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Development of a Heart Failure Detection Kit Based on Antibody Against Amino-Terminal Pro-Brain Natriuretic Peptide (NT-proBNP): Identification and Immunogenicity Exploration of NT-proBNP Protein

Andreas Budi Wijaya[1*], Dina Fauziah[1], Karina Nilasari[1], Editya Fukata[1], Dyta Loverita[1], Taurisma Aulia Nanda Wibisono[1], Farsya Hidayah Septiananda[1], Rajendra Aulya Gilardino[1]

[1] Faculty of Medicine, Universitas Negeri Malang, Malang, East Java, Indonesia.

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Corresponding Author:
Andreas Budi Wijaya
andreasbudiwijaya.fk@um.ac.id

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Abstract: Heart failure (HF) remains a major public health problem with high morbidity and mortality worldwide. The use of cardiac biomarkers such as N-terminal pro–B-type natriuretic peptide (NT-proBNP) has been established as a reliable tool for HF diagnosis and prognosis. However, the high cost and technical requirements of conventional immunoassays limit their accessibility in low-resource settings. This study aims to produce and characterize polyclonal antibodies against NT-proBNP as a preliminary step toward developing an affordable, locally produced detection kit suitable for primary healthcare use in Indonesia. Three male Oryctolagus cuniculus rabbits were immunized intramuscularly with recombinant human NT-proBNP antigen emulsified in Freund's adjuvant. Immunogenicity was assessed using indirect ELISA at 405 nm absorbance. The ELISA results demonstrated a progressive increase in NT-proBNP-specific antibody titers from week 1 to week 4, with peak levels observed after the second booster (week 7). This pattern reflected the establishment of both primary and secondary humoral immune responses. The absorbance values indicated that NT-proBNP antigen is immunogenic and capable of inducing significant polyclonal antibody production in rabbits. The resulting polyclonal antibodies have multi-epitope recognition potential, which is advantageous for developing point-of-care immunoassays such as lateral flow tests. These results represent an essential foundation for subsequent phases, including antibody purification, affinity testing, and prototype development of an NT-proBNP-based heart failure detection kit optimized for Indonesian healthcare settings. Human NT-proBNP is confirmed to be a highly immunogenic protein capable of inducing the production of specific polyclonal antibodies in rabbits.

Keywords: ELISA; Heart failure; Immunogenicity; Polyclonal antibody; NT-proBNP

Introduction

Heart failure (HF) is a global health problem with high morbidity and mortality rates in both developed and developing countries, including Indonesia. This condition is characterized by the inability of the heart to pump blood effectively, resulting in decreased tissue perfusion and clinical symptoms such as shortness of breath, fatigue, and peripheral edema. Early detection and accurate diagnosis play a crucial role in determining patient management and prognosis (Chowdhury et al., 2023).

Standard diagnostic modalities such as echocardiography have limitations, including the need for skilled personnel, limited availability of equipment, and relatively high costs (Delalic et al., 2023). Therefore, cardiac biomarkers such as N-terminal-pro-brain natriuretic peptide (NT-proBNP) are widely used in

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clinical practice. NT-proBNP is produced by the ventricles in response to increased myocardial wall pressure, and elevated NT-proBNP levels correlate not only with the occurrence of heart failure but also with disease severity, hospitalization rate, and mortality, making it a valuable diagnostic and prognostic biomarker (Ali et al., 2025).

However, NT-proBNP testing using conventional immunoassay techniques requires sophisticated laboratory facilities and is costly (Reeves et al., 2018). This poses a challenge in expanding the coverage of heart failure screening, particularly in areas with limited healthcare resources. This situation highlights the need for simpler, more efficient, and affordable diagnostic technology innovations.

One potential approach is the development of an antibody-based detection kit for NT-proBNP. The production of polyclonal antibodies specific to NT-proBNP could be the first step in designing a rapid test kit that allows for self-testing or testing at primary health care facilities. Such kits are expected to increase screening coverage, accelerate diagnosis, reduce examination costs, and reduce dependence on imported products.

