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 (^{32}P)-based PCR-dot blot hybridization method on sputum samples from patients in Jakarta, Indonesia. Bacterial DNA was extracted using BOOM method. *KatG* and *rpoβ* were amplified by PCR and katG315 or rpoβ531 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 *rpoβ*, 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

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caused by a mutation (T**C**G⟶T**T**G) at codon 531 of *rpoβ* encoding RNA polymerase subunit β and a mutation (A**G**C ⟶A**C**C) 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 (^{32}P)-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 H_{37}Rv 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 14,000*g* 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 CCG CGA TCA AGG AGT-3′) primer were used for PCR amplification of *rpoβ*, while forward (5′-TCG GGG TCG TTG ACC TCC CA-3′) and reverse (5′-TGG CCG CGG 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 $MgCl_2$ (*rpoβ*) or 2.0 mM $MgCl_2$ (*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 *rpoβ* was amplified by using the following thermalcycling conditions: 95ºC for 15 minutes; 40 cycles of 94ºC for 55 seconds, 58ºC for 55 seconds, and 72ºC for 2 minutes; followed by one cycle of 72ºC for 10 minutes. As for *katG*, the gene was amplified by the following conditions: 95ºC for 15 minutes; 40 cycles of 94ºC for 1 minute, 64ºC for 1 minute, and 72ºC for 2 minutes; followed by one cycle of 72ºC for 10 minutes. The PCR amplicons were analyzed by 1.5% agarose gel electrophoresis and visualized under ultraviolet light.

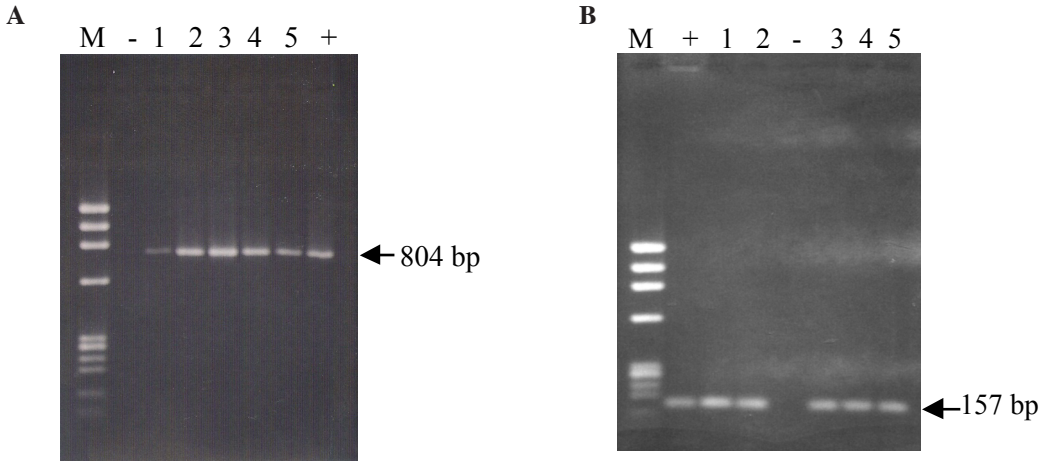

Fig 1–Analysis of the PCR amplicons of *katG* (**A**) and *rpoβ* (**B**). Lanes 1-5, the five examples with PCR positive results showing the 804 bp DNA of *katG* or 157 bp DNA of *rpoβ*; lane M, DNA ladder; lane -, negative control; lane +, positive control.

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

rpoβ531mu (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 rpoβ531mu or katG315mu, 60 µCi of ^{32}P-labeled gamma-ATP, 20 U T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 minutes and the reaction was terminated by incubating at 72°C 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 100°C and immediately placed on ice. DNA was blotted onto membrane using a dot blotter (Bio-Rad, Hercules, CA**)**. The blotted membrane was heated at 80°C for 2 hours and then soaked in hybridization solution (5x SSPE, 5x Denhardt and 0.5% SDS) at 58°C (rpoβ531mu) or 56°C (katG315mu) overnight (16-18 hours). Forty ml of the hybridization solution containing 1 µM ^{32}P-labeled oligonucleotides were incubated with membrane at 58°C and 56°C for 1-2 hours for the rpoβ531mu 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 69°C and 68.5°C for 15 minutes for rpoβ531mu and katG-315mu, respectively. The radiolabelled dots on the membrane were detected with autoradiography.

