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The specific DNA region of esxA gene for the target of PCR to determine *Mycobacterium tuberculosis* accurately

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ABSTRACT

The *esxA* gene is a potent virulence gene and also known as conserved gene potential as a target to diagnose *Mycobacterium tuberculosis* complex (MTBC) especially *Mycobacterium tuberculosis* (*M. tuberculosis*). The aim of this study was to evaluate the fidelity of specific DNA region of *esxA* gene as target for PCR to identify and determine species MTBC among *Mycobacteria* clinical isolates, compare with the culture method. Clinical isolate samples were collected randomly from January 2016 until July 2016 among *Mycobacteria* sputum isolates of suspected pulmonary tuberculosis (TB) patients in Dr. Soetomo Hospital, Surabaya, Indonesia. Samples were detected and identified using BACTEC MGIT 960 system (BD) culture method, then identified the MTBC TB Ag MPT 64 (SD Bioline). Furthermore, clinical isolates from culture method were amplified by using PCR based on *esxA* as gene target. Primer for *esxA* gene region was designed using Clone Manager 6, version 6.00. The amplicon was confirmed as positive result as DNA band appeared in 339bp. *M. tuberculosis* H37Rv strain was used as positive control, PCR mixture without DNA, and *Mycobacterium fortuitum* (*M. fortuitum*) isolates as negative control. Total 37 clinical isolates from sputum specimens which had been analyzed using PCR revealed that all clinical isolates were positive and in concordance with the result of standard culture method, MGIT. The conserved and specific DNA region of *esxA* gene with size of 339 bp for PCR have high accuracy for identification of M. tuberculosis that is important in determining diagnosis of MTBC infection.

Keywords: *esxA* gene, *Mycobacterium tuberculosis*, PCR, Identification
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INTRODUCTION

Tuberculosis caused by intracellular pathogen MTBC is one of the chronic diseases caused burden and adverse for productive people, therefore it is still a major health problem in almost all over the world. South-East Asia and Western Pacific Region contributed around 58% of the world's TB cases in 2014. Based on WHO Global Tuberculosis Report of 2015, there were 9.6 million new TB cases and 1.5 million death caused by TB in 2014. Meanwhile, in 2015 there were 10.4 million new TB cases with 1.4 million mortality. Approximately around one-third of the world population was infected by *M. tuberculosis*, mainly in developing countries, including Indonesia.[1,2,3,4]

Indonesia carried second highest TB burden in the world. In 2014, the estimated TB prevalence in Indonesia Including *human immunodeficiency virus* (HIV) infected patient with TB was 647 per 100,000 population. The incidence rate for TB including HIV+TB was 399 per 100,000 population, and the mortality number was 41 per 100,000 in 254 million population.[2] In addition, incidence rate includes HIV+TB in 2015 was 395 per 100,000 population with mortality was 40 per 100,000 population.[1] WHO also stated that estimation of TB patient living with HIV in 2014 was 1.2 million (12%) out of 9.6 million people with new TB cases. One of the strategic program that recommended by WHO to resolve those issues was to implement the strengthening TB diagnostic method.[1]

Rapid and accurate diagnostic method to detect *M. tuberculosis* played important role to control TB cases and reduce case of TB transmission. *Polymerase Chain Reaction* (PCR) is one of the methods that can detect and identify *M. tuberculosis* accurately. Several studies showed that all type of PCR could be applied as a rapid diagnostic tool to accompany culture method which usually consumed a lot of time, around three to eight weeks, to get the result.[5,6,7] There were some methods that able to be used as diagnostic tools other than PCR, such as Immunochromatographic Test (ICT), Enzyme-Linked Immunosorbent (ELISA), Interferon-Gamma Release Assays (IGRA), and ELISPOT that mostly using ESAT-6 and CFP-10 as an antigen target.[8,9,10,11,12] However, PCR was well known as more sensitive and simple procedure.[13,14]

