

Equine BNP measurement using a porcine BNP enzyme-linked immunoassay: a pilot study

*Analyse van equine BNP met een porciene BNP “enzyme-linked immunoassay”:
een pilootstudie*

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ABSTRACT

Brain natriuretic peptide (BNP) is used in human medicine for the diagnosis of congestive heart failure. Since BNP is species-specific and no equine assay is available, BNP has never been determined in horses. Because there is more than 90% homology between porcine and equine BNP, a porcine BNP enzyme-linked immunoassay (ELISA) was used in the present study to measure BNP in plasma of healthy horses (group 1; n=20), horses with cardiac disease without (group 2a; n=8) and with atrial dilatation (n=8), ventricular dilatation (n=1) or both (n=1) (group 2b; n=10). Samples were stored at -20°C and -80°C to study the influence of storage temperature. No significant differences were found between the BNP concentrations of group 1 (77.79; 37.20-513.36 pg/mL), group 2a (52.02; 24.69-268.37 pg/mL) or 2b (94.73; 42.88-470.66 pg/mL). Samples stored at -80°C showed significantly (72.19, 24.69-513.36 pg/mL; P=0.001) higher concentrations than samples stored at -20°C (47.35, 24.69-430.60 pg/mL). In this pilot study, it is suggested that the porcine BNP assay does not allow accurate detection of equine BNP. An equine specific BNP assay should be developed to study BNP concentrations in horses.

SAMENVATTING

“Brain natriuretic peptide” (BNP) wordt gebruikt in de humane geneeskunde voor de diagnose van congestief hartfalen. Vermits er geen BNP-test voor paarden beschikbaar is, werd BNP nog nooit bepaald bij paarden. Op basis van de 90% homologie tussen equine en porciene BNP, werd in de voorliggende studie het plasma BNP-gehalte van gezonde paarden (groep 1; n=20), paarden met een hartaandoening zonder (groep 2a; n=8) en met atriale dilatatie (n=8), ventriculaire dilatatie (n=1) of beide (n=1) (groep 2b; n=10) bepaald met een porciene BNP “enzyme-linked immunoassay” (ELISA). Er werd geen significant verschil gevonden tussen de BNP-concentratie van groep 1 (77,79; 37,20-513,36 pg/mL), groep 2a (52,02; 24,69-268,37 pg/mL) of 2b (94,73; 42,88-470,66 pg/mL). In deze pilootstudie wordt aangetoond dat deze porciene BNP ELISA-test geen accurate detectie van BNP bij paarden toelaat. Een specifieke equine BNP-test zou dus ontwikkeld moeten worden om de BNP-concentratie bij paarden te meten.

INTRODUCTION

Natriuretic peptides (NPs) have become an essential aid to establish a proper diagnosis and prognosis and to monitor patients with heart failure (Dickstein et al., 2008). Atrial and brain NPs are the two most important members of the NP family, which contains a typical 17-amino acid ring closed by a disulfide binding between two cysteine-molecules (Stein and Levin, 1998). Both atrial and brain NPs are released

in case of cardiac dilatation and subsequently broken down into a stable, inactive NH₂-terminal molecule (NT-proANP and NT-proBNP) and an unstable, active molecule (ANP and BNP). Atrial NPs are mainly secreted in case of atrial dilatation (Hayek and Nemer, 2010; Hori et al., 2011; Trachsel et al., 2012), while brain NPs are more related with ventricular pathologies (Goetze, 2004). Due to their longer half-life, brain NPs are often preferred for the diagnosis of heart failure in human clinical practice (Woodard and Rosado,

2007; Ray et al., 2008). A half-life of 2-5 min (Ruskoaho, 1992) and of 55-60 min (Ikeda et al., 2007) have been reported for human ANP and NT-proANP, respectively, while BNP and NT-proBNP have a half-life of 12-20 min and 60-120 min, respectively (Pemberton et al., 2000; Kemperman et al., 2004; Kimura et al., 2007; Kroll et al., 2007).

