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Sequence Analysis of the Gene Region Encoding ESAT-6, Ag85B, and Ag85 C Proteins from Clinical Isolates of *Mycobacterium tuberculosis*

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Abstract

Mycobacterium tuberculosis secreted proteins in culture filtrate and early phase of infection, such as early secretory antigen target 6 (ESAT-6), culture filtrate protein 10 (CFP-10), and antigen 85 complex i.e. Ag85A, Ag85B, and Ag85C which played roles in adherence, invasion, cytolysis, and evading cytosol of macrophage, were virulence factors that determined the immune responses important on pathogenesis of Tuberculosis (TB), including granuloma formation or tissue that determine the degree of disease. The purpose of this research was to analyze the gene region sequence encoding ESAT-6, Ag85B, and Ag85C of *Mycobacterium tuberculosis. Mycobacterium tuberculosis* strain analyzed was taken from sputum of pulmonary TB patients in East Java, Indonesia. Sequenced DNA analyzed using GENETYX Ver.10. There were no SNPs both inside and outside epitope region of gene encoding ESAT-6, Ag85B, and Ag85C from clinical isolates of *Mycobacterium tuberculosis*. From this study, it could be concluded that the highly conserved gene region encoding ESAT-6, Ag85B, and Ag85C revealed no sequence polymorphism SNPs in epitope regions among *Mycobacterium tuberculosis* clinical isolates from sputum specimens of pulmonary TB patients.

Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)

Keywords: *Mycobacterium tuberculosis*; ESAT-6; Ag85B; Ag85C; the region gene

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Nomenclature

ESAT-6	early secretory antigen target	*esxA*	ESAT-6
CFP-10	culture filtrate protein	*fbpA*	secreted fibronectin-binding protein antigen 85-A
Ag85A	Antigen 85A	*fbpB*	secreted fibronectin-binding protein antigen 85-B
Ag85B	Antigen 85B	*fbpC*	secreted fibronectin-binding protein antigen 85-C
Ag85C	Antigen 85C	CMI	Cell-mediated immunity
TB	Tuberculosis	DTH	Delayed-type hypersensitivity
Px	Patient	PAM	Peptidylglycine α-amidating monooxygenase
MTBC	*Mycobacterium tuberculosis* complex	SNPs	Single nucleotide polymorphisms

1. Introduction

As high TB burden, high HIV burden, and high MDR-TB burden country, Indonesia still possessed large health concern[1]. TB burden estimation in 2013 revealed prevalence of 680.000 (340.000-1.100.000) with rate per 100.000 population of 272 (138-450), including HIV+TB, in 250 million population with 71% case detection; while incidence amounted 460.000 (410.000-520.000) and rate of 183 (164-207), including HIV+TB, and mortality of 64.000 (36.000-93.000) with 25 (14-37) rate that excludes HIV+TB[1]. During 2014 in East Java, the total numbers of TB patients being treated are 42.222 (second after West Java with number 61.721) with mortality is 1.308, and children TB patients are 2.342[2]. In 2014, total population of East Java is 804 per 4.104 km^2 with the crowded population is located at big cities such as Surabaya[3].

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* complex (MTBC) as intracellular pathogen which had a long duration of time and invaded various organs, with pulmonary parenchyma as the highest cases of organ infected and the leading cause of mortality and spreading.

The effort to resolve this global problem had been focused on several important strategies, which were to improve the diagnosis method and effective vaccines applied[4]. TB pathogenesis was based on the stage of the complex mechanism of CMI with ensuing DTH reaction as the sequence interaction outcome of antigen molecules PAMs that predominantly acted as secretory virulence effectors. These molecules were translocated through cell wall multiprotein of MTBC which would then affected RPR receptors or adhesin molecules of the host cells, prolonging the latent and active progress of disease.

Analyses of MTBC molecular biological characteristics have identified several antigens as virulence agents with important role in TB pathogenesis[4]. ESX-1 system encoded by *esx* gene family allowed specialized protein secretion, 6-kDa early antigenic target (ESAT-6) and 10-kDa culture filtrate protein (CFP-10) through mycobacterial cell envelope; ESAT-6 (*esxA* or Rv3875) and CFP-10 (*esxB* or Rv3874) found in the phagosome; ESAT-6 is a secretory effector inducing membrane lytic of macrophage apoptosis that enable phagosomal escape, cell entry, and intercellular spread, intracellular survival, and as a major pathogenic determinant[5,6,7,8]. The *esx* gene family is dynamic, and sequence changes likely lead to immune variation; nevertheless there was no or less variant found in *esxA* and *esxB*[9]. Human T cell epitopes of MTBC were evolutionarily hyperconserved, distinguished by lack of antigenic variation and immune evasion, however there were polymorphisms found in the nucleic acid sequence encoding significant virulence factors, which may alters the antigens produced, and also may cause change in function, or allowing immune evasion [4,5,9]. Previous study conducted by Davilla *et al.* (2010) reported that no DNA polymorphism in *esxA* and *esxH* gene and only one SNP change (C to A) in *fbpB* gene among 39 (44.3%) of the 88 strains[10].

