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SINGLE NUCLEOTIDE POLYMORPHISM OF *ECCB$_5$* GENE OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ISOLATES FROM SUSPECTED PULMONARY TB PATIENTS IN SURABAYA INDONESIA

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Abstract

Background: *Mycobacterium tuberculosis* Complex (MTBC) is a group of Mycobacterium that causes tuberculosis (TB). TB is an infectious disease that remains a global health problem. Indonesia is one of the five countries in the world where TB is the most prevalent and became the country with tle second largest rate of TB in 2014 and 2015. MTBC has high pathogenicity that can cause infections in animals and humans. The most common route of transmission is via airborne droplet nuclei and contact with animals or humans infected with TB. MTBC has many virulence factors. One of these factors is EccB$_5$ that is encoded by *eccB$_5$* gene. EccB5 is a transmembrane protein-conserved membrane protein and could play a role in inducing damage in host cells, macrophage infection, and may correlate with active disease. The characterization of *eccB$_5$* gene needs to be studied to determine the nucleotide sequences, which may be associated with active disease. The aim of this research was to analyze the nuclotide sequences of *eccB$_5$* gene of MTBC from suspected pulmonary tuberculosis patients, SNPs of *eccB$_5$* gene and possible correlation with the disease, especially in Indonesia.
Materials and Methods: Samples were collected from the Tuberculosis Laboratory, Clinical Microbiology of Dr. Soetomo Hospital Surabaya Indonesia. DNA extraction used boiling extraction method and continued nucleic acid amplification using PCR techniques. Primer pairs used eccB$_5$ SK.. The positivity of DNA specific revealed amplicon in 1592 bp. PCR product was sequenced by 1st Base (First BASE Laboratories Sdn Bhd, Selangor, Malaysia). The sequence analysis used Genetyx-Win version 10.0 (Genetyx Corporation, Tokyo, Japan).
Results: Total isolates of *Mycobacterium* spp. were 28 and those that showed positive MTBC were 24 isolates and 4 nontuberculosis mycobacteria (NTM) using immunochromatographic test (ICT). The amount of homology from MTBC using blast NCBI was 99%-100%. Two SNPs were found in position c.1277 which revealed replacement of amino acid in 426 of codon position.
Conclusion: The sequence of *eccB$_5$* gene of MTBC showed high significant homology, while proposed non-synoymous single nucleotide polymorphisms (nsSNP) may associated with clinical outcomes.

Keywords: Pulmonary Tuberculosis, *Mycobacterium tuberculosis* Complex, *eccB$_5$* gene, SNPs

Introduction

Mycobacterium tuberculosis Complex (MTBC) is a zoonotic bacteria that causes tuberculosis (TB) in humans and animals (Smith, 2003; Carroll et al., 2016). In 2011 new cases of TB worldwide were more than 9 million, resulting in 1.4 million deaths as a result of TB. In 2013, the prevalence was 11 million and the incidence was 9 million (WHO, 2014). Indonesia was one of five countries with the largest rate of TB incidence in 2014 and 2015. Indonesia also in had the second highest global occurance of TB globally in 2015 (WHO, 2015; WHO, 2016). The estimated mortality rate in 2015 of TB with HIV was 26,000 and the mortality rate with HIV negative status was 100,000 (WHO, 2016).

Pathogenesis of pulmonary tuberculosis transmission is initiated by the bacterial spread from TB-positive individuals to other persons through the air by coughing and sneezing (Collins et al., 2004; CDC, 2013). Transmission occurs by direct contact of droplet nuclei containing MTBC via inhalation. MTBC is deposited into the pulmonary alveoli and multiplicates intracellularly. MTBC also induces and manipulates the immune system response of the host and secretion of the host's pro-inflammatory cytokines (TNF, IL-6 and IL-12) post-infection to macrophages (Bottai et al., 2012; Nunes-Alves et al., 2014; Boradia et al., 2014). Typical clinical symptomps of TB patients are coughing for more than 3 weeks or longer, respiratory disease, chest pain, coughing containing blood and/or sputum, fever, and night sweats (CDC, 2013; Nunes-Alves et al., 2014).

MTBC has many virulence factors such as type VII secretion system (T7SS) and consists of ESX-1 to ESX-5 system (Houben et al., 2012). ESX-5 could manipulate the macrophage immune response. Several genes are known to encode this system, especially *eccB*$_5$ gene in the ESX-5 system. EccB$_5$ is a conserved membrane protein encoded by the *eccB*$_5$ gene and could play a role of inducing damage in host cells and macrophage infection and also severe disease process (Abdallah et al., 2009; Abdallah et al., 2011; Shah et al., 2015). Otherwise reported by Di Luca et al., 2012 revealed that slow-growing mycobacteria consist ESX-5 system, but not found in fast growing. The characterization of nucleotide sequences of the *eccB*$_5$ gene needs to be investigated, especially by using isolates from suspected pulmonary tuberculosis patients in Indonesia, which up to the moment have not been clearly identified. This study aims to define and determine genetic nucleotide sequences, single nucleotide polymorphisms (SNPs) and homology of the *eccB*$_5$ gene and their correlation to active disease.

