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Recombinant Protein Production from TPO Gen Cloning and Expression for Early Detection of Autoimmune Thyroid Diseases

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Abstract. Autoimmune Thyroid Disease (AITD) is an autoimmune disease that has many clinical symptoms but is difficult to detect at the onset of disease progression. Most thyroid autoimmune disease patients are positive with high titre of thyroid autoantibodies, especially thyroid peroxidase (TPO). The detection AITD are still needed because these tests are extremely high cost and have not regularly been performed in most of clinical laboratories. In the past, we have explored the autoimmune disease marker and it has been developed as source of polyclonal antibodies from patient origin. In the current study, we develop recombinant protein which resulted from cloning and expression of TPO gene from normal person and AITD patients. This work flows involves: DNA isolation and PCR to obtain TPO gene from human blood, insertion of TPO gene to plasmid and transformation to *E. coli* BL21, Bacterial culture to obtain protein product, protein purification and product analysis. This products can use for application to immunochromatography based test. This work could achieved with the goal of producing autoimmune markers with a guaranteed quality, sensitive, specific and economically. So with the collaboration with industries these devices could be used for early dete

Keywords: recombinant protein, TPO gene, Autoimmune thyroid diseases (AITD)ction of the diseases in the community.

1. Introduction

Autoimmune thyroid disease (AITD) is a disorder in the activity of the thyroid gland. Its most commonly found in women, and is still a problem that has not been addressed comprehensively because of delays in diagnosis. The incidence of AITD in the world is reported to be more prevalent in adult women than in men, and affects approximately 2-5% of the world's population [1]. (Based on US and European population screening data, AITD reportedly found in 27% of adult women with increasing frequency at age above 50 years and only 7% occurred in adult males [2]. Ministry of Health the Republic of Indonesia in 2013 indicates that thyroid abnormalities still occupy the top 5 non-communicable diseases in Indonesia, with prevalence of about 0.4-0.7% [3]. Manifestations

and complications caused by autoimmune thyroid abnormalities have been widely reported, especially during pregnancy will produce poor outcomes for the mother and foetus [4,5,6].In order to support one of Indonesian's government program of Sustainable Development programs (SDG's), Improving maternal and child health quality is implemented by early detection and treatment of AITD. Early detection and good treatment of pregnant women with AITD will manifest to improved fetal growth. Pregnant women with AITD rarely show typical clinical symptoms, making it difficult to identify early in pregnancy. If not treated early enough will increase the risk of miscarriage and preterm birth [7], low birth weight [6], as well as manifestations of disability and impaired fetal brain development [4]. Therefore, a proper routine screening is needed to identify potential pregnant women with AITD, so that early care can be undertaken by a doctor prior the manifestation of maternal and fetal health occurs.

In the blood serum of patients with AITD found several antibodies that can be used as a serological marker, such as thyroid peroxidase (TPO), thyroid stimulating hormone receptor (TSHR) and thyroglobulin (TG). The formation and enhancement of autoantibodies titre to both TPO and TSHR appear to be more dominant and directly involved in AITD patho-mechanism. Antibodies titre to TPO and TSHR can be detected earlier before alteration of T3 and T4 hormone levels. It is appropriate to monitor both hypothyroid and hyperthyroid conditions, which is generally without any specific signs that appear. This phenomenon supports to create a design for early diagnosis in the phase of pre-disease of AITD to determine the progression of the disease towards prevention as well. A tool to diagnose the disease at early stage is more focused to detect the presence of autoimmune markers of TPO, which has a specificity and sensitivity better than available commercial detection kit. This work proposes to produce recombinant protein of thyroid peroxidase (TPO) as a rapid test material for early detection AITD.

2. Methods

This study has been running for more than 15 years to look for markers of autoimmune diseases and develop it into a Rapid Test, conducted at several laboratories in UB, which has been certified by ISO and KAN. The uses of a sample of human blood were approved by the Research Ethics Committee, Brawijaya University No. 109/EC/KEPK/04/2012).