Determining target gene of *M. tuberculosis* was an important step in developing diagnostic method for PCR. Understanding virulence and immunogenic gene were one of the ways to determine target gene. It has been reported that *Mycobacteria* have at least four pathways secreting proteins called Sec, SecA2, twin-arginine translocase, and the early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1). However, there was another study that only classifies it into 3 major types of protein secretion, namely general secretory pathways (Sec), twin-arginine pathways (TAT) and T7SS that transported protein through mycobacterial cell envelope. *M. tuberculosis* had five T7SS, namely ESX-1, ESX-2, ESX-3, ESX-4, and ESX-5.[15,16]

ESX-1 region consisting *esxA gene* was considered a major pathogenic determinant of *M. tuberculosis*, and its absence, due to the deletion of the RD1 region, from the *M. bovis* bacillus Calmette-Guérin (BCG) vaccine was thought to substantially contribute to the attenuation and safety of this strain. RD1 was consistently deleted from BCG but present in all virulent isolates of *M. bovis* and *M.tuberculosis*.[17,18,19]

Much attention has been focused on *esxA* gene (ESAT-6) because it was required for virulence and major target of immune response in infected individuals. *esxA* played crucial role in *M. tuberculosis* invasion, was able to modulate activation of macrophage, and affect host signaling pathways, such as TCR pathways, TLR-NF,κβ, focal adhesion pathways, and MAPK. A dual knockout of *esxA* and *esxB* in *Mycobacterium bovis* resulted in decreased virulence of the pathogen, indicating that the two molecules might play important roles in immunopathogenesis and virulence. Previous studies stated that *esxA* was a conserved gene in *M. tuberculosis*.[20,21,22,23]

Developing a simple, sensitive and specific method for diagnosis TB was critically needed. Moreover, *esxA*, as a target gene in PCR method, offered a promising approach for diagnosis TB infection. Detection and identification *esxA* gene in the specimen could be a crucial role in determining MTBC especially *M. tuberculosis* diagnostic. The aim of this study was to evaluate accuracy of PCR method using the specific DNA region of *esxA* gene as a target to detect and identify DNA of *M. tuberculosis* in the clinical isolates from pulmonary TB specimens.

MATERIALS AND METHOD

A collection of clinical isolates of *Mycobacteria* were collected in random sampling from sputum specimens of suspected pulmonary TB patients in Dr. Soetomo Hospital, Surabaya, Indonesia in January 2016 until July 2016. The study was approved by the ethics committee in health research of Dr. Soetomo Hospital General Hospital, Surabaya in Indonesia No. 124/ Panke. KKE/ II/ 2014. The gold standard method for isolation, detection, and identification of MTBC from sputum was conducted using standard culture method by BACTEC MGIT 960 system (BD), Löwenstein-Jensen (LJ) medium as solid medium for collecting isolates then samples identified by TB Ag MPT 64 (SD Bioline) in TB laboratory at Dr Soetomo Hospital. Parallel test for identification of Mycobacteria clinical isolates was examined for *M. tuberculosis* by PCR method in TB laboratory at Institute of Tropical Disease, Universitas Airlangga. The clinical isolates were subculture on LJ medium at 37°C for 3 weeks.

One loop colony of fresh mycobacteria isolates sample in LJ medium was dissolved in TE buffer suspension. The DNA was extracted by modified heat shock (boiling) method as follows: sample was aliquoted in 1.5 ml tube, centrifuged at 6000 rpm for 10 min, then supernatant was discarded and TE buffer was added in to it, then mixed by vortex. The solution in tube was heated at 98-100°C for 20 min. After 20 min, sample was vortexed and second heat shock was performed at 98-100°C for 15 min. While the solution maintained in hot condition, the DNA in the 500 µl was moved to the new microtube and used for PCR.[24,25]

The primers were designed by using Clone Manager software 6, version 6.00. The primers used were esxA (ESAT-6) F: 5'-GCATGACAGAGCAGCAGTGGAAT-3' as forward primer and esxA (ESAT-6) R: 5'-GAGGAGAAGCCCGGTTGCCCTTTCGCT ATTCTACG-3' as reverse primer. PCR Master Mix that used was KAPA2G Fast Ready Mix PCR Kit. In 50 µl solution was containing 25 µl KAPA2G Fast Ready Mix PCR Kit, 20 µl nuclease free water, 1 µl of each primer and 3 µl DNA.

