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Development of immunoassays for quantification of NT-proBNP in canine blood

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Introduction

NT-proBNP measurement has been introduced into veterinary practice during the last decade (reviewed in Ref. 1). Amino acid sequences of human and canine NT-proBNP differ considerably. This makes impossible use of human NT-proBNP immunoassays for determination of canine NT-proBNP concentration in blood. Therefore, species-specific NT-proBNP immunoassays are required for the measurement of canine NT-proBNP.

One of the major challenges with commercially available canine NT-proBNP assays is their sensitivity to proteolytic degradation of NT-proBNP in blood samples (2). In 2005, we have demonstrated that the apparent stability of human NT-proBNP is dependent on the epitope specificity of the antibodies utilized in the assay (3). Therefore, the aim of the present study was to develop monoclonal antibodies (mAbs) for canine NT-proBNP assay that is less sensitive to NT-proBNP degradation in a sample.

Another limitation of the existing canine NT-proBNP immunoassays is the narrow measurable range. Immunoassays with wider dynamic range are required for stratifying disease severity and deriving prognostic information in dogs. Therefore, we intended to obtain mAbs that can be used for the development of highly-sensitive immunoassays with wide dynamic range.

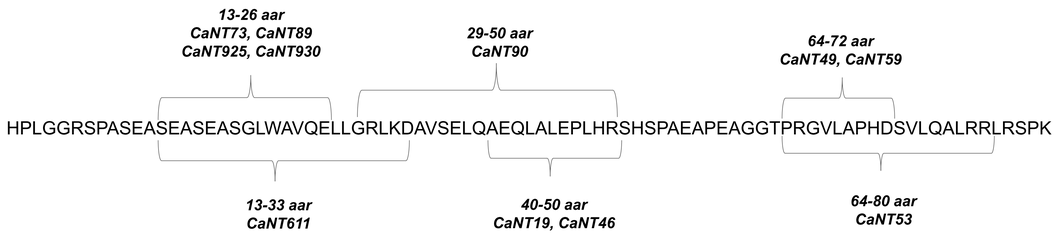

Figure 1. Epitope location of selected anti-canine NT-proBNP mAbs.

Materials and methods

Standard protocol was used for mouse monoclonal antibody (mAb) development.

Recombinant canine NT-proBNP$_{1\text{-}85}$ expressed in E. *coli* (HyTest, Finland) was used as a calibrator in assays.

DELFIA technology was used for development of sandwich immunoassays. Detection MAbs were labeled with stable europium chelate.

Blood from healthy dogs and dogs with heart disease was collected into Vacuette® EDTA tubes and than centrifuged at 2000 g for 10 min at room temperature. Samples were stored at -70°C before use.

For the stability study pooled EDTA plasma of dogs with heart disease was incubated at two different temperatures (+4°C and +20°C) for different time intervals.

References:

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2. Collins SA, Patteson MW, Connolly DJ, Brodbelt DC, Torrance AG, Harris JD. Effects of sample handling on serum N-terminal pro-B-type natriuretic peptide concentration in normal dogs and dogs with heart disease. J Vet Cardiol. 2010 Apr;12(1):41-8.

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Results and discussion

A large panel of 65 mAbs specific to different regions of canine NT-proBNP was obtained. Antibody epitopes were distributed almost along the whole NT-proBNP molecule.

All mAbs were tested as capture and detection antibody in sandwich immunoassays with both recombinant NT-proBNP and pooled plasma from dogs with heart disease (source of endogenous NT-proBNP). From this broad panel of mAbs we selected eleven antibodies that demonstrated the highest signal with native NT-proBNP (Fig. 1).

Six two-site combinations demonstrated the highest sensitivities to both recombinant and native antigens: **CaNT611-CaNT19, CaNT90-CaNT89, CaNT90-CaNT53, CaNT73-CaNT59, CaNT53-CaNT930 and CaNT19-CaNT89.** These combinations were further evaluated with regard to working range and analyte stability.

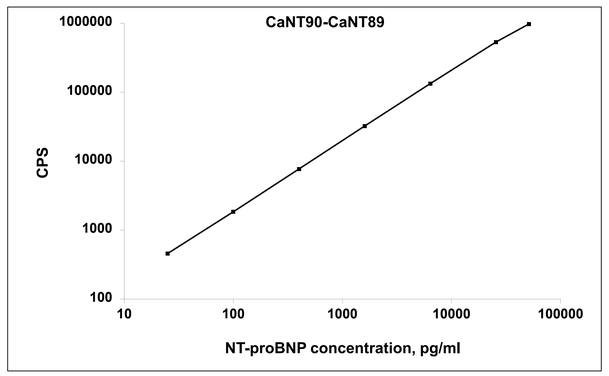

Figure 2. Calibration curve for NT-proBNP sandwich immunoassay (one-step). The mixture of biotinylated capture mAb (CaNT90) and detection mAb (CaNT89) were incubated in a streptavidin-coated plate together with calibrator (50 µL) for 40 min at room temperature.