RESULTS

PCR positive was defined as a 157-bp (*rpoβ*) 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

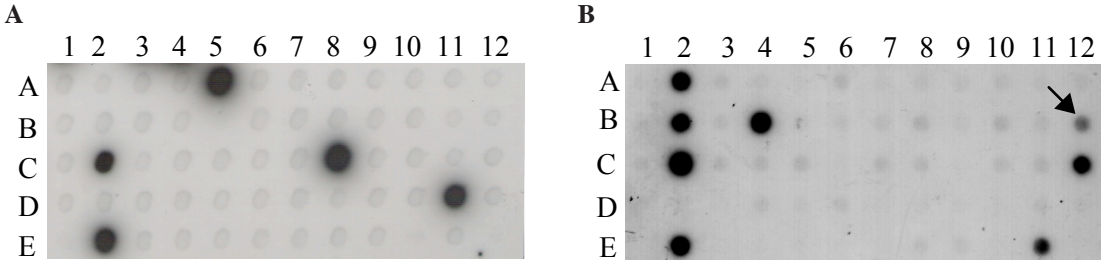

Fig 2–Radioisotope (^{32}P)-based dot blot hybridization tests. Presence of mutation katG315 (A) and rpoβ531 (B) is shown as black dot. Arrow marks a strain giving dot with low intensity. For the katG315 mutation assay (A), A5 and E9 is mutation-positive and mutation-negative control, respectively. E10 is negative control without any DNA. For the rpoβ531 mutation assay (B), C12 and E9 is mutation-positive and mutation-negative control, respectively. E10 was negative control without any DNA.

100 sputum samples were PCR positive for both *rpoβ* and *katG*. The PCR solutions were dot blotted onto membrane and hybridized with ^{32}P-labelled katG315mu and rpoβ531mu oligonucleotides. Of the 100 samples, 11% and 22% of the samples showed presence of mutation at codons 315 of *katG* (Fig 2A) and at 531 codon of *rpoβ* (Fig 2B). Five percent of of the 100 samples showed both mutations. Positive controls were MTB isolates that have mutations at codon 531 of *rpoβ* and codon 315 of *katG*, confirmed by DNA sequencing (data not shown).

DISCUSSION

As the conventional drug susceptibility testing takes 3 weeks (Zhang *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 *et al*, 2002; Wu *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 (^{32}P)-based PCR-dot blot hybridization method for detection of MTB strains resistant to rifampicin and INH (Victor *et al*, 1999). The method is rapid and only takes 2 days for detection of MDR-TB. Also, the radioisotope (^{32}P)-based assay is much more sensitive than the non-radioisotope assays (Valentine-Thon *et al*, 1991).

Of 100 samples, the dot blot hybridization method detected 11% and 22% of MTB strains having mutated at codon 315 of *katG* and codon 531 of *rpoβ*, 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 *rpoβ* 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 AG**C** (Ser)⟶AG**G**/AG**A** (Arg), A**G**C (Ser)⟶ A**A**C (Asn), **A**GC (Ser)⟶**G**GC (Gly), and A**G**C (Ser)⟶A**T**C (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 (A**G**C⟶A**C**C) of *katG* and codon 531(T**C**G⟶T**T**G) of *rpoβ* 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 531of *rpoβ*, 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 migth be caused by polymorphism. It means that the sample contains a mixture of MTB strains, of which the strains that have mutation of T**C**G to T**T**G at codon 531 of *rpoβ* 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 succesfully employed radioisotope (^{32}P)-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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