Amplification of PCR was started from pre-denaturation at 95°C, 3min; denaturation at 95°C for 10sec; annealing at 59°C, 10sec; extension at 72°C, 15sec; with 35 cycles and followed by final extension at 72°C, 10min. Afterward, the PCR result was determined by electrophoresis and read using UV transluminator. The positive result indicated by DNA band in 339 bp. The *esxA* gene with size of 339 bp was specific DNA region of *esxA* gene that can determine MTBC species. Positive control used was *M. tuberculosis* H37Rv ATCC 27294 and *Mycobacterium fortuitum* (*M. fortuitum*) ATCC 6841 species alongside PCR mixture without DNA was used as negative control.

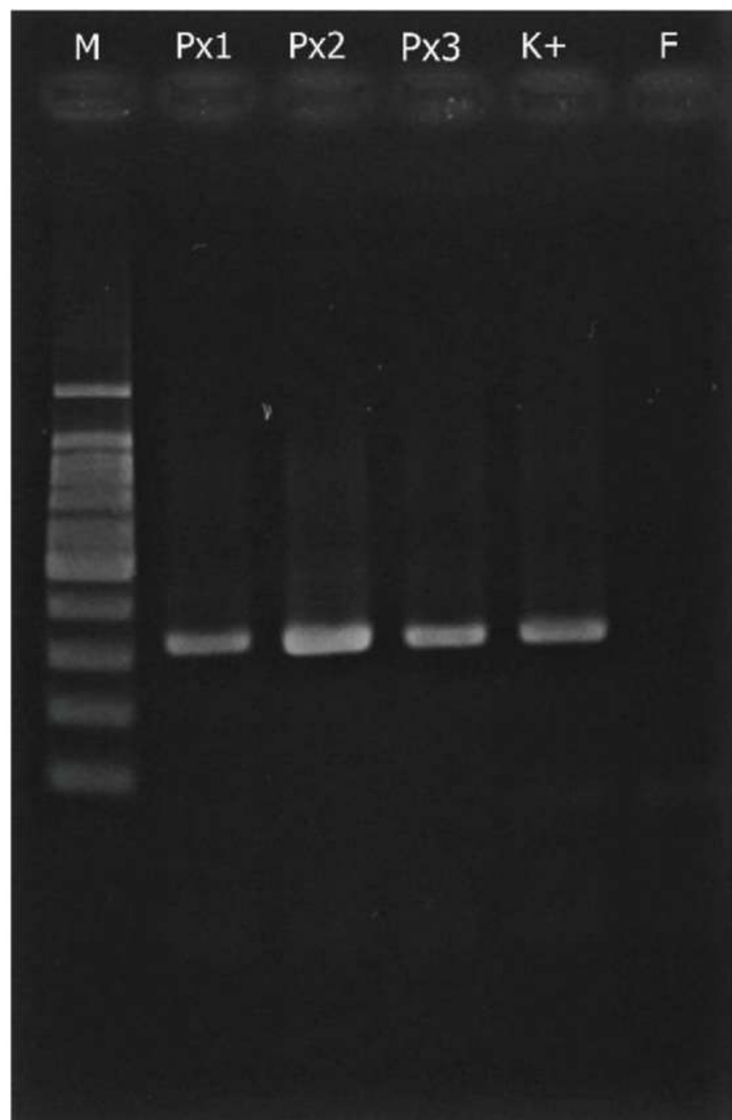

Figure 1 Visualization of electrophoresis from PCR products. DNA band in 339 bp indicating positive result of *esxA* gene specific DNA region. All samples (Px1, Px2, and Px3) showed positive result. M = DNA marker Sizer™-100 bp; K+ = *M. tuberculosis* H37Rv ATCC 27294 as positive control; F = *M. fortuitum* ATCC 6841 as negative control

Table 1 The accuracy of in-house PCR method based on the specific DNA region of *esxA* gene compared to MGIT Method along with TB Ag MPT64 for *M. tuberculosis* identification

Test		MGIT + TB Ag MPT64	
		+	-
In-house PCR method	+	30	0
	-	0	7
Total		37	

RESULT

A total of 37 clinical specimens were tested, 30 samples showed positive result and 7 isolates with negative result, no band in 339bp, were categorized as non-tuberculous *mycobacteria* (NTM) species (Figure 1, Table 1).