Human and equine ANP have 100% homology (Van Der Vekens et al., 2013). This explains why ANP has already been successfully determined in horses using human assays. A significant correlation between ANP plasma levels and left atrial dilatation has already been demonstrated (Gehlen et al., 2007; Trachsel et al., 2012; Trachsel et al., 2013; Trachsel et al., 2014). In contrast to ANP, the molecular structure of BNP and NT-proBNP is more variable amongst species (Van Der Vekens et al., 2013). Recently, a new canine and feline NT-proBNP assay (Cardiopet, IDEXX, Hoofddorp, the Netherlands) has been developed and successfully used for the detection of cardiac disease in dogs and cats (Oyama and Singletary, 2010; Tominaga et al., 2011; Hassdenteufel et al., 2013). However, equine studies regarding brain NPs are still missing. Since NT-proBNP has a longer half-life and is currently clinically the most important molecule in dogs and cats (Oyama, 2013), equine NT-proBNP measurement might be the best choice for future use in clinical practice. However, compared to NT-proBNP, the shorter BNP molecule seems to have more homology between some species (Van Der Vekens et al., 2012). Therefore, similarly to ANP, only for BNP, there is a chance that assays validated for other species might be useful to detect equine BNP. Human and equine BNP have ±75 % homology (Van Der Vekens et al., 2013). Hence, successful use of human BNP assays in horses seems unlikely. The difference between equine and porcine BNP is much smaller: only 3 of the 32 amino-acids of porcine and equine BNP differ (Van Der Vekens et al., 2013) (Table 1). Thus, a porcine assay might be suitable for the measurement of BNP in horses. Brain NP-like activity has been demonstrated in equine atrial tissue using antiserum against porcine BNP (Mifune et al., 1995), but a porcine BNP assay has never been used to measure BNP in horse plasma. The objectives of this pilot study were (1) to use a porcine BNP assay for the measurement of BNP in plasma of healthy horses and horses with cardiac disease and (2) to determine whether sample storage temperature has an important effect on analysis results.

MATERIALS AND METHODS

Study population

The study was approved (EC2012/57) by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University. All horses were privately owned and all examinations were performed with the owner's informed consent. The study population consisted of 20 healthy horses (group 1) and 18 horses with cardiac disease (group 2). The horses of group 1 were subjected to a thorough examination, including medical assessment of case history, clinical examination, echocardiographic examination and registration of electrocardiography (ECG) at rest and during a standardized lunging exercise test, which consisted of a five-minute walk, a ten-minute trot, a four-minute canter and a one-minute gallop (Verheyen et al., 2013). In order to be included in group 1, the horses had to be healthy, trained 2-4 times a week and had to be free of cardiorespiratory disease.

The horses of group 2 were presented at the Faculty of Veterinary Medicine, Ghent University (Merelbeke, Belgium) with a cardiac murmur. Only horses, in which moderate to severe regurgitation at one or more cardiac valves could be visualized by means of cardiac ultrasound, were included. All horses of this group underwent echocardiography and ECG recording. Based on echocardiographic measurements, the horses were classified into two subgroups: group 2a included the horses with valvular regurgitation without cardiac dilatation and group 2b included the horses with valvular regurgitation and either atrial dilatation or ventricular dilatation or both.

Echocardiographic measurements

Transthoracic echocardiographic examination included two-dimensional B-mode, M-mode and color Doppler (GE Vivid 7 Dimension, GE Healthcare, Diegem, Benelux) using a phased-array transducer (3S Phased Array Transducer, GE Healthcare, Diegem, Benelux) at a frequency of 1.7/3.4 MHz. A modified base-apex ECG was recorded simultaneously and at least three cardiac cycles from each view were stored. Off-line analysis was performed using dedicated software (EchoPAC software version 12, GE Healthcare, Diegem, Benelux). For each parameter, the average of three cardiac cycles was determined. The left atrial

Table 1. Comparison of the amino-acid sequence of equine and porcine brain natriuretic peptide. The amino-acids in red are different between horses and pigs.

