

CLONING AND EXPRESSION OF MCE1A GENE FROM *MYCOBACTERIUM TUBERCULOSIS* BEIJING AND H37RV STRAIN FOR VACCINE CANDIDATE DEVELOPMENT

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Abstract

Background: Tuberculosis remains the leading cause of death in the world, especially wherever poverty, malnutrition and poor housing prevail. *Mycobacterium tuberculosis* Beijing strain is the most common strain that causes tuberculosis in Indonesia. The wide spread of tuberculosis has been further aggravated by HIV-AIDS and drug resistance. Unfortunately, Bacille Calmette-Guerin (BCG) as the current vaccine has different protection function and efficacy. According to function analysis, *mce1A* gene was predicted to have a role in host invasion and survival of *Mycobacterium tuberculosis* in human macrophages.

Materials and Methods: We performed cloning and protein expression of *Mce1A* gene of *Mycobacterium tuberculosis* Beijing strain as local isolate and standard strain H37Rv as a comparison on the expression system *Escherichia coli* BL21(DE3). *Mce1A* gene from the strains were amplified by PCR and inserted into the vector pET28a. Each resulting recombinant plasmid was subsequently transformed into *E. coli* BL21(DE3) and *Mce1A* protein was expressed with IPTG induction.

Results: *E. coli* BL21(DE3) was successfully transformed with a recombinant plasmid that contains the *Mce1A* gene insert with correct orientation and reading frame. There was no mutation found in the amino acids sequence for B and T cell epitope. *Mce1A* expression in *E. coli* BL21(DE3) showed a protein band that was higher than expected. The protein was confirmed with Western blotting using anti-His detector.

Conclusion: We assumed that *Mce1A* recombinant protein that has been expressed in *E. coli* BL21(DE3) is in their dimeric form or alternatively formed aggregates of different sizes.

Key words: *Mce1A* gene, vaccine, *Mycobacterium tuberculosis*, Beijing strain

Introduction

From past centuries up to the present day, tuberculosis (TB) has remained the leading cause of death in the world from infectious disease. It kills about 3 million people and infects almost 9 million others every year wherever poverty, malnutrition and poor housing prevail (Goering et al, 2008, Lönnroth et al, 2010). Ninety percent of infected individuals are latently infected where they harbor the pathogen in its dormant form, whereas the remaining 10 percent suffer from active disease (Kaufmann et al, 2010, Thaïss, 2010). WHO annual report on 2013 estimated that in 2012 approximately 8.6 million people developed TB and of those, 1.3 million died from the disease in the world. Indonesia is placed at ninth in the world after China, Philipines, Brazil, Russia, Tanzania, Kenya, Thailand and Vietnam in terms of number of cases of tuberculosis. In Indonesia, it was estimated for new cases and relapse infection of about 297 cases per 100,000 people while the mortality rate was about 27 cases per 100,000 people each year (WHO, 2013).

The wide-spread of TB has been further aggravated by other infectious diseases such as HIV-AIDS. An estimated 1.1 million (13%) of the 8.6 million people who developed TB in 2012 were HIV-positive (WHO, 2013). The compromise of immune mechanism in human immunodeficiency virus (HIV)-infected individuals that leads either to reactivation of old tuberculous infections or to increased susceptibility to new infection is a major contributor to the increasing incidence of tuberculosis (Raviglione et al, 1995). Another factor is the emergence of multidrug-resistant tubercle bacilli (Aziz et al, 2006). Globally in 2012, an estimated ~450,000 people developed MDR-TB and there were an estimated 170,000 deaths from MDR-TB (WHO, 2013). Therefore, current antibiotics TB treatment has become ineffective.

Many strains of *Mycobacterium tuberculosis* caused tuberculosis infection in Indonesia, but the Beijing strain is the most common. A study by van Crevel using spoligotyping technique showed that 34 percent of *M. tuberculosis* isolate from Indonesia tuberculosis patients is the Beijing strain. Meanwhile in several countries like China, Mongolia, South Korea, Thailand and Vietnam, the percentages for the Beijing strain are about 85, 50, 43, 37 and 34 respectively. This facts suggests that this clone spreads in Southeast Asia, where TB is endemic (van Crevel et al, 2001). Previous studies stated that the Beijing strain is highly prevalent (Anh et al, 2000), more virulent and responsible for drug resistance (Kurepina et al, 1998). Beijing genotype strains account for a substantial proportion of multidrug resistant TB cases in Azerbaijan, Estonia and Cuba (Marttila et al, 1998, Niemann et al, 1997, Diaz et al, 1998).

