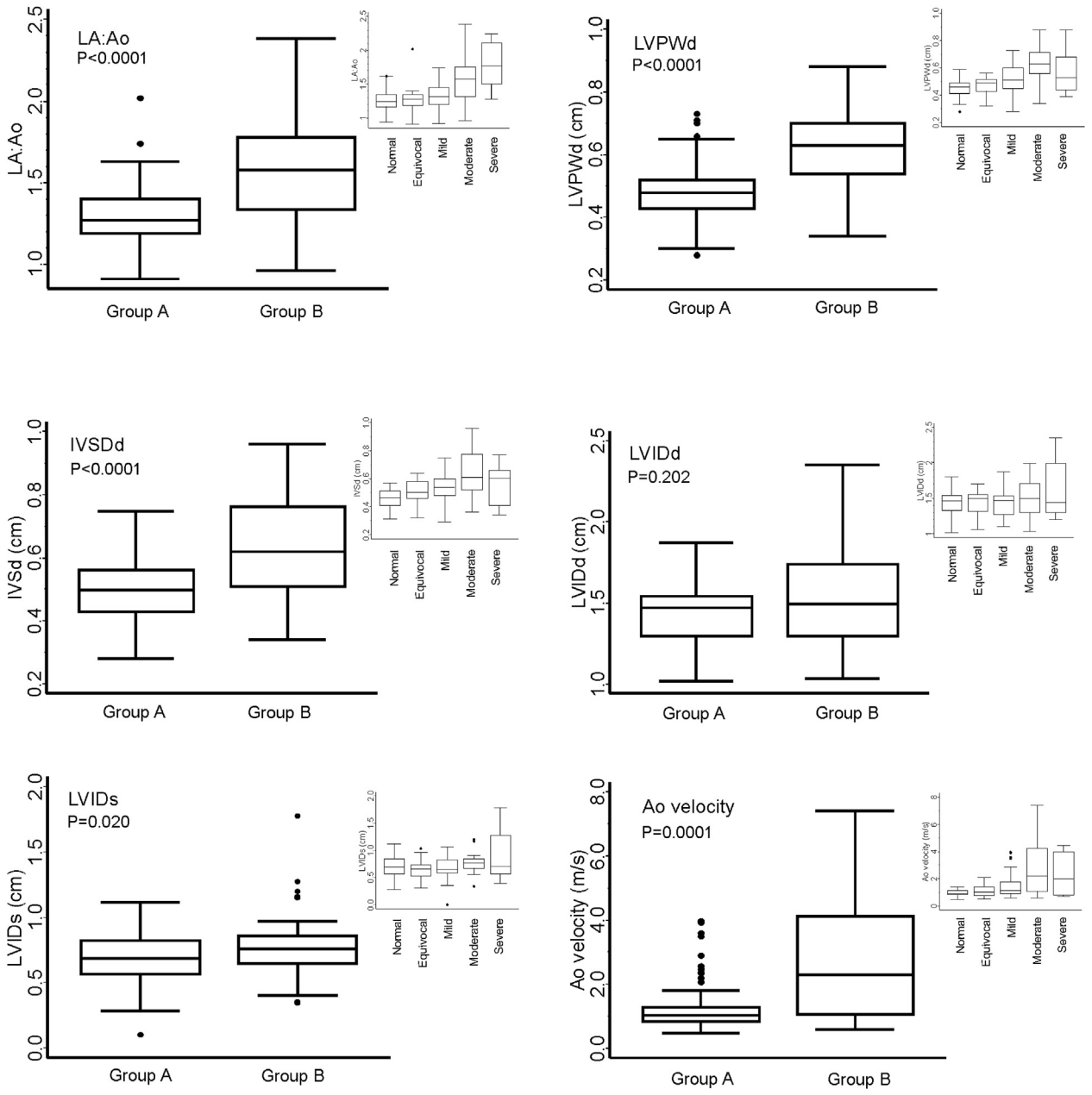

Figure 1 Box and whisker plots of echocardiographic characteristics in 109 cats diagnosed as normal or with equivocal or mild occult (pre-clinical) heart disease (Group A) and in 37 cats diagnosed with moderate or severe occult heart disease (Group B) that underwent a point-of-care NT-proBNP ELISA assay. The insets are plots of each of the 5 subgroups. The boxes encompass the interquartile range and the line within the box denotes the median value. The whiskers extend to 1.5 times the interquartile range. The circles denote data points that lie >1.5 times the interquartile range above the box.