The limit of quantification of the best mAb combinations was 25 pg/ml. The working range was up to 50,000 pg/ml. These combinations can be used in one-step immunoassay format without loss in sensitivity or assay performance (Fig. 2). The incubation time can be limited to 20 minutes that allows development of the rapid immunoassays.

When plasma samples from healthy dogs and dogs with heart disease were tested using the selected assays, NT-proBNP concentrations were within the working range in all tested assays. There was no need for dilution of samples even for dogs with advanced heart failure. NT-proBNP concentrations determined by selected immunoassays were significantly higher in the group of dogs with heart disease than in a group of healthy dogs (Fig. 3).

Canine NT-proBNP is considered to be an unstable molecule. Using commercially available immunoassays, it was demonstrated that NT-proBNP immunoreactivity decreases during plasma or serum sample storage because of proteolytic degradation (2, 4). Therefore, we tested ability of the selected assays to detect endogenous NT-proBNP after incubation of pooled canine plasma sample at +4°C and room temperature. When immunoreactivity of endogenous NT-proBNP was determined in samples after incubation, there was no considerable difference between results of measurements in different assays. At +4°C endogenous NT-proBNP remained stable for at least 72 hours (95-105% of initial immunoreactivity, Fig. 4A).

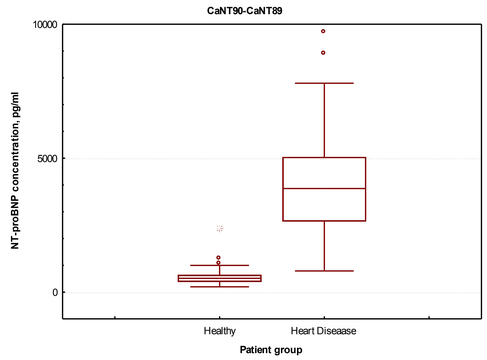

Figure 3. A box plot demonstrating the concentration of NT-proBNP in EDTA plasma from healthy dogs (N=31) and dogs with heart disease (N=26). NT-proBNP concentration was determined using assay CaNT90-CaNT89.

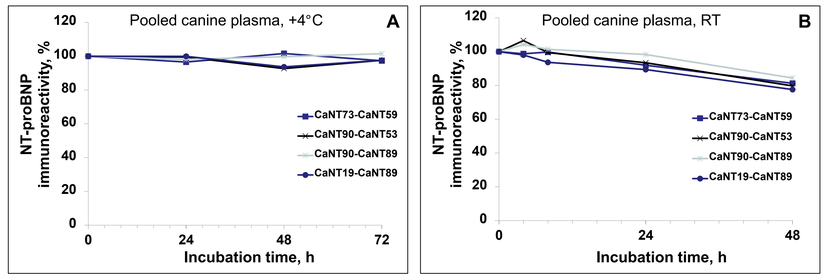

Figure 4. Immunoreactivity of endogenous canine NT-proBNP in pooled EDTA plasma sample incubated at +4°C for 24, 48 and 72 hours (A) or room temperature for 4, 8, 24, and 48 hours (B). NT-proBNP concentration in pooled plasma was 9 ng/ml.

When plasma was incubated at room temperature, 89-98% of initial immunoreactivity was detected in the sample after 24 hours of incubation (Fig. 4B).

The possible explanation of the discrepancy between the results of stability studies described in literature and those obtained in the current study is that epitopes of all mAbs used in the current study were remote from the N-terminus and located either in the central or in the C-terminal part of canine NT-proBNP. According to our earlier data, N-terminal region of human NT-proBNP is very unstable, whereas stability of central and C-terminal parts of human NT-proBNP is much higher (3).

Thus stability data indicates that EDTA plasma could be stored at +4°C for at least 72 hours with little to no loss in the immunoreactivity of endogenous canine NT-proBNP if measured by selected immunoassays.

Conclusions

- **Broad monoclonal antibody panel specific to canine NT-proBNP was developed;**
- **Six matched mAb pairs could be used for the development of rapid and sensitive immunoassays with wide dynamic range for canine NT-proBNP measurements;**
- **Selected mAb pairs were less susceptible to the proteolytic degradation of NT-proBNP than previously described immunoassays.**

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