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Original Research Article

Characterization of Pili Protein as Cell Adhesion Molecules of *Mycobacterium Tuberculosis* H37RV to Mouse Enterocytes: In vitro Study

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ABSTRACT

Tuberculosis (TB) is an important public health problem in Indonesia. The number of patients suffering from the disease is the fifth largest in the world. *Mycobacterium tuberculosis* is a bacterial pathogen that can invade and colonize host cells. The bacterium has pili on its surface that facilitate its adhesion to host cells. Thus, identification and characterization of *M. tuberculosis* pili proteins can provide insight that may be useful in developing new strategies for the disease control. The aim of present study was to determine hemagglutinins and adhesins in the shaved pili of *M. tuberculosis* H37rv by using hemagglutination and adhesion tests. This experimental study employed a control group and three observational groups by using a CRD (completely randomized design) approach. The pili of *M. tuberculosis* were mechanically shaved from the surface by using a pili cutter. The pili proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and were purified by electroelution. The purified proteins were then mixed with mouse erythrocytes to obtain a hemagglutination titer. Following this, adhesion tests were performed to study the ability of pili proteins to attach to mouse enterocytes. It was observed that several pili proteins with molecular weights of 63-kDa, 37-kDa, and 11-kDa were able to attach to mouse enterocytes. A hemagglutination test confirmed that the 11-kDa pili protein was a hemagglutinin. An adhesion test showed that 11-kDa pili protein has the ability to attach on mouseenterocytes. These results were found to be statistically significant using ANOVA ($p < 0.05$). Overall, the data indicate that *M. tuberculosis* H37Rv produces an 11-kDa pili protein that plays an important role in hemagglutination and adhesion.

Keywords: Adhesin protein, Hemagglutinin protein, *Mycobacterium tuberculosis* strain H37rv.

INTRODUCTION

In Indonesia, tuberculosis (TB) is a major public health problem. In 2009, over 500,000 new cases of TB were reported with 25% mortality. Further, the percentage of Tuberculosis-Human Immunodeficiency Virus (TB-HIV) co-infection was about 3.6 % and that of multi-drug resistant TB cases was about 2.3%. [1] TB is an infectious disease caused by *Mycobacterium tuberculosis* and it primarily infects lungs, which act as the main route for bacterial infection in more than 98% cases of TB. Once in the respiratory tract, the bacteria reach the alveoli of the lungs after passing through the bronchial mucociliary defense system. In the lungs, the bacteria are phagocytosed by alveolar macrophages, but some may evade the macrophages, resulting in replication. Eventually, this leads to the formation of colonies known as the primary focus. [2,3]

M. tuberculosis is a bacterial pathogen that has the ability to proliferate

intracellularly in the host. Several strains of this bacterium are found in humans and animals; the human-specific strains are the primary cause of the disease. [2,4] These bacteria are aerobic, non-spore forming, rod-shaped of 0.2-0.6µm width and 1-4µmlength. The bacteria appear red with a blue background when stained using the Ziehl-Neelsenstain. *M. tuberculosis* can be cultured on Lowenstein-Jensen medium (an egg-based medium) and Middlebrook medium by incubation at 37°C for 2-3 weeks. [2]

Bacterial attachment to the mucosal surface is a pivotal step inits pathogenesis. Adhesins are specific attachment factors that facilitate theinteraction of bacteria with host cells. [4]

Structurally, there are two types of adhesion molecules: afimbriae (mostly surface-exposed proteins) and fimbriae or pili. Pili are hair-like appendages on the bacterial surface and are composed of repeating subunits of pilin. Pili are straight or flexible filaments of 1-10-nm width and 0.07-µm length, and often help the bacteria bind to the host's receptor. Additionally, hydrophobic properties of pili further streng then these attachments to the host cells. As virulence factors, pili have the ability to agglutinate human and animal erythrocytes, form bacterial aggregates, form biofilms, and attach and colonize mucosal surfaces. [5,6]

Many gram-negative bacteria have more than one type of pili. *M. tuberculosis* has two different types of Mycobacterium tuberculosis pili(MTP) type IV pili and curli-like pili. [7] Type IV pili play an important role in bacterial attachment to host cells, co-aggregation, immunomodulation, motility, and DNA uptake. [8] The MTP serve as attachment factors during pathogenesis and stimulate the humoral immune response. [9]

Since pili play an essential role in bacterial pathogenesis, they are considered important targets for vaccine development. [6,10] In addition, previous studies have reported that adhesion proteins are also potent immunogens, and thus can be targetedfor the development of new vaccines. [11]

To date, there is insufficient evidence for the induction of protective cellular immune response by *M. tuberculosis*piliagainst bacterial infection. Furthermore, not many studies have been conducted targeting specific bacterial adhesions. [7,12] While there is more research in immunogenicity, bacterial virulence factors, genetic factors, and pathogenesis of infection are needed for vaccine development. [13] In this study, the piliproteins of *Mycobacterium tuberculosis* strain H37Rv were isolated and assessed for their hemagglutination (HA) and adhesion properties.

