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Original Article

Salivary sIg-A response against the recombinant Ag38 antigen of *Mycobacterium tuberculosis* Indonesian strain

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Abstract: An evaluation of the humoral response based on secretory immunoglobulin A levels in the saliva of pulmonary tuberculosis (TB) acid-fast bacillus-positive (TB-AFB+) patients against a recombinant 38 kDa antigen (Ag38-rec) is reported. A total of 60 saliva samples consist of 30 TB-AFB+ patients and 30 healthy controls were tested against 500 ng of semi-purified antigen using the dot blot method. Results showed that the protein antigen could differentiate between healthy individuals and TB-AFB(+) patients. Whole saliva demonstrated better reactivity than centrifuged saliva. The Ag38-rec protein indicated statistically comparable sensitivity (80% versus 90%), but lower specificity (36.6% versus 70%) compared with purified protein derivative (PPD). Surprisingly, both antigens similarly recognized secretory immunoglobulin A in the saliva of the healthy group (50% versus 50%, respectively). These findings suggest that the Ag38-rec protein originating from a local strain of *Mycobacterium tuberculosis* may be used for TB screening, however require purity improvement.

Keywords: Secretory Ig-A, saliva, antigen 38 recombinant

Introduction

Tuberculosis (TB) has been a serious threat in developing countries, particularly in peripheral (i.e., suburban and rural) areas, primarily due to a lack of facilities able to affordably and quickly diagnose the disease. Clinical examination, the acid-fast bacillus (AFB) test and culture are the gold standard methods for the diagnosis of TB. However, because bacterial culture is costly, with results taking up to two months to obtain, most TB patients in suburban or rural areas rely solely on the AFB test. A rapid, simple and inexpensive method to confirm Mycobacterium tuberculosis (Mtb) infection, therefore, is required to slow or prevent the spread of the disease. A serodiagnostic approach focusing on TB antibodies in TB patients has been considered to be the simplest and quickest method and, therefore, has been extensively studied [1-3].

Antibodies present in saliva have shown great promise in diagnosing many diseases such as malaria and rubella, among others. Unlike blood sampling, saliva sampling is noninvasive and relatively quick. Saliva contains 85% secretory immunoglobulin A (sIg-A), which is produced by B lymphocytes at the ductus saliva [4, 5]. IgA contributes to only 13% of total antibody in human serum and is predominantly found in extravascular secretions. IgA is the main imunoglobulin isotype in saliva and other secretions [6-9]. The sensitivity of sIg-A has been demonstrated in several fields and has been suggested as a diagnostic test in areas where microscope is not available [10, 11].

In a previous study, we successfully expressed a recombinant *pab* gene coding for the 38 kDA antigen (Ag38-rec) from Mtb using a heterologous system in *Escherichia coli (E. coli)*. The *pab* gene was isolated from patients with severe pulmonary TB from Malang, Indonesia [12]. The 38 kDa protein from Mtb contains B-cell epitopes and has been shown high specificity for *Mycobacterium* complex [13, 14]. In the present study, the Ag38-rec from Indonesia

was purified and its immunogenicity was evaluated against sIg-A isolated from the saliva of TB patients.

Materials and methods

Strain and plasmid

The *E. coli* BL21-(DE-3) strain used in the present study was purchased from the Central Laboratory of Basic Science, Brawijaya University, Malang, Indonesia. Informed written consents were obtained from the patients. The study was approved by the Ethics Committees of Dr. Saiful Anwar General Hospital, Malang, Indonesia.

Production of Ag38-rec

To produce recombinant Ag38-rec, *E. coli* BL21-(DE3)/pMBhis were cultured at 37°C in Luria Broth medium containing ampicillin (100 mg/mL) to an optical density (600 nm) of 0.6. pMBhis is a plasmid carrying the *pab* gene fused with the *trx* gene [12]. The expression of the *pab-trx* fusion product was induced by the addition of 0.5 mM isopropyl-B-D-thiogalactopyranoside (IPTG). After 3 h, cells were harvested by centrifugation at 8000 x *g* for 10 min at 4°C. The supernatant was removed and the pellet was washed with phosphate buffer (pH 7.2) and frozen in liquid nitrogen before being stored at -20°C. The following day, the pellet was disrupted by sonication.