Result revealed that PCR method using specific DNA region of *esxA* gene for *M. tuberculosis* identification had 100% specificity and 100% sensitivity compared to standard culture method MGIT, Bactec System (BD) and TB Ag MPT64 (SD Bioline). Seven samples were from mycobacteria clinical isolates of sputum specimen reported as NTM species.

DISCUSSION

PCR method needed a primer to detect gene accurately and to design primer, a conserved target gene with some prior sequence information was necessary.[26] Some studies reported that *esxA* was able to impair the activity of host macrophage, induce apoptosis, and disrupt host immunity. *esxA* also played crucial role in *M. tuberculosis* invasion, enabled it to diffuse from one macrophage to another. Deletion of ESAT-6 could stop *M. tuberculosis* spreading into uninfected macrophages but not affected its multiplication.[18,20,27,28,29]

M. tuberculosis genome had five copies of ESAT-6 loci cluster gene showed that ESAT-6 play important role in maintaining the viability of bacteria. Sequence gene of *esxA* or amino acids of ESAT-6 was highly conserved, specific and considered as dominant antigen because a large amount of memory T cells acquired by an already cured host from *M. tuberculosis* infection was found to be specific for ESAT-6 antigen major epitope.[30,31]

Other studies also showed that *esxA* gene was one of the gene potentials as diagnostic target. Turbawaty *et al.*[8] revealed that ESAT-6, CFP-10 and MPT-64 could be used as diagnostic target for ICT TB antigen cocktail. Besides that, various researches on the validity of IGRA using ESAT-6 along with CFP-10 has been conducted and ESAT-6 has been known as important antigen stimulating IFN-γ to produce T-cells in the first phase of TB infection. *esxA* that encode ESAT-6 was crucial for virulence and immunity stimulation on infected host.[11,12,20]

ICT was a simple and rapid test able to detect *M. tuberculosis* and TB Ag MPT 64 (SD Bioline) was one of the diagnostic tools based on immunochromatographic. Although TB Ag MPT 64 (SD Bioline) was known as rapid and simple tool, its sensitivity needed further investigation. The molecular method offered a better solution for that

issue. PCR was one of rapid, simple, and sensitive diagnostic method that has been widely used. In addition, PCR could also be used as an accurate and rapid method accompanied culture method as gold standard for TB identification.[13,32,33]

Current research revealed that all results from PCR method based on the specific DNA region of *esxA* gene was found to be similar to MGIT Bactec System (BD) and phenotypic test TB Ag MPT 64 (SD Bioline) results (Table 1), their concordance was 100%. Clinical samples used was obtained from identification in suspected *Mycobacteria* colony isolates that grown in LJ and had confirmed by BACTEC MGIT 960 system (BD). Seven sample that detected as negative result was reported as NTM species.

TB Ag MPT 64 (SD Bioline) was one of the immunochromatographic assay reported as simple, rapid, and inexpensive method able to differentiate *M. tuberculosis* and NTM. Several studies reported that ICT TB Ag MPT 64 (SD Bioline) has high sensitivity and specificity. Value of sensitivity was varied, ranging from 99.2% to 100%, while its specificity ranging from 96.4% to 100%.[34,35,36,37,38] However, ICT couldn't stand alone as sole TB diagnosis method, it should be supported by other diagnostic tools. Diagnostic test recommended by WHO was microcopy technique, culture and species identification, drug-suseptibility testing, and molecular testing such as PCR and GeneXpert.[2]