MATERIALS AND METHODS

Bacterial and mouse strain

In this study, *M. tuberculosis* H37rv strain was used for piliisolation. Animal studies were carried out on 10-12 weeks old male Balb/c mice.

M. tuberculosis H37rv piliisolation

M. tuberculosis H37rv was cultured on Middlebrook medium at 37°C, while shaking for 48-72 hours. The starter culture was inoculated on a Middlebrook7H11 agar plate containing Oleic Albumin Dextrose Catalase (OADC) and incubated at 37°C for two weeks. The bacteria were than harvested and suspended in a beaker containing 200 ml PBS. The bacterial suspension was split into 25-ml aliquots, and the pili were shaved using a pili cutter. The cutting was performed in six rounds, and each round was followed by centrifugation at 6,000 rpm for 30 min at 4°C. The details of each round of cutting are as follows:5,000 rpm for 30 s; 5,000 rpm for 1 min; 5,000 rpm for 2 min; 10,000 rpm for 1 min; 10,000 rpm for 2 min; and 10,000 rpm for 2 min. The pili-containing supernatants were collected after each round. The pellets obtained were then resuspended in PBS until the sixth round. Following this, all the pili-containing supernatants were centrifuged at 12,000 rpm

for 15 min at 4°C. The lipid contaminants were removed using chloroform: ethanol (2:1). The suspension was then centrifuged at 18,000 rpm for 30 min. The aqueous and interphase fractions were transferred into another tube and extracted twice. Finally, the aqueous phase and interphase were ultracentrifuged at 120,000 rpm, 4°C, for 3 hours. The pellets were resuspended in PBS and stored at -20°C.

***M. tuberculosis* H37rv pili purification**

Pili proteins were separated using SDS-PAGE electrophoresis, according to Laemli (1970). The desired protein bands were sliced and put into cellophane membranes (cut off 14-kDa). The proteins were purified using electroelution at 125 mV for 25 min.

HA assay

The HA assay was conducted to specify the titer of *M. tuberculosis* H37rv pili proteins based on their ability to attach to the receptor protein present on mouse erythrocytes. This assay was carried out according to the Hanne and Finkelstein (1982) method. Mouse erythrocytes were washed three times in PBS and final concentration of 0.5% was prepared in Phosphat Buffer Saline(PBS)To a round-bottomed 96-well dish was added 50 µl PBS. Thereafter, 50 µl pili protein was added to the first well and serial dilutions were made to the right. The twelve well were used as control. After that, into each well were added 50µl mouse erythrocytes. The plate was than incubated for15 min at room temperature. Results are negative if dots of erythrocyte are found at the center of round-bottomed plates. Positive results are confirmed if reddish color appears in the suspension. Protein concentrations with the highest titer were used for further assays. [14]

Balb/c mouseenterocytes isolation

The mouseenterocytes were isolated according to Nagayama et al., (1995). The mice were sacrificed using saturated chloroform in a closed jar. The small intestine was removed and sliced into 5-cm fragments. Thereafter, the lumen was opened by cross-sectioning and was washed with PBS (pH 7.4, containing dithiothreitol 1 mM). After that, the lumen tissue was placed in buffered solution containing KCl 1.5 mM, NaCl 9.6 mM, sodium citrate 2.7 mM, KH_2PO_4 8 mM, and$Na_2HPO_4$5.6 mMpH 7.3. The lumen was then incubated at 37°C for 30 min with gentle shaking. The supernatant was transferred into PBS (pH 7.4), containing EDTA 1.5 mM and dithiothreitol0.5 mM (pH 7.4), and was vigorously shaken for 30 min at 37°C. The supernatant was than centrifuged in cooling centrifuge at 1000 rpm for 10 min. The pellets were again washed with PBS (pH 7.4). The pellets were then resuspended in basal medium containing 20% new calf serum. The enterocyte concentration was made up to 10^6 cells/ml for the subsequent steps. [15]