Purification of Ag38-rec using Ni-TED columns

In the previous study, pMB38 was constructed by fusing the *pab* gene with a 6 x histidine tag located at the N-terminus. Purification was performed using a Ni-TED column according to the manufacturer's protocol (Protino, Dueren, Germany). The pellet was resuspended in 2.5 volumes of 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF) (Sigma, USA) and disrupted using a sonicator at 40% power for 20 s three times. The cell extract was then centrifuged for 15 min at 10,000 x *g*. The supernatant was subsequently loaded onto an Ni-TED column and washed twice with phosphate buffer containing 10 mM imidazole. To elute the protein bound to the nickel column, phosphate buffer containing 250 mM imidazole was applied. Protein elution was repeated three times. To remove the imidazole, the eluent was dialyzed against the same buffer used for protein elution, using dialysis tube with a pore size of 12.5 kDa. The buffer was changed three times in 3 h intervals and was then left overnight. Finally, all fractions were analyzed using 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The presence of Ag38-rec was confirmed using Coomassie blue stain.

Clinical specimens

Saliva specimens (n=60) were obtained from 30 pulmonary TB patients from primary health care clinics in Malang City, Indonesia, and 30 healthy controls. Specimens were collected between December 2012 and April 2013. The diagnosis of TB was established by clinical presentation, chest radiograph examination and sputum smear positivity. Clinical data were re-evaluated by a Pulmonologist. Clinical assessments included were patient history; signs and symptoms; and follow-up information. Patients were enrolled in the study if they met the following inclusion criteria: pulmonary TB confirmed by the Ziehl-Neelsen method; individuals older than 17 years of age; and individuals who had not been previously treated for TB.

Serological test

The dot blot is a serological test to detect the specific reaction between an antigen and an antibody and is based on the same working principle as the Western blot; however, the dot blot requires a smaller amount of antigen. Nitrocellulose membrane was cut into 7.5 cm × 11 cm sheets and then soaked in sterile water for 30 min before mounting onto the dot blot apparatus. Through a hole in the apparatus, a 20 L volume containing 1 g of antigen was dropped onto the prewetted membrane (using Tris-Cl buffer [pH 7.4] then incubated overnight at 4°C with blocking buffer. The next day, the blocking buffer was removed and replaced with TBS buffer (50 mM Tris base, 0.2 M NaCl, 5% skim milk, pH 7.4) and gently shaken for 10 min at 4°C. After the blocking agent was removed, 50 µL of primary antibody was applied to the membrane and incubated for 2 h at room temperature with gentle shaking. The solution was then removed and the membrane was washed three times with 0.05% TBS Tween-20. The secondary antibody was diluted 1:500 in Tris-Cl

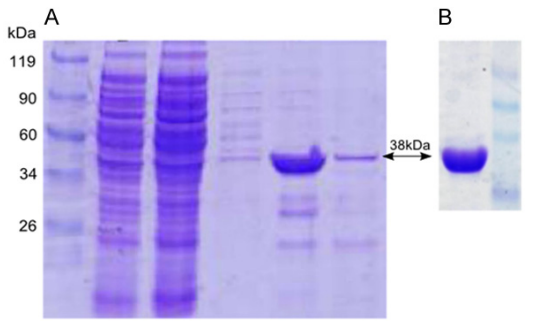

Figure 1. Purification of protein Ag38-rec using Ni-TED kit: A: Marker protein (1) Flow through (2) Wash 1 (3) wash 2 (4) elution 1-2 (5-6). B: Protein Ag38-rec after dialysis.

and the membrane was incubated in the solution at room temperature for 1 h on a shaker, followed by three washes using 0.05% TBS Tween-20. Finally, a chromogenic substrate (BCIP-NBT) was applied to the membrane in the darkroom at room temperature for 30 min. The reaction was stopped by the addition of H_2O. The Corel Draw (Corel, USA) program was used to interpret the color range of the spot(s). A positive result was defined as a dark blue or dark purple spot (>50%) on the blot. As control positive PPD (protein purified derivative) from Mtb (Serum Staten Insitute, Danemark) was used.

Saliva collection

To avoid problems with analyte retention or the introduction of contaminants, saliva was collected using a passive drool method according to a manual from Oasis Saliva Collection (USA). To lessen possibility of contamination from substances in the saliva that may interfere the immunoassay the following precautions were applied for research participants: no alcohol for 12 h before sample collection; no consumption of a significant meal within 60 min of sample collection; no dairy products for 20 min before sample collection; no foods with high acidity, or high sugar or caffeine content, immediately before sample collection; and rinsing mouth with water to remove food residue before sample collection.

