IDENTIFICATION OF Mycobacterium tuberculosis ADHESION PROTEIN IN SPUTUM OF TUBERCULOUS PATIENTS

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ABSTRACT

The pulmonary tuberculosis is an endemic disease in Indonesia. Pulmonary tuberculosis diagnosis at this moment confirmatory using clinical manifestation with detection and identification mycobacteria. Detection and identification of Mycobacteria with culture method, examination microscopy and molecular detection with DNA or RNA Mycobacteria. Pendekatan diagnosis tuberculosis paru secara serologis menggunakan berbagai macam antigen. Adapun tujuan umum dari penelitian ini adalah untuk mengetahui deteksi Protein Pili pada s-IgA pada sputum penderita tuberculosis dengan pemeriksaan Immunoblotting sedangkan tujuan khusus dari penelitian ini adalah mengetahui berat molekul Protein Pili dan mengetahui sensitivitas dan spesifisitas Protein Pili dengan menggunakan metode Immunoblotting. Pada penelitian ini digunakan Pili Mycobacterium tuberculosis sebagai antigen dan s-IgA (secretory IgA) sebagai antibodi untuk diagnosis tuberculosis paru. Pada penelitian ini dilakukan isolasi Pili dan menentukan berat molekulnya dengan elektroforesis SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). Hasil penelitian ini ditemukan Pili Mycobacterium tuberculosis yang merupakan molekul hemaglutinin dan didapatkan band (pita) berat molekul Pili sebesar 63,63 kDa yang direspon oleh s-IgA. Selanjutnya dilakukan uji pada 96 sampel sputum pasien yang tersedia yang dicurigai menderita tuberculosis paru dengan metode Immunoblotting dan dengan table Mc.Nemar hasinya didapatkan sensitivitas 71,42% dan spesifisitas sebesar 63,93% pada cut-off (titik potong) 121,24.

Keyword: adhesin, hemaglutinin, sIgA, Pili, M. tuberculosis

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INTRODUCTION

Tuberculosis is a disease caused by the bacterium Mycobacterium tuberculosis. Commonly infect the lungs, although it can also infect other organs. Other properties of bacteria are aerobic. These properties indicate that the germ is more like a network of high oxygen content. In this case the pressure of the lung apex is higher than in other parts, so that the apex is a place of predilection of tuberculosis. Tuberculosis remains a health problem in the world today (Gilbert 2003).

In Indonesia, tuberculosis is an endemic disease re-emerged as the leading cause of death after heart disease and respiratory tract. The number of TB cases were found to increase significantly in recent years. Figures discovery of new smear positive cases increased from 38% in 2003 to 54% in 2004. The incidence of smear positive (infectious) in 2005 estimated 107 new
cases/100,000 population (246,000 new cases each year) and prevalence of 597,000 cases in all cases. In Indonesia, it is estimated that there are approximately 583,000 new pulmonary TB patients who appear every year and 140,000 of them died from the disease every year. In the province of Jakarta in 2003 the TB cure rate is still below the national target (<85%) (Paradise 2007).

Tuberculosis is caused by a bacterial pathogen Mycobacterium tuberculosis complex (TBC) consisting of M. tuberculosis, M. africanum, and M. bovis. Including M. microti and M. canettii rare. Differentiation of Mycobacterium tuberculosis complex is very important to do because they relate to the success of patient therapy and for epidemiological purposes, especially in areas where tuberculosis has become an epidemic and is a major health problem for humans (Paul 2003).

M. tuberculosis is inhaled into the lungs and be ingested by alveolar macrophages, the macrophages will then perform three important functions, namely: 1) produce proteolytic enzymes and other metabolites that have the effect miko-bactericidal, 2) produce soluble mediators (cytokines) in response to M. tuberculosis in the form of IL-1, IL-6, TNF (Tumor Necrosis Factor alpha), TGF (Transforming Growth Factor beta) and 3) process and present mycobacterial antigens to T lymphocytes. Cytokines produced by macrophages have the potential to suppress the effects immunoregulator and cause clinical manifestations of tuberculosis. IL-1 are endogenous pyrogens cause fever as a characteristic of tuberculosis. IL-6 increases the production of immunoglobulins by B cells are activated, causing hyper-globulinemia are often found in patients with tuberculosis. TGF function together with IFN to increase the production of nitric oxide metabolites and kills bacteria and is required for granuloma formation to cope with mycobacterial infection. Besides TNF can cause pathogenesis effects such as fever, weight loss and tissue necrosis that are characteristic tuberculosis. TNF in patients with tuberculosis also serves to enhance T cell susceptibility to apoptosis either spontaneously or by stimulation of M. tuberculosis in vitro. IL-10 inhibits cytokine production by monocytes and lymphocytes, whereas TGF suppress T cell proliferation and inhibit macrophage effector functions (King 2004).