Adhesion Assay

M. tuberculosis H37rv was grown in Middlebrook 7H9 broth containing Tween 20 at 37°C for two weeks. The bacteria were then pelletedusing refrigerated centrifugation at 6,000 rpm for 15 min. The pellet was resuspended in PBS-BSA (1%) and final bacterial concentration 10^8 cells/ml (OD = 1, λ = 600 nm = 10^9 cells/ml) was made. [16] The hemagglutinin protein solution was prepared in test tubes with the following concentrations: 0 µl (control), 25 µl, 50 µl, 100 µl, 200 µl, and 400 µl in 300 µl PBS. In each test well,300 µl of mouseenterocyte solution was added, and well were incubated in water bath at 37°C for 30 min with gentle shaking. Thereafter, 300 µl bacterial suspensions was added into each test tube and incubated for 30 min under previous conditions. The suspension was again pelleted in the cooling centrifuge at 1,500 rpm for 3 min and was washed twice with PBS. The pellet was smeared on a slide and was stained according to the Ziehl-Neelsen method. The adhesion index is the average number of total bacteria attached to the enterocytes.

Statistical analysis

The adhesion index was performed using Tukey test and one way ANOVA.

RESULTS

Identification of *M. tuberculosis* H37rv pili

M. tuberculosis H37rv grown on Middlebrook 7H11 medium was identified morphologically as white, colony-forming bacteria with a rough surface (Figure 1).

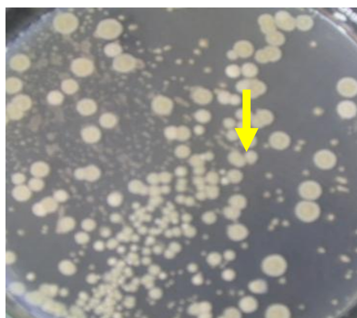

Figure 1.White and rough-surfaced colonies of *M. tuberculosis* H37rv (marked by yellow arrow) on Middlebrook 7H11 medium.

M. tuberculosis H37rv pili were purified using chloroform: methanol (2:1), and their protein components were analyzed by SDS-PAGE electrophoresis to confirm their molecular weight. The pili proteins shaved from first three cycles showed several bands, with the most intense bands being those of molecular weight 63kDa, 37KDa, and 11kDa, as presented in Figure 2. These data were similar to those reported by a previous study. [9] Lanes 2 to 4 shows results of crude pili, without chloroform-methanol extraction. Lanes 5 to 7 show results of the extracted pili proteins. For the HA assay, the desired separated proteins were sliced and purified by electroelution.

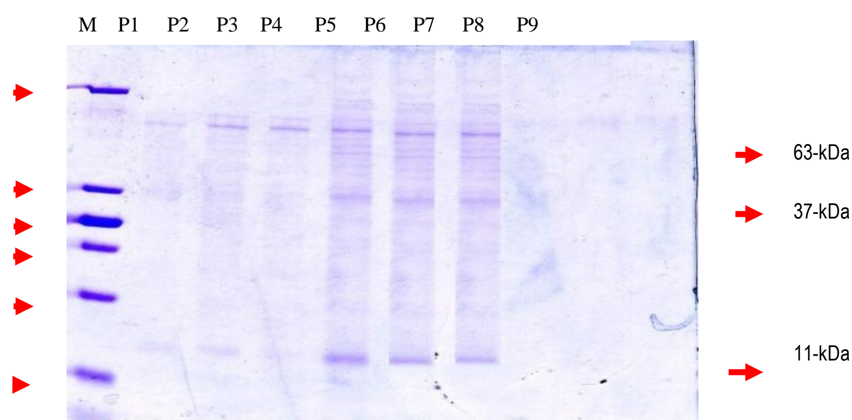

Figure 2. SDS-PAGE electrophoresis profile of *M. tuberculosis* H37Rv pili protein subunits
Lane 1: Protein marker (Fermentas); Lanes 2 to 4: shaved pili proteins from first three cycles without chloroform-methanol extraction; Lanes 5 to 7: shaved pili proteins from first three cycles after chloroform-methanol extraction.