Brain natriuretic peptide	
Equine	S P K M M R N S G C F G R R L D R I G S F S G L G C N V L R R Y
Porcine	S P K T M R D S G C F G R R L D R I G S L S G L G C N V L R R Y

size was evaluated by the measurement of the end-systolic left atrial-to-aortic root diameter ratio (LA/Ao) from a short axis image. The short axis systolic diameter of the left atrium (LA) was measured by placing two calipers in a line extending from and parallel to the commissure between the non-coronary and left coronary aortic valve cusps to the distant margin of the left atrium. The internal short axis diameter of the aorta at valvular level was determined by placing calipers along the commissure between the non-coronary and right coronary aortic valve cusps (De Clercq et al., 2008). If the LA/Ao was > 1.25 , the horses were diagnosed as having atrial dilatation and thus categorized in group 2b. Similarly, ventricular dilatation was evaluated by the measurement of the left ventricular internal diameter during diastole (LVID_d) from a short-axis M-mode view of the left ventricle at chordal level. Horses with a LVID_d > 13.30 cm were considered having left ventricular dilatation (Grenacher and Schwarzwald, 2010), and therefore categorized in group 2b. A subjective assessment of the dimensions of the right atrium and the right ventricle was performed on a long axis four chamber view.

Blood sampling and analysis

Two ethylenediaminetetraacetic acid (EDTA) tubes with aprotinin (Vacutainer 1.6 mg K3 EDTA and 50 KIU aprotinin/mL blood, BD diagnostics, Erembodegem, Belgium) were collected by puncture of the jugular vein with a venoject (Venosafe, Terumo Europe, Leuven, Belgium). Aprotinin (Buckley et al., 1999) is a proteinase inhibitor that might improve sample stability. From ten healthy horses, EDTA samples without aprotinin were also collected and stored at -80°C to study the influence of this proteinase inhibitor. The blood samples were directly stored on ice and centrifuged (1000g, 15 min, $2-8^{\circ}\text{C}$) within 30 minutes. Next, the plasma was harvested and transferred into two cryovials (Cryovials, 2 mL, VWR international, Pennsylvania, USA). One cryovial was stored at -80°C and one at -20°C until analysis. Time until analysis was 1 (1-2) day in group 1, 7 (1-17) days in group 2a and 9 (1-31) days in group 2b. Sample analysis was performed with a porcine competitive inhibition enzyme-linked immunoassay (ELISA, E90541Po, Usen, Wuhan, China), in which a monoclonal antibody, specific for porcine BNP, was pre-coated on the plate and attached to BNP present in the sample. Next, biotin-labeled porcine BNP was added, which began a competition with the BNP present in the sample. Finally, avidin bound to horse radish peroxidase and 3,3', 5,5'-tetramethylbenzidine (TMB) substrate were added to establish a color reaction. The intensity of the color reaction was reverse proportional to the BNP concentration in the sample. The inter-assay and intra-assay coefficients of variation (CV) were $< 12\%$ and $< 10\%$ according to the

manufacturer's instructions. The detection range was between 24.69 and 2000.00 pg/mL. For the analysis of all 76 samples (38 samples stored at two different temperatures), three different ELISA plates were used. All samples were analyzed in duplicate and the samples from the horses of each group were included in each plate. For each horse, the -20°C and -80°C samples were analyzed within the same plate to avoid inter-assay variation in order to allow comparison of storage temperature.

Statistical analysis

Data analysis was performed using commercially available computer software (SPSS Statistics 22.0, Chicago, IL). The level of significance was set at $P=0.05$. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to test normal distribution of data. Normally distributed data were expressed as mean \pm standard deviation and non-parametric data were expressed as median (range). The mean age and weight of groups 1 and 2 were compared by a student's t-test for independent samples. The median BNP concentration and the storage time between groups were compared by a Mann Whitney U-test. Comparison between the BNP concentration of samples stored at -20°C and -80°C and of samples with and without aprotinin was done by the Wilcoxon signed rank test. The influence of the storage temperature was further studied by Bland Altman analysis. Samples with a concentration under the limit of detection (< 24.69 pg/mL) were assigned the value of the limit of detection. The association between BNP and echocardiographic measurements was examined by the Spearman correlation coefficient ($P=0.01$).

RESULTS

Clinical, echocardiographic and electrocardiographic examination

Group 1 consisted of 20 Warmblood horses: 10 geldings, 9 mares and 1 stallion (age: 8 ± 4 years, weight: 563 ± 52 kg, height: 168 ± 5 cm). The LA/Ao and LVID_d were 1.15 ± 0.05 and 11.33 ± 1.19 cm, respectively. The exercise ECG was normal in 18/20 horses. One horse had one atrial premature depolarization (APD) and another horse had two ventricular premature depolarizations (VPDs) during exercise.