Predominantly secreted proteins in MTBC culture filtrate and early secreted protein in MTBC infection are early secretory antigen target (ESAT-6), culture filtrate protein (CFP-10), antigen 85 Ag85A, Ag85B, and Ag85C which played role in adherence, invasion, cytolysis, evasion of cytosol in macrophage, acted as virulence factors determining granuloma formation or tissue necrosis or severity of disease [4,5,9]. Ag8B is one of the three homologous proteins part of Ag85 complex, constituting up to 41% of total mycobacterial proteins in log-phase culture[11]; vaccination by BCG recombinant that overexpresses Ag85B able to induce better protection compared to traditional BCG vaccine[12].

Polymorphism able to change protein structure and function could be determined as antigenic variant that functioning on immune evasion. Antigen 85 complex consisted of three dominant secreted proteins i.e: Ag85A,

Ag85B, and Ag85C, which played role in TB pathogenesis, possessed an enzymatic mycolyltransferase activity, involved in cell wall synthesis; also contributed in mycobacteria adherence, invasion, and dissemination in host cells; induced Th1-type immune responses, control intracellular infections. Genetic diversity on sequence variation could affect immune recognition[4]. The purpose of this research was to analyze the nucleotide sequence of gene region encoding ESAT-6, Ag85B, and Ag85C among clinical isolates of *Mycobacterium tuberculosis* from pulmonary TB patients. Isolates selected were sensitive to first line anti-TB drugs.

2. Methods

Clinical isolates of *Mycobacterium tuberculosis complex* taken from sputum specimens of pulmonary TB patients in Dr Soetomo Hospital, Surabaya; patients mainly came from East Java area in Indonesia. Clinical isolates collected from January 2014 until December 2014, then three isolates sensitive to anti-TB first line (Isoniazid, Rifampicin, Ethambutol, Streptomycin, and Pyrazinamide) were chosen via randomized sampling for PCR with gene region encoding ESAT-6, Ag85B, and Ag85C proteins as target. DNA products then sequenced and sequence were analyzed then translated to proteins. Translated proteins and identified human T cell epitope were analyzed using GENETYX Ver.10. Primers used were designed using Clone Manager software according to *Mycobacterium tuberculosis* H37Rv with gene encoding ESAT-6, Ag85B, and Ag85C as the region target i.e.:
ESAT-6, length 351 bp of *esxA* gene, primers,
5'- GAG GAG AAG CCC GGT TGC CCT TTC GCT ATT CTA CG -3'R
5'- GAC GAC GAC AAG ATG ACA GAG CAG CAG TGG AAT -3'F
Ag85B, length 1032 bp, primers,
5'- GAC GAC GAC AAG ATG ACA GAC GTG AGC CGA AAG ATT CGA G -3'F
5'- GAG GAG AAG CCC GGT TAA GCA ACC CTT CGG TTG AT -3'R
Ag85C, length 1206 bp, primers,
5'- GAC GAC GAC AAG ATG ACG TTC TTC GAA CAG GTG CG-3'F
5'- GAG GAG AAG CCC GGT AAC CAA TTA CGG GTC GAG TTA G -3'R

3. Results and discussion

Fig. 1 (a, b, c) were PCR products of the target DNA region of the gene encoding ESAT-6, Ag85B, Ag85C from three clinical isolates.