Early diagnosis of M. tuberculosis infection is often based on clinical data, but to establish a definitive diagnosis required the isolation and identification of M. tuberculosis laboratory. Limitations of this culture requires a long time and not at all sufferers can be obtained a positive result, then clearly we need a diagnostic tool to detect active TB, especially in developing countries (Nugraha 2007).

An understanding of the mechanisms and bacterial factors responsible for the ability of M. tuberculosis in causing disease in humans is important for the development of increased treatment strategies. Many bacterial pathogens use pili as adherence factors for the colonized host. Found that M. tuberculosis produce something that is fine (width of 2 to 3 nm), fused, flexible pili that are recognized by IgG antibody-containing serum obtained from patients with active tuberculosis, indicating that the bacteria produce pili or pili associated with antigen during human infection (Cristopher 2007).

MTP Association (Mycobacterium tuberculosis Pili) into the extracellular matrix protein laminin in vitro, assuring that MTP possess adhesive easily. Mutants lost the ability to produce Isogenitik MTP MTP in vitro and showed reduced laminin binding ability. Part MTP in morphology, biochemical, and functional properties associated with bacterial pili. Therefore, it was shown that MTP are previously unidentified as host colonization factors of M. tuberculosis (Cristopher 2007).

Many of mycobacterial antigens have been identified either semipurified or purified and used as immunodominant commercial kits such as antigen protein 16kDa, 24 kDa, 38 kDa, Esat-6, but less sensitivity. Protein is an immunodominant 38 kDa lipoprotein antigen which is the fifth component of the antigen with an affinity that is specific only to chromatografi Mycobacterium tuberculosis-complex (Senoi 2007).

Use of Mycobacterium tuberculosis antigen by dot blot method on several previous studies provide information for further diagnostic techniques. Secretory IgA (mainly sIgA2) is immunoglobulin the airway mucosa. IgA provides protection against harmful antigens, and IgA can cause a systemic response through the role of cell surface receptors on macrophages and leukocytes. IgA mainly plays a role in mucosal defense.

This study is exploratory and experimental laboratories, including isolation of antigens of Mycobacterium tuberculosis strains H37Rv and sputum IgA from patients infected with M. tuberculosis. Nitroselulose membrane (NC) was cut 7.5 x 11 cm and soaked first in sterile H2O for 30 minutes. Then mounted on the tool immunoblotting. Through hole devices, the membrane that has been moistened with TBS, soaked with antigen 50? L (in tris buffered saline pH 7.4), incubated overnight at 4? C until completely absorbed antigen into the NC membrane. Then performed blocking with blocking buffer TBS (containing 50 mM Tris base, 0.2 M NaCl, 0.5% skim milk pH 7.4), incubated overnight at 4? C, blocking solution was discarded. The next stage
in the membrane drops sputum were tested by 50? L, incubated for two hours at room temperature and placed on a shaker. Solution was discarded, and then washed three times with TBS-0.05% Tween-20. Secondary anti-mouse Ab was added to the dilution 1: 2500 in tris saline solution, incubated at room temperature for one hour, on a shaker. Washed again three times with TBS-0.05% Tween-20. Subsequently incubated at room temperature for 30 minutes. The reaction was stopped by adding H2O.

Initially, M. tuberculosis H37Rv grown in 7H9-OADC-Tw at 37 °C with vibration for 48-72 hours. At the beginning of the culture used for inoculation of 7H11 to plate 100, as a medium, modified by the expenditure of OADC and incubated for 2 weeks as described above. Bacteria were using a sterile glass spreader and diluted into 200 ml PBS. Dilution is divided into 25-ml aliquots, and pili are cut mechanically from the bacterial surface with powerful vortexing for 5 minutes in a 50 ml conical tubes containing 1 cc of sterile 3 mm glass beads. After cutting, the suspension of bacteria which had been centrifuged at 3000 rpm for 1 hour, and the resulting supernatant was collected. Pelleted bacteria were washed with PBS to obtain more pili, and the supernatant collected. Remaining bacterial cells and debris cleared by two centrifuge at 3000 rpm, followed by centrifuge at 18,000 rpm for 10 minutes. Contamination of fat has been removed after mixing with a volume ratio of chloroform / methanol (2:1) for one hour. After centrifuged at 18,000 rpm for 30 min to partition the 35-ml centrifuge tubes and liquid fractions Interphase carefully collected and extracted two more times, and soluble material (lipids) in the organic solution discarded. Finally the liquid and the fraction of interphase duiultrasentrifus at 120 000 rpm for 3 hours at 4 °C in the Ti-56 fixed angle rotor. The result MTP-pellets that have been diresuspensi in PBS and analyzed by 16% SDS / PAGE (49) or Tricine-PAGE. Protein concentration determined by protein assay (Cristoper 2007).

