DETECTION OF MULTIDRUG-RESISTANT MYCOBACTERIUM TUBERCULOSIS DIRECTLY FROM SPUTUM SAMPLES OF PATIENTS FROM JAKARTA, INDONESIA BY RADIOISOTOPE-BASED PCR-DOT BLOT HYBRIDIZATION

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Abstract. The problem of eradicating tuberculosis (TB) has become more complicated by the emergence of multidrug resistant TB (MDR-TB). Any rapid laboratory method that can be used to detect drug susceptibility of Mycobacterium tuberculosis (MTB) is urgently needed. In this study, we employed the radioisotope (³²P)-based PCR-dot blot hybridization method on sputum samples from patients in Jakarta, Indonesia. Bacterial DNA was extracted using BOOM method. KatG and rpoB were amplified by PCR and katG315 or rpoB531 mutations were identified by dot blot hybridization. Of 100 samples, 11% and 22% showed presence of mutation at codons 315 (AGC→ACC) of katG and 531 (TCG→TTG) of rpoB, respectively. Five percent of the samples showed both mutations. This method is rapid, sensitive, and reliable and can be used to screen large numbers of samples in epidemiological studies.

Keywords: M. tuberculosis, MDR-TB, radioisotope, dot blot hybridization

INTRODUCTION

Tuberculosis (TB) is caused by Mycobacterium tuberculosis (MTB) and poses a major public health problem around the world. In 2007, Indonesia was the third country on the list of 22 high burden TB countries in the world following China and India (WHO, 2009). Globally, 9.27 million new cases of TB occurred in 2007 (139 per 100,000 population), and the incidences of this disease increased during 2006 to 2007 (WHO, 2009). One of the causes of failure in controlling and managing of TB is the increase of MTB isolates that are resistant to anti-TB drugs (ATDs). The problems become more complicated with the emergence of multidrug-resistant tuberculosis (MDR-TB).

MDR-TB is defined as TB that is resistance to both rifampicin and isoniazid (INH) with or without resistance to other anti-TB drugs (Rattan et al, 1998; Zhang et al, 2005). MTB isolates that are resistant towards rifampicin and INH are mostly
caused by a mutation (TCG→TTG) at codon 531 of rpoB encoding RNA polymerase subunit β and a mutation (AGC→ACC) at codon 315 of katG encoding peroxide catalase, respectively (Sajduda et al, 2004; Abe et al, 2008). The availability of appropriate techniques for the detection of the MDR-TB is a critical factor in order that any eradication program of MDR TB to be achieved successfully.

Up to now, detection of the MDR-TB is performed by using conventional culture technique, which is time consuming as it takes at least two weeks (Zhang et al, 2005). Molecular biology techniques have been applied for the rapid detection of the MDR-TB. Several PCR-based techniques that have been developed include single strand conformation polymorphism (SSCP), direct sequencing, PCR-restriction fragment length polymorphism (PCR-RFLP), and dot-blot hybridization (Telenti et al, 1993; De Beenhouwer et al, 1995; Felmlee et al, 1995; Victor et al, 1999).

Of those, the dot-blot hybridization is a more suitable method particularly for a large number of samples and epidemiological studies. Here, we developed a radioisotope (32P)-based PCR-dot blot hybridization assay and evaluated it using sputum samples obtained from patients in Jakarta, Indonesia.

MATERIALS AND METHODS

Bacterial isolates and clinical samples

M. tuberculosis strain H₃₇Rv was used as the standard bacterium for wild-type control. Two other isolates obtained from the Clinical Microbiology Laboratory, Faculty of Medicine, University of Indonesia, were used as positive controls for rifampicin and INH resistances. One hundred sputum samples were obtained from Center of Public Health for Respiratory Disease, Jakarta, Indonesia. All samples were acid-fast bacilli (AFB).

Specimen preparation and DNA extraction

Seven hundred and fifty µl aliquot of sputum was homogenized with 750 µl of suspension solution containing 1% N-acetyl-L-cysteine and 2% NaOH. The suspension was centrifuged at 14,000g for 1 minute and pellet was extracted using the BOOM method with 40 µl of final elution (Garcia de Viedma, 2003). Five µl of the final elution was used as template for PCR assay.

PCR

A forward (5’-TGC ACG TCG CGG ACC TCC A-3’) and a reverse (5’- TCG GGG TCG TTG ACC TCC CA-3’) primer were used for PCR amplification of rpoB, while forward (5’-TCG GGG TCG TTG ACC TCC CA-3’) and reverse (5’-TGG CCG CGG TCG ACA TT-3’) primers for amplification of katG (Victor et al, 1999).

