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IMMUNOGENICITY ASSAY OF KatG PROTEIN FROM *Mycobacterium tuberculosis* IN MICE: PRELIMINARY SCREENING OF TB VACCINE

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The tuberculosis (TB) disease is still widely found even though BCG vaccine given to many people. Ineffectiveness of the BCG vaccine is one of causes that make the difficulties in preventing TB transmission. Objective of the research was to determine the immunogenicity of KatG protein of M. tuberculosis clinical isolate L19 in mice. The KatG protein as antigen was prepared by expression of the katG gene of M. tuberculosis clinical isolate L19 in Escherichia coli BL21 using pColdII-DNA vector. After purification by affinity chromatography, the KatG was vaccinated to mice to detect its immunogenicity. The expression of katG in E. coli BL21 could result in KatG protein with molecular weight 80 kDa in sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). The pure KatG protein could significantly stimulate the immune response of mice by triggering the antibodies production of IgG1, IgG2a, IgG2b, IgG2c, IgG3, and IgM. The highest antibody level was obtained when the mice were vaccinated by KatG L19 with the dose of 45 μg/ml. Of the antibodies, the IgG2c isotype was dominantly produced in the blood serum. The KatG protein exhibited a high immunogenicity in mice, so it is possible to develop as a vaccine candidate for TB. A clinical test should be performed in a future to ensure its safety as a therapeutic protein.

K e y w o r d s : KatG, immunogenicity, M. tuberculosis, vaccine, clinical isolate.

Tuberculosis (TB) is a major health problem throughout the world causing a large number of deaths, more than any other single infectious disease, that is caused by *Mycobacterium tuberculosis* infection. There were strategies to reduce the spread of TB including the use of Anti TB therapeutic, but the pandemics of Multidrug-Resistant TB (MDR TB) have emerged since 1990 that causing TB more difficult to treat [1]. Tuberculosis control with preventive actions through the development of a vaccine became one of the main concerns in current TB control program. Bacillus Calmette Guerin (BCG) is a TB vaccine that contains live bacteria that have been weakened (attenuated), it can stimulate the immune system but do not cause disease in healthy people. The BCG vaccine can not be given to people who are clinically immunosuppressed [2]. The BCG is currently used in many countries with a high prevalence of TB to prevent childhood tuberculous meningitis and miliary disease [3]. It is also approved by the FDA for vaccination against tuberculosis and for the treatment of bladder cancer [4]. In spite of the BCG vaccine is used throughout the world, the facts showed BCG is still not effective because the TB cases are still high. The protection provided by BCG varies widely from 0-80%, then it continuously decrease up to 17% in the next 15 years [1]. In an effort to answer those problems, is necessary to develop a new TB vaccine.

DNA vaccine encoding the type of protein naturally EAST - 6 and KatG has been tested on mice and it reported to increase the immune response in the mouse cells producing antibodies, then they were classified as intracellular pathogenic proteins. Although DNA vaccine may provide an immune response that is quite good, the use of a DNA vaccine becomes ineffective if the DNA cannot be expressed into proteins in the host cell. Therapeutic proteins and peptides have the potential to elicit immune responses resulting in anti-drug antibodies [5].

The *katG* gene from *M. tuberculosis* clinical isolate L19 has been cloned in in *Escherichia coli* using – DNA vector [6]. The gene that encodes an intracelular protein with molecular weight 80 kDa has been reported as one of pathogenic intracelular protein [6-9]. The KatG is also classified by TubercuList as a virulence factor that participates in the response to stress and host oxidative reactions. The KatG protein also represents a catalase-peroxidase of Rv1908c which is grouped as one of the surface-exposed proteins that is involved in cell wall biogenesis and maintenance [10, 11]. The surface-exposed proteins are excellent targets for the host adaptive immune system, so they are potentially used for the development of vaccines for mycobacterial disease [10]. The *katG* gene expression is needed to provide its protein, then further used to search its potential as TB vaccine.