Group 2 consisted of 14 Warmblood horses, 1 Arabian horse, 1 Friesian, 1 Spanish horse and 1 mixed breed and included 8 geldings, 8 mares and 2 stallions (age: 13 ± 6 years, weight: 566 ± 90 kg; height: 167 ± 9 cm). The mean age ($P=0.006$) in group 2 was significantly higher than the mean age in group 1. No difference in weight was found. A systolic murmur was detected in 13/18 horses and a diastolic murmur in

6/18 horses. Echocardiographic examination demonstrated a moderate or severe regurgitation of one (n=13) or more valves (n=5) in all of the horses. The mitral valve was mostly affected (n=10), followed by the aortic (n=7), tricuspid (n=4) and pulmonary valve (n=2). Two horses also showed a ventricular septal defect and one Friesian was diagnosed with an aortopulmonary fistula. Based on the LA/Ao and the LVID_d, eight horses had normal left atrial and left ventricular diameters and were categorized in group 2a. One horse had a markedly enlarged right atrium, and was therefore classified in group 2b. The remaining nine horses had a LA/Ao>1.25 (n=8) or a LVID_d>13.30 (n=2), and were therefore categorized in group 2b. The mean LA/Ao and LVID_d in group 2a were 1.16±0.06 and 12.41±0.83 cm, respectively. A mean LA/Ao of 1.40±0.18 and LVID_d of 12.53±1.54 was demonstrated in group 2b. Electrocardiographic examination demonstrated atrial fibrillation in three horses. An exercise ECG was performed in 15/18 horses. No abnormalities were found in 4/15 horses. Six horses had between one and nine APDs and eight horses between one and four VPDs during exercise.

BNP comparison between groups

When samples were stored at -80°C (BNP₋₈₀), the median BNP concentration in group 1 was 77.79 pg/mL (37.20-513.36) pg/mL. No significant (P=0.135) difference was found with the BNP₋₈₀ concentration of group 2a (52.02 pg/mL, range 24.69-268.37 pg/mL) or group 2b (94.73 pg/mL, range 42.88-470.66 pg/mL) (Figure 1). There was no correlation between the BNP₋₈₀ concentration and the LA/Ao or LVID_d (Table 2).

Influence of sample storage temperature and aprotinin addition

When samples were stored at -20°C (BNP₋₂₀), a significantly (P<0.001) lower BNP concentration (47.35 pg/mL, range 24.69-430.60 pg/mL) was found than when samples were stored at -80°C (72.19 pg/mL, range 24.69-513.36 pg/mL). Similarly as for BNP₋₈₀, no significant (P=0.792) BNP₋₂₀ difference was found between group 1 (48.84 pg/mL, range 24.69-139.63 pg/mL), group 2a (51.85 pg/mL, range 24.69-430.60 pg/mL) or group 2b (40.80 pg/mL, range 24.69-409.13 pg/mL) (Figure 2). A significant correlation (P<0.01) existed between BNP₋₂₀ and BNP₋₈₀ (Table 2). The mean difference between the BNP₋₈₀ and BNP₋₂₀ concentrations was 62.69±149.61 pg/mL (Figure 3). Almost all (n=32) of the BNP samples stored at a temperature of -80°C had a higher concentration than when stored at -20°C.

The median BNP concentration was significantly (P=0.037) higher in EDTA samples with aprotinin (240.86, 95.15-513.36 pg/mL) than in EDTA samples without aprotinin (147.54, 75.27-227.98 pg/mL).

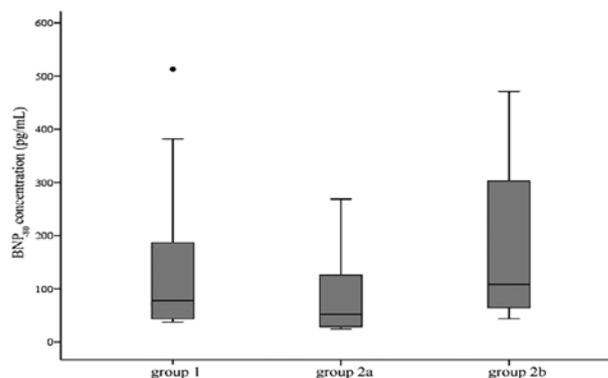


Figure 1. Brain natriuretic peptide concentrations when samples were stored at -80°C (BNP₋₈₀) of group 1 (healthy horses), group 2a (horses with regurgitation without atrial or ventricular dilatation) and group 2b (horses with regurgitation with atrial or ventricular dilatation).