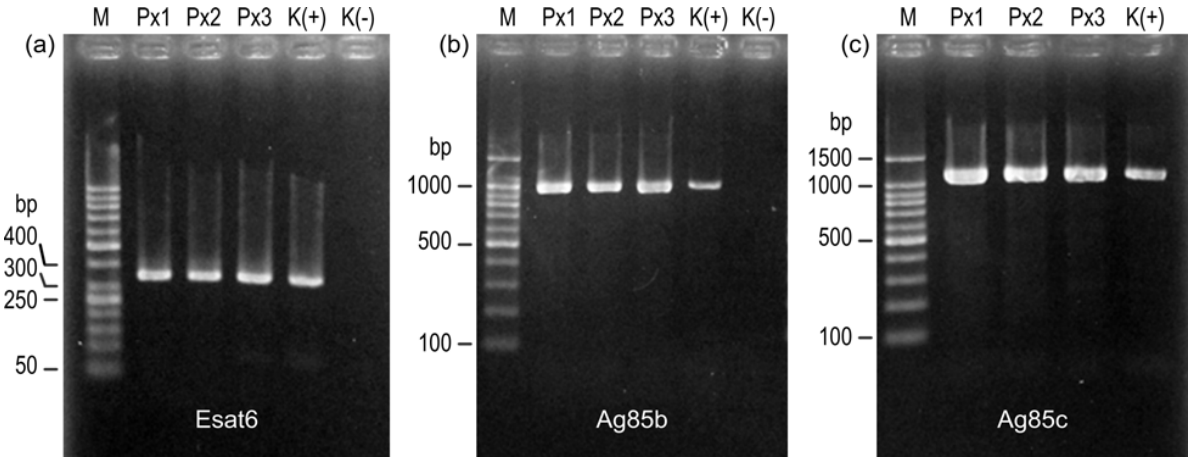

Fig. 1. (a) PCR product of the gene region of *esxA* (ESAT-6) in *Mycobacterium tuberculosis* that shows DNA bands in 351 bp; (b) PCR product ofAg85B *(fbpB)* in *Mycobacterium tuberculosis* showing DNA bands in 1032 bp*;* (c) PCR product of Ag85C (*fbpC*) in *Mycobacterium* tuberculosis that show DNA bands in 1206 bp.

The analyzed nucleotide sequence showed no SNP in the *esxA*, *fbpB,* and *fbpC* gene region of *Mycobacterium tuberculosis* clinical isolates collected from sputum specimens of pulmonary TB patients sensitive to first line anti-TB drugs. All of nucleotide sequences of these genes were 100% homologous or identical.

T cell epitopes identified using IAd pattern revealed 4 epitopes in ESAT-6, 7 epitopes in Ag85B, and 7 epitopes in Ag85C proteins, all of the three samples (Px1, Px2, Px3) were found identical to wild type *Mycobacterium tuberculosis* strain H37Rv (Table 1).

Table 1. T cell epitopes identified in ESAT-6, Ag85B, and Ag85C protein region of *Mycobacterium tuberculosis* clinical isolates from sputum specimens of pulmonary TB patients in East Java Indonesia, 2014

Gene	T cell epitopes (IAd Pattern Position)	
	Amino acid position	Amino acid sequence
Esat 6 gene (*esxA/ rv* 3875) of Px1, Px2, Px3	4-9	ASAIQG
	11-16	VTSIHS
	26-31	TKLAAA
	79-84	VTGMFA
Ag85B gene (*fbpB/ rv*1886*c)* of Px1, Px2, Px3	6-11	IGTAAA
	21-26	AGGAAT
	68-73	LDGLRA
	138-143	LSANRA
	146-151	PTGSAA
	175-180	AGSLSA
	200-205	AGGYKA
Ag85C gene (*fbpC/ rv*0129*c*) of Px1, Px2, Px3	1-6	LRSAAT
	48-53	LQVPSA
	74-79	LDGLRA
	167-172	ALILAA
	269-274	LRTNQT
	306-311	LVAMKA
	320-325	ATPPAA

Results revealed no SNP or polymorphism found in *esxA*, *fbpB,* and *fbpC* gene region of *Mycobacterium tuberculosis* clinical isolates from sputum specimens of pulmonary TB patients sensitive to first line anti-TB drugs, which represent of virulence strains from clinical severe diseases. Those *Mycobacterium tuberculosis* gene region of *esxA*, *fbpB,* and *fbpC* were revealed to be highly conserved, indicate that this finding was important for diagnostic and vaccine development.

Solans *et al*. (2014) have researched about a specific polymorphism in *Mycobacterium tuberculosis* H37Rv and revealed that there are polymorphism in *whiB6* as a part of the PhoP regulon that have important role in ESAT-6 protein expression at transcriptional regulation[5]. On the other hand, Davila *et al*. (2010) reported that among the 88 strains, genetic analysis of *esxA* and *esxH* revealed no nucleotide polymorphisms in the genes encoding for ESAT-6 and TB10.4 proteins, besides that, the analysis of *fbpB* revealed only one synonymous SNPs change C to A, located at position 714 bp of the gene sequence, among 39 (44.3%) of the 88 strains. It is also showed that Ag85B-ESAT-6 and Ag85B-TB10.4 vaccine candidates may be effective in geographically distinct areas of the world because *esxA*, *esxB,* and *fbpB* genes are highly conserved in two distinct populations[10]. Comparing the result in this study, research conducted by Davila et al (2010) has similarity with present study.