PCR was performed in 50 µl of reaction mixture with the following compositions: 1x HotStar PCR buffer; 2.25 mM MgCl₂ (rpoB) or 2.0 mM MgCl₂ (katG); 1x Q solution; 200 µM of each dGTP, dCTP, dATP and dTTP; 0.2 µM of each primer, 1.5 U HostStart Taq DNA polymerase (Qiagen, Hilden, Germany), and 5 µl of DNA template. The rpoB was amplified by using the following thermal cycling conditions: 95°C for 15 minutes; 40 cycles of 94°C for 55 seconds, 58°C for 55 seconds, and 72°C for 2 minutes; followed by one cycle of 72°C for 10 minutes. As for katG, the gene was amplified by the following conditions: 95°C for 15 minutes; 40 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 2 minutes; followed by one cycle of 72°C for 10 minutes. The PCR amplicons were analyzed by 1.5% agarose gel electrophoresis and visualized under ultraviolet light.
Direct Detection of MDR-TB in Sputum Samples

Dot blot hybridization using $^{32}$P-labelled probes

$rpo\beta531mu$ (5'-AGC GCC GAC TGT TGG CGC TG-3') and $katG315mu$ (5'-GAT'CACC ACC GGC ATC GAG G-3') oligonucleotides were used as hybridization probes to rifampicin and INH resistance, respectively (Victor et al., 1999). Each oligonucleotide was labeled with $^{32}$P at its 5' end in 50 µl reaction mixture containing 1x kinase buffer, 1 µM $rpo\beta531mu$ or $katG315mu$, 60 µCi of $^{32}$P-labeled gamma-ATP, 20 U T4 polynucleotide kinase. The mixture was incubated at 37ºC for 30 minutes and the reaction was terminated by incubating at 72ºC for 10 minutes.

Dot blot hybridization was performed as described previously (Victor et al., 1999). In brief, 10 µl aliquot of PCR solution was added with 190 µl of the dot buffer (0.4 N NaOH and 25 mM EDTA), and the mixture was heated at 100ºC and immediately placed on ice. DNA was blotted onto membrane using a dot blottter (Bio-Rad, Hercules, CA). The blotted membrane was heated at 80ºC for 2 hours and then soaked in hybridization solution (5x SSPE, 5x Denhardt and 0.5% SDS) at 58ºC ($rpo\beta531mu$) or 56ºC ($katG315mu$) overnight (16-18 hours). Forty ml of the hybridization solution containing 1 µM $^{32}$P-labeled oligonucleotides were incubated with membrane at 58ºC and 56ºC for 1-2 hours for the $rpo\beta531mu$ and $katG315mu$, respectively. The membrane was washed twice with washing buffer (2x SSPE and 0.1% SDS) at room temperature for 30 minutes, followed by a final wash (1x SSPE and 0.1% SDS) at 69ºC and 68.5ºC for 15 minutes for $rpo\beta531mu$ and $katG315mu$, respectively. The radiolabeled dots on the membrane were detected with autoradiography.

RESULTS

PCR positive was defined as a 157-bp ($rpo\beta$) and 804-bp ($katG$) DNA fragments visualized on 1.5% agarose gel (Fig 1). The PCR showed the high amplification efficiency based on the band intensity and one specific DNA band (Fig 1). All
100 sputum samples were PCR positive for both \textit{rpo}\textsubscript{b} and \textit{kat}G. The PCR solutions were dot blotted onto membrane and hybridized with \textsuperscript{32}P-labelled katG315mu and rpo\textsubscript{b}531mu oligonucleotides. Of the 100 samples, 11\% and 22\% of the samples showed presence of mutation at codons 315 of \textit{kat}G (Fig 2A) and at 531 codon of \textit{rpo}\textsubscript{b} (Fig 2B). Five percent of of the 100 samples showed both mutations. Positive controls were MTB isolates that have mutations at codon 531 of \textit{rpo}\textsubscript{b} and codon 315 of \textit{kat}G, confirmed by DNA sequencing (data not shown).