HA assay of *M. tuberculosis* H37rv pili

The HA assay of the 63kDa, 37kDa, and 11kDapili proteins revealed that these proteins were HA-positive. They were found to have the ability to bind to the receptor present on the erythrocyte surface. Thus, they prevented erythrocyte sedimentation at the bottom of the plate (Figure 3). However, the 11-kDa pili protein showed the highest titer (1/32). Therefore, the 11-kDa pili protein was identified as the hemagglutinin protein.

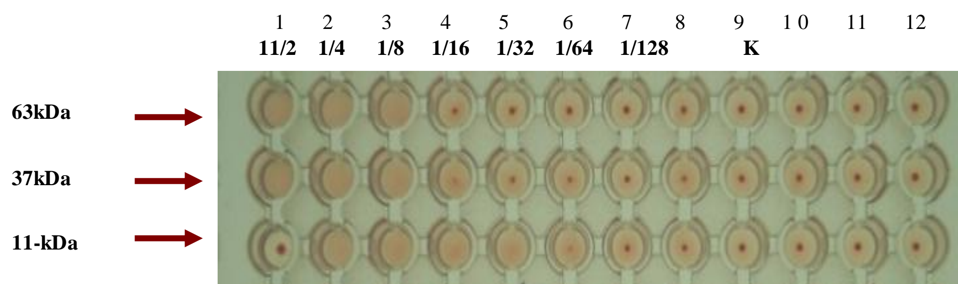

Figure 3. Results of the HA assay of HA-positive 63kDa, 37kDa, and 11kDapili proteins of *M. tuberculosis* H37Rv. The 11-kDa protein showed the highest titer.

Adhesion assay of *M. tuberculosis* H37rv pili proteins on mouseenterocytes

In the control group, mouseenterocytes were directly exposed to *M. tuberculosis* H37rv. Bacterial adherence to enterocytes was viewed at 1000x magnification. As shown in Figure 4a, mouseenterocytes (blue) were completely covered by *M. tuberculosis* H37rv (red). It was speculated that *M. tuberculosis* H37rv have adhesin proteins that facilitate their attachment to mouseenterocytes. Since the HA assay results showed that the 11-kDa pili protein was hemagglutinin, we checked the ability of this protein to block bacterial attachment to mouseenterocytes. After covering mouseenterocytes with the 11-kDa pili protein (at a 1/1 dilution), the cells were challenged with *M. tuberculosis* H37rv. Only a few bacteria were able to attach to the mouseenterocytes, indicating that these enterocytes have a receptor for this 11-kDa protein (Figure 4b).

In the P1 group, in which the 11-KDa pili protein at a 1/1 dilution covered the mouseenterocytes, showed the lowest adhesion index (Figure 5). The adhesion index is the mean value of the counted mouseenterocytes covered by bacteria per hundred cells.

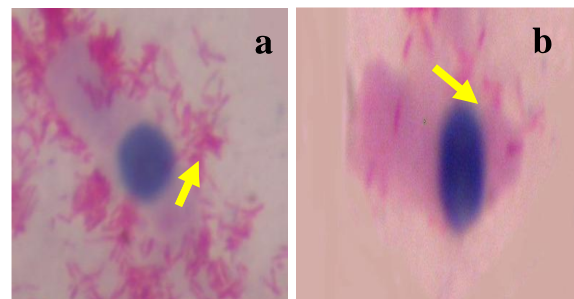

Figure 4a. *M. tuberculosis* H37rv attachment (yellow arrow) to mouseenterocytes (control group). **Figure 4b.***M. tuberculosis* H37rv attachment (yellow arrow) to mouseenterocytes, which are covered by the 11-kDa pili protein at a 1/1 dilution. The preparation was stained by the Ziehl-Neelsen technique.

The adhesion index in different groups using serial dilutions of 11-kDa protein varied significantly ($p < 0.05$). Tukey HSD analysis showed that the lowest index adhesion was obtained in the group with the 1/1 dilution of the 11-kDa pili protein. The results of this group were non-significant with respect to 1/2 dilution group but significant with respect to the 1/4, 1/8, and 1/16 dilution and control group.