The effectiveness of a vaccine depends upon the level of specificity of protein antigens to induce the immune system to produce specific antibodies, so it is necessary to create a pure protein to be used as a vaccine. Immunogenicity of protein vaccine candidate should be investigated in the target population since animal testing and *in vitro* models cannot predict immune response in humans. In addition, immunogenicity has a role in demonstrating product comparability following manufacturing changes and similarity in the context of biosimilar development [11]. Currently, detection of a product's immunogenicity involves measuring antibodies specifically generated against the vaccine candidate. The vaccine is said to be ideal if the concentration is low enough effective to induce high antibody production and be able to induce the formation of a specific type of antibody which has a half-length.

Immunogenicity of KatG L19 protein from *Mycobacterium tuberculosis* has been unknown. Based on the case, it was made the purification of crude extract of KatG protein from *Mycobacterium tuberculosis* local clinical isolate and followed by the immunogenicity assay in mice. The immunogenicity test was conducted to determine the presence (or absence) of antibodies based on the ability of the antibodies to recognize the KatG protein in mice. Additionally, it was also prepared to detect antibodies with desired specificities such as IgM, IgG subclasses etc [5].

Materials and Methods

Samples. The *Escherichia coli* BL21 (DE3) carrying of pCold II-*katG* L19 recombinant was obtained from the laboratory of Biochemistry, Faculty of Sciences and Technology, Airlangga University, used to produce the KatG protein. Female mice *Mus musculus* strain Balb/C, 8-10 weeks old, weighing 25-30 gr, were obtained from the PUSVETMA Surabaya.

Expression of KatG protein. The recombinant of *E. coli* [pCold II-*katG* L19] was cultured in Luria Bertani (LB) medium composed of 0,5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl and 100 μg/ml of ampicillin, shaking it at 150 rpm and at a temperature of 37 °C until obtained the optical density of cells 0.5-0.6 at λ 600 nm [6-8]. The culture was then moved to incubator at 15 °C for 30 min without shaking, added with 0.1 mM IPTG and reincubated at 15 °C with shaking speed at 150 rpm for 24 hours. The cells were separated by centrifugation at 5.000 g at 4 °C for 10 min, and washed with lysis buffer (50 mM Tris-Cl pH 7.4; 200 mM NaCl), and centrifugated at 5.000 g at 4 °C for 10 min. The cell pellet was resuspended in 7-10 ml of 0.02 M phosphate buffer pH 7 and lysed in the sonicator at 60 Hz for 10 min. The supernatant containing of KatG was centrifuged at 10.000 g in 4 °C for 20 minutes and stored at -20 °C [6-8].

Protein purification. Purification of KatG protein was carried out by affinity chromatography method using HisTrap HP column containing Ni-Sepharose matrix. Protein sample first setted to pH 7.4 in sodium phosphate buffer containing 25-50 mM NaCl and 10 mM imidazol. Before the protein sample was filled in HisTrap column, first the column was washed and balaced respectively with milli-Q water and binding buffer (50 mM NaH_2PO_4 pH 7.4, 25 mM NaCl, 10 mM imidazole) as much as 5 times of the column volume. After protein sample was flowed in column, the liquid that came out of the column was accommodated. The protein was eluted with binding buffer containing of 50-200 mM imi-

dazole [6, 9]. Each fraction was accommodated per 1 ml, then the presence of KatG protein in the fraction was detected by sodium dodecyl sulfate - polyacrylamide gel electrophoresis. (SDS PAGE) [6, 8].

SDS-PAGE. The KatG protein was analyzed by SDS PAGE using 12% (w/v) and 4% (w/v) acrylamide for separating and stacking gel respectively based on Sambrook method [12].

Protein Concentration Assay. A total of 5 µl protein sample coupled with 795 µl of distilled water, then added with Bradford reagent until a final volume of 1 ml. After the mixture was incubated for 5 min at room temperature, the absorbance of the mixture was read by UV-Vis spectrophotometer at 595 nm. The BSA was used as standar in the assay [12].

Production of Antibody. Activation as a vaccine. The KatG protein as antigen protein was added with complete Freund's adjuvant (CFA) (ratio 1 : 1), then mixed in vortex for 30 mins. The active antigen protein was further used as a vaccine for the experimental animal [12, 14].