Ninety-six of 146 (65.8%) POC ELISA assays were visually assessed as negative and 50/146 (34.2%) as positive. There was no systematic difference in optical densities between paired serum and plasma samples (mean difference, 0.0262 O.D.; 95% CI, −0.0385—0.0908 O.D.; $n = 36$). A positive POC ELISA result was associated with a median NT-proBNP concentration significantly greater than that associated with a negative POC ELISA result (positive POC ELISA: median [IQR] 540 [284—1044] pmol/L vs. negative POC ELISA: median [IQR] 40 [25—68] pmol/L; $P < 0.0001$) (Fig. 3). The upper 95th percentile of NT-proBNP concentration associated with a negative POC ELISA result was 122 pmol/L while the lower 5th percentile of NT-proBNP concentration from a positive POC ELISA

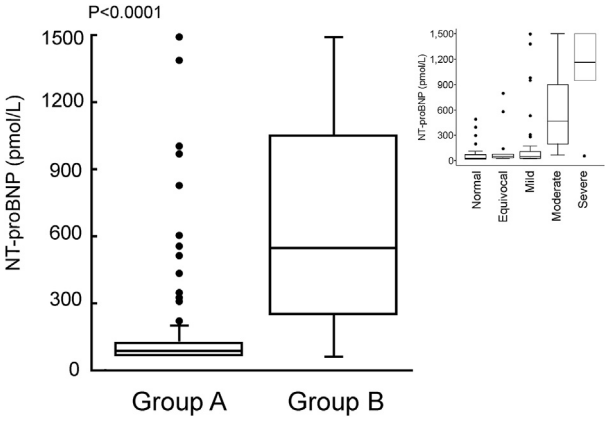

Figure 2 Box and whisker plot of NT-proBNP concentration using a second-generation NT-proBNP ELISA plate assay in 146 cats. Group A includes normal cats or those with equivocal or mild occult (preclinical) heart disease (OcHD) and Group B includes those with moderate to severe OcHD. The inset is a plot of the 5 subgroups. The boxes encompass the interquartile range and the line within the box denotes the median value. The whiskers extend to 1.5 times the interquartile range. The circles denote data points that lie >1.5 times the interquartile range above the box.