To protect unstable analytes and to prevent bacterial growth, all samples were maintained at 4°C for no longer than 2 h before freezing at or below -20°C. Freezing and centrifugation also helped to precipitate mucins in the samples, which facilitated pipetting. On the day the

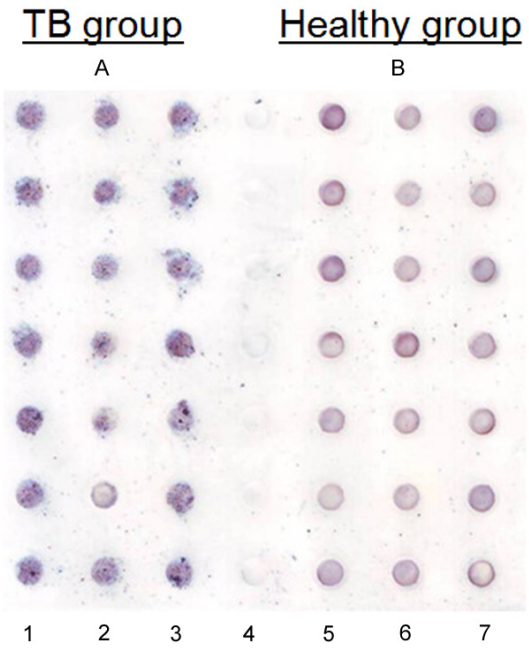

Figure 2. Immune response of protein Ag38-rec against sIg-A from saliva TB-(AFB+) patients (A) and healthy person (B) using DotBlot method: saliva TB patients (column 1 and 5), pellet (column 2 and 6), supernatant (column 3 and 7) blank (column 4), saliva of healthy person, pellet and supernatant (column 5-8).

samples were analyzed, they were brought to room temperature, vortexed and then centrifuged for 15 min at approximately 3000 rpm (1500 x g). Assays were performed using only clear saliva, pipetted slowly to avoid the formation of bubbles.

Statistical analysis

Each assay was repeated in triplicate. The sensitivity and specificity of the Ag38-rec dot blot were calculated by comparison with the smear results and dot blot of purified protein derivative (PPD) using the McNemar test. Statistical significance of differences between groups was analyzed by using Student's t test ANOVA analysis. A value of $P<0.05$ was thought of as statistically significant.

Results

Purification Ag38-rec

Purification of Ag38-rec was achieved using a Ni-TED column, yielded 3 mg/g wet pellet, with 80-90% purity (**Figure 1A**). Several other co-

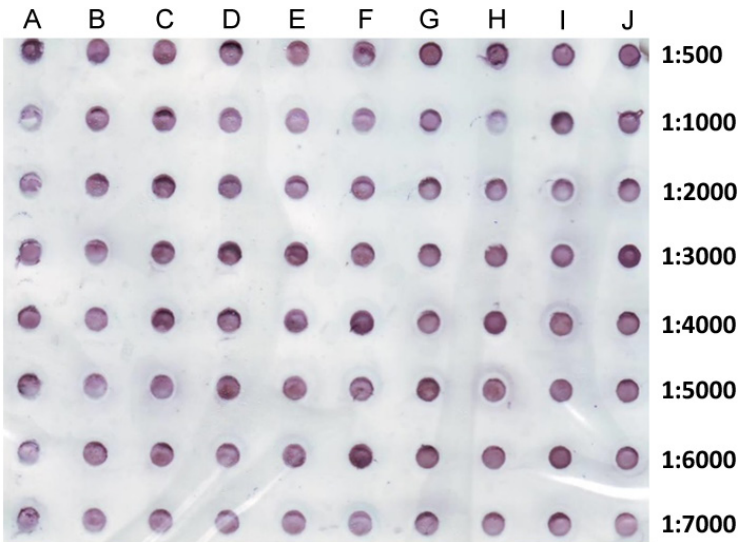

Figure 3. Immune response of Ag38-rec antigen against sIg-A from saliva with varied canncentration. Saliva without dilution (A), ½ (B), 1/3 (C), ¼ (D), 1/5 (E), 1/6 (F), 1/7 (G), 1/8 (H), 1/9 (I), 1/10 (J) dilution.

Table 1. Comparison of the immune response of sIg-A in saliva of TB-AFB(+) group of patient and healthy control towards Ag-38 rec as compared to PPD

Antigen	PPD		Ag38-rec	
	Healthy (n=30)	TB-AFB(+) (n=30)	Healthy (n=30)	TB-AFB(+) (n=30)
	9 (+)	27 (+)	19 (+)	24 (+)
	21 (-)	3 (-)	11 (-)	6 (-)
Total	30	30	30	30

purified proteins, which appeared on visualization with SDS-PAGE and Coomassie blue staining, remained present after the elution step. However, after dialysis, purity increased to almost 90%, indicated by the disappearance of some of the lower protein bands on SDS-PAGE (**Figure 1B**). Confirmation of whether the band was purified Ag38-rec was conducted using a Western blot against anti-Ag38. A single band in the high range between 32 kDa and 52 kDa appeared after incubation with chromogenic substrate (data not shown).