Bacille Calmette-Guerin (BCG) is the current vaccine for TB. It has been used for decades to prevent TB on uninfected individuals. BCG is impressive with respect to its low cost and its high safety (Ho et al, 2010). It also has been used in many countries although its weak protective function varies from 77 percent in United Kingdom to 0 percent in Chinglepur, India to even negative efficacy in some studies performed in the US (Fine, 1989, Bannon, 1999). The difference in protective function of the vaccine perhaps was caused by the difference of the BCG strains that have been used (Behr et al, 1999), beside patients co-infection factor by parasites (Malhotra et al, 1999, Elias et al, 2005). It was assumed that some of the BCG strains that have been used for vaccines lost the genes responsible for synthesizing proteins that stimulate protective antibody generation (Svenson et al, 2010). BCG vaccine limitations have urged many researchers to work on further vaccine improvement by proposing new vaccine candidates from recombinant proteins or recombinant strains.

Several proteins of *M. tuberculosis* are required as a defense mechanism inside host cells, particularly in the macrophages or as disease progression. Srivastava, et al. reported that several genes that have been fused with *gfp* gene were expressed inside macrophage, such as Rv0619 (*mce1A*), Rv3097c(PE_PGRS63), Rv2232, Rv1026, Rv1635, *viuB*, Rv2231 (*cobC*), and Rv0997 gene. *Mce1A* gene showed the highest expression level whereas PE_PGRS63 gene was the lowest. According to a function analysis, *mce1A* gene was predicted to display a role in host invasion by *M. tuberculosis* and survival of the pathogen in the human macrophages, while PE_PGRS63 gene is probably a triacyl glycerol lipase (esterase) (Srivastava et al, 2007).

Studies on protein expression of *mce1A* gene of *Mycobacterium tuberculosis* Beijing strain as local isolates have not been done much in Indonesia. Therefore, in this article we report cloning and expression of *mce1A* gene of *M. tuberculosis* Beijing strain as local isolate and standard strain H37Rv as a comparison on the expression system of *Escherichia coli* BL21(DE3). The results of this study are expected to open up opportunities for further research on the effects of the expressed protein to the immune system and to provide a possibility to develop TB subunit vaccine candidates.

Materials and Methods

Strain, plasmid and media

M. tuberculosis Beijing strain was from the Laboratory of Bacteriology, Puslitbang Kemenkes RI while the *M. tuberculosis* H37Rv strain was from the Laboratory of Tuberculosis, Microbiology Department, University of Indonesia; *E. coli* DH5 α and the plasmid pET-28a were purchased from Novagen, USA; *E. coli* BL-21(DE3) was from Promega, USA.

The Beijing and H37Rv strains were grown on Lowenstein-Jensen agar at 37°C, while *E. coli* DH5 α and BL21(DE3) cells were in Luria-Bertani (LB) broth or on LB agar in presence of kanamycin (30 μ g/ml) wherever appropriate.

M. tuberculosis Beijing strain and H37Rv *mce1A* gene synthesis and cloning into *E. coli* strain DH5 α and BL21(DE3)

Mce1A gene from H37Rv (Rv0619) and Beijing strains were each amplified by PCR technique using FastStart DNA Polymerase (Roche Applied Science, Germany). The DNA sequences of forward (F) and reverse (R) primers for the amplification of *mce1A* gene are shown below.

mce1A F: 5'-**GAATTC**ATGACGACGCCGGGG-3'

mce1A R: 5'-**CTCGAG**TCATGGGTTGATCGTG-3'

Forward primer contained an *Eco*RI restriction enzyme site (shown in bold) whereas the reverse primer contained a *Xho*I restriction site (shown in bold). The reaction mixtures in a final volume of 50 μ l contained; *M. tuberculosis* genomic DNA, 5 μ l of 10x polymerase buffer (Roche), 20 pmol of each primer and 100 μ mol of dNTPs. The cycle parameters consisted of thirty five (35) cycle of 5 min at 95°C; 30 s at 95°C; 30 s at 55°C; 1 min 30 s at 72°C and finalized with 72°C for 7 min. The amplified products were resolved by agarose-gel electrophoresis. The PCR resulted in amplification of a single DNA product of the expected size, the gel slice was excised and the DNA fragment was purified.