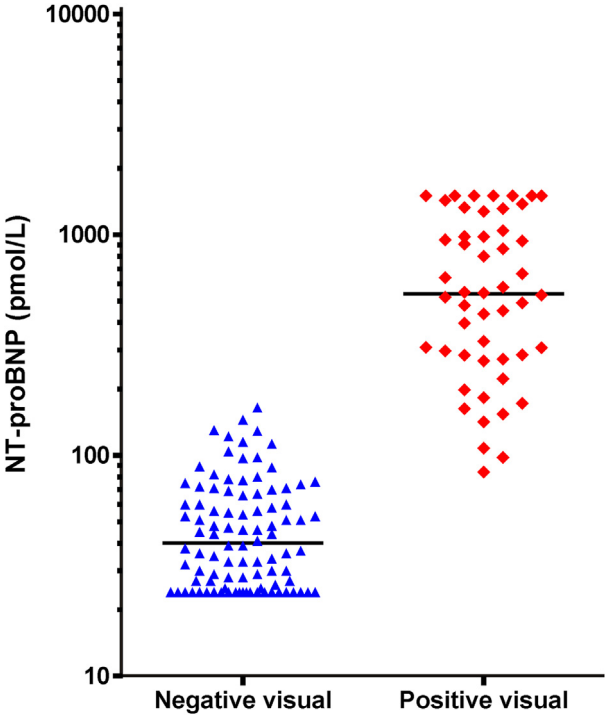

Figure 3 NT-proBNP concentration measured using a second generation NT-proBNP plate assay from 96 cats with a negative visual assessment of a point-of-care NT-proBNP ELISA (blue triangles) and from 50 cats with a positive visual assessment of a point-of-care NT-proBNP ELISA (red diamonds). The lines represent the median values within each group. Note the use of a log scale on the *y*-axis.

result was 108 pmol/L, indicating that the approximate threshold for visual interpretation of the POC ELISA as either negative of positive occurred between these two values. The proportion of cats with positive visual POC ELISA results increased with severity of disease. Visual POC ELISA results were positive in 5/43 (11.6%) normal cats, 2/16 (12.5%) equivocal cats, 12/50 (24.0%) mild OcHD, 26/31 (83.9%) moderate OcHD, and 5/6 (83.3%) severe OcHD (Fig. 4). Visual POC ELISA differentiated cats in Group B from those in Group A with a sensitivity of 83.8% and specificity of 82.6% (Table 2). The PPV was 62.0%, the NPV 93.8% and the overall accuracy was 82.9%. The PPV and NPV varied with disease prevalence (Fig. 5). A positive visual POC ELISA result increased the probability of moderate to severe OcHD by a factor of 4.8 as compared to cats with a negative POC ELISA result. The distribution of POC ELISA results based on echocardiographic findings was visually assessed for each cat using a scatterplot of LA:Ao versus the sum of IVSd and LVPWd, and categorized by POC ELISA result (Fig. 6). Receiver operator curve results were similar in the subset of cats with cardiomyopathic forms of OcHD with a sensitivity of 88.6%, specificity of 81.3%, and overall accuracy of 83.2% (Table 2). A positive visual POC ELISA result increased the probability of moderate to severe cardiomyopathic forms of OcHD by a factor of 4.7 as compared to cats with a negative POC ELISA result. The sensitivity and specificity of the quantitative plate ELISA assay using a cut-off of 100 pmol/L was similar to results of the POC ELISA (Table 2).

Visual assessment of the POC ELISA resulted in 19 false positives in 5 normal, 2 equivocal, and 12 mild OcHD cats. The 5 false positive normal cats

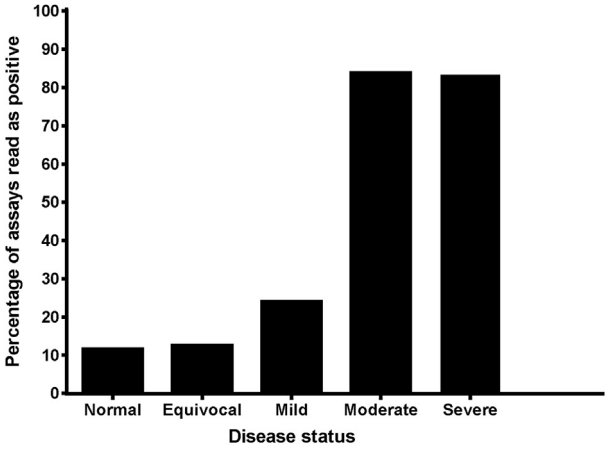

Figure 4 Percentage of point-of-care NT-proBNP ELISA assays read as positive by visual interpretation in normal cats and cats with equivocal, mild, moderate, and severe occult (preclinical) heart disease.

Table 2 Sensitivity and specificity of visual and automated point-of-care ELISA assay and ELISA plate assay (≥100 pmol/L) to detect moderate or severe occult heart disease (OcHD) or moderate or severe cardiomyopathic forms of OcHD only.

