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Multiplex PCR for Rapid Detection of Rifampin and Isoniazid Resistance in *Mycobacterium tuberculosis* Isolated from Bandung, Indonesia

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Mycobacterium tuberculosis resistant to rifampin and isoniazid, known as multidrug-resistant *M. tuberculosis* (MDR-TB) strains, is an emerging problem of great importance to public health, with higher mortality rates than for drug-sensitive strains. Rifampin resistance is due to mutations on the hot spot region of the *rpoB* gene, especially at positions 526 and 531, and isoniazid resistance is due to mutation on *katG* at position 315. Mechanisms of resistance are an appropriate target for molecular genotyping diagnostic methods. Here we examined the multiplex PCR assays for the rapid detection targeting *rpoB526, rpoB531,* and *katG* mutations. Sixty-one *M. tuberculosis* strains were studied based on *rpoB526*, *rpoB531,* and *katG315* assays employing multiplex PCR. Of the 61 strains, the susceptibility tests determined 42 isolates were MDR-TB strains, 10, 4, and 5 isolates were resistant to rifampin, isoniazid, and at least to six drugs which prescibed for TB, respectively. The mutation profiles of the 42 MDR strains assayed by multiplex PCR were 81 and 38.1% on *rpoB* and *katG*, respectively. Six rifampin-resistant isolates (60%) had a mutation on *rpoB*, 25% isoniazid-resistant isolates had mutation on *katG*, and 20% of the isolates that were sensitive to all drugs tested had a mutation on *rpoB*. Sequencing analysis revealed sensitivity of the multiplex PCR assay for *rpoB* was 98.4% and was 100% for *katG*. There was a 19% difference between phenotype and genotype properties of all isolates detected. In conclusion, the sensitivity of multiplex PCR method was sufficient for preliminary detection of *rpoB* and *katG* mutations, but resistance *M. tuberculosis* to rifampin and isoniazid were not always conferred by mutated alleles on *rpoB* and, especially, on *katG*.

Key words: multiplex PCR, *rpoB*, *katG*, sequencing

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is still the single most deadly infectious disease in developing countries. The spread of MDR-TB, defined as *M. tuberculosis* resistant to at least isoniazid (INH) and rifampin (RIF), is worsening and emerging as a global emergency. It has increased worldwide and reached epidemic proportions in many countries (Fang *et al.* 1999; Rie *et al*. 2001; Wei *et al*. 2003). Indonesia is third in the rank of the highly burdened countries, after China and India. In 2001, the WHO estimates there are 122 TB patients per 100 000 people. The mortality rate is at least 140 000 people each year in Indonesia (WHO 2006).

RIF in combination with INH is a frontline anti-tuberculosis agent that is prescribed daily in millions of doses worldwide. Resistance to RIF is conferred by mutations in the *rpoB* hot spot region, encoding the subunit-b of RNA polymerase. The two most frequent mutations in the *rpoB* are mutations at codon *rpoB526* and *rpoB531* (Gillespie 2002; Mokrousov *et al*. 2003; Van der Zanden *et al*. 2003; Werngren and Hoffner 2003). INH is a prodrug, which is peroxidatively activated by the *M. tuberculosis* catalase-peroxidase *katG* to produce damaging cell wall of the bacteria (Whitney and Wainberg 2002). Mutations at codon S315T (AGCaACC) were found in 60-70% of INH resistant strains (Van Doorn *et al*. 2001; Ramaswamy *et al*. 2003). This mutation was reported to be associated with intermediate or high levels of resistance to INH (1.0 to 10 mg l^{-1}) (Mokrousov *et al*. 2002a; Caws *et al*. 2006). The *inhA* and *kasA* genes may participate in INH resistance.

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Recent advances in the development of rapid and reliable diagnostic methods have allowed for detection of resistance to anti-tuberculosis drugs without the need for a viable culture. In this work we examined multiplex PCR assay as a means for rapid detection of *rpoB526, rpoB531,* and *katG315* mutation related to RIF and INH resistance, respectively. The aim is to confirm results from molecular assays of multiplex PCR by nucleotides sequencing.

