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T CELL EPITOPES OF THE *ESXA* FULL GENE OF *MYCOBACTERIUM TUBERCULOSIS* FROM SPUTUM OF MDR-TB PATIENTS

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Abstract

Background: In 2015, World Health Organization (WHO) discovered 10.4 million tuberculosis (TB) cases around the world. Multidrug-resistant tuberculosis (MDR-TB) became a threat because it has high mortality number. There were 480,000 new MDR-TB cases in 2015. Based on those problems, diagnostic development to detect *M. tuberculosis* rapidly and accurately is needed. The importance of detecting epitope expression of *esxA* full gene because there was a potential of complexity over the protein structure and might affect the protein concentration. By knowing epitope prediction, there's an expectation that it can help the development of TB diagnostic. This research was aimed to determine the T cell epitope prediction of *esxA* full gene from MDR-TB patients
Material and Methods: Total of 24 MDR-TB sputum isolate from TB patients at Dr. Soetomo Hospital were collected from September to December 2016. Samples were confirmed as MDR-TB using GeneXpert and Bactec MGIT 960. Those samples tested using PCR targeted 580 bp of *esxA* gene and sequencing. Gene sequence was aligned against wild type using Bioedit program version 7.2.5 and NCBI BLAST. T cell epitope prediction was analyzed by GENETYX version 10.
Results: Epitope predictions that could be obtained were IEAAAS, ASAIQG, VTSIHS, TKLAAA, VTGMFA based IAd Pattern Position and EAAAS based Rothbard/Taylor Pattern Position. Those prediction epitopes can determine the severity of disease, therefore full gene of *esxA* could be used as diagnostic target.
Conclusion: This research discovered five specific T cell epitope prediction based on IAd Pattern Position and one epitope prediction according to Rothbard/Taylor Pattern Position.

Keywords: *esxA* gene, ESAT-6, T cell epitope prediction, MDR-TB

Introduction

Tuberculosis (TB) is one of deadly infectious diseases transmitted by air. WHO reported that there were 10.4 million TB cases with 1.4 million deaths in 2015. Not only that, there were an additional 0.4 million deaths because of TB in HIV patients (WHO, 2016). In addition, there were 480,000 new cases of multidrug-resistant tuberculosis (MDR-TB) with 1,000 people infected by rifampicin-resistant (RR-TB) needed second line antibiotic (WHO, 2016).

Indonesia was one of the countries with high burden of TB and ranked as second highest TB burden country in the world for two years consecutively, in 2014 and 2015. In 2015, new MDR/RR-TB cases in Indonesia was 2,8%. Incidence rate of all TB cases in Indonesia were about 395 cases per 100,000 populations in 2015. In addition, only 52% of MDR-TB patients were successfully treated, mostly because of high number of mortality and patients with loss to follow up (WHO, 2015; WHO, 2016).

Because of this problem, good management of TB patients and developing an accurate diagnostic to detect *M. tuberculosis* is needed. The *esxA*, a gene which encodes 6kDa early secretory antigenic target (ESAT-6), is one of the most important virulence factors of *M. tuberculosis* and has a potential to be the target of TB diagnostic.

The *esxA* is known as a conserved gene in *M. tuberculosis* and belong to region of difference 1 (RD-1). The RD-1 region is removed to create BCG (Bacillus Calmette-Guerin) vaccine (Ganguly et al., 2008; Callahan et al., 2010). ESAT-6 is known to inhibit macrophage activation, induce apoptosis, and interfere with host immunity. ESAT-6 is also a membrane lytic factor that allows *M. tuberculosis* avoiding phagosomal attack because ESAT-6 was able to suppress autophagosome formation (Solans et al., 2014; Yu and Xie, 2012).

However, MDR-TB cases are making diagnostic development of TB quite hard to do. Some studies stated that resistance of bacteria towards antibiotic was correlated to fitness cost of the bacteria (Melnyk et al., 2014; Cohen at al., 2003; Salvatore et al., 2016). Previous study stated that resistance caused by mutation was associated with bacteria's fitness cost because the mutation targeted gene encoded important cellular function (Cohen at al., 2003). There's another study which also said that resistant *M. tuberculosis* was found to be less viable than antibiotic-sensitive *M. tuberculosis* both in vivo or in vitro. Several cases showed that resistant *M. tuberculosis* also had lower transmission rate (Kim et al., 2006; Comas et al., 2012). However, Cohen *et al*. (2003) reported that mutant with both lower and equal fitness cost value than antibiotic-sensitive *M. tuberculosis* had been found.