	All forms of OcHD ($n = 146$; prevalence, 25.3%)			Cardiomyopathic OcHD only ($n = 131$; prevalence, 26.7%)		
	Visual	Automated	Plate	Visual	Automated	Plate
Sensitivity (%)	83.8	81.1	86.5	88.6	85.7	91.4
Specificity (%)	82.6	82.6	78.0	81.3	81.3	77.1
Accuracy (%)	82.9	82.2	80.1	83.2	82.4	80.9
LR+	4.81	4.65	3.93	4.72	4.57	3.99
LR−	0.20	0.23	0.17	0.14	0.18	0.11

LR+, positive likelihood ratio; LR−, negative likelihood ratio.

were between 10 and 17 years of age and included 4 domestic shorthair cats and one Exotic shorthair cat. Eleven of the 12 false positives with mild OcHD were diagnosed as having mild HCM and had ELISA plate NT-proBNP concentrations ranging from 108 to 1500 pmol/L. Visual assessment of the POC ELISA yielded 6 false negative results including 5 moderate OcHD cats and 1 severe OcHD cat. Three of the 5 false negatives with moderate disease were diagnosed with HCM and had NT-proBNP concentrations of 122, 130, and 165 pmol/L. The remaining 2 false negatives were in 2 cats diagnosed with 3rd degree heart block with NT-proBNP concentrations of 66 and 77 pmol/L. The 1 false negative with severe OcHD had ARVC and a NT-proBNP concentration of 26 pmol/L.

There was a significant curvilinear relationship between the optical density results from the automated POC ELISA reader and ELISA plate NT-proBNP concentration (Fig. 7). Ninety-eight of 146 (67.1%) POC ELISA assays were assessed as negative and 48/146 (32.9%) as positive by the automated POC ELISA reader. The median automated POC ELISA optical density was significantly different between groups (Group A: 0.412 O.D., [0.248—0.642 O.D.] vs. Group B: 1.50 O.D., [1.11—1.96 O.D.]; $P < 0.0001$).

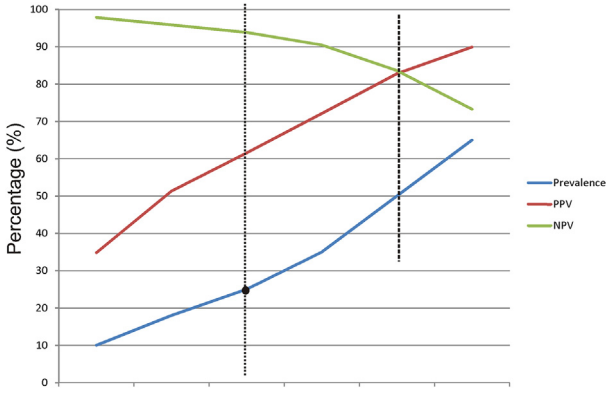

Figure 5 Positive predictive value (PPV) and negative predictive value (NPV) of visual interpretation a point-of-care NT-proBNP assay in 146 cats calculated across a range of occult heart disease (OcHD) prevalence values. Due to the reciprocal nature of PPV and NPV, PPV is very low but NPV is very high if disease prevalence is low, and PPV is very high and NPV relatively low if disease prevalence is high. The circle and dotted line represent the prevalence of OcHD found in the current study (25.3%) and the resulting PPV and NPV values, respectively. Note that PPV and NPV are maximized at a prevalence of approximately 50% (dashed line) but are low if the assay is used in populations with very low prevalence of OcHD, reinforcing the need to carefully select the patient population to be tested.