Materials and Methods

Samples

Samples were collected from suspected pulmonary tuberculosis patients from the Tuberculosis Laboratory, Department of Clinical Microbiology, Dr. Soetomo Hospital, Surabaya, Indonesia, from January 2016 until January 2017. All samples of sputum were prepared for staining with Ziehl Neelsen (ZN) method, and standard culture method in Lowenstein Jensen (LJ) media (WHO, 1998a; WHO, 1998b). Sputum samples were performed to decontamination using Sodium hydroxide (NaOH) with a concentration of 4% and prepared to culture in LJ media (WHO, 1998b).

Phenotypic identification MTBC : Isolates from LJ media were prepared for differentiating MTBC and NTM according to the procedure of SD-Bioline test (Standard Diagostic, inc, Republic of Korea) (Arora et al., 2015).

DNA extraction : DNA extraction used boiling extraction method (Aldous et al., 2005).

PCR : Nucleic acid amplification used PCR techniques and primer pairs *eccB*$_5$ SK forward and *eccB*$_5$ SK reverse. PCR Master Mix used GoTaq® (Promega, USA). DNA thermal cycler Bio-Rad (Bio-Rad laboratories) was set to perform initial denaturation at 95°C for 3 minutes, denaturation 95°C for 10 seconds, annealing 53,8 ^{o}C for 15 seconds followed by 35 cycles, extension 72°C for 15 seconds and final extension 72°C for 10 minutes. Positive amplification showed amplicon in 1592 bp under UV trans illuminator.

Bioinformatic analysis : PCR products were sequenced by 1stBASE Sequencing INT (First BASE Laboratories Sdn Bhd, Selangor, Malaysia). Sequences data were aligned to reference strain from gene bank NCBI (2016) *Mycobacterium tuberculosis* H37Rv (RefSeq: NC_000962.3) using Genetyx-Win versi 10.0 (Genetyx Corporation, Tokyo, Japan).

Ethical Approval: Ethical clearance no 532/Panke.KKE/IX/2016, was issued by the Ethical Committee of Health Research of Dr. Soetomo Hospital.

Result

The number of total sputum samples were 28 from suspected pulmonary tuberculosis patients. All samples were identified by microscopy using ZN staining method and showed positive for 28 samples. There were 28 samples of culture using LJ media that resulted in a growth of colonies, and brief use of SD-Bioline test resulted in 24 positive MTBC and 4 NTM (Table 1) . All positive samples of MTBC were subjected to amplification using PCR technique and sequencing.

Table 1. The result of several test methods in sputum samples of suspected pulmonary tuberculosis patients in Dr. Soetomo Hospital Surabaya Indonesia

Methods	Positive	Negative	Total
ZN staining	28	0	28
LJ media	28	0	28
SD-Bioline test	24	4	28
PCR	24	4	28