Our findings had similar concordance with other studies that showed variable sensitivity and specificity of in-house PCR used to analyze various kind of TB samples with varied primers. Several studies used *gyrB* as target of primer and revealed that 41 clinical isolates and 30 sputum from patient suspected of pulmonary TB were correctly detected as positive result by using it as target gene at 1020 bp.[5,39]

Another study in the USA has reviewed 84 in-house PCR method to detect *M. tuberculosis* in sputum samples and showed that 97% of summary receiver operator characteristic (SROC), representing that the method has high accuracy. Other results from that research also signify that the use of IS6110 target sequence and the use of nested PCR method can significantly increase the accuracy of PCR.[14] However, a research in Uganda during September 2007 to February 2008 showed that in-house PCR that used IS6110 as gene target has 75% of sensitivity while the specificity was 35.9%. That research showed that IS6110 alone cannot improve the accuracy of PCR diagnostic.[33]

Another study in Shaanxi Provincial People's Hospital, China showed that detection of ESAT-6 encoded by *esxA* gene could be an indicator of early diagnosis of renal injury induced by TB. The *esxA* gene was found to possess high specificity and potential to become diagnostic tools for monitoring active TB and even for early diagnostic of TB-induced renal injury.[40] Singh *et al.*[41] reported that primer set of *esxA* gene has 100% sensitivity in *M. tuberculosis* strains detection and all samples of NTM species showed 100% negative result in its specificity.

The specific DNA region of *esxA* gene primers with size of 339 bp was expected to be more specific and accurate for determining MTBC compared to esxA gene primers with size 300 bp, 306 bp, and 320 bp because it could cover the full gene of *esxA* in MTBC.[21,41,42] Previous researches showed that primer set amplifying *esxA* gene was a unique and specific target for rapid diagnostic to distinguish *M. tuberculosis*. Development of in-house PCR using *esxA* gene to detect *M. tuberculosis* needed to be taken into consideration because improvement of its accuracy and detection ability could be enhaced, resulting in TB cases could be controlled better.[14,41]

Some studies also reported that *esxA* was a conserved and specific gene in *M. tuberculosis* potentially able to be a gene target for primer. Davila *et al.*[43] reported that there was no gene variation found in *esxA* gene from previously examined 88 strains. The same study also revealed that there were no polymorphisms found in *esxA* gene among all examined clinical samples. It was in agreement with a study conducted by Uplekar *et al.*[44] on 108 samples. This study revealed that out of 108 samples analyzed, no esxA sequence variation was found, and it even showed that *esx* genes within ESX-1 to ESX-4 gene cluster exhibited less variation compared to *esx* gene located outside those loci.

Another study also showed that from all examined samples, no single nucleotide polymorphism (SNP) on *esxA* gene along with *fbpB* and *fbpC* genes region was found from *M. tuberculosis* clinical isolates. Result also showed that all clinical isolate sample analyzed have the same four T cell epitopes in *esxA* gene.[45] ESAT-6 amino acid encoded by *esxA* gene was also found to be highly conserved among mycobacterium complexes.[20]

Gene encoded ESAT-6 was represented as specific antigen in *M. tuberculosis* that found to be absent in BCG species and nonpathogenic *Mycobacteria*. It was recognized by immunity system from infected host thus many studies associate ESAT-6 as antigen expression or strong serodiagnostic antigen to differentiate infected TB and BCG-vaccinated with TB,[21,41] however on current research we tried to use *esxA* gene as a diagnostic method to identify

M. tuberculosis species because of its specific and conserved character.

The specific DNA with the size of 339 bp of *esxA* gene for PCR has high accuracy for identification of *M. tuberculosis* that is important in determining diagnosis of MTBC. Identification until species level was crucial for its correlation with the pathogens, pathogenesis, therapeutic determination, and clinical outcome. Thus, the specific DNA region of MTBC for biomarker to detect and identify MTBC in the specimen from TB patients, have big advantages as the viable marker, virulence, and activity of MTBC multiplication that could be correlated with the activity of disease. This research still needs nucleotide sequence analysis in the specific region of *esxA* gene as confirmation in homology level or differentiation level among MTBC species or other *Mycobacteria*.

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