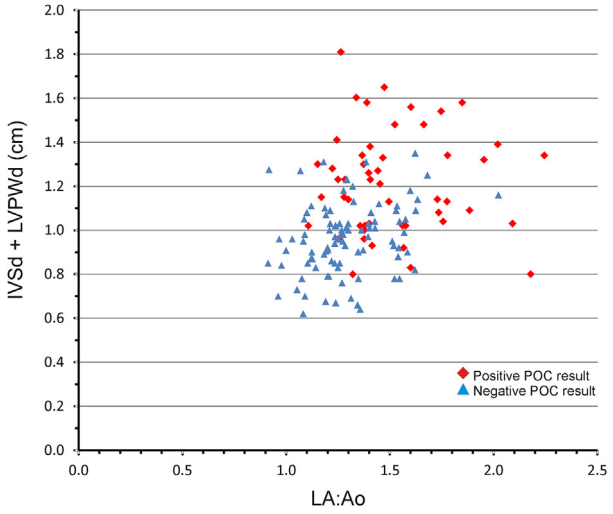

Figure 6 Echocardiographic left atrial to aortic root dimension ratio (LA:Ao) vs. the sum of the diastolic thickness of the left ventricular interventricular septum (IVSd) and left ventricular posterior wall (LVPWd) in 50 cats that tested positive (red diamond) and 96 cats that tested negative (blue triangle) using a point-of-care NT-proBNP ELISA assay. Cats with larger LA:Ao and diastolic wall thickness tended to possess positive results as compared to cats with smaller LA:Ao and thinner diastolic wall thickness.

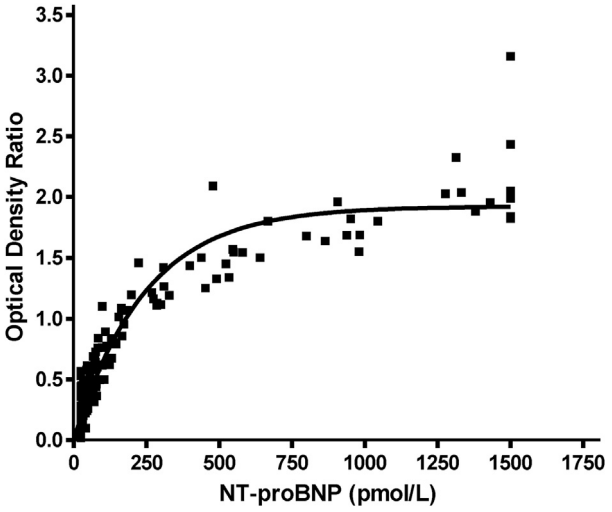

Figure 7 Plot of the optical density ratio of a point-of-care NT-proBNP assay as determined by an automated optical scanner vs. the concentration of NT-proBNP determined using a second generation NT-proBNP plate assay. The optical density ratio is calculated by the automated reader as the optical density of the patient spot over the optical density of the reference spot. The assay demonstrates good linearity over a range of NT-proBNP concentrations up to 400 pmol/L. $R^2 = 0.906$.

Results were similar if only cats with cardiomyopathic forms of OcHD were included in the analysis (Group A: 0.397 O.D., [0.248—0.670 O.D.]; $n = 96$ vs. Group B: 1.549 O.D., [1.163—1.961 O.D.]; $n = 35$; $P < 0.0001$). Automated POC ELISA differentiated cats in Group B from those in Group A with a sensitivity of 81.1%, specificity of 82.6%, and overall accuracy of 82.2% (Table 2). A positive automated POC ELISA result increased the probability of moderate to severe OcHD by a factor of 4.7 as compared to cats with a negative automated POC NT-proBNP ELISA result (Table 2). Results in the subset of cats with moderate to severe forms of occult cardiomyopathy were similar (Table 2). In 143/146 (98.0%) cats, the visual and automated interpretation of the POC ELISA were concordant. The 3 cases in which visual and automated results did not agree involved 1 healthy cat and 2 cats with cardiomyopathy. These three cats yielded ELISA plate results of 237 pmol/L (visual, normal; automated reader, abnormal), and 142 and 84 pmol/L (visual, abnormal; automated reader, normal), respectively.