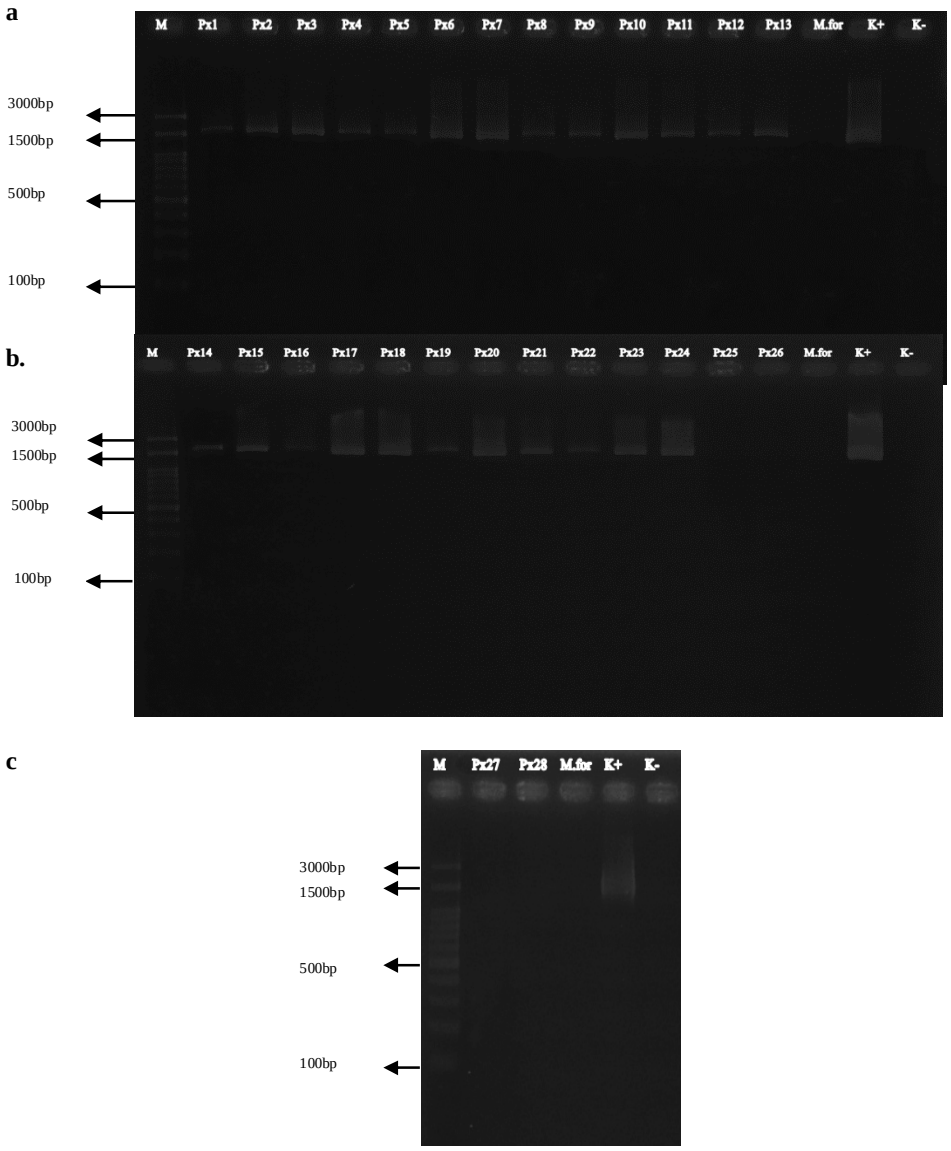

Figure 1. PCR product amplification, **a**, M : Marker; Px1-Px13 isolates of MTBC; M.for : *Mycobacterium fortuitum*; K+ : *Mycobacterium tuberculosis* H37Rv as a positive control; K- : negative control. **b**, M : Marker; Px14-Px24 isolates of MTBC; Px25-px26 isolates of NTM; M.for : *Mycobacterium fortuitum*; K+ : *Mycobacterium tuberculosis* H37Rv as a positive control; K- : negative control. **c**, M : Marker; Px27-Px28 isolates of NTM; M.for : *Mycobacterium fortuitum*; K+ : *Mycobacterium tuberculosis* H37Rv as a positive control; K- : negative control.

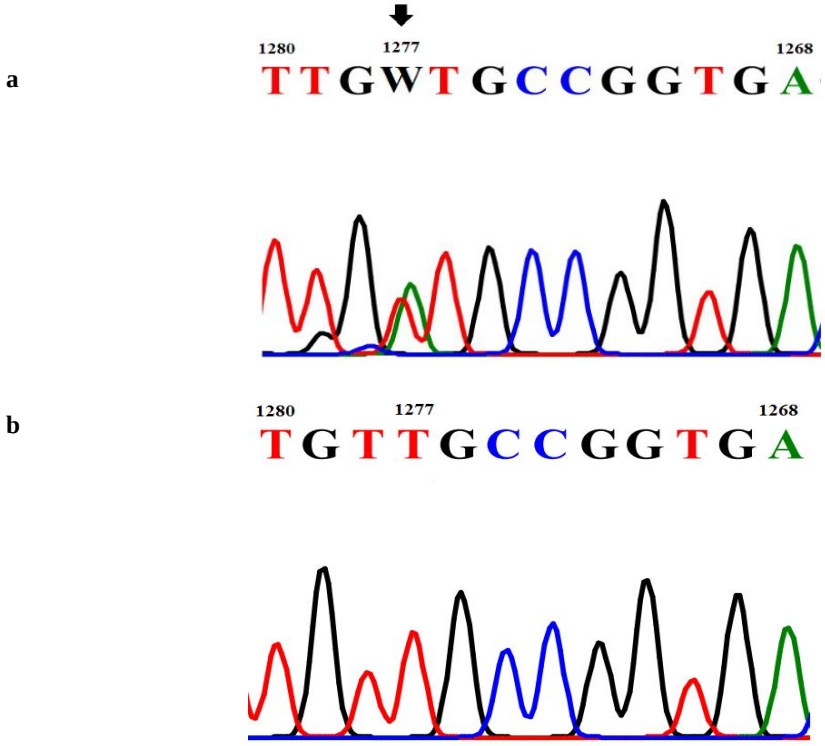

Figure 2. Sequence electropherograms result in reversed primer of PCR product, **a**, sequence electropherogram from sample with SNP (c.1277 T/A), **b**, sequence electropherograms from sample without SNP W= T and A