Mice immunization and serum isolation. Immunization was done three times to female mice Balb/C, aged 8-10 weeks with KatG antigen. The work was clustered in 4 treatments with 6 repetitions. First immunization, the mice were injected intraperitoneally by mixture of KatG in varing concentration 5, 15, and 45 µg/ml with CFA (ratio 1 : 1) in total volume of 100 µl. For control, it was used 6 mice with a strain, sex, and the same age, but injected with phosphate buffer saline (PBS) without antigen. The second immunization was done 14 days after the first immunization. The mice were injected by a mixture of KatG with incomplete Freud's adjuvant (ICFA) in the ratio 1 : 1. The third immunization was completed after 28 days, and the mice were only injected by KatG antigen in PBS without adjuvant [12-14].

Five days after the last immunization, the blood of the mice was drawn from their heart, then collected into tubes and stand at room temperature for 2 h to coagulate the red blood cells. Furthermore, the blood was centrifuged at 3000 rpm at 4 °C for 10 min. Serum was collected to measure the antibody titers and serotyping assay [12, 15].

Determination of antibody content. The measurements of antibody levels was detected with Protein Detector™ Peroxidase ELISA Kits, KPL Inc. One hundred µl of recombinant proteins was filled in the microplate with 96 wells, incubated at 4 °C for 24 hours to coat the plate well, then added with 200 µl 10% (w/v) of BSA as blocking reagent and re-incubated for 15 min at room temperature. To each of the wells was added 100 µL of primary antibody in PBS derived from serum, then incubated at room temperature for 1 hour and followed by washing the each well with 300 µl wash solution. To each of the wells was added with 100 µl of HRP - anti-mouse IgG conjugate and incubated for 1 h at room temperature, then washed with 300 µl wash solution and repeated three times. Additionally, each of the wells was added 100 µl substrate of 3,3′5,5′-tetramethylbenzidine (TMB). Finally, it was added 100 µl stop solution to every well. Optical density of antibody was measured at 450 nm [12, 13, 15].

Antibody isotyping assay. The 100 µl of serum samples and positive control working solution filled respectively to the test samples wells, then it sealed by a cover and incubated at 37 °C for 90 min. Subsequently, the cover was removed and the plate content discharged. The 100 µl of biotin – Anti – mouse IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgM antibodies working solution was added to the test samples wells respectively. The 6 Biotin – antibodies were also reacted to the positive control. The plate was sealed with a cover and incubated at 37 °C for 60 min. Subsequently, the cover was removed and plate washed 3 times with wash buffer. The 100 µl of HRP – Streptavidin Conjugate (SABC) working solution was added to each well, the the plate was covered and incubated at 37 °C for 60 min. The cover was removed, and plate washed 5 times with wash buffer. The 90 µl of TMB substrate was added to each well and incubated at 37 °C in dark within 15 min. The 50 µl of stop solution was added to each well and mix thoroughly. The absorbance of yellow product was recorded at a wavelength (λ) of 450 nm [12, 15, 24].

Results and Discussion

The best and potential antigens for subunit vaccine against TB are continuously sought after sequencing of the *M. tuberculosis* genome. However the antigen candidate should be released by intracellular mycobacteria which has many promiscuous epitopes to be presented to MHC class I and MHC class II, and can emerge a strong Ag-specific type 1 immune response [16-20]. This paper showed that KatG of *M. tuberculosis* has a potential antigen for vaccine candidate, because it can induce a high antibody response in the experimental animal.