In addition, results in this study are also in agreement with Uplekar *et al.* (2011) concluding that there is no sequence variation has yet been observed in *esxA*. Uplekar *et al*. (2011) reported that there is an amino acid substitution (E68K) in *esxB* that appear in 18 of the 108 strains representing diverse lineages, this substitution occurs within a known human T cell epitope. All 23 esx genes from 108 clinical samples were sequenced to identify substitutions that may give an effect on the immunogenicity or function of Esx proteins. Esx genes encoded within the ESX-1 to ESX-4 loci displayed less variation than the esx genes located outside these loci. Result from Uplekar *et al*. (2011) also showed that three out of five *esxA* paralogs in all clinical data set encoded by the ESX-1 to ESX-5 loci were invariant. There was also an absence of silent substitutions in the esx components of the ESX-1 to ESX-4 loci with the exception of esxD[9].

esxA (*rv*3875) genes encoding ESAT-6 protein is a member of *esx* genes family, located adjacent to CFP-10-encoding *esxB* gene which were cotranscribed. Gene sequence analyzed from *Mycobacterium tuberculosis* clinical isolates revealed no *esxA* variation and absence of silent substitutions. Other member of *esx* genes family (*esxB* to *esxW*) were dynamic, homologous recombination frequently occurred in those multigenes family with potential antigenic variability. There are a number of SNPs in highly immunogenic Esx family proteins, including the one found in epitope regions which may effected the immunogenicity [5,9,13]. Multiple T cell epitopes can be found in the ESAT-6 protein and recognized by IFN-γ-secreting T cell lines with various HLA-DR phenotypes[14].

The SNPs profile as the variant of strains, could cause protein changed that altered the structure and function of secretory protein. These diversity of secretory protein could affects immune response variation i.e. diversity of human T cell epitopes significant to determine the outcome of pathogenesis process causing higher severity of disease, either latent infection or active TB until severe diseases occurred[14,15,16,17].

Many studies have reported about SNPs profile and T cell epitope of Ag85 complex, Jiang *et al*. (2015), for example, reported that Ag85A, Ag85B and Ag85C showed lower substitution of amino acid in T or B cell epitopes and lower polymorphisms; Ag85 were hyperconserved in T or B cell epitopes and the genes were more likely to be under purifying selection, indicate a suitable diagnostic marker and vaccine for TB[4]. Antigen 85 complex is a family of fibronectin-binding proteins which possessed potential as virulence factors, encoded by *fbpA*, *fbpB*, and *fbpC* genes. These proteins acted as mycolyl transferase, have role in cell wall synthesis by catalyzing mycolic acid transport and resulting in trehalose dimycolate (TDM), an envelope lipid essential for virulence. 30 kDa Ag85B is the most abundant and the most copious extracellular protein, it plays role in cord factor biosynthesis[15,16,17]. Ag85A and Ag85B are essential secreted proteins, while Ag85C is associated with cell envelope. Ag85C localization is less exposed, thus evolutionary pressure on its gene was fewer[18].

Human T cell epitopes of *Mycobacterium tuberculosis* were evolutionarily hyperconserved, characterized by lack of antigenic variation and immune evasion[4]. Ag85 complex contained species-specific and shared epitopes, providing a universally present target[11], activating naive T cells, inducing T cell differentiation, elevating production of cytokines important for macrophage activation[19].

Effective TB control strategy require understanding of the antigen-specific immune responses, especially mediated by human $CD4^+$ and $CD8^+$ T cells essentials in protective immunity; several secreted proteins have been showed to induce strong CMI due to the short peptides bearing epitopes that bind to MHC molecules recognized by T cells[9].

4. Conclusion

The region of protein ESAT-6 and Ag85 are highly conserved in T cell epitopes, there is no SNPs polymorphisms found in gene encoding ESAT-6, Ag85B and Ag85C among *Mycobacterium tuberculosis* clinical isolates of sputum specimens from pulmonary TB patients in East Java Indonesia. These strains are sensitive to first line anti-TB drugs.

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