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 their measurable range. Immunoassays with wider dynamic range are required for determination of NT-proBNP in biological samples and they should preserve immunoassay stability and sensitivity. For these reasons, we tested the possibility of using NT-proBNP immunoassays with wider dynamic range for the measurement of canine NT-proBNP concentration in blood.

Results and discussion

Materials and methods

Standard protocol was used for mouse monoclonal antibody (mAb) development. Recombinant canine NT-proBNP was expressed in E. coli (Hytest, Finland) was used as an antigen for the development of sandwich immunoassays. DELFIA technology was used for development of sandwich immunoassays.

Blood from healthy dogs and dogs with heart disease was collected into Vacutette® EDTA tubes and then 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 from dogs with heart disease was incubated at different temperatures (4°C and +20°C) for different time intervals.

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 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: CaNT61-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.

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 heart disease. NT-proBNP concentrations determined by selected immunoassays were significantly higher in the group of dogs with heart disease than in the group of healthy dogs (Fig. 3).

Canine NT-proBNP is considered to be an unstable molecule. Using commercially available immunoassays, it was demonstrated that from NT-proBNP immunoassay becomes unstable after incubation of pooled canine plasma sample at +4°C and room temperature. Therefore, we tested the proteolytic degradation of NT-proBNP and we demonstrated that from NT-proBNP immunoassay becomes unstable after incubation of pooled canine plasma sample at +4°C and room temperature. When immunoassay 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 was stable for at least 72 hours (95-105% of initial immunoassay, Fig. 4A).

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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