**DISCUSSION**

As the conventional drug susceptibility testing takes 3 weeks (Zhang \textit{et al}, 2005), a rapid laboratory method is urgently needed in order to achieve the control program of TB. Many studies have reported the importance of sequence analysis for identification of MTB clinical strains. However, sequence analysis is expensive because it requires an automated sequencer, which is very limited in developing countries including Indonesia. On the other side, genotypic assays, including the real-time PCR assay coupled to fluorescence detection and reverse line blot method, have been developed (Garcia de Viedma \textit{et al}, 2002; Wu \textit{et al}, 2009). However, these assays are not suitable for routine clinical services because the assays can process a few number of samples, whereas the dot blot hybridization is suitable for surveillance and epidemiological studies because it can handle a large number of samples in one test. Therefore, we employed the radioisotope (\textsuperscript{32}P)-based PCR-dot blot hybridization method for detection of MTB strains resistant to rifampicin and INH (Victor \textit{et al}, 1999). The method is rapid and only takes 2 days for detection of MDR-TB. Also, the radioisotope (\textsuperscript{32}P)-based assay is much more sensitive than the non-radioisotope assays (Valentine-Thon \textit{et al}, 1991).

Of 100 samples, the dot blot hybridization method detected 11\% and 22\% of MTB strains having mutated at codon 315 of \textit{kat}G and codon 531 of \textit{rpo}\textsubscript{b}, respectively. The results do not reflect the all resistant
patterns because there are other mutations eg, katC, inhA, oxyR, ahpC, furA, and kasA, besides the katG and rpoB that can cause the MTB strains to be resistant to INH and rifampicin (Gillespie, 2002; Zhang et al, 2005; Bostanabad et al, 2008; Wu et al, 2009). In addition, the mutation patterns at codon 315 of katG could be different (6% mutation frequency), such as AGC (Ser)→AGG/AGA (Arg), AGC (Ser)→AAC (Asn), AGC (Ser)→GGC (Gly), and AGC (Ser)→ATC (Ile) (Bostanabad et al, 2008). Therefore, even though the results do not reflect all resistant patterns, the method detected the majority of the INH or rifampicin resistant MTB strains because the mutation frequencies at codon 315 (AGC→ACC) of katG and codon 531(TCG→TTG) of rpoB are more than 64.9% and 61.7%, respectively (Abe et al, 2008; Bostanabad et al, 2008).

Five percent of strains showed mutations at both codons 315 of katG and codon 531 of rpoB, MDR-TB. In European countries, MDR-TB incidence is low, less than 1% in France (Robert et al, 2003), 3.88% in Italy and 5.75% in Germany (Migliori et al, 2007). Overall, the proportion of patients with MDR-TB is 4.0% and the proportion of new cases with MDR-TB ranges from 0% to 17% (Chiang et al, 2010). In Asian countries, MDR-TB cases are also variable from less than 1.0% in Hong Kong, SAR, Japan, New Zealand and Singapore; 4.3% in the Philippines; to 7.2% and 7.3% in Heilongjiang and Inner Mongolia Autonomous Region of China ( Rivera et al, 2000; Chiang et al, 2010).

MDR-TB results from accumulation of mutations in individual drug target genes, at least those genes conferring rifampicin and INH resistance (Rattan et al, 1998). Several factors can cause MTB to be MDR-TB, including poor national program in particular countries, poor patient management, patients’ noncompliance and inappropriate administration of drugs by clinicians, alcoholism, drug addiction, and poverty and poor understanding of side effects (Davies, 2001; Faustini et al, 2006; Su et al, 2008; Prabhudesai and Singh, 2009).

Technically, there was a dot with lower intensity compared with that of positive control (Fig 2). This might be caused by polymorphism. It means that the sample contains a mixture of MTB strains, of which the strains that have mutation of TCG to TTG at codon 531 of rpoB were few in numbers. This notion was based on the high intensity of its DNA band on agarose gel (data not shown). The phenomenon also has been reported by other researchers (Victor et al, 1999; Chaoui et al, 2009). Another report showed dots with low intensities for heterozygous samples, while homozygous samples showed dots with high intensities (Rabachini et al, 2010). The issue is important clinically, because an early detection of resistant MTB strains before they spread into the general population is a proper way to prevent the transmission of TB and to eradicate the drug resistant MTB strains.

In summary, we have successfully employed radioisotope (³²P)-based PCR-dot blot hybridization for detection of drug resistant MTB from sputum samples directly. This method is rapid, sensitive, and reliable for large numbers of samples and epidemiological studies. A limitation of this method is that we only used samples obtained from Jakarta; thus, the results are not representative data for Indonesia. In the future, we will evaluate the method for larger numbers of samples from several areas in Indonesia.
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