Discussion

Results of the present study indicate that in cats referred specifically for cardiac evaluation, a POC NT-proBNP ELISA distinguished cats with high and low likelihoods for an echocardiographic diagnosis of moderate and severe OcHD when compared to healthy cats with equivocal or mild OcHD. In the current study, POC assays and quantitative ELISA were based on a second-generation set of anti-NT-proBNP ELISA antibodies that have not been previously described for clinical use. Prior studies[4—7] using the first-generation ELISA plate assay to detect occult cardiomyopathy reported sensitivity and specificity between 70.8% and 92.4%, and 93.9% and 100%, respectively. Sensitivity and specificity are values that describe test performance within the study population or sample and are not directly applicable to any one individual, whereas PPV and NPV indicate the probability of a true positive or negative in a single individual. In the present study, POC ELISA results indicated that animals testing negative had a 93.8% probability to be free of moderate to severe OcHD. The PPV of the POC ELISA was much lower indicating that cats with a positive result had a 62.0% probability of moderate to severe OcHD. Thus, in the population studied, false negative results were less common than false positive results. As such, the POC ELISA appeared to be a more useful test to rule out OcHD in the study population vs. a test to rule in OcHD.

Testing cats with an NT-proBNP POC ELISA device has not been previously reported. Compared to prior versions of the quantitative ELISA assay, results were obtained using either serum or plasma samples without a need for special sample handling or the use of a protease inhibitor. Technical performance of the assay was demonstrated by the linearity of optical density vs. NT-proBNP concentration. The cut off value at which the device was interpreted as positive was generally similar to reported cut-offs of >95—100 pmol/L using the first generation assay.[4—7] Visual and automatic optical scanning of the POC ELISA yielded similar results.

The POC ELISA has several potential advantages compared to the quantitative reference laboratory assay. By obtaining results at the time of examination, the need for additional diagnostic tests can be quickly assessed rather than having to wait up to 72 h for results. In the present study, the likelihood of a false positive was higher than the likelihood of a false negative. As such, the authors' caution that a positive POC ELISA result does not necessarily indicate the presence of moderate or severe OcHD, and pet owners should consider pursuing definitive diagnostics, such as echocardiography, in a cat with a positive result. NT-proBNP is constitutively produced by the myocardium and released along a continuum of values. Previous studies indicated that NT-proBNP is correlated to traditional measures of disease severity such as ventricular wall thickness and left atrial size.[5,7] Thus, a

quantitative ELISA assay might be beneficial as a follow up to a positive POC ELISA if the need for echocardiography remains uncertain. Very elevated concentrations would represent more compelling evidence for the presence of disease in comparison to a value at or near the cut-off.

Within the study population, cardiomyopathic forms of OcHD (HCM, RCM/UCM, ARVC) were the most prevalent form of OcHD. In this and other studies,[4–7] the clinical utility to detect non-cardiomyopathic forms of cardiac disease such as primary arrhythmias, valvular disease, congenital heart disease, or morphologic changes secondary to hyperthyroidism or systemic hypertension, has not been fully evaluated. In the present study the small percentage of cats with non-cardiomyopathic disease did not markedly change the assay's overall performance as results were similar whether these cats were included or excluded from the analysis.