2.1. Chemical and sample collection

Sample was taken from 20 of patient's blood serum which had been confirmed with gold standard of Mab-human Thyroid peroxidase (TPO), phosphate buffer pH 7, phenimethylsulfonil fluoride (PMSF), sodium etilene diamineacetate (NaEDTA), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate (Na_2HPO_4), glycerol, sodium hydroxide (NaOH), chloric acid, ammonium sulphate ($(NH_4).2SO_4$), Barium Chloride ($BaCl_2$), Tris-Cl, Tween-20, polyacrilamide, bis-acrylamide, Comassie-blue R-250, Sephadex-G75, tris-glycine buffer, pottasium carbonate, TPO gene primer; Forward TPO: 5' ATG GAATTC ATG GCTTCTTGTACAACGGGTTC 3' and Reverse TPO: 5' ATG GTCGAC TTA GTCGTGGTC GATGTATTGTC 3', PstI restriction enzyme, DNAeasy blood and tissue kit (Qiagen), agarose, Oneshoot® cell, Super Optimum Broth medium with catabolite repression (SOC) Medium, Luria Berthani medium and agar Medium from Oxoid, *E. coli BL21* previously preserved in our laboratory, pQE-T4, distilled water are used throughout this works.

2.2. Instruments

Centrifuge used was Tomy High Speed Sentrifuge MX-305, horizontal electrophoresis used from Biorad, miniprotean electrophoresis used from Biorad electrophoresis, Biorad-Mini transblot, thermocycler used C1000 thermal cycler from Biorad, and Shaking Waterbath from Memmert, Biologic Duolflow from Biorad, and Gel documentation from Biostep.

2.3. DNA isolation and PCR to obtain TPO gene from human blood

DNA isolation was conducted based on protocol of DNAeasy blood and tissue kit (Qiagen). Then it was continued with amplification of DNA target gene with programs of: pre-denaturation at 95 ºC for 7 min; 35 cycles of denaturation at 95 ºC for 30 sec, annealing on 60 ºC for 20 sec, and elongation at 72 ºC for 30 sec, then it was maintained at 60 ºC. The gene encoding TPO was then separated by PstI enzyme, then it was analyzed with agarose gel electrophoresis. The used of human samples for this research were approved by Ethical Committee of Faculty Medicine Brawijaya University and were completed with inform consent (No. 109/EC/KEPK/04/2012).

2.4. Insertion of TPO gene to plasmid and Transformation to E. coli BL21

2 µL of PCR product was mixed with a 0.50 of salt solution and 0.50 µL of pQE-T7 vector, then it were blended carefully and incubated for 5 min at room temperature (22-23 ºC). Before the transformation process, the waterbath was set at 42 ºC, S.O.C medium was warmed to room temperature, then the tube then be applied was warmed to 37 ºC for 30 min. 1 vial of Oneshot® cell then immersed in ice for each transformation. 2 µL of pQE T-7® cloning reaction from the previous stage then was added to the One Shot® then mixed slowly, then it was incubated in ice for 5 to 30 min.

The cells were given a heat shock for 45 seconds at 42 ºC without being shaken, then the tube was transferred to ice for 2 min, added 250 µL S.O.C. medium at room temperature. Tubes were closed tightly and centrifuged at 200 rpm. A total of 10-50 µL of the transformed was cultured on a selectively preheated agar plate and incubated overnight at 37 ° C. To ensure even small breeding, 20 µL added S.O.C. medium.

2.5. Bacterial culture to obtain protein product

400 µL of LB medium was acclimatized at 37 C for 1 h, then it was added with 100 µL cells which passed of colonic selection. Then it was incubated at 37 ºC with at 250 rpm shift speed, 24 h.

2.6. Protein purification and product analysis

Competent cell culture media added with PBST containing 4mM PMSF as much as 5 times the volume. The homogenized sample was then centrifuged at 6000 rpm, 4 ºC for 15 min, and supernatant was collected. Supernatant then was added with cold ethanol with a ratio of 1:1, kept for 24 h in the refrigerator. Then it was centrifuged at 10,000 rpm for 10 minutes at 4 ºC. Ethanol then was removed, the precipitate was dried and added with Tris Cl buffer of 50 µl. The recombinant TPO protein iwas stored in -20 ° C. To obtain TPO recombinant protein with uniform size then the recombinant product was purified using Biologic Duo Flow Biorad

2.7. Application to immunochromatography based test

The application of recombinant TPO to reverse-slow immunochromatography based test is conducted based on this schematic ilustration in Figure 1. This components consist of 2 (two) main parts sections are test area which consits of polyester membrane, nitrocellulose membrane to place the test components are control line (goat anti mouse IgG) as well as test (recombinant protein of TPO) and sample pad. The observation area is consist of absorbent paper and signal reagent area to put signal reagent and buffer to let the immunochromatography system works.

