

Random amplified polymorphic DNA analysis of *Mycobacterium tuberculosis* isolates resistant to Isoniazid in Indonesia

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ABSTRACT

Background: *M. tuberculosis* is the most important etiological factor of tuberculosis. One of the factors that make TB hard to eradicate is the emergence of *M. tuberculosis* drug resistance. Drug resistance in *M. tuberculosis* is attributed primarily to the accumulation of mutations in the drug target gene. **Objectives:** to analyze profile of Random Amplified Polymorphic DNA (RAPD) in *M. tuberculosis* isolates resistant to Isoniazid and found RAPD marker. **Methods:** seven Isoniazid resistant isolate of *M. tuberculosis* from Makassar, Indonesia strain were analyzed by RAPD method using primers OPN 02, OPN 09, OPN 20, BG 65, N 9, that amplification fragment DNA than as molecular marker. **Results:** The results of the present study showed high degree of polymorphism in the *M. tuberculosis* strains in the population, and found that specific DNA fragment at Isoniazid resistant isolates using primer N 9 is 1450 bp as a marker. **Conclusion:** This study gives information about RAPD marker of *M. tuberculosis* strain to Isoniazid resistant.

Keywords: *Mycobacterium tuberculosis*; RAPD; Isoniazid Resistant

1. INTRODUCTION

Tuberculosis (TB) is a bacterial infection disease cause by acid fast bacilli—*Mycobacterium tuberculosis* (*M. tuberculosis*). This bacillus currently infect one third of the world's population. According to the World Health Organization report, 8.8 million new cases of tuberculosis

occur each year, resulting in 3 million deaths [1,2]. It is estimated 95% cases of TB and 98% deaths caused by TB occur in developed countries [3].

Indonesia is the fifth country with largest number of TB cases in the world. One of the factors that make TB hard to eradicate is the emergence of *M. tuberculosis* drug resistance.

The estimated 75% of TB patients are in the economically productive age of 15 to 50 years. Multidrug resistant TB (MDR-TB) is defined as resistance to at least Isoniazid and Rifampicin, the two most important drugs in the treatment of TB [1].

Evidence collected between 1994 and 2002, within the framework of the Global Project on anti TB Drug Resistance Surveillance and from other sources, has confirmed previous assertions that in resource—limited countries, drug resistant TB, including MDR-TB is ubiquitous [4].

China and India, the countries with the highest TB incidence in the world, also have documented MDR-TB among new TB cases in some of their geographical areas [4,5].

Drug resistance in *M. tuberculosis* is attributed primarily to the accumulation of mutations in the drug target gene; these mutations lead either to an altered target (e.g., RNA polymerase and catalase—peroxidase in Rifampicin and Isoniazid resistance, respectively) [6].

A major development in the diagnosis of TB was the introduction of several nucleic acid amplification (NAA) techniques, such as the polymerase chain reaction (PCR) that has been widely evaluated [2].

Random Amplified Polymorphic DNA (RAPD), also known as arbitrarily primed PCR, allows the detection of polymorphisms without prior knowledge of the nucleotide sequence. The polymorphisms may be used as genetic markers and the construction of genetic maps. This

method utilizes short (C10 nucleotides) primers of arbitrary nucleotide sequence that are annealed in the first few cycles of PCR at low stringency. RAPD is also a technique ideally suited to fingerprinting applications because it is fast, requires little material and is technically easy [7].

The other study that showed correlation with RAPD method and genetic diversity at *M. tuberculosis* was studied by: 1) Richner, S. *et al.*, in South Africa (1999); 2) Bardakci, in Turkey (2001); 3) Tazi, L. *et al.*, in Casablanca, Morocco, (2004); 4) Korzekwa, K. *et al.* in Olaszyn (2006); 5) Singh, J. P. N., Verma, R., Chaudhurt, P., in India (2006); 6) Jain, A. *et al.* in Luknow, India (2008); 7) Va'zquez-Marrufo, G. *et al.*, in Mexico (2008); 8) Rodriguez, J. C., Royo, G. and Rodriguez-Valera, F. in Spain (2009) [8-15].