Based on those studies, it is suspected that mutation on genes involved in anti-TB drugs such as *rpoB*, *katG*, and others can affect its secretion of virulence genes, causing different severity degrees to manifest on MDR-TB patients. Detection of epitope expression of full gene *esxA* as virulence gene was important to do because there was a potential of complexity over the epitope and could affect the protein structure along with the level of protein concentration which played an important role in the pathogenesis of TB. By knowing epitope prediction, there's an expectation that it can help the development of TB diagnostic. The aim of this study was to determine the T cell epitope prediction of *esxA* full gene from MDR-TB patients.

Materials and Methods

Total of 24 MDR-TB sputum isolates samples from pulmonary TB patients in Dr. Soetomo Hospital, Surabaya, Indonesia were collected on September to December 2016. Samples were confirmed as MDR-TB using GeneXpert and Bactec MGIT 960 test (BD system) (Cepheid, 2015; Siddiqi and Rüsch-Gerdes, 2006; Hasan et al., 2013). Samples were then tested using the polymerase chain reaction (PCR) performed at the ITD Laboratory, Universitas Airlangga, Surabaya. The study was approved by ethics committee in health research of Dr. Soetomo Hospital with ethical clearance number 541/Panke.KKE/IX/2016 and has been approved at 27 September 2016.

Sputum was decontaminated and concentrated using Alkali Petrof method recommended by WHO, process was first done before extraction and PCR process (WHO, 1998). DNA was extracted using QiAmp DNA Kit (DNeasy Blood & Easy Kit, Cat No. 69504) and followed with PCR process. Primer for PCR process was designed using Clone Manager software 6, version 6.00 targeted at 580 bp. KAPA2G Fast Ready-Mix PCR Kit was used as PCR Master Mix. The solution mixture consisted of 25 µl KAPA2G Fast Ready-Mix PCR Kit, 1 µl of 10 µM pair of primer, 20 µl nuclease-free water, and 3µl of DNA template.

The amplification reaction in thermal cycle started with pre-denaturation at 95°C for 3 minutes, continued by denaturation at 95°C for 10 seconds, annealing at 58.3°C for 10 seconds, and extension at 72°C for 15 seconds. Denaturation, annealing and extension process was done for 35 cycles and finished with final extension at 72°C for 10 minutes. Positive amplicon results were confirmed by the presence of DNA bands at 580 bp.

Sequencing processes performed by sending PCR products to 1st BASE. 1st BASE used ABI PRISM 3730xl Genetic Analyzer developed by Applied Biosystem. Samples sequence was then analyzed using Bioedit program version 7.2.5 and NCBI BLAST to determine homology percentage between the sample and the wild-type *M. tuberculosis* H37Rv (NC_000962.3) sequence referenced from GenBank. T cell epitope prediction was analyzed by GENETYX version 10.

Results

In this study, total of 24 sputum samples from MDR-TB patients previously examined using PCR were found positive of full gene *esxA* (100%), as indicated by 580 bp DNA band. All samples then went into sequencing process in 1st BASE, Singapore. The result of alignment in Bioedit version 7.2.5 showed that all samples sequenced had 100% homology to wild-type *M. tuberculosis* H37Rv (NC_000962.3) sequence.

Samples sequence were also analyzed using the NCBI BLAST program, which result was that all examined samples had a 100% homology against *M. tuberculosis* H37Rv. Samples also had 100% homology percentage on *Mycobacterium tuberculosis* complex (MTBC) systems, such as *Mycobacterium bovis*, *Mycobacterium caprae*, and *Mycobacterium africanum*. Three other strains were discovered to have 99% homology with the sample, such as *M. tuberculosis* strain 96121, *M. tuberculosis* RGTB327, and one other organism from MTBC, namely *M. canetti* CIPT 140060008 (Table 1).

Table 1. Homology of MDR-TB sputum sample isolate with bacteria in NCBI database

No	Bacteria species	*Query coverage (%)*	*E value*	*Identity* (%)
1.	*M. caprae* (MTBC)	100	0,0	100
2.	*M. bovis* (MTBC)	100	0,0	100
3.	*M. africanum*	100	0,0	100
4.	*M. tuberculosis* H37Rv	100	0,0	100
5.	*M. tuberculosis* strain Beijing	100	0,0	100
6.	*M. canetti* (MTBC)	100	0,0	100
7.	*M. tuberculosis* H37Ra	100	0,0	100
8.	*M. tuberculosis* strain 96121	100	0,0	99
9.	*M. canetti* CIPT 140060008 (MTBC)	100	0,0	99
10.	*M. tuberculosis* RGTB327	100	0,0	99

By using GENETYX ver. 10, epitope prediction of all the 24 samples were IEAAAS, ASAIQG, VTSIHS, TKLAAA, VTGMFA based on *IAd Pattern Position* and EAAAS based on *Rothbard/Taylor Pattern Position*. Those prediction epitopes can determine the severity of disease, therefore full gene of *esxA* could be used as diagnostic target (Table 2).