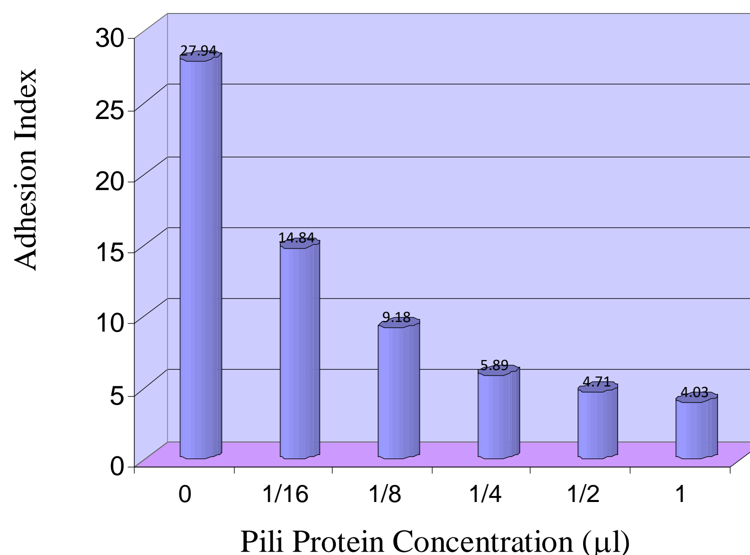

Figure 5. Adhesion index of the 11-kDa pili protein for mouseenterocytes. The greater the protein dilution, the higher the adhesion index value.

DISCUSSION

Most bacterial pathogens have adhesive organelles called pili or fimbriae that help the bacteria attach to the host receptors and play an important role in bacterial colonization. [9,17] Owing to

important role of piliin bacterial pathogenesis, they may serve as targets in vaccine development. [9] Our study showed that *M. tuberculosis* H37Rv forms colonies that appear white on Middlebrook medium. UsingZiehl-Neelsen staining, the cultured cells appear red-colored and rod-shaped. Previously, it had been reported that *M. tuberculosis* H37Rv cultured on Middlebrook 7H10 medium or Mycobacteria agar for 2-3 weeks and observed with an electron microscope forms a thin, dense mesh (2-3 nm) with a coiled set of fibers resembling pili that extend out from the bacterial surface. This organelle was called *Mycobacterium tuberculosis* pili or MTP. [9]

M. tuberculosis H37Rv pili were isolated by mechanical shaving using a pili cutter. The lipid components present in the *M. tuberculosis* cell walls were removed using chloroform/methanol (2:1). The pili proteins were separated by SDS-PAGE electrophoresis to determine their molecular weight. The proteins of interest were purified using electroelution. The SDS-PAGE electrophoresis results showed that pili contain subunit proteins with molecular weights of63 kDa, 37kDa, and 11kDa. Another study has reported that the molecular weight of the pilin monomer was about 10-25 kDa. [9] It has also been reported that pili have the ability to agglutinate animal and human erythrocytes. In this study, pili subunits with molecular weight of 63kDa, 37kDa, and 11kDa were tested for their capacity to agglutinate mouse erythrocytes. It was found that only 11-kDa protein subunit was able to agglutinate erythrocytes, with the highest titer of 1/32 (Figure 4). These findings indicate that the pili 11-kDa subunit has the ability to attach to the receptors present on mouse erythrocytes. In another study, the attachment of intestinal bacteria to mammalian cells was studied through the process of agglutination. When the bacterial cells were mixed with erythrocytes of different species or with yeast cells, agglutination occurred with different patterns, [18] probably owing to the specific bond formation between lectin and carbohydrate moieties like mannose, which function as recognition molecules in lectinophagocytosis. [19] In addition, attachment between enterobacter cells and target cells occurs owing to a reaction between adhesion molecules and sugar moieties such as glycolipids and glycoproteins present on the target cells. [20,21]

In our study, we also showed that the 11-kDa pili protein subunit can act as a hemagglutinin as well as an adhesin (figure 5). It was found that greater the dilution of the subunit protein, the higher the number of *M. tuberculosis* bacteria attached to mouseenterocytes. The adhesion of the subunit protein shows that the mouseenterocytes have receptors for 11-kDa pili protein subunit. These findings were further supported by adhesion index results: the 11-kDa piliprotein subunit with a 1/1/ dilution (P1) had the smallest adhesion index, which increased on increasing the dilution of the protein (figure5).

The above findings indicate that *Mycobacterium tuberculosis* H37Rv produces an 11-kDa pili protein that acts as hemagglutinin as well as an adhesin. There, it was concluded that the 11-kDa pili protein is the specific adhesin (may be the only one) in *Mycobacterium tuberculosis* H37Rv and this protein plays a vital role in its pathogenesis. [11]

CONCLUSION

This study concluded that *Mycobacterium tuberculosis* H37Rv has an 11-kDa pili protein that serves as a hemagglutinin and as an adhesin.

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