Each resulting amplicons were subsequently inserted into the pET-28a (Novagen) plasmid inframe with the T7 promoter and the T7 terminator region to generate the recombinant plasmids pET28a/Mce1Abeijing and pET28a/Mce1AH37Rv respectively with hexa histidine tags. To illustrate the schematic construction of plasmid is shown as reference in Fig.1. Finally, plasmids pET28a/Mce1Abeijing and pET28a/Mce1AH37Rv were transformed

into DH5 α and BL21(DE3) strains of *E. coli* for expression studies. The presence of the inserts was confirmed by PCR colony, plasmid isolation and DNA sequencing.

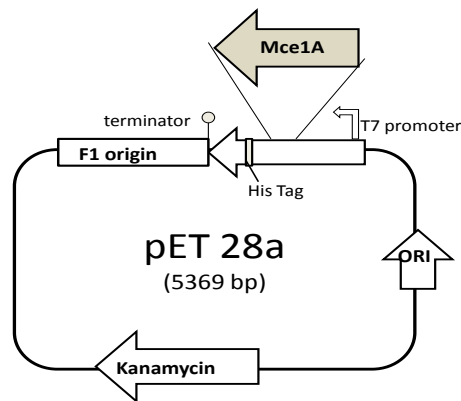


Figure 1: Schematic construction of recombinant plasmid pET28a/Mce1A. The reading frame consist of: T7 promoter, Mce1A gene insert, His-tag and T7 terminator. Kanamycin resistance gene as selection marker and Origin of Replication site (ORI).

Expression of recombinant Mce1A in *E. coli* BL21(DE3)

An overnight culture of *E. coli* BL21(DE3) carrying the recombinant expression plasmid pET28a/Mce1A-Beijing was diluted 1:10 in fresh LB broth containing kanamycin and grown with vigorous shaking at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.4. Isopropyl- β -D-1-thiogalactopyranosida (IPTG, Sigma) was added to the culture to a final concentration of 1 mM and the induced cultures were grown for an additional 4 h. Cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and the supernatant was discarded. The pellet was subsequently washed in sterile cold distilled water and then centrifuged at 5,000 rpm for 10 min at 4°C and the supernatant was discarded. The pellet was resuspended in sterile, cold distilled water and analysed on SDS-PAGE for expression of the recombinant protein. The expressed Mce1A protein was confirmed with a Western blot experiment using anti-Hisdetector™ Nickel-HRP conjugate (KPL). The recombinant Mce1A-H37Rv was also expressed and confirmed using the same protocol.

Results

Analysis of Mce1A gene in the recombinant plasmids

The presence of *mce1A* gene in each recombinant plasmid, *i.e.* pET28a/Mce1ABeijing or pET28a/Mce1AH37Rv, was verified by using PCR colony technique using 5'vector primer and 3'insert primer and visualized using an agarose gel. The band of the resulting amplicon appeared on the gel as a single band at the size as it should be, suggesting that there is an insert of *mce1A* gene. Further analysis was performed by isolating the DNA plasmid and visualizing it in agarose 0,8%. The band of the recombinant plasmids were bigger in size than the *wild type* plasmid. Analysis for orientation and framing of *mce1A* gene in each recombinant plasmid was performed with DNA sequencing. Based on these results, the *mce1A* genes were successfully fused in the recombinant plasmids with T7 promoter and T7 terminal region (data not shown).

Expression of recombinant protein Mce1A in *E. coli* BL21(DE3)

The expression of Mce1A from each recombinant plasmids was studied in *E. coli* BL21(DE3) strain. By detecting proteins using SDS-PAGE 15%, it appeared that both plasmids in this strain were able to express His-tagged Mce1A at high level. Interestingly, the molecular weight of the expressed proteins was higher than expected. They were observed at ~95 kDa (Figure 2) instead of 47 kDa as the calculated molecular weight for Mce1A. For further confirmation we detected the proteins using anti-His-tag monoclonal antibody by Western blot technique. The molecular weight of the proteins was are also higher than expected (Figure 3).