2. MATERIALS AND METHODS

The samples were seven resistant isolates from human patients (sputum) diagnosed suffering from pulmonary tuberculosis obtained at the Provincial Health Laboratory of South Sulawesi and Biotechnology laboratory of Research Centre Hasanuddin University, Indonesia. All isolates were cultured on Lowenstein-Jensen medium and identified as *Mycobacterium tuberculosis*, then continued with susceptibility test on Lowenstein-Jensen medium and anti-tuberculosis drug namely Isoniazid, for analyzing resistance or sensitivity to Isoniazid [16,17]. This study used seven Isoniazid resistant isolates of *Mycobacterium tuberculosis*.

The DNA was extracted by Wizard Genomic DNA [18]. PCR using RAPD method was performed with 5 primers according to Singh *et al.* (2006) and Tazi *et al.* (2004) (Table 1). Amplification of Mycobacterium DNA, uses total volume of 25 µl. The reaction mixture contained 1 unit of Taq DNA polymerase 0.125; MgCl₂ 3, dNTP 0.5; 10 × buffer 2.5; template DNA 5 µl; ddH₂O 12,875 µl and primers 30 pmol. Amplification was carried out in a thermal cyler (AB Applied Biosystem). The cycling condition consisted of 45 cycles of an initial denaturation (pre PCR) step for 5 min at 94°C, follow by denaturation step for 1 min at 94°C, an annealing step for 1 min at 36°C, and an extension step for 1 min at 72°C, and a final

Table 1. Sequence of the 5 RAPD Primers used in this study.

RAPD Primers	Sequence	Reference
OPN 02	5' ACCAGGGGCA 3'	Singh <i>et al.</i> , 2006
OPN 09	5' TGCCGGCTTG 3'	Singh <i>et al.</i> , 2006
OPN 20	5' GGTGCTCCGT 3'	Singh <i>et al.</i> , 2006
BG 65	5' CTCGAGCGGC 3'	Singh <i>et al.</i> , 2006
N 9	5' TGCCGGCTTG 3'	Tazi <i>et al.</i> , 2004

extension (post PCR) for 5 min at 72°C [12,19,20].

Electrophoresis that was performed after RAPD-PCR, used a 2% agarose gel stained with Etidium bromide and 100 bp marker. Subsequently, the gel was visualized and photographed using a gel documentation and analysis system. The dendrogram program (<http://www.expasy.ch/tools/>). A data matrix composed of numerals 1 and 0 was built on the basis of presence: 1, or absence: 0.

3. RESULTS

This study used seven Isoniazid resistant isolates, I1, I2, I3, I4, I5, I6, I7, by using five short primers (see Table 1). The primers have been showed in Table 1. The results of RAPD-PCR are shown in Figures 1-5. In this study, all seven isolates of *M. tuberculosis* showed several fragments. All five primers revealed discriminating patterns.

The dendrogram analysis, using primer OPN 02, formed five clusters. The larger cluster consisted of 3 isolates,

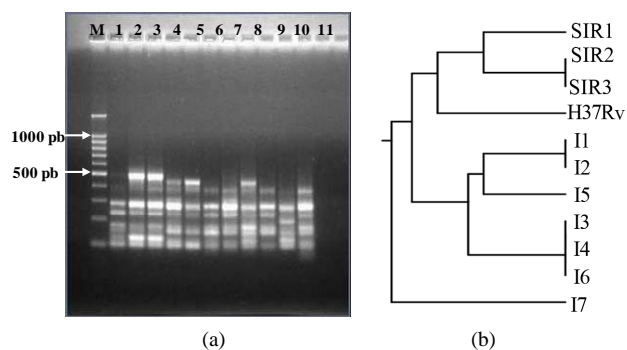


Figure 1. (a) RAPD Profiles of *M. tuberculosis* with primer OPN-02. M = 100 bp DNA marker, 1 - 3 Isoniazid and Rifampicin sensitive, 4 -10: Isoniazid resistant, 11: H37Rv. (b). Dendrogram *M. tuberculosis* Isoniazid resistant isolates, SIR1 - SIR3: Isoniazid and Rifampicin sensitive, I1 - I7: Isoniazid resistant.