Therefore, this study aims to produce polyclonal antibodies against NT-proBNP in experimental animals and further investigate its immunogenicity through ELISA method. The results of this study will form the basis for the development of a prototype NT-proBNP antibody-based heart failure detection kit that is practical, affordable, and suitable for the needs of the Indonesian healthcare system.

Method

Research design

This study used a true experimental laboratory research design, including: production of NT-proBNP-based polyclonal antibodies in experimental animals, and testing of antibody immunogenicity using the ELISA method.

Samples and Subjects

Rabbits (*Oryctolagus cuniculus*) were used as hosts for the production of polyclonal antibodies against NT-proBNP. All experimental procedures involving animals have been granted ethical approval from the Health Research Ethics Committee of Malang State University (No. 27.03.06/UN32.14.2.8/LT/2025).

Research Location and Duration

The research was conducted at the Central Biomedical Laboratory of the Faculty of Medicine, Brawijaya University (FKUB), the Biochemistry Laboratory of the Faculty of Mathematics and Natural Sciences, UB. Clinical samples were collected at RSSA Malang. The research series took place from May 2025 to May 2026.

Research Procedures

For production of polyclonal antibodies, 300 µL of recombinant human NT-proBNP antigen (Cat no. RPA485Hu01; Cloud-Clone Corp, Wuhan, China) was mixed with Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA) (3:1 ratio) and vortexed until a stable emulsion was formed. The emulsion was injected intramuscularly with an optimized rabbit immunization protocol (week 1: Ag+CFA; week 3: Ag+IFA1; week 7: Ag+IFA2). Bleeding procedures were performed periodically (pre-immunization to week 12) for serum collection. Antibodies were purified using the SAS (saturated ammonium sulfate precipitation) method to obtain the IgG fraction.

For immunogenicity test, indirect ELISA is used to determine antibody titers. A microtiter plate is coated with NT-proBNP antigen (50 µL, inactivated in coating buffer pH 9.6) and incubated overnight. After blocking with 1% BSA, rabbit serum containing polyclonal antibodies is added and incubated for 2 hours at room temperature. This is followed by alkaline phosphatase-labeled anti-IgG (1:2500), then PNPP substrate (1 mg/mL) is added. The reaction is stopped with 1N NaOH and the absorbance is read at a wavelength of 405 nm.

Data Analysis

ELISA absorbance values were analyzed to determine the immune response (antibody titer) produced. Immunogenicity effectiveness was determined based on absorbance values relative to baseline.

Result and Discussion

Result

In this study, three white Oryctolagus cuniculus rabbits were used as research subjects. They were male and two years old. Two rabbits were used to obtain NT-pro BNP immunization treatment in this study, and one rabbit was used as a control. A total of 300 µL of NT-pro BNP antigen was added to 100 µL of CFA adjuvant (ratio 3:1) and vortexed at full speed for approximately 2 hours to form a perfect emulsion. The injection volume for each rabbit was 400 µL. At this point, the actions that had been carried out in week I included: pre-immunization bleeding, CFA immunization, IFA 1, and bleeding 1, which would later be continued until week 12. Antibody levels against NT-pro BNP were measured using ELISA, read at a wavelength of 405 nm.

Measurement of NT-pro BNP antibodies shows that the NT-pro BNP antigen present in patient serum is antigenic and capable of triggering antibody production. The highest titer of polyclonal antibodies obtained from induced NT-pro BNP protein was measured using the ELISA method. This method can detect polyclonal antibodies from the blood collection process that requires the highest titer (Figure 1). The graph shows the anti-NT-pro BNP titer in rabbit blood serum ten times after bleeding induced with NT-pro BNP protein. It was found that the concentration of anti-NT-pro BNP increased gradually from the first week to the fourth week and reached its highest level after the booster. The highest titer indicates an increase in the number of antibodies as a primary immune response. The rabbit immune system recognizes the human NT-pro BNP antigen, so the immune response continues to synthesize specific antibodies to form anti-NT-pro BNP.