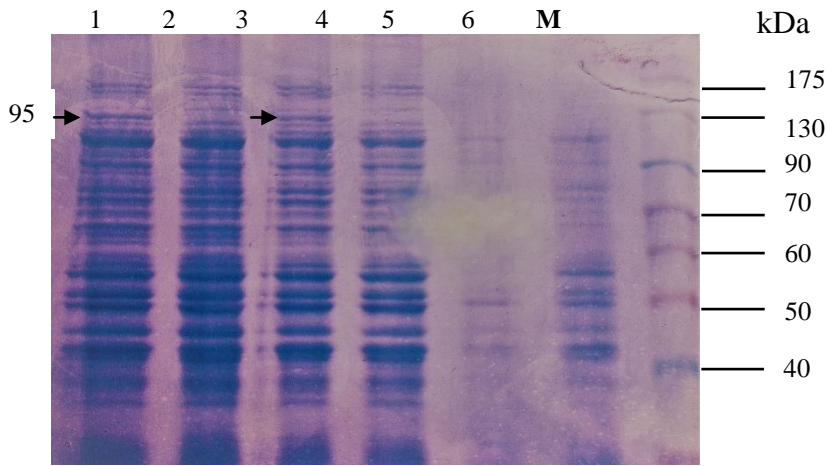


Figure 2: SDS-polyacrylamide gel electrophoresis of whole-cell lysates of uninduced and induced recombinant *E. coli* BL21(DE3) cells. Lane M: markers; their molecular weight is indicated in kDa on the right side. Lane 1 and 2: lysate of *E. coli* BL21(DE3) cells carrying pET28a/Mce1A-Beijing with and without IPTG induction respectively; Lane 3 and 4: lysate of *E. coli* BL21(DE3) cells carrying pET28a/Mce1A-H37Rv with and without IPTG induction respectively; Lane 5: lysate of IPTG-induced *E. coli* BL21(DE3) containing wild type pET28a; Lane 6: lysate of IPTG-uninduced *E. coli* BL21(DE3) containing wild type pET28a. Expressed proteins are indicated by an arrow.

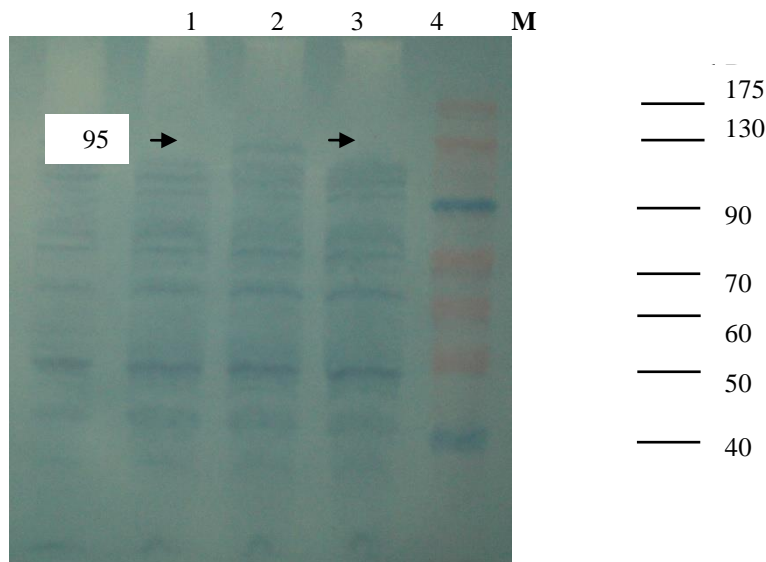


Figure 3: Western blot analysis. Proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. Monoclonal antibody against His-tag was used at a dilution of 1:8000 to detect signal of the recombinant proteins. Lane M: protein markers; their molecular weight is indicated in kDa on the right side. Lane 1 and 2: lysate of *E. coli* BL21(DE3) cells carrying pET28a/Mce1A-Beijing with and without IPTG induction, respectively; Lane 3 and 4: lysate of *E. coli* BL21(DE3) cells carrying pET28a/Mce1A-H37Rv with and without IPTG induction, respectively; Expressed proteins are indicated by an arrow.

Discussion

The initial step in the pathogenesis by intracellular pathogens is the invasion of the host cell. In case of *M. tuberculosis*, Mce1A protein of *mce1* operon was the first protein of the pathogen to be implicated in its invasion and survival inside the macrophages (Ahmad et al 2004, Harboe et al 2002). Mammalian cell entry (Mce) proteins are a family of proteins that encodes *invasin/adhesins*-like proteins with hydrophobic amino acid regions at the N-terminus, possibly representing signal sequences. These proteins are located on the bacterial cell surface (Ahmad et al, 2005).