MATERIALS AND METHODS

Strains Collection. *M. tuberculosis* clinical isolates were recovered from different patients originating from Bandung, West Java with new or previous diagnosed pulmonary TB. Clinical specimens were collected from sputum, pleural exudate, and cerebrospinal fluid of patients between January and November 2005 at Balai Pengembangan dan Latihan Kesehatan (BPLK) laboratory for Mycobacteria, Department of Health and Rotinsulu Lung Diseases Hospital, Bandung, West Java.

Susceptibility Testing. Drug susceptibility was determined using the proportion method on Lowenstein-Jensen medium with the following critical concentration of the drugs: INH (0.2 mg l^{-1}), RIF (40 mg l^{-1}), streptomycin (10 mg l^{-1}), kanamycin (40 mg l^{-1}), ethambutol (10 mg l^{-1}), and pyrazinamide (40 mg l^{-1}). The control was a tube of the medium without drugs inoculated by the strains of *M. tuberculosis* being tested. All the tubes were incubated at 37 °C, growth was monitored after 6-8 weeks of incubation and susceptibility results were then read. An isolate was considered resistant if the bacterial growth in the culture

tube was ≤1% compared with bacterial growth in the respective control tube (Kumari and Ichhpujani 2000).

Preparation DNA Samples of *M. tuberculosis*. *M. tuberculosis* colonies were lysed by 20 µl lysis buffer (50 mM Tris-HCl pH 8.5; 1 mM EDTA pH 8.5; 0.5% Tween-20); and 4 ml Proteinase-K, using a 58 °C incubation temperature for an hour. The lysate was heated to 95 °C to disrupt Proteinase K, and the supernatant was then collected for further analysis. For both multiplex PCR and PCR for nucleotides sequencing, we used 5 ml DNA samples from this preparation.

Multiplex PCR. The multiplex PCR assay (modification of Mokrousov *et al*. 2002b; Mokrousov *et al.* 2003) was employed for mutational assay on *rpoB526, rpoB531*, and *katG315*. Multiplex PCR, either for *rpoB* or *katG*, consisted of three primers targeting codons *rpoB526*, *rpoB531*, and *katG315*. A negative control was amplified using a standard strain of *M. tuberculosis*, H37Rv. The primer sequences that used are shown in Table 1.

The inner forward primers are positioned so that their 3'-OH ends pair with the second bases of the respective codons in the wild-type allele. If a mutation occurs, this results in a mismatch at the 3'end of the inner primer sequence, in the absence of the variable PCR product (181 bp for *rpoB526*, 167 bp for *rpoB51,* and 292 bp for *katG315*). The invariable fragments of 249 and 435 bp are amplified by two outer primers, forward and reverse, flanked the *rpoB* hot spot and *katG* region, respectively. The amplification a pair of forward and reverse primer is used to control the quality of the multiplex PCR. Meanwhile, the amplification employing similar forward and reverse primer without inner primer is used for nucleotide sequencing analysis.

The multiplex PCR reactions for *katG315* were carried out in a thermal controller (Perkin-Elmer PCR system 2400, Inc.) under the following conditions: initial denaturation at 96 °C for 3 min; 30 cycles at 94 °C for 1 min, 58 °C for 40 s, and 72 °C for 30 s with the final elongation at 72 °C for 3 min. The PCR reactions for *rpoB526* and *rpoB531* were carried out under the following conditions: initial denaturation at 96 °C for 3 min; 5 cycles of 95 °C for 45 s, 60 °C for 1 min, and 72 °C for 30 s. Then 5 cycles at 95 °C for 40 s, 59 °C for 50 s, and 72 °C for 30 s. Then 25 cycles at 94 °C for 50 s, 55 °C for 40 s, and 70 °C for 30 s with the final elongation at 72 °C for 3 min. The PCR product was electrophoresed on 1.5% w/v agarose gel incorporating 0.5 g ml^{-1} ethidium bromide for visualization using a UV transilluminator.

RESULTS

Sixty-one strains of *M. tuberculosis* were collected from two laboratories and analyzed for their phenotype and genotype properties. The susceptibility tests showed 42 strains were resistant to at least RIF and INH (MDR-TB), 10 strains were sensitive to INH but resistant at least to RIF, 4 strains were sensitive to RIF but resistant at least to INH, and 5 strains were considering sensitive to all of the drugs tested. The results of susceptibility test are shown in Table 2.