Table 2. Prediction of T cell epitope *esxA* gene in sputum sample of MDR-TB patients

Gene	T cell epitope			
	IAd Pattern Position		Rothbard/Taylor Pattern Position	
	Amino acid position	*Sequence*	Amino acid position	*Sequence*
esxA	11-16	IEAAAS	12-16	EAAAS
	15-20	ASAIQG		
	22-27	VTSIHS		
	37-42	TKLAAA		
	90-95	VTGMFA		

Discussion

Several studies have suggested that mutations occurring in bacteria could interfere with their fitness cost. Previous research explained that resistant *M. tuberculosis* strain in clinical sample has different fitness cost from that of mutated strain in laboratory. There is no depression of the growth from resistant *M. tuberculosis* in clinical sample strain and still possessed the same transmission power as antibiotic-sensitive bacteria (Cohen et al., 2003; Melnyk et al., 2014).

In addition, mutations in gene targeted by anti-TB drugs possibly affecting secretion of the virulence genes such as *esxA* gene. Thus, there is a possibility that *esxA* gene could also be mutated and affected the virulence power of MDR-TB. However, the result of this experiment showed the opposite. The sequence of *esxA* gene showed that all samples had 100% homology with *M. tuberculosis* H37Rv (NC_000962.3) along with MTBC group and there was no similarity found towards NTM (Nontuberculous mycobacteria).

This indicated that *esxA* gene is a conserved gene even in the MDR-TB sample. Previous research analyzing ESAT-6, Ag85B, and Ag85C sequence was in agreement with this research. In another study, mutation was also not found in *esxA* gene observed from 38 patients with MDR-TB (Mertaniasih et al., 2016; Uplekar et al., 2011). Another study also reported that there is no variation found in *esxA* gene from 88 clinical isolates (Davilla et al., 2010). Many previous studies stated that esx group from ESX-1 to ESX-4 gene cluster had fewer variation (Uplekar et al., 2011; Solans et al., 2014).

Besides the similarity of *esxA* gene sequence with the wild type *M. tuberculosis* H37Rv, the result also showed that there is similarity to the functional structure of the epitope protein which is the determinant of immunodominant among the samples. Studying the function of the *M. tuberculosis* epitope could be a step to acknowledge the immunopathogenesis of TB, diagnostic development and vaccine development (Ivanyi, 2014).

Understanding the epitope prediction of *esxA* gene is important to comprehend the immune response of human towards *M. tuberculosis*. A study stated that cellular immunity mediated by $CD8^+$ T cells is important to control the latent *M. tuberculosis* infection (Zhai *et al*., 2016). In this study there were 5 epitope T cells analyzed by IAd Pattern Position and 1 epitope with Rothbard / Taylor Pattern Position. This result showed that the protein could be recognized by T cell lymphocytes and has high possibility to be used as vaccine candidate.

However, the results of the epitopes in this study showed different result with those reported by Mertaniasih et al. (2016) which acquired the epitope T cell prediction by IAd Pattern Position for only four epitopes. This is possible because the target gene captured in this study is much longer than that done by previous studies that only got 351 bp.

One of the T cells epitope predictions in this research used Rothbard/Taylor Pattern which was calculated by the possible pattern of 4 (charged or glycine, hydrophobic, hydrophobic, polar or glycine) and 5 amino acids (charged or glycine, hydrophobic, hydrophobic, hydrophobic or proline, polar or glycine) that occurred in 15 random amino acid peptides. That pattern can be used to predict areas within protein that can be recognized by MHC class I and II accurately (Rothbard and Taylor, 1988).

This study showed that the T-cell epitope predictions discovery was different from previous studies. This is a recent study of *esxA* gene exploration in patients with pulmonary tuberculosis in Indonesia. Those prediction epitopes can determine the severity of disease, therefore full gene of *esxA* could be used as diagnostic target. This research discovered five specifics T cell epitope prediction based on IAd Pattern Position and one epitope prediction according to Rothbard/Taylor Pattern Position.

T cell's response targeted a conserved epitope and it could be a benefit for the pathogen. Nayak *et al*. (2015) discovered several novel MTB proteins recognized by human T cells which could be a candidate for TB vaccine. ESAT-6 known as potent virulence factor in *M. tuberculosis* has a high potential as vaccine and diagnostic target for TB, thus knowing epitope prediction from *esxA* gene from various places especially countries with high incidence of TB is important.

Conclusion

This research proved that there were no variation or mutation found from nucleotide sequence among the MDR-TB isolates of sputum. In addition, we discovered five specifics T cell epitopes prediction based on IAd Pattern Position and one epitope prediction according to Rothbard/Taylor Pattern Position which was different from previous study.

Conflict of interest: Authors declare that they have no conflict of interest.

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