Immune response test of antigen Ag38-rec

To evaluate the ability of Ag38-rec to recognize the sIgA-antibody, a dot blot analysis was performed. This antigen can differentiate between healthy and TB-AFB(+) patients' sera (**Figure 2**). The protein reacting with sIg-A of TB-AFB(+) patients appeared dark purple in color, in contrast to the saliva from healthy individuals, which appeared pale purple. Whole saliva produced the strongest color (**Figure 2**, columns 1 and 5) followed by saliva supernatant (**Figure 2**, columns 3 and 7). In contrast, pelleted saliva showed weak color (**Figure 2**, columns 2 and 6). Attempts were made to reduce the concentration of recombinant antigen for the blot as well as the amount of saliva through serial dilution. The variation of antigen concentration from 500 ng to 20 ng did not affect the color of the dot (**Figure 3**).

After a preliminary test, Ag38-rec was further tested in all patients using the dot blot method, with a concentration of 500 ng for each dot blot. Surprisingly, protein concentrations varied between as well as within the patient groups. Dots that turned dark purple with >50% darkness were unevenly distributed among saliva from both healthy and TB-AFB(+) patients. The same result was also observed in the healthy sample using PPD. Among the 60 samples, eight healthy samples were positive in both PPD and Ag38-rec dot blots, while six healthy were negative in both PPD and Ag38-rec dot blots (**Table 1**). A positive dot blot was found in 21 saliva samples from TB patients, which reacted with both PPD and Ag38-rec. **Table 1** presents a comparison of the performance of Ag38-rec and PPD dot blot. Apparently PPD demonstrated higher (90% with 95% coefficient of Interval [95% CI]) but statistically not significant ($p>0.05$) sensitivity than that of Ag38-rec (80% with [95% CI]) (**Table 2**). The same result applied for specificity i.e 70% versus 36.6%, respectively (**Table 2**). Surprisingly the positive dan negative predictive value (PPV and NPV) of both antigens was not significantly differ statistically ($p>0.05$) (**Table 3**).

Discussion

The 38-kDa Mtb antigen is the most frequently studied serological TB antigen and the main

Table 2. Sensitivity and specificity of Ag38-rec against sIg-A of TB patients

Sample type	No (%) of positive cases	Sensitivity % (95% CI)	Specificity % (95% CI)
PPD	27	90 (80.86-99.13)	70 (56.04-83.95)
Ag38-rec	24	80 (67.82-92.18)	36.6 (21.92-51.27)
P		0.28	0.01

Table 3. Positive and negative predictive values of Ag38-rec against sIg-A in TB patients

	PPV %, (95% CI)	NPV %, (95% CI)
PPD	75.0 (60.2-89.79)	73.0 (73.89-100)
Ag38-rec	62.79 (47.99-77.58)	64.7 (51.09-78.30)
P	0.180	0.180

component in several commercial serological TB tests [15, 16]. The potency of Ag38-rec, generated from the *pab* gene that was isolated from the Mtb local strain, to detect the humoral response sIg-A in the saliva of pulmonary TB-AFB(+) patients was evaluated. Antigen preparation began with purification of Ag38-rec. In the previous study, the plasmid carrying the *pab* gene was fused with a 6 x histidine tag at the N-terminus, enabling a straightforward purification using the His-tag column. With such a construct, it was expected that the Ni+ resin of the Ni-TED column would be sufficiently adequate to produce high-purity antigen. Unfortunately, during the purification process, there were several proteins co-purified with the target band, resulting in a purity of only 80% to 90%. Increasing the concentration of imidazole (253 mM) in the elution buffer (to increase the competition with histidine-containing nontarget proteins to bind to the Ni-TED resin) did not result in higher purity. We suggest conducting the purification of antigen using Fast Performance Liquid Chromatography (FPLC) in addition to spin column.