Table 1 Nucleotide sequences of primers used in the multiplex PCR assay and PCR for nucleotide sequencing

Primer	Nucleotide sequences
Forward *rpoB*	5'-GTCGCC GCGATCAAGGA-3'
Reverse *rpoB*	5'-TGACCCGCGCGT ACA C-3'
Inner *rpoB531*	5'-ACAAGCGCCGAC TGT C-3'
Inner *rpoB526*	5'-GTCGGGGTTGACCCA-3'
Forward *katG*	5'-GCAGATGGGGCTGATCTACG-3'
Reverse *katG*	5'-AACGGGTCCGGGATGGTG-3'
Inner *katG315*	5'-ATACGACCT CGATGCCGC-3'

Molecular analysis of *M. tuberculosis* is a promising alternative to bacteriological assays. The genetic basis for RIF and INH resistance are mutations in the *rpoB* and *katG* genes, respectively. Using the method PCR-based, therefore the time to identify RIF and INH resistant strains were further is shortened to 2 h. In addition, it does not require any special probe preparation and can be applied to simultaneous analysis of several variable segments of the bacterial genome. Results of the multiplex PCR assays are shown in Fig 1 and 2.

Based on multiplex PCR results, this study determined the existance of various genotype combinations of *M. tuberculosis* strains as shown in Table 3.

DISCUSSION

There are many tools developed to simplify methods on TB diagnostics. The susceptibility test remains the gold standard method for phenotyping of *M. tuberculosis*. In total, drug-susceptibility testing of 61 *M. tuberculosis* isolates showed that resistance to RIF was the most frequent (85.2%) in this study, followed by INH resistance (75.4%). This study identified 42 strains of MDR-TB with nine of them (21.4%) resistant to six drugs tested. Resistance to ethambuthol, streptomycin, kanamycin, and pyrazinamide was observed for 60.7, 41.0, 26.2, and 21.3%, respectively.

Taniguchi *et al*. (1996) suggest that there was a strong correlation of specific amino acid substitutions of *rpoB* and *katG*, and its minimum inhibitory concentration to RIF and INH. This study revealed a correlation between the level of resistance and specific mutation on *rpoB526, rpoB531,* and *katG315*. Twenty one strains of MDR-TB had the capability to grow on media containing RIF 300 µg ml^{-1} and 19 strains

Table 2 Resistance pattern of MDR *M. tuberculosis* strains iso

No. of strains[a]	RIF	INH	ETM	STR	KN	PYR
9	R	R	R	R	R	R
8	R	R	R	R	-	-
8	R	R	R	-	-	-
7	R	R	-	-	-	-
5	R	-	R	-	-	-
3	R	R	-	R	-	-
2	R	R	R	-	R	-
2	-	R	-	-	-	-
1	R	R	-	-	-	R
1	R	R	-	R	R	-
1	R	R	R	-	-	R
1	R	R	R	-	R	R
1	R	R	-	R	-	R
1	R	-	R	R	R	-
1	R	-	R	R	-	-
1	R	-	-	R	-	-
1	-	R	R	-	-	-
1	-	R	-	-	R	-
1	R	-	-	-	R	-
1	R	-	-	-	-	-
5	-	-	-	-	-	-
Total	52	46	37	25	16	13

[a]n = 6, RIF: rifampin, INH: isoniazid, ETM: ethambutol, STR: streptomycin, KN: kanamycin, PYR: pyrazinamide.

of MDR-TB has the capability to grow on media containing INH 10 µg ml^{-1} (data not shown). *Mycobacterium tuberculosis* strains carrying a mutated allele on *rpoB531* had a high level resistance to RIF (MICe"100 mg l^{-1}) compared with strains carrying a mutated allele on *rpoB526*. Likewise, *M. tuberculosis* strains carrying a mutated allele on *katG315* had a high level resistance to INH (MIC between 1 mg l^{-1} and e"100 mg l^{-1}) than strains that had no carrying mutated allele on *katG315* (MIC 0.2-10 mg l^{-1}).