Recombinant proteins can be expressed using prokaryotic systems, eukaryotic systems or *in vitro* systems. We chose the prokaryotic systems because we would like to express a protein of prokaryotic origin and the obvious choice is to use *E. coli*. The *E. coli* system is the first-choice host for the initial screening of recombinant protein expression, because these cells can be readily manipulated, are cultured inexpensively and grow rapidly. An *E. coli* expression vector possesses the same features found in any vector, such as a selection marker, origin of replication,

transcriptional promoter, 5' untranslated region (5'UTR) and translation initiation site. The pET expression system featuring the T7 promoter is by far the most widely used system for heterogeneous expression in *E. coli*. (Jia B, Jeon CO, 2016).

We also used *E. coli* strain BL21(DE3) as expression vector that allow high-efficiency protein expression of any gene that is under the control of a T7 promoter and has a ribosome binding site. It is lysogenic for λ -DE3, which contains the T7 bacteriophage gene I, encoding T7 RNA polymerase under the control of the *lacUV* promoter. T7 promoter activity is strong, and a recombinant protein can accumulate to up to 50% of total cellular proteins. T7 expression hosts such as DE3 strains contain a chromosomal copy of the T7 phage RNA polymerase gene under control of the *lac* promoter derivative *lacUV5*. When Isopropyl- β -D-1-thiogalactopyranosida (IPTG) is added, LacI binding to the *lac* operator is inhibited, allowing for the expression of T7 polymerase, which transcribes the target gene and leads to recombinant protein production (Jia B, Jeon CO, 2016).

In the present study we have generated recombinant *Escherichia coli* BL21(DE3) clones in which *Mycobacterium tuberculosis* Beijing and H37Rv strain Mce1A transgene are integrated. SDS-PAGE and Western blot analysis suggested that the molecular weight of the recombinant protein from both clones are about twice (~95 kDa) the calculated molecular weight for Mce1A from *M. tuberculosis* (47 kDa). Several explanations in regard to this observation may be considered. Mammalian cell entry (Mce) proteins are a family of proteins that encodes *invasin/adhesins*-like proteins with hydrophobic amino acid regions at the N-terminus, possibly representing signal sequences. These proteins are located on the bacterial cell surface (Ahmad et al, 2005). Generally, monomeric Mce1A was difficult to be obtained in a pure form as the protein showed the tendency to degrade and formed aggregates of different sizes. This resulted in the appearance of protein bands of lower or higher molecular weight than the 47 kDa protein band expected from monomeric Mce1A (Ahmad et al, 1999). Another study reported that in sonicates of *M. tuberculosis*, the Mce1A protein band was located at a high position on the gel, corresponding to the 250 kDa molecular mass marker. This position did not change in different experiments that used increased concentrations of SDS in the application buffer. These findings may indicate a type of complex formation between Mce1A and other component(s) via covalent bonds. Also, there is a tendency of free Mce1A to aggregate into higher molecular weight forms (Harboe et al, 1999). The amino acid identity exhibited by various Mce proteins of each *mce* operon is similar to that exhibited by other conserved proteins and indicates a common evolutionary origin from an ancestral *mce* gene. (Ahmad et al, 2005). Further study on Mce3 proteins encoded by the *mce3* operon of *M. tuberculosis* also showed that these Mce3 proteins exhibited greater tendency to produce dimeric forms which persisted even under the reducing and denaturing conditions of SDS-PAGE (Ahmad et al, 2004). Another study on purified Mce1A protein detected the protein as doublet. Since anti His-tag antibodies detected the lower molecular weight band, it is probably due to partial degradation (Saini, 2008).

Conclusion

We successfully cloned and expressed *mce1A* (Rv0169) genes of *M. tuberculosis mce1* operon from Beijing and H37Rv strains in *E. coli*. The recombinant protein molecular weight from both clones are about twice (~95 kDa) as the calculated for Mce1A from *M. tuberculosis* (47 kDa). We assumed that the recombinant protein is in their dimeric form or alternatively formed aggregates of different sizes. This explanation should be valuable in further studies of expression at the protein level and exposure of proteins on the cell surface of *M. tuberculosis* under different experimental conditions.

Conflict of Interestt: The authors hereby declare that there is no competing interest.

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