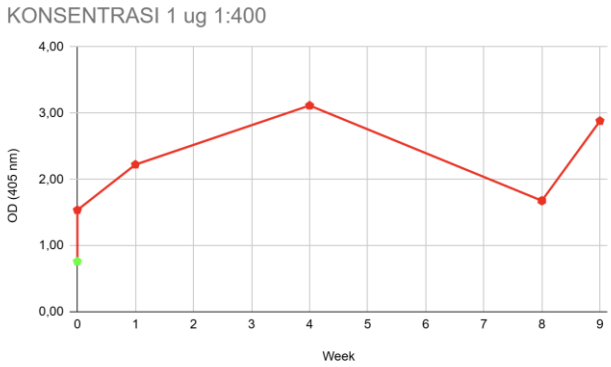


Figure 1. Antibody Titer Graph Against NT-pro BNP Results from Indirect ELISA

Boosters were administered in weeks 3 and 7, and in the ninth bleeding process, an increasing trend was observed in NT-pro BNP antibody titers. This increase in absorbance is a secondary immune response, in which B cells are reactivated to produce anti-NT-pro BNP after rabbits are re-immunized with NT-pro BNP and incomplete adjuvant.

Antibody concentrations peaked as a manifestation of the primary immune response. Memory B cells involved in the secondary immune response showed more progressive proliferation compared to naive B cells. This phenomenon results in accelerated secondary immune responses accompanied by a significant increase in antibody production. Evaluation of antibody titers in the fifth week indicated a significant decrease compared to the fourth week. This degradation can be attributed to the termination of anti-NT-pro BNP synthesis, which is a component of the primary immune response, followed by the catabolism of primary antibodies in the rabbit circulatory system.

Quantitative analysis using the ELISA method validated that human NT-pro BNP has immunogenic characteristics that include humoral and cellular immune responses. The induction of these immune responses resulted in the formation of specific polyclonal anti-NT-pro BNP antibodies in rabbit serum, which is a consequence of the antigen's capacity to induce a cascade of immune system responses. This phenomenon confirms the immunogenicity of human NT-pro BNP, which is capable of triggering the activation of the body's defense system through specific antigen recognition mechanisms.

Discussion

Heart failure is one of the leading causes of cardiovascular morbidity and mortality worldwide, including in Indonesia. Early diagnosis of heart failure is often hampered by limited access to specific biomarker tests such as B-type Natriuretic Peptide (BNP) and N-terminal pro-B-type Natriuretic Peptide (NT-proBNP), which require laboratory facilities with advanced immunochemical equipment such as electrochemiluminescence immunoassay (ECLIA) (Asta et al., 2025). In fact, these biomarkers have high diagnostic and prognostic value, as NT-proBNP levels increase significantly with increased ventricular wall pressure and myocardial stress (Yancy et al., 2013). Therefore, the development of Point-of-Care Testing (POCT) based on antibodies against NT-proBNP is a strategic step to expand the coverage of heart failure screening, especially in areas with limited laboratory facilities.

Biologically, NT-proBNP is an inactive fragment of the prohormone BNP produced by ventricular cardiomyocytes in response to cardiac wall stretching (Daniels et al., 2007). The NT-proBNP molecule is relatively stable in circulation compared to active BNP, as it has a longer half-life (60–120 minutes vs. 20 minutes) and is resistant to proteolytic degradation (Clerico et al., 2011). This makes it an ideal target for immunological detection. In the context of diagnostic kit development, the NT-proBNP antigen can be used to induce an immune response in experimental animals such as rabbits, to produce polyclonal antibodies that have a high affinity for the immunogenic epitopes of the protein.