Rapid tests are generated based on the concept in Figure 1. The series of detection devices to be used using nitrocellulose membranes, polyester membranes and adsorbent paper on the plastic base. Nitrocellulose membrane was used as a place of attachment antigen (recombinant human protein TPO) and the control line (goat anti mouse IgG), on the top and bottom membranes attached polyester with a width of 0.5 cm, to cover the nitrocellulose membrane. Antigen attached to a linear manner over the nitrocellulose membrane as a test line. while goat anti-mouse IgG attached to a nitrocellulose membrane as a standard or control lines. A total of 20 microliters of serum sample is dripped onto polyester pads near the control zone. Then dropped as much as 10 microliters of a solution of colloidal

gold on polyester over the observation window and dried. A total of 1 drop of buffer solution is dripped onto the same pads and 2 drops of buffer on a polyester cushion coloidal gold signal reagent, then incubated a few moments to penetrate the sample and buffer boundaries. Test card immediately closed and the observed results of the examination of the observation window 20 to 30 minutes later. A positive result achieved if it appears 2 red lines on the control line and a test line. Negative results achieved if the red lines appear first on the control line. The results appear invalid if tidal lines at all or only a line appears on the test [10,11].

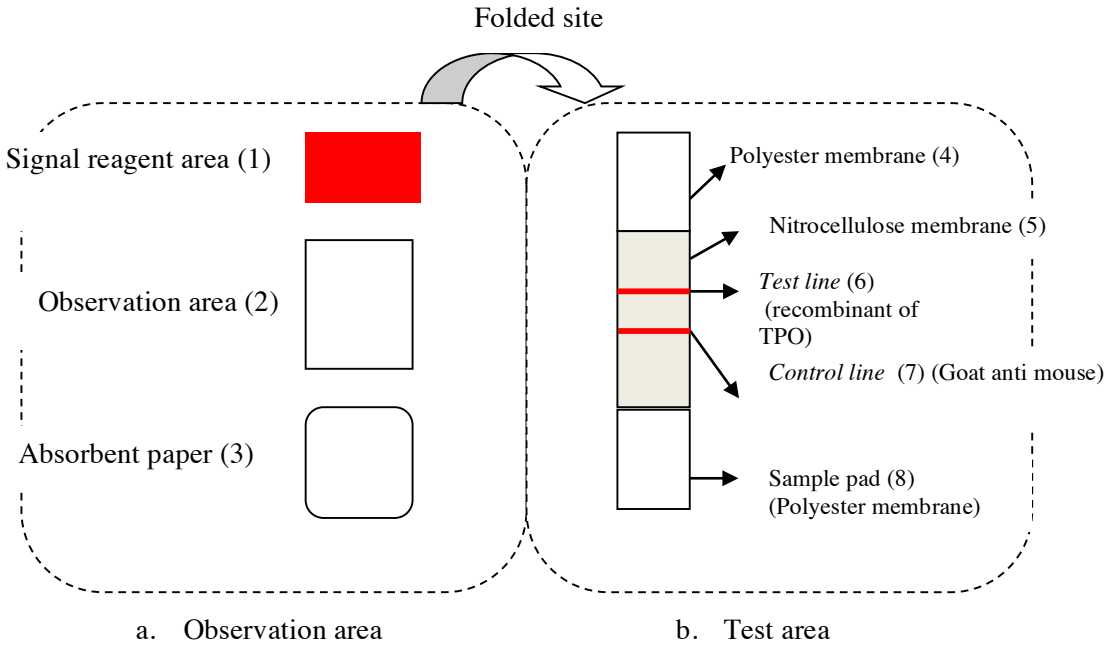

Figure 1 Schematic illustration of reverse flow-immunochromatography based test for detecting TPO antibody from sera

3. Result and discussions

A pair of primers used to detect the high-sensitizing protein coding of TPO gene in DNA of AITD patients by PCR method, and below is PCR product resulted from Forward TPO: 5' ATG GAATTC ATG GCTTCTTGTACAACGGGTTC 3' and Reverse TPO : 5' ATG GTCGAC TTA GTCGTGGTC GATGTATTGTC 3'. TPO gene characterization by PCR method that has a gene length of ± 700 bp (Figure 2).