Isolates with positive *eccB*$_5$ gene as the target showed amplicon of 1592bp (Figure 1) and were sequenced by 1st Base Sequencing INT (First BASE Laboratories Sdn Bhd, Selangor, Malaysia). Alignment for sequences had similarity of 100% and three isolates had 99% identity compared to *Mycobacterium tuberculosis* H37Rv (RefSeq: NC_000962.3). Based on the result of nucleotide sequences analysis of the isolates, we identified SNPs (Figure 2) in the same position. This research took into account the indication of amino acid change (N/I) or non-synonymous SNPs in 426 codon position (from the 1521 of full gene of *eccB*$_5$). This research also showed that *eccB*$_5$ gene was not amplified in four isolates of NTM (Figure 1) and fast growing mycobacteria (*Mycobacterium fortuitum*) using multiple alignment program of Genetyx-Win version 10.0 (Genetyx Corporation, Tokyo, Japan).

Discussion

There is an increasing mortality rate due to infectious disease caused by members of the MTBC and this has a correlation with a severe disease that remains a global health problem (WHO, 2015; WHO 2016). The trend of increasing TB incidence could be assosiated with virulence factor of MTBC such as type T7SS (consists of ESX-1 to ESX-5 system) (Forrellad et al., 2013; Houben et al., 2014; Simeone et al., 2015). EccB5 is a transmembrane protein that is part of the ESX-5 system and encoded by the *eccB*$_5$ gene and the larger membrane complex translocation channel. All components in ESX-5 and also *eccB*$_5$ gene correlate with nutrition uptake, substrate transport, and viability of MTBC. They may play a role in active disease and process of infection (Abdallah et al., 2011; Bottai et al., 2012; Di Luca et al., 2012; Stoop et al., 2012; Simeone et al., 2015).

The finding of single nucleotide polymorphisms (SNPs) in the *eccB*$_5$ gene could be associated with TB clinical outcome, correlated with a severe disease procces and virulent strains. The SNPs of nucleotide sequences provide the basic information, especially from clinical isolates that found from pulmonary tuberculosis patients in Indonesia. In this research, we show SNPs with subtitution of nucleotide sequences at position 1277 from the 1521 full gene of *eccB*$_5$ and replacement of the amino acid or non-synoymous SNPs (nsSNPs) (N/I) at 426 codon position. Asparagine (N) is common involved in binding site that has a polar side chain and generally on the protein surface. In contrass, isoleucine (I) is hydrophobic amino acid and has several function in substrat recognition (Bett and Russell, 2003). Accordingly Beckham et al., (2017), one of the core protein of ESX-5 (EccB$_5$) was significant lower of expression but affects the formation complex and stability. Several other studies demonstrated that *eccB*$_5$ gene was found SNPs in 2017898 from whole gene of *Mycobacterium tuberculosis* with amino acid T/T (Satta et al., 2016). Most nsSNPs produce modification of an amino acid (Gutacker et al., 2006). Based on the report from our research, the proposed SNPs, whose functions are still unknown, had association with the disease proccess in patients who showed severe

clinical symptoms such as haemoptysis, asphyxia and were suspected of TB mediastinum. These SNPs may correlate with the level of virulence factor, such as nutritional uptake and cell host damage.

Ramón-García et al., (2012) showed that several gene such as *eccB*$_5$, *eccCb*$_5$, *eccA*$_5$, *esxJ, esxM, esxN* and *esxU* are included in the immunologically active ESAT-6 family of protein. As previously described, the ESX-5 system among *eccB*$_5$ gene had effect in the substrate transport through cytosolic mechanism and possibly induce cell death (Abdallah et al., 2009 ; Abdallah et al., 2011). The *eccB*$_5$ gene also affects the activation of phosphate and stability of cell wall of MTBC (Ates et al., 2015; Elliot and Tischler, 2016).

The present study also found that *eccB*$_5$ gene as long as 1521bp were not amplified in *Mycobacterium fortuitum* and in some NTM members. The final result of blast and alignment from NCBI showed that no significant similarity was found in reference strain of *Mycobacterium fortuitum* Complex (taxid:1866885) and *Mycobacterium* sp. MOTT36Y_CP003491.1 only has similarity 79%. Based on this result, *eccB*$_5$ gene used with this primer pairs could also differentiate the member of NTM and the member of MTBC.

Conclusion

The nucleotide sequence of *eccB*$_5$ gene of MTBC showed high significant homology. This study found the presence of nsSNPs and replacement of amino acid (N/I) in 426 codon position, which may be proposed as SNP in MTBC strains from pulmonary tuberculosis patients in Surabaya, Indonesia. The nsSNPs may associated with clinical outcome in suspected pulmonary tuberculosis patients.

Conflict of interest: No conflict of interest exists concerning this research.

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