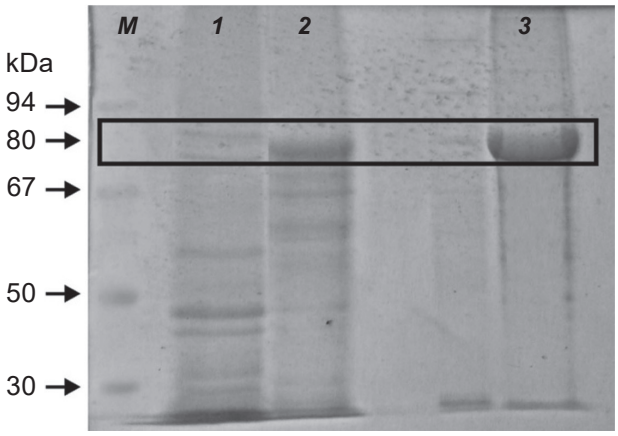

Fig. 1. Profile of KatG L19 protein in SDS–PAGE electropherogram before and after purified by affinity chromatoghraphy method. Lane M, protein marker; lane 1, extract of protein from E. coli had no a katG recombinant; lane 2, extract of protein from E. coli containing a katG recombinant; lane 3, pure KatG protein (80 kDa) after eluted with 150 mM imidazole from column

The antigen of KatG L19 protein. The KatG protein used for antigen was produced by expression of *katG* gene (2.2 kb) of *M. tuberculosis* clinical isolate L19 in *E. col*i BL21(DE3) using pCold II-DNA vector. The KatG protein 80 kDa was found in extract of *E. coli* BL21(DE3) recombinant after it analyzed by SDS PAGE. The KatG protein was absent in extract from non recombinant of *E. coli* BL21(DE3) (Fig. 1, lane *1* and *2*). The KatG L19 protein in extract then was purified by using affinity chromatography based on Immobilized Metal Affinity Chromatography (IMAC). The pure of KatG L19 protein resulted a band 80 kDa in SDS-PAGE after the protein extract eluted with sodium phosphate buffer containing 150 mM imidazole (Fig. 1, lane *3*). The pure of KatG L19 was further used as antigen for immunization in mice.

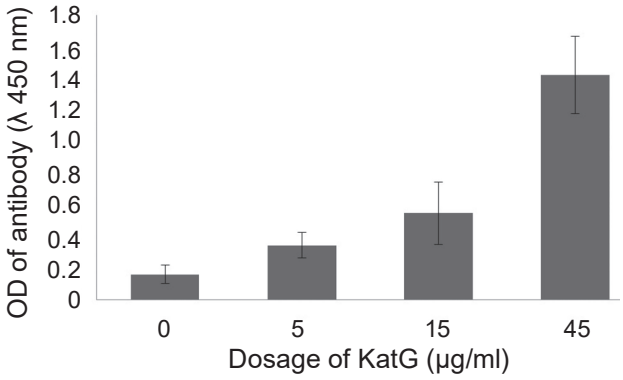

Fig. 2. Grafic of antibody levels produced by mice after injected with KatG L19 in various dosages. The KatG with dosage of 45 µg/ml gave a high response for antibody production in mice

Antibody Response of Mice Against to KatG L19. Immunization of mice with pure KatG L19 protein was performed in various doses 5, 15 and 45 µg/ml. The antibody product of each threatment was recorded by ELISA method. The KatG can elicite a high antibody response in mice after the experimental animal injected by the protein in various doses. The highest antibody in our research was reached when the mice vaccinated by KatG L19 in the dose of 45 µg/ml (Table and Fig. 2). Antibody response against to KatG at various doses of 0, 5, 15 and 45 µg/ml showed a significant differences among all treatments. The Anova and Duncan tests also confirmed the differences with score of $P < 0.05$, except for antibody product from the KatG antigen with doses 0 and 5 µg/ml (Table).

The optimal of antigen doses is a crutial to be investigated in the vaccination process, since the antigen dose significantly affects T cell functional avidity, differentiation status, and their subsequent protection against TB. Optimal protective capacity of T cells against *M. tuberculosis* infection relies on

The optical density (OD_{450nm}) of antibody response in mice after injected by KatG L19 in various doses

Treatment	Repetition						everage (χ)	SD
	1	2	3	4	5	6		
0 µg/ml	0.266	0.112	0.172	0.160	0.141	0.107	0.159	0.057[a]
5 µg/ml	0.446	0.270	0.322	0.252	0.340	0.430	0.343	0.080[a]
15 µg/ml	0.633	0.335	0.477	0.419	0.896	0.521	0.546	0.197[b]
45 µg/ml	1.824	1.192	1.373	1.170	1.557	1.424	1.423	0.244[c]