This amplification process plays a role in the provision of TPO gene encoding as the main antigenic substance in the test in the form of recombinant proteins. The plasmid choosen in this work also have a role in gene transcription while the gene were inserted and transporter to cells. The principal components of plasmid vector having influence to gene transcription include the origin of replication (ORI), promoter, antibiotic selection marker and transcription terminators [8]. Below the schematic illustration of plasmid reconstruction to obtain TPO gene insertion area (Figure 3).

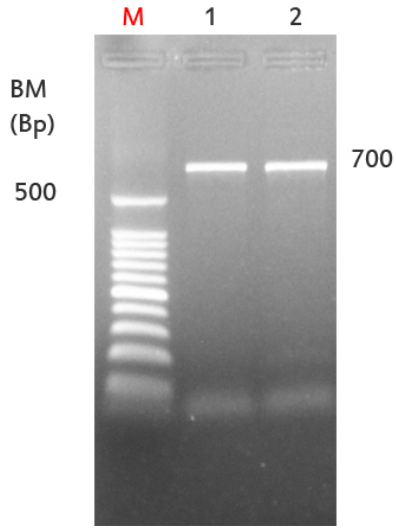

Figure 2 PCR product of TPO gene amplification (Agarose 2%); M: Marker, C: 1-2: sample

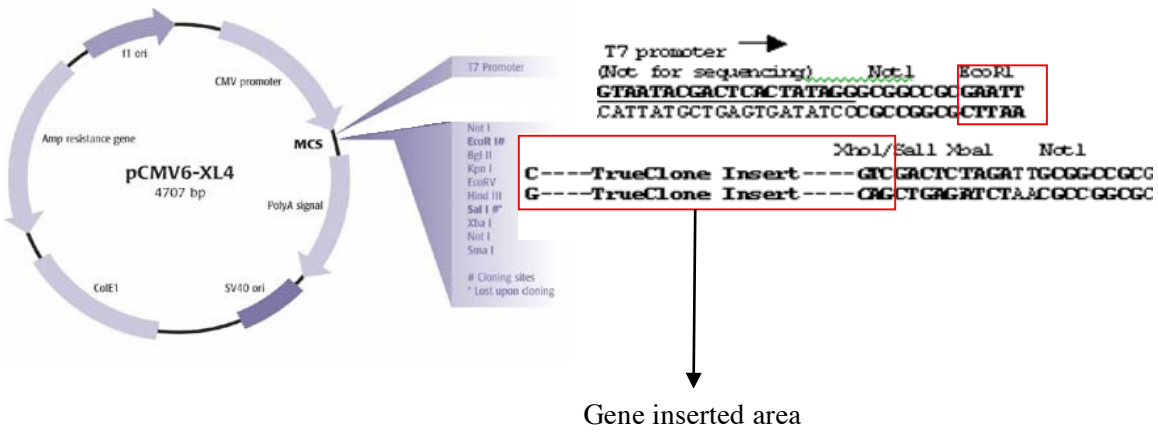

Figure 3 Plasmid reconstruction for TPO-gene insertion

After the plasmid reconstruction, TPO gene is inserted into the Plasmid vector with DNA ligase. After the insertion is successful then the Plasmid is transformed to a competent BL / 21 cell for the cloning process. After a competent cell was transformed, it was continued for selection with IPTG and X-GAL administration to obtain white and blue colonies. Recombinant protein then was isolated from competent cells of *E coli* BL21 then it was characterized with Western Blotting. Then the protein with molecular weight approximately 51-52 kDa were purified to apply to immunochromathography test strip [9].

The main advantage of the immunochromatography method is the simplicity of the test, which mixes the specimen with complex particles with antibodies, inserts the mixture into the membrane and waits for several minutes (10-15 minutes) until the chromatographic process is complete [12].

4. Conclusion and future works

This work succesfully obtain rapid diagnostic kit to detect TPO antibody with have more advantage over the commercial kit available in market. The further studies for clinical test to obtain specificity and sensitivity are needed to implement as well the kit stability test.

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