Preliminary findings demonstrated that recombinant NT-proBNP antigen could be successfully expressed and purified using an Escherichia coli expression system. The expressed protein showed a molecular weight within the expected range of approximately 8.5 kDa, consistent with the predicted molecular mass of recombinant human NT-proBNP reported in the literature (Tarmizi et al., 2014). After repeated immunization with purified recombinant NT-

proBNP antigen, rabbit serum showed a significant increase in anti-NT-proBNP antibody levels as determined by indirect ELISA, indicating successful induction of an antigen-specific humoral immune response. This finding is consistent with the study by Lee et al., (2021) which demonstrated that recombinant NT-proBNP antigen could generate highly reactive antibodies and showed favorable antigen–antibody binding performance in ELISA-based screening and plasma samples from patients with heart failure. This binding pattern is important for diagnostic validity, especially when the developed kit is designed in a sandwich immunoassay format on a lateral flow device, where sensitivity is highly dependent on the capture antibody and detection antibody pair (Posthuma-trumpie & Korf, 2009).

Several similar studies have developed NT-proBNP-based point-of-care testing platforms, including fluorescence immunochromatographic assays. Li et al., (2016) demonstrated that a fluorescence immunochromatographic assay achieved a low limit of detection of 11 pg/mL and a wide linear detection range of 20–30,000 pg/mL, indicating strong analytical performance and potential applicability for rapid heart failure screening compared with conventional laboratory-based immunoassays. However, commercial products are still dominated by imported technologies (e.g., Roche, Abbott, and Alere), with relatively high prices and cold chain requirements that limit their use in resource-constrained areas. Therefore, the development of local antibodies against NT-proBNP has two main advantages: (1) it supports national diagnostic independence and (2) it allows for the adaptation of kits to Indonesia's tropical climate, which can affect reagent stability.

In this study, the identification of the immunogenicity of the NT-proBNP protein opens up opportunities for the next stage of development, namely the formulation of a rapid, easy-to-use lateral flow immunoassay (LFIA)-based detection kit that can be applied in primary care. Such prototypes have been proven globally to accelerate the screening of chronic diseases, as they only require <15 minutes and do not require complex laboratory equipment (Posthuma-trumpie & Korf, 2009).

Challenges that still need to be further evaluated include optimizing antigen purity, increasing antibody affinity through specific IgG fraction purification, and clinical validation of heart failure patient samples with varying degrees of severity (NYHA I–IV). Environmental factors such as temperature and humidity also affect reagent stability, as reported by Zhang et al., (2023) that storing antibodies at temperatures >30°C for 7 days reduced reactivity by up to 20% (Zhang et al., 2023). Therefore, buffer and stabilizer formulations must be adjusted for tropical conditions. Thus, the development of an anti-NT-proBNP antibody-based heart failure detection kit is an important step toward expanding screening of high-risk populations in Indonesia, reducing diagnostic delays, and strengthening the capacity for domestic diagnostic biotechnology innovation. The success of the identification and exploration of NT-proBNP immunogenicity serves as the foundation for developing a national POCT prototype that has the potential to be commercialized and integrated into early heart failure detection programs in primary healthcare facilities.

After immunizing rabbits with NT-proBNP antigen combined with CFA/IFA adjuvant, the immune response formed showed a classic pattern of humoral immunity activation. Based on the indirect ELISA results, there was a progressive increase in absorbance from the first week to the fourth week, reaching a peak after the booster (week 7). This phenomenon indicates that the first immunization with CFA triggered a primary immune response, characterized by the activation of naïve B cells and the formation of low levels of antibodies. Boosters with IFA in weeks 3 and 7 induced a secondary immune response, in which memory B cells underwent rapid proliferation, producing antibodies with high affinity for NT-proBNP. This pattern is consistent with the kinetics of polyclonal antibody production commonly found in rabbit immunization (Deluca et al., 2022). Increased absorbance values in ELISA reflect increased concentrations of anti-NT-proBNP specific antibodies in serum, suggesting that NT-proBNP is immunogenic and capable of inducing significant polyclonal antibody formation. These results support the initial hypothesis that NT-proBNP protein can be used as a target antigen for the development of antibody-based point-of-care testing (POCT) kits.