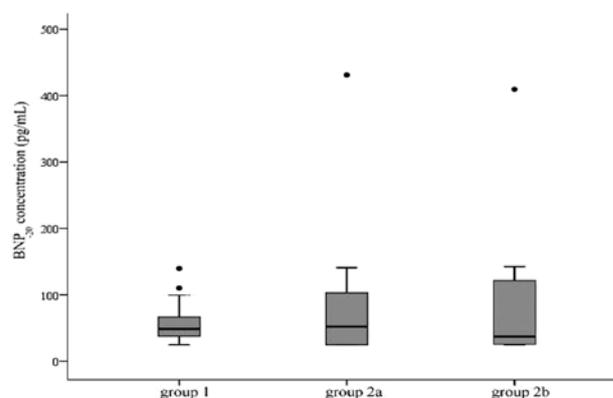


Figure 2. Brain natriuretic peptide concentrations when samples were stored at -20°C (BNP₋₂₀) of group 1 (healthy horses), group 2a (horses with regurgitation without atrial or ventricular dilatation) and group 2b (horses with regurgitation with atrial or ventricular dilatation).

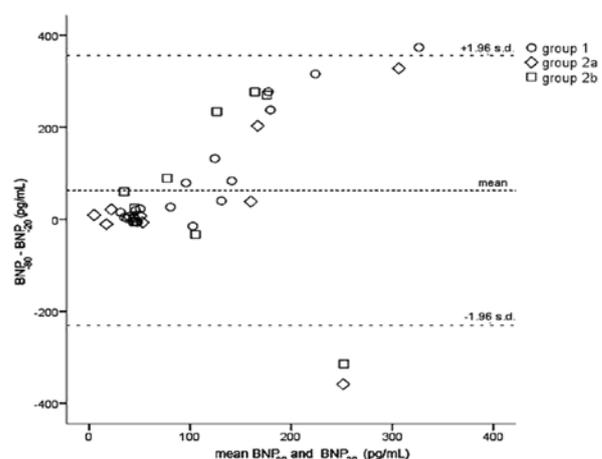


Figure 3. Bland-Altman plot showing the relationship between the difference (BNP₋₈₀ - BNP₋₂₀) of brain natriuretic peptide (BNP) relative to the mean BNP concentration when samples stored at -80°C and -20°C were analyzed at the same time (group 1= healthy horses; group 2a= horses with regurgitation without atrial/ventricular dilatation; group 2b= horses with regurgitation and atrial or ventricular dilatation; BNP₋₈₀: BNP concentration stored at -80°C; BNP₋₂₀: BNP stored at -20°C).

Table 2. Spearman correlation coefficients between brain natriuretic peptide concentrations stored at -80°C (BNP₋₈₀) and -20°C (BNP₋₂₀) and echocardiographic measurements (n = number of horses, LA/Ao: left atrial-to-aortic root ratio, LVID_d = left ventricle internal diameter during diastole, * = P<0.01).

	BNP ₋₈₀	BNP ₋₂₀	LA/Ao	LVID _d
BNP ₋₈₀ n	1 38	X	X	X
BNP ₋₂₀ n	0.505* 38	1 38	X	X
LA/Ao n	0.096 37	-0.300 37	1	X
LVID _d n	0.202 38	-0.112 38	0.123 37	1