The prevalence of moderate or severe OcHD in the study population was 25.3%, which is likely higher than that seen in populations of cats examined in a primary care practice, but is lower than the prevalence of OcHD in previous studies. In three previous studies,[5–7] prevalence of OcHD in the study population ranged from 38.9% to 76.1%. A population's prevalence of disease affects the subsequent interpretation of test results (Fig. 5) and ignoring the pre-test likelihood for disease can lead to erroneous assumptions and unnecessary testing or diagnostic errors.[10,11] In the current study, the study population included cats with a higher proportion of OcHD compared to what would be expected in populations of cats seen by general veterinary practitioners. The chance that a positive assay result is a true positive is directly proportional to the prevalence of disease; therefore indiscriminant testing of cats with very low likelihood of heart disease will result in a higher rate of false positives (Fig. 5). Thus, the POC ELISA is most effectively used in cats suspected as having heart disease based upon physical exam findings including heart murmur, gallop rhythm, arrhythmia, or radiographic evidence of cardiomegaly, as opposed to any cat that might be examined or subjected to anesthesia. In the present study, cats presented with any number of different combinations of these findings. Further studies are needed to better characterize each of these subgroups so that more specific estimates of pre-testing prevalence can be made. In humans, NT-proBNP testing of first-degree relatives of patients with HCM was 92% sensitive and 96% specific for identifying preclinical disease.[12] However, the prevalence of HCM in the study population was 34%, which is much higher than the estimated prevalence of 0.2% in the general population,[13] and is one reason why the NT-proBNP assay was so effective in the closely-related study population. The results of our study can provide estimates of PPV and NPV in populations with different prevalence values (Fig. 5), but little is known about the specific prevalence of OcHD in cats based on age, breed or physical exam findings. Cross-sectional studies are needed to supply more specific pre-test probabilities across a range of suspicious findings so that assay results in any individual cat, say one who is female, 6 years of age, and with a heart murmur, can be most accurately interpreted.

This study has several important limitations. The batched POC ELISA analysis used in the current study did not exactly mimic the proposed use in the field wherein veterinarians would be expected to run the assay very shortly after sample collection. Future studies should involve on-site recruitment of patients in routine general practice where the majority of POC ELISA tests would be anticipated to be performed. The current gold standard for diagnosis and staging of OcHD in cats is echocardiography yet there are no standardized criteria for what constitutes mild vs. moderate vs. severe disease. In the current study, the attending cardiologist was asked to assimilate the global clinical profile for each cat using medical history, physical examination, and echocardiogram, and using this collective database, to classify the extent of heart disease based upon their clinical experience. With regard to echocardiography, LA:Ao, LVPWd, IVSd, and aortic velocity were the parameters with greatest degree of significance between Group A vs. B (Fig. 1), suggesting that clinicians were heavily influenced by these variables when assigning disease severity. Another limitation is that diagnosis of heart disease was based on the opinion of a single blinded investigator and future studies might have investigators review each other's cases to achieve a consensus opinion. Within Group B, the number of cats with severe disease was small and future studies are needed to better distinguish NT-proBNP assay results between cats with moderate and severe disease. Comorbidities such as hyperthyroidism and chronic kidney disease are relatively common in geriatric cats, and the clinical utility of POC ELISA in this subset of cats requires additional investigation. The number of cats with non-cardiomyopathic forms of disease in the study was small and the utility of the assay in cats with arrhythmias, valvular, or congenital disease requires further study. One important goal of testing in the preclinical phase is to apply an intervention that reduces future

morbidity or mortality in the subjects testing positive. The efficacy of medications such as angiotensin converting enzyme inhibitors, beta-blockers, and antithrombotics to alter the natural history of disease in the preclinical phase is unknown. How NT-proBNP assays relate to these potential therapies, as well as to simple monitoring strategies such as increased owner vigilance for subtle respiratory signs or more frequent veterinary examinations, requires further study.

In conclusion, a POC NT-proBNP ELISA identified cats with high and low likelihood of moderate to severe OcHD in a selected population. Negative POC ELISA results indicated that OcHD was highly unlikely. Positive results should be interpreted in the clinical context of the individual's pre-testing likelihood for OcHD and be used to encourage owners and veterinarians to pursue definitive diagnostics such as echocardiography. Thus, POC testing provides timely information that can help guide clinical decisions.

Conflict of interest

Disclosures with relation to IDEXX Laboratories, Inc:

Consulting: MAO, RLS, PRF, JER, SGG

Speaker honoraria: MAO, RLS, PRF, JER, SGG

Reimbursement for travel: MAO, RLS, MCM, SEA, PRF, JER, SGG

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Stock or other investments: None

Nothing to declare: HK, PML

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