The test of ANOVA and Duncan presented a significance difference when the p score achieved at least 5%. No significant difference of antibody product was found in antigen dosages of 0 and 5 µg/ml, whereas the others had significant value

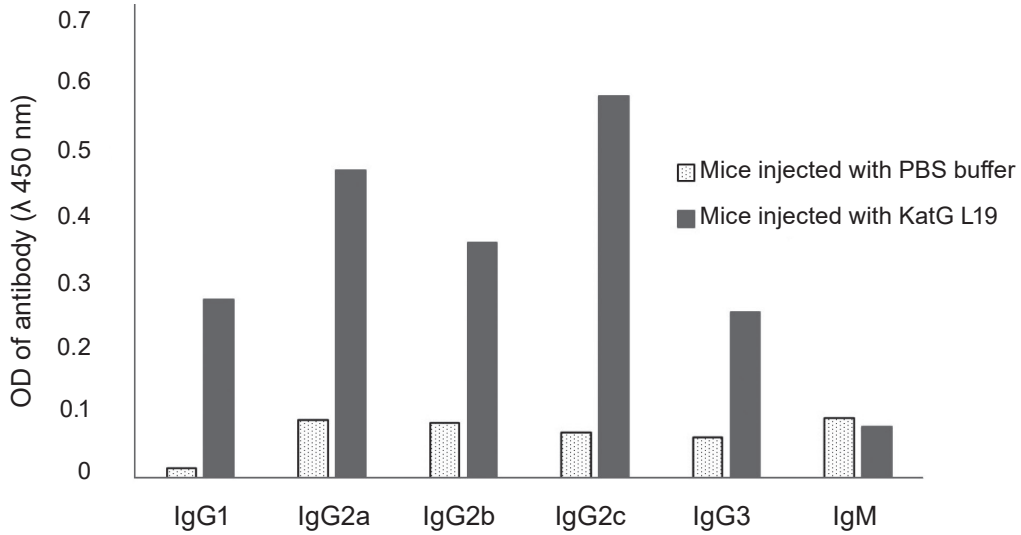

Fig. 3. Histograme of antibody types which produced by mice with present and absent of KatG L19 protein. The KatG L19 protein induced significantly the production of IgG1, IgG2a, IgG2b, IgG2c, IgG3 in mice

the ability of T cells to home into the lung parenchyma to make close contact with granuloma resident Mtb-infected host cells. The quality of T cells becomes very important in the protection of tuberculosis vaccines which provided by a significant role of antigen doses in vaccine-mediated protection [11].

Immunogenicity profile of KatG L19. The imunogenicity analysis of KatG requires information of the present specific immunoglobulin of IgG in serum which does a main role against to pathogens. In human, the immunoglobulin is the most abundant antibody isotype which found in the circulation and provide the majority of antibody-based immunity against invading pathogens [21-23].

The KatG which injected in mice stimulated the expression of six antibody types, i.e IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgGM. Of the antibodies, the IgG2c and IgG2a isotypes emerged higher that other, whereas IgM was expressed in the lowest content (Fig. 3). The first and second highest OD of immunoglobulin were found on the type IgG2c and IgG2a isotypes in all treatment groups for KatG doses of 5, 15 or 45 μg/ml. The data suggest that KatG elicits a high immunogenicity effect through the formation of IgG which is subsequently used to realize an immune response, so it is a possible to be deveoped as vaccine candidate in future.

The IgM antibody was produced quite a bit in mice after it immunized by KatG for 35 days. The IgM is classified as the first-line antibody for defense against infection from antigen proteins. High production of IgM can occur when the first week of infection, then decrease in the remaining weeks [17, 23]. Because of the blood isolation in this study was performed on day 35, so the obtained IgM level was low. The IgG2c and IgG2a subclasses were the highest immunoglobulin that appears in blood serum of mice, this might be due to their abilities to respond antigen proteins. Immunohistochemistry assay is developed in future to detect the lymphocytes level of CD4 and CD8 to provide well on the relationship of the antibody response with the number of lymphocyte cells.