Immunoglobulin (antibody) was isolated from the sputum of patients suspected of being infected M. tuberculosis. Sputum of patients added to PBS containing 4 mM PMSF and 0.02% NaN3 with a ratio of 1:1, vortex until homogeneous and then centrifuge at 3000 rpm, 15 min, 4oC. The pellets formed discarded. Supernatant was carried out precipitation with cold absolute ethanol, 4oC overnight, and then centrifuge 12,000 rpm, 15 min, 4oC. Formed supernatant discarded. The pellets are stored in Buffer 0.5 M Tris-Cl; pH 6.8;-40oC.

After data collection was completed, a tabulation of diagnostic tests with the gold standard examination (sputum smear, SPS) with a standard table dummy. To get research results s-IgA used Corel Photoplain12 program to obtain accurate data about the thickness of thin color on nitrocellulose membrane. Computer program was obtained from data in the form of the mean, standard deviation, median, and pixels. Clinical outcome can be known about how the sensitivity and specificity of this research.

RESULTS

Figure 1. The results stain a positive smear

Figure 2. Electrophoregram Pili by staining kromasi

Figure 3. The results of hemagglutination with erythrocytes Pili Mice

Identification of acid-resistant bacteria carried by acid-fast staining Ziehl-Neelsen stain on sputum after homogenization and decontamination processes. The results of staining seen rod-shaped bacteria (bacilli) red with a blue background. The procedure at this stage
aims to determine the band profile Pili antigens. Profile antigen bands that emerged then calculated molecular weight by calculating the correlation regression compared with markers. Hemagglutination test whether pili aims to receptors on red blood cells found on the surface of red blood cells.

Figure 4. The results of immunoblotting Crude Pili Mycobacterium tuberculosis with s-IgA antibodies in the sputum of patients

Table 1. Calculation of sensitivity and specificity MTP with s-IgA

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<tbody>
<tr>
<td>79.63</td>
<td>22.85</td>
<td>90.16</td>
</tr>
<tr>
<td>93.91</td>
<td>42.85</td>
<td>81.96</td>
</tr>
<tr>
<td>121.24</td>
<td>71.42</td>
<td>63.93</td>
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</tbody>
</table>

Figure 5. Calculation of cutoff with ROC curve

This test also can find the optimal levels of pili that can react with the antibody on the surface of red blood cells that can agglutinate red blood cells. In the present study used blood cells of mice. Immunoblotting test conducted to test whether Pili Mycobacterium tuberculosis sensitive and specific to the s-IgA are present in the sputum of patients. Carried out on 96 sputum samples of patients suspected of suffering from pulmonary tuberculosis and is obtained from dr.Saiful Anwar Hospital Malang. Presentation of the results of sensitivity and specificity immunoblotting with Mycobacterium tuberculosis Pili blotter tool with s-IgA from the sputum of tuberculosis patients with primary antibodies. Calculation of cut-off (cut point) is done by looking at the coordinates of points on the y-axis sensitivity and 1-specificity on the x axis.

DISCUSSION

Phase I study aims to identify Mycobacterium tuberculosis Pili and to determine their molecular weight. Mycobacterium tuberculosis bacteria that have been cultured in Middlebrook medium was taken and carried out by mechanical isolation, whereas pili were isolated using a method that is chemically Cristopher (2007). Pili molecular weight measurements performed by the method of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel Elecrophoresis) and performed the way Laemli kromasi staining. The results obtained Pili major band with a molecular weight of 63.63 kDa.