The antibody response of sIg-A from the saliva of pulmonary TB patients against Ag38-rec was characterized using the dot blot method. This method is considered to be simple, inexpensive and fairly sensitive, and should be a suitable serodiagnostic method in peripheral (ie, suburban and rural) areas. Our observation showed that the reactivity of the A38-rec antigen was quite high, resulting in a dark purple color when it was tested with saliva from TB-AFB(+) patient both as a whole saliva suspension and as a supernatant (**Figure 2**). In contrast, a pale purple color appeared in the dot blots of healthy individual. Given that the purity of our antigen was <90%, it appears that Ag38-rec was fairly successful as a candidate of serodiagnostic agent in the initial attempt because it could recognize sIg-A antibody in the saliva of TB patient but not in the healthy control. Whole saliva demonstrated slightly better performance than when it was centrifuged and only supernatant was used. The weakest color was generated by pelleted saliva. Hagewald found that supernatant from saliva that had been centrifuged at 6000 x g for 15 min contained more sIg-A compared with a suspension of saliva [6]. This difference result in our study could be due to the handling saliva process prior analysis. Instead of fresh saliva, we used frozen saliva that might somehow reduce the quality of immunoglobulin.

The Ag38-rec protein was then further evaluated to recognize sIg-A in all saliva samples from pulmonary TB patients. We found fairly high sensitivity, although this was still lower than PPD (80% versus 90%) but both antigens not significantly differ upon statistical analysis ($p>0.05$). This result supported the dot blot data, demonstrating the positive response of the antibody to this antigen despite great heterogeneity in responses among TB patients. The high sensitivity may be due to the selection of sample patients we used i.e AFB(+) and have not received antituberculosis yet, assuming that the mycobacterium was still very active [19]. Other reason could have been partly dependent on the purity of the antigen. Since Ag38-rec has only 80-90% purity it is possible that several proteins from host were also co-purified. The protein of *E. coli* i.e the host in which Ag38-rec was produced may have also been recognized by the sIg-A antibody. One literature stated that heterogeneity of antigen recognition by antibodies from serum of TB patient lead to the difficulty to detect specific antibody responses when unpurified antigens of Mtb were used [20]. Whether this also applies to sIg-A in saliva is not known.

In contrast we found that antigen 38-rec has a low specificity (36%), which was much lower than PPD (70%) ($p<0.05$). The low specificity suggests that a substantial number of individuals produced the same antibodies against the 38 kDa antigen, which was also detected in the saliva of healthy patients (8 out of 24). The immune system in TB patient do not react to all antigenic substances of the tuberculous bacilli, consequently the specificities of the antibodies vary among patients. The variation occurs from person-to-person upon antigen recognition, rather than recognition of particular antigens [22]. The variation of the antibody response to *M. tuberculosis* is suggested to be regulated by human leukocyte antigen (HLA) types [21]. In this study the TB patients were recruited from an endemic area, thus some of the healthy individuals might had been TB contacted and, thus, were producing antibody responsive to Ag 38-rec. Our result was similar with the previous study using serum samples [22] in which the highest proportion of positive antibody responses was detected from regions where TB is highly endemic.

Overall the high sensitivity and specificity of an antigen to recognize an antibody depend on several factors such as protein purity, immunogenicity and the native form of the antigen [17]. In the literature, serological tests involving adult populations have shown high specificity and a much lower sensitivity. The sensitivity of the tests was affected by the stages of the disease and on the presence of mycobacteria in sputum. In chronic and culture-positive cases, antigenic stimulation occurred continuously and, particularly, increased in respond to specific antigens [18]. The patients we investigated in the present study were all AFB(+) and newly diagnosed. The result of culture test and type of lung lesion were not accessed. To evaluate further the sensitivity of the test, we suggest including patients samples with different disease severity. Significant variance in the serological results could be obtained by using even the same antigen with samples from different population.

Our results indicate that a reliable serodiagnostic test for TB will be difficult to realize even with the use of the disease-related 38 kDa protein. The main limiting factor was the apparent antibody response in the saliva of both infected and healthy individuals from endemic areas. It may worth to try the sample from non endemic area, whether it turned to pale purple. PPD was used as positive control and produced the same result despite the fact that the PPD contained a mixture of many proteins, suggesting that the problem was not merely due to the antigen itself. Because saliva samples were collected from patients from endemic areas, many healthy people may have had contact with TB patients without becoming sick [19]. We did not perform the tuberculin test, which is not routinely performed in urban areas; consequently we could not ascertain whether dark colors in the dot blots of healthy persons were the result of these individuals being tuberculin positive.

Conclusion

Our results demonstrated that evaluation of Ag38-rec yielded an acceptable level of sensitivity and but specificity still need to be improve to differentiate TB patients from healthy individuals. Better purification of the antigen may increase the specificity.

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Disclosure of conflict of interest

None.

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