DISCUSSION

Human assays have been used for equine ANP (Gehlen et al., 2007; Trachsel et al., 2012; Trachsel et al., 2013) and NT-proANP measurement (Kokkonen et al., 2002) based on the interspecies sequence homology between molecules (Gehlen et al., 2007; Trachsel et al., 2012; Trachsel et al., 2013). A sequence homology of 100% and 80-90% has been described for equine ANP (Richter et al., 1998) and NT-proANP (Kokkonen, 2002), respectively. Since BNP and NT-proBNP are species-specific molecules, and no equine BNP or NT-proBNP test exists, the determination of brain NPs in horses remains impossible. However, brain NPs determination might be helpful as screening or follow-up test for cardiac disease in horses, especially if cardiac ultrasound is not available. Since equine and porcine BNP have a more than 90% homology (Van Der Vekens et al., 2012), a competitive porcine BNP ELISA was used in an attempt to measure equine BNP. Firstly, normal values were determined in 20 healthy horses and a median BNP concentration of 77.79 (37.20-513.36 pg/mL) was found. In comparison to humans and small animals, the BNP concentration was unexpectedly high and had a broad range. However, the results of the present study should be interpreted with caution as the porcine BNP assay was not validated for use in horses. Different BNP cut-off values have been described depending on the disease, which has to be detected. In humans, a cut-off value of 17.9 pg/mL for the detection of left ventricular systolic dysfunction has been described (McDonagh et al., 1998). In dogs, a cut-off value of 23 pg/mL (MacDonald et al., 2003), 24.6 (DeFrancesco et al., 2007) and 6.21 pg/mL (Oyama et al., 2007) has been described for the detection of cardiac disease, for the distinction between cardiac and non-cardiac dyspnea and for the detection of dilatory cardiomyopathy, respectively. However, the clinical utility of BNP for the detection of cardiac disease in dogs has been questioned, since a lot of healthy dogs also have

BNP concentrations above the cut-off value (Lee et al., 2011) and NT-proBNP measurement might be more useful for the detection of cardiac disease in clinical practice (Oyama, 2013). The maximal BNP concentration and the BNP range of these dogs were still lower than the values in the present study. Although it is possible that the BNP concentration is higher in horses than in humans or dogs, also cross reactivity with other NPs, such as proBNP, NT-proBNP or other degradation products (Goetze, 2004) may lead to increased BNP detection values. The presence of cross reactivity in BNP and NT-proBNP assays has also been described in human medicine and makes clinical interpretation of assay results challenging (Luckenbill et al., 2008). In dogs, this cross reactivity might be decreased in a second generation ELISA (Canine Cardiopet, IDEXX, Hoofddorp, the Netherlands), which uses specific capture and detection antibodies targeted against canine NT-proBNP epitopes (Fox et al., 2014). Heterophile antibodies can cause false positive reactions, and might therefore also explain the high BNP concentration in the present study (Solter et al., 2008). However, a competitive ELISA was used and normally, heterophile antibodies are too weak to compete with the high affinity antigens in these assays and cause only false positive results when sandwich ELISAs are used (Kaplan and Levinson, 1999). Another possibility is that the monoclonal antibodies did not attach to equine BNP. Although there is only a limited difference between the sequence of equine BNP and porcine BNP (Table 1), it remains possible that the monoclonal antibodies of the porcine BNP assay were developed against a BNP region where the equine and porcine BNP amino-acid sequences differ. Since many samples had a BNP concentration above the limit of detection and because a significant difference between sample storage temperatures was detected, it seems likely that the monoclonal antibodies did detect equine BNP or at least an equine BNP-like molecule. Cross reactivity with another equine molecule might explain why no differ-