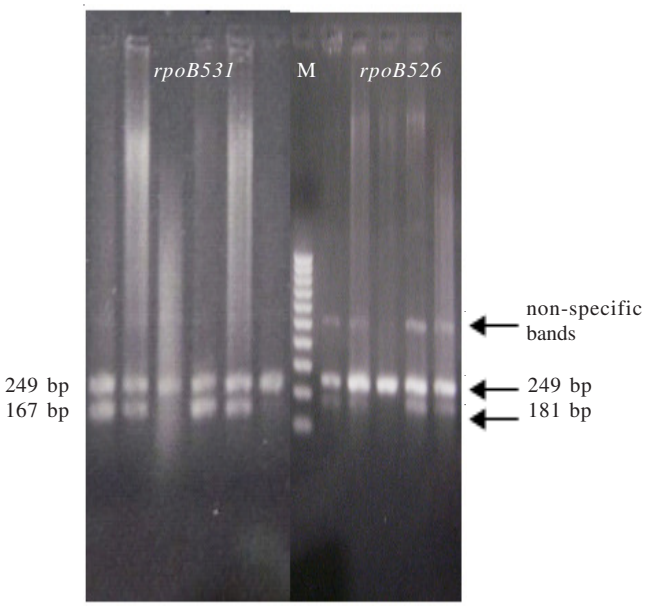

Fig 1 Profiles generated by multiplex PCR targeting *rpoB526* and *rpoB31* (note: samples which amplified a product of 167 and 181 bp were considered to be wild-type *rpoB531* and *rpoB526*, the strains with no amplified product of 167 and 181 bp were considered to be mutant, M: 100 bp marker lane).

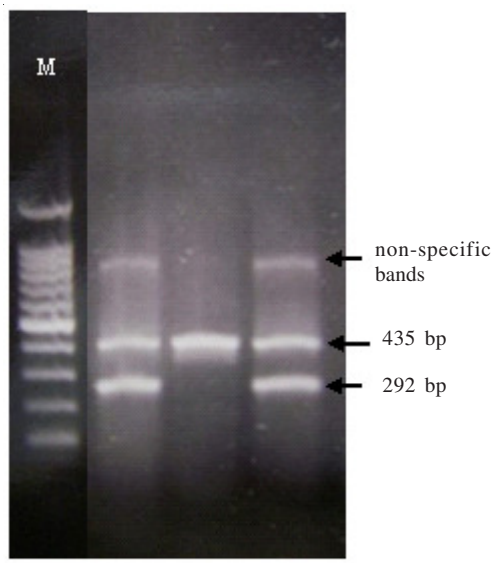

Fig 2 Profiles generated by multiplex PCR targeting *katG315* (note: the strains with amplified product of 292-bp were considered to be wild-type, the strains with no amplified product of 292 bp were considered to be mutant, M: 100 bp marker lane).

Table 3 Genotype combinations of MDR *M. tuberculosis* strains isolated from Bandung employing multiplex PCR

No. of strains (n=61)	Genotype		
	katG315	*rpoB526*	*rpoB531*
21	W	W	W
13	W	T	W
8	T	W	T
8	W	W	T
7	T	T	W
2	W	T	T
1	T	W	W
1	T	T	T

W: wild-type allele; T: mutant allele.

Multiplex PCR assays for RIF and INH resistance tests rely on detection of mutations on *rpoB526, rpoB531,* and *katG315*. Of the 61 *M. tuberculosis* strains, approximately 68.9% were carrying mutated alleles of *rpoB526* and *rpoB531*, whereas 27.9% carrying mutated allele of *katG315*. Mutation on *rpoB526* and *rpoB531* in this study were lower than reported earlier, with more than 95% of the rifampin-resistant *M. tuberculosis* strains caused by these mutations (Van der Zanden *et al*. 2003). Forty-two MDR-TB strains consisted of 16 (38.1%), 15 (35.7%), and 3 (7.1%) strains carrying the mutated allele of *rpoB526*, *rpoB531,* and both *rpoB526* and *rpoB531*, respectively. Eight strains of MDR-TB were not detected carrying mutated alleles at either codon 526 or 531. Meanwhile, of the 42 MDR-TB strains analyzed by multiplex PCR for *katG315*, only 16 strains (38.1%) were detected carrying mutated allele of *katG315*. The results of genotype analysis on *katG315* also were lower than reported in previous studies, which showed a 60-70% frequency for *katG315* mutation. Mokrousov *et al*. (2002a) found a 93.6% prevalence of the *katG315* mutation in strains from patients with both new and previous diagnosed cases of TB.