Immunologically, the polyclonal antibodies produced from this immunization have the advantage of multi-epitope binding capacity to the NT-proBNP antigen, thereby increasing detection sensitivity. This is highly relevant for the development of lateral flow immunoassay (LFIA) methods, which require antibodies with high affinity and the ability to recognize antigen conformation variations in biological samples (Zhang et al., 2023). The consistency of ELISA results for NT-proBNP indicates that the antibodies produced have sufficiently high titers and specificity. Based on the significant increase in absorbance pattern after the second booster, antibodies can be said to reach optimal protective titers in weeks 7 to 9. This point is the basis for the ideal time to collect serum with maximum titers for IgG antibody purification.

This immunogenicity test proves that human NT-proBNP can trigger the activation of the rabbit immune

system through the recognition of certain antigenic conformational epitopes. This activation includes a humoral immune response with the production of specific antibodies that can be used to detect NT-proBNP antigens in biological samples from heart failure patients. These findings are also consistent with a report by (Lee et al., 2021). stating that NT-proBNP is a stable target with linear epitopes that are easily recognized by immunized animal antibodies.

The results of this study have direct implications for the development of a rapid detection kit prototype based on anti-NT-proBNP antibodies. With the proven immunogenicity of the NT-proBNP protein and the successful production of polyclonal antibodies, the next stage can focus on antibody purification and characterization (using Protein A/G affinity chromatography and cross-reactivity testing); construction of a rapid detection system, for example using a lateral flow assay method compatible for screening in primary health facilities; and clinical validation testing of serum from patients with echocardiographically confirmed heart failure diagnoses, to determine diagnostic sensitivity and specificity values. In the Indonesian context, this approach can reduce dependence on imported reagents and expand the coverage of heart failure screening in primary care, especially in areas with limited advanced diagnostic facilities.

Several limitations to note include: affinity testing or epitope mapping has not been performed to determine antibody specificity, and quantitative data on NT-proBNP levels in patient serum have not been correlated with immunogenicity results. Further research is recommended to include cross-reactivity testing against BNP homologous proteins, clinical trials of rapid detection kits, and optimization of antibody conjugation with gold nanoparticles (AuNP) for lateral flow immunochromatography systems.

The results of this study show that NT-proBNP protein isolated from human serum has high immunogenicity and is capable of inducing the formation of anti-NT-proBNP polyclonal antibodies in rabbits. The immune response shows an increase in antibody titer after booster, indicating effective immune memory formation. The antibodies produced have the potential to be developed into the main component of a rapid diagnostic kit for heart failure screening in Indonesia, which is more economical and easier to use than conventional laboratory methods such as ELISA or echocardiography.

Conclusion

In conclusion, NT-pro BNP protein is immunogenic, with the highest production of antibodies occurring after the booster. These findings confirmed that NT-proBNP can serve as an effective antigen for antibody-based detection systems.

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Author Contributions

Andreas Budi Wijaya: Conceptualization, Methodology, Project Administration, Supervision, Writing, Review & Editing.
Dina Fauziah: Methodology, Validation, Formal Analysis, Supervision, Writing, Review & Editing.
Karina Nilasari: Resources, Supervision, Writing, Review & Editing.
Editya Fukata: Funding Acquisition, Resources, Supervision, Writing, Review & Editing.
Dyta Loverita: Validation, Formal Analysis, Supervision, Writing, Review & Editing.
Farsya Hidayah Septiananda: Investigation, Data Curation, Formal Analysis, Writing Original Draft.
Taurisma Aulia Nanda Wibisono: Investigation, Visualization, Data Curation, Writing Original Draft.
Rajendra Aulya Gilardino: Investigation, Validation, Formal Analysis, Writing Original Draft.

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Conflicts of Interest

The authors declare no conflict of interest.

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