Conclusion. The KatG L19 from clinical isolate of *M. tuberculosis* stimulate significantly immune response in mice to produce antibody. The protein induces the formation of immunoglobulin IgG1, IgG2a, IgG2b, IgG2c, IgG3, and IgM, where the IgG2c isotype produced dominantly. The high immunogenic exposed by KatG L19 encourages its development as a protein candidate for TB vaccine, so it needs to develope a clinical testing for the protein in future.

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АНАЛІЗ ІМУНОГЕННОСТІ ПРОТЕЇНУ KatG ІЗ *Mycobacterium tuberculosis* У МИШЕЙ: ПОПЕРЕДНІЙ СКРИНІНГ ВАКЦИНИ ТВ

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Туберкульоз залишається одним із поширеніших захворювань, хоча багато людей пройшли вакцинацію БЦЖ. Неефективність вакцини БЦЖ є однією з причин, які ускладнюють профілактику поширення туберкульозу. Метою дослідження було визначення імуногенності протеїну KatG в клінічному ізоляті L19 штаму *M. tuberculosis* у мишей. KatG, як антиген, отримували експресією гена *katG* клінічного ізоляту L19 *M. tuberculosis* в *Escherichia coli* BL21 із використанням вектора pColdII-DNA. KatG після очищення афінною хроматографією використовували для вакцинації мишей. Експресією katG в *E. coli* BL21 одержували протеїн KatG із молекулярної масою 80 кДа за електрофорезу на PAGE-SDS. Очищений протеїн KatG значно стимулював імунну відповідь мишей, спричинюючи продукування антитіл IgG1, IgG2a, IgG2b, IgG2c, IgG3 і IgM. Найвищий рівень антитіл спостерігали у разі вакцинації мишей KatG L19 в дозі 45 мкг/мл. Серед антитіл IgG2c-ізотип переважно продукувався в сироватці крові. Показано, що протеїн KatG виявляє високу імуногенність у мишей і може бути запропонований як потенціальна вакцина проти туберкульозу. Необхідно в подальшому провести клінічні дослідження для оцінки безпеки його застосування як терапевтичного протеїну.

Ключові слова: KatG, імуногенність, *M. tuberculosis*, вакцина, клінічний ізолят.

АНАЛИЗ ИММУНОГЕННОСТИ ПРОТЕИНА KatG ИЗ *Mycobacterium tuberculosis* У МЫШЕЙ: ПРЕДВАРИТЕЛЬНЫЙ СКРИНИНГ ВАКЦИНЫ ТВ

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Туберкулез остается одним из широко распространенных заболеваний, хотя большинство людей вакцинированы БЦЖ. Неэффективность вакцины БЦЖ является одной из причин, которые затрудняют профилактику распространения туберкулеза. Целью исследования было определение иммуногенности протеина KatG в клиническом изоляте L19 штамма *M. tuberculosis* у мышей. KatG в качестве антигена получали экспрессией гена *katG* клинического изолята L19 *M. tuberculosis* в *Escherichia coli* BL21 с использованием вектора pColdII-DNA. KatG после очистки аффинной хроматографией использовали для вакцинации мышей. Экспрессией katG в *E. coli* BL21 получали протеин KatG с молекулярной массой 80 кДа при электрофорезе на PAGE-SDS. Очищенный протеин KatG значительно стимулировал иммунный ответ мышей, вызывая продуцирование антител IgG1, IgG2a, IgG2b, IgG2c, IgG3 и IgM. Самый высокий уровень антител наблюдали при вакцинации мышей KatG L19 в дозе 45 мкг/мл. Среди антител IgG2c-изотип преимущественно продуцировался в сыворотке крови. Показано, что протеин KatG проявляет высокую иммуногенность у мышей и может быть предложен в качестве потенциальной вакцины против туберкулеза. Необходимо в дальнейшем провести клинические исследования для оценки безопасности его применения в качестве терапевтического протеина.

Ключевые слова: KatG, иммуногенность, *M. tuberculosis*, вакцина, клинический изолят.

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