Of the ten strains sensitive to INH, but resistant to RIF when subjected to assay, seven were not demonstrated as having variable amplified product of 181 or 167 bp. This means that only three strains were detected carrying mutated alleles on *rpoB526* and/or *rpoB531*. Interestingly, one strain sensitive to RIF but resistant to INH, and one strain sensitive to all six drugs, demonstrated had no amplified 181 bp product. Both strains that sensitive to RIF were considered to carrying mutated alleles on *rpoB526*. Those two strains might develop resistance to RIF at a low level (<40 mg l^{-1}) so that in the susceptible test to RIF (40 mg l^{-1}) they show as sensitive. Meanwhile, only one of four strains sensitive to RIF, but resistant to INH, was detected carrying a mutated allele on *katG315*.

There was a 500 and 1 000 bp of non-specific band in a few products of multiplex PCR for both *rpoB526* and *rpoB531*, and *katG315*, respectively. The unspecific binding of primers or the abundant of DNA templates might cause this non-specific band (measurement of DNA samples were not done so that the concentration of DNA in every sample was different).

All the isolates were sequenced in order to avoid misdiagnosis through false positive and negative results of multiplex PCR, especially *M. tuberculosis* strains which demonstrated that no mutation occurred. However, sequencing analysis revealed approximately 98% correlation between multiplex PCR of *rpoB526, rpoB531, katG315,* and sequencing. The sequencing showed one *M. tuberculosis* MDR strain had a mutated allele carrying CAC (his) to CGC (arg) at codon *rpoB526*, but this was not detected by multiplex PCR although it was re-confirmed a second time. In the absence of the allele-specific PCR product, this can be

caused by inappropriate positioning of the PCR primers, which resulted in an underestimation of the prevalence of this mutation. Although Mokrousov *et al*. (2003) had been stably adjusted to assess reproducibility of multiplex PCR, it might be that the inner primer sequence and the effectiveness of the thermal controller was the crucial factor which affected the process of the multiplex PCR. The remaining isolates showed similarity between multiplex PCR and sequencing. This finding demonstrates that although the level of sensitivity of multiplex PCR to identify *rpoB526, rpoB531,* and *katG315* mutations was not 100% because it was based on the specificity of multiplex PCR. However, this method can be used as preliminary assay for the detection of mutation on *rpoB526, rpoB531,* and *katG315*.

It is logical that a mutation would not be selected for in a population if it did not confer some form of selective advantage. The sequencing analysis recognized new mutated alleles in this study. Four of the eight MDR-TB strains carrying wild-type alleles on *rpoB526, rpoB531,* and *katG315* had a mutation at a position both outside and within the hot spot of *rpoB*, either *rpoB526* and *rpoB531*. It is remain unclear and undetermined what is the resistance mechanism of the remaining four MDR-TB strains. Interestingly, one strain sensitive to at least six anti-TB drugs demonstrated a new mutated allele carrying CCC (Pro) to CAC (His) at codon *rpoB535*, but the clinical significance of this mutation is not clear.

The high percentage of isolates lacking mutations suggests that phenotyping methods remain an important complement to genotyping methods for drug susceptibility testing. The genotype analysis determined 15 of the 52 (28.8%) strains which have phenotype resistant to RIF had no mutation on both *rpoB526* and *rpoB531*. There were also 29 of the 46 (63%) strains phenotype resistant to INH which had no mutation on *katG315*. Therefore, this study revealed a 19% difference between phenotype and genotype properties of all isolates detected by multiplex PCR of *rpoB526, rpoB531,* and *katG315*. Our results suggest that this molecular method only determines the expected mutations, especially on *rpoB526* and *rpoB531*. Susceptibility tests for the detection of INH resistance remain the most crucial assay because of the frequency of resistance corresponding to *katG315* mutation being low. Therefore, although the molecular methods may aid in the rapid detection of mutations associated with drug resistance, the results always need to be confirmed by phenotyping methods.

In conclusion, the molecular analysis of *M. tuberculosis* is a promising alternative to bacteriological assays. The study demonstrates multiplex PCR is rapid, easy to perform, and sufficiently accurate for preliminary assay. It was essential that the test always be verified by standard culture-based methods because there were resistant strains of *M. tuberculosis* that were carrying no mutated alleles, whether on *rpoB* or *katG*. It is hope that the development of novel molecular methods using a rapid and reliable genetic approach will facilitate the appropriate and timely delivery of anti-tuberculosis therapy.

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