ence was found between horses of groups 1 and 2, and why no correlation with echocardiographic parameters was found. Antibodies against porcine BNP have been used to detect BNP-like activity in atrial cardiac myocytes (Mifune et al., 1995). In the study by Mifune et al. (1995), atrial natriuretic peptide reactivity was detected in these same myocytes. Therefore, a cross-reaction between ANP and the anti-BNP antibodies could possibly explain the BNP immunoreactivity, which was found with the anti-BNP antibodies. However, the hypothesis was tested and no such cross-reactivity could be detected (Mifune et al., 1995). Another explanation for the lack of difference between groups and the wide BNP range in healthy horses is the presence of confounding factors. Age, gender and breed have been described to affect the BNP concentration: a higher BNP concentration has been found in older patients and in women (Redfield et al., 2002). Similarly, high NT-proBNP values have been found in Labrador retrievers and Newfoundland dogs (Sjostrand et al., 2014). A negative correlation between BNP and obesity has also been reported (Wang et al., 2004). However, in the present study, no correlation was found between the BNP concentration and gender, age or weight in the group of healthy horses. Since all healthy horses were Warmblood horses, the influence of breed could not be examined. However, 14/18 horses with cardiac disease were also Warmblood horses. Therefore, the influence of breed on the results seems limited. The difference in sample storage time might also influence results. The samples of group 1 were stored for a significantly shorter time than samples of groups 2a and 2b. If the BNP concentration in samples of group 2a or 2b had been reduced due to longer storage, the BNP difference between healthy horses and horses with cardiac disease would have become smaller. However, according to manufacturer's instructions, samples can be stored for 2 months at -80°C , and the BNP concentration in the present study seemed surprisingly high in comparison to other species. Therefore, the influence of sample storage seemed limited. The presence of a large inter-assay variability could also explain the results. The inter-assay CV was determined by the manufacturer for the porcine BNP molecule. A different inter-assay CV may be applied when measuring equine BNP. A large inter-assay CV means that repeated measurement of the same samples gives different results. Three different ELISA plates were used for analysis of all samples. If there had been a large inter-assay variation between the three plates, the difference between healthy horses and horses with cardiac disease would have become smaller. Samples from healthy horses analyzed with plate 1 ($n=9$; 190.57 pg/mL , range $95.15\text{-}513.36\text{ pg/mL}$) had a very high BNP concentration in comparison to plates 2 ($n=9$; 42.48 pg/mL , range $37.20\text{-}61.74\text{ pg/mL}$) and 3 ($n=2$; 69.10 pg/mL , range $44.35\text{-}93.84\text{ pg/mL}$). When only samples from plates 2 and 3 were included in this

study, a significant difference between horses with cardiac dilatation (94.37 pg/mL , range: $43.77\text{-}470.66\text{ pg/mL}$) and healthy horses (44.35 pg/mL , $37.20\text{-}93.84\text{ pg/mL}$, $P=0.014$), and between horses with dilatation and horses with regurgitation without dilatation (49.56 pg/mL , $24.69\text{-}179.30\text{ pg/mL}$, $P=0.043$) was found. However, a correlation with echocardiographic measurements was still not found, and only a small number of horses were included (group 1= 11, group 2a= 7 and group 2b= 9). Therefore, from these results, it could not be concluded that BNP increases in case of cardiac dilatation. Finally, BNP is especially correlated to ventricular dilatation and most of the horses of group 2 had atrial dilatation. Only 2 of the 10 horses of group 2b had ventricular dilatation. The BNP₋₈₀ concentrations of these horses were 88.79 and 243.35 pg/mL . Therefore, future studies on BNP or NT-proBNP measurement in horses should include more horses with ventricular dilatation.

Sample storage temperature had a strong influence on sample concentration: samples, which were stored at -80°C had a significantly ($P<0.001$) higher concentration than samples stored at -20°C . Since both samples from the same horse were analyzed on the same ELISA plate, the inter-assay CV could not have influenced these results. Previous studies in human medicine have also shown that BNP is unstable at -20°C (Mueller et al., 2004). As it is possible that in the present study, a BNP-like molecule was measured instead of BNP, it could not be determined whether the difference was fully attributable to BNP sample stability.

This pilot study is the first, in which measurement of equine plasma BNP using a porcine assay was attempted. The major limitation of the study was the use of a non-validated BNP assay. BNP-like immunoreactivity in the samples was measured but it was not proven whether it concerned BNP or not. The possible existence of peptide cross-reactivity, sample instability and assay variability further complicated the results. These three factors should also be taken into account when measuring ANP and NT-proANP in horses. Currently, only one ANP assay validation study has been published (Trachsel et al., 2014) and shows that there is a high inter-assay variability and that different ANP assays are poorly comparable. Therefore, there is a need for a validated, species-specific equine BNP /NT-proBNP test and a reliable ANP or NT-proANP assay for the use in clinical practice.

CONCLUSIONS

Brain natriuretic peptide-like activity was found in equine plasma samples using a porcine BNP assay. Despite the high homology ($>90\%$) between equine and porcine BNP, the porcine BNP ELISA could not differentiate healthy horses from horses with cardiac disease, probably due to a high inter-assay CV and

cross-reactivity with other plasma proteins. A major limitation of the study is that a non-validated assay was used. Therefore, further studies on assay validation, imprecision and accuracy should be performed. Ideally, equine specific BNP and NT-proBNP assays should be developed in order to study horses with and without cardiac disease.

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