Phase II is a hemagglutination test. Hemagglutination test aims to detect whether the bond antigen (Pili) with the receptor present in the surface of red blood cells can agglutinate red blood cells. By doing this test, can also be determined the highest titer of an antigen as the haemagglutinin molecule. For antigen dilution was performed (pili) in serial. Dilution also aims to reduce excess levels of antigen, so that the antigen-antibody bonds formed can be optimal. Hemagglutination test interpretation is as follows when the antigen (pili) that can agglutinate red blood cells of mice mean hemagglutination reaction is positive and if the antigen (pili) are not able to agglutinate red blood cells of mice mean hemagglutination reaction negative. Of hemagglutination test, the positive results seen in the AC line which is started at a dilution titer ¼ Crude Pili. While on the line DE is a positive titer at dilution 1 / 64. On pellets (line FG) positive results began to show dilution 1/64.Ini Pili isolation results with Christopher have hemagglutinin properties. This is an initial step to further prove Pili as adhesion molecules. Adhesion molecules is an important key in microbial infection, because infection is preceded by attachment of microbes on the host through adhesion molecules Tandya (2006).

Phase III trials is a diagnostic test Pili sputum Mycobacterium tuberculosis on 96 patients suspected of suffering from pulmonary tuberculosis by using dot blot method. Immunoblotting is an easy test methods to detect the presence of antigen. This method is faster because without a preceded by SDS-PAGE (sodium dodecyl sulfate gel electrophoresis Polycrylamide), but the antigens to be tested directly in-coating the membrane with a ratio of 1:100. To get sIgA in sputum of patients, each patient's sputum sample preparation
performed by the addition of a solution of PBS (phosphate buffer saline), carried out mechanically by vortex homogenasi and given ethanol. After antigen being coated into the membrane, then the primary antibodies from patients with sputum sIgA 1:200 ratio dropped into each appliance sinks in the dot blotter. While the secondary antibody used was anti-human IgA monoclonal antibody with a ratio of 1:500. Purple color formed is complex antigen-primary antibody-secondary antibody labeled with alkaline phosphatase Western Blue substrate. This suggests that the antigen can be recognized by primary antibody. Bright dark color dot depends on the specificity of the antigen to be recognized by the antibody.

Calculation of cut-off (cutoff) performed using Mc.Nemar table. In the calculation of cut-off, obtained sensitivity of 71.42% and specificity of 63.93% at a mean of 121.24. This sensitivity value will be higher, if the cut-off we raise. Selection of cut-off values, depending on the purpose of our diagnostic test. If the diagnostic test to establish disease, are advised to choose a cut-off with high sensitivity and specificity that is not too high. Conversely, if the purpose of diagnostic aims to get rid of the disease (screening), it is advisable to choose the cut-offs with higher specificity values. However, good research is that having a high sensitivity and specificity. Therefore, in this study, the value of the proposed cut-off is 121.24 which gives the value of 71.42% sensitivity and specificity of 63.93%.

This study has several drawbacks, namely that cross-reactions may still occur in some samples of sputum smear negative, which probably caused the low number of bacteria Mycobacterium tuberculosis in sputum so it is not detected in the smear readings and possible bias is so high that on the ROC curve of the degree of strength BTA immunoblotting results are relatively weak but significant for specificity (screening).

**CONCLUSION**

In this study Pili s-IgA protein can be detected by immunoblotting. Pili of Mycobacterium tuberculosis molecular weight of s-IgA response by sputum is 63.63 kDa. With dot blot examination based on the gold standard smear readings (Acid Resistant Bacteria), in 96 sputum Mycobacterium tuberculosis Pili obtained by sIgA is able to respond to the diagnosis (upholding the disease) on the cut-off (cutoff) with a sensitivity of 71.42 121.24 % and a specificity of 63.93%. In this study also found that the pili of Mycobacterium tuberculosis is the hemagglutinin molecule. There are significant differences between mean values Pili-sIgA immunoblotting results between the groups with negative AFB smear-positive group. It is hoped this research can be continued using a larger sample to test the possibility of cross reactions. The results could be further developed for diagnostic means of pulmonary tuberculosis. It is hoped this research can also proceed to find the specific structure of Mycobacterium tuberculosis Pili as adhesion molecules, as usually is the molecular adhesion molecule agglutinin too. When Pili proved an adhesion molecule, then the Pili Mycobacterium tuberculosis can be used as a candidate molecule in the prevention and treatment of Mycobacterium tuberculosis. Antibody and antigen needed a more pure for the preparation of antibodies and antigens. For further research is recommended indirect detection of monoclonal antibodies.

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