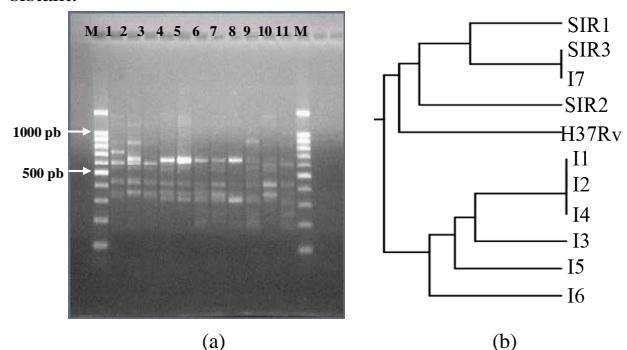


Figure 2. (a). RAPD Profiles of *M. tuberculosis* with primer OPN-09. M = 100 bp DNA marker, 1 - 3: Isoniazid and Rifampicin sensitive, 4 - 10: Isoniazid resistant, 11: H37Rv. (b). Dendrogram *M. tuberculosis* Isoniazid resistant isolates, SIR1 - SIR3: Isoniazid and Rifampicin sensitive, I1 - I7: Isoniazid resistant.

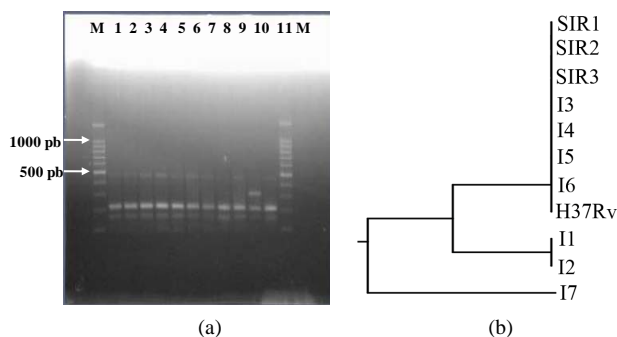


Figure 3. (a). RAPD Profiles of *M. tuberculosis* with primer OPN-20. M = 100 bp DNA marker, 1 - 3: Isoniazid and Rifampicin sensitive, 4 - 10: Isoniazid resistant, 11: H37Rv. (b). Dendrogram *M. tuberculosis* Isoniazid resistant isolates, SIR1 - SIR3: Isoniazid and Rifampicin sensitive, I1 - I7: Isoniazid resistant.

the smaller cluster consisted of one isolate. The genetic relatedness was closest among isolates within clusters (**Figure 1**).

Figure 2(b) using primer OPN 09 were divided into two populations that were sensitive (comparison) and H37Rv (reference) was with Isoniazid resistant, whereas each population were divided into three subpopulations.

Figure 3(b) using primer OPN 20, formed two clusters. The larger cluster consisted of 8 isolates (3 sensitive isolates, 1 H37Rv, 4 Isoniazid resistant isolates), and the smaller cluster consisted of one isolate (resistant to Isoniazid).

Figure 4(b) using primer BG 65 showed four clusters, whereas the larger cluster consisted of 3 isolates (resistant to Isoniazid), and the smaller cluster consisted of one isolate (resistant to Isoniazid).

The dendrogram analysis with primer N 9, formed six clusters with two populations that are sensitive, H37Rv

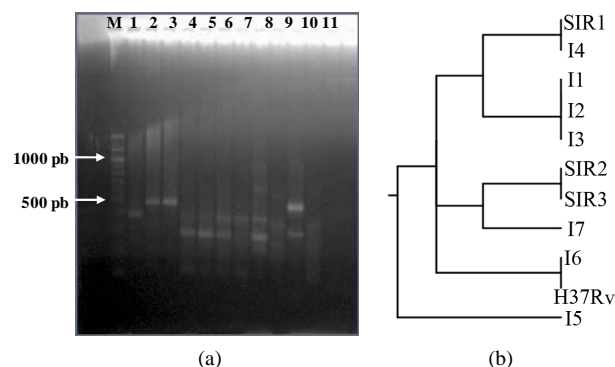


Figure 4. (a). RAPD Profiles of *M. tuberculosis* with primer BG-65. M = 100 bp DNA marker, 1 - 3: Isoniazid and Rifampicin sensitive, 4 - 10: Isoniazid resistant, 11: H37Rv. (b). Dendrogram *M. tuberculosis* Isoniazid resistant isolates, SIR1 - SIR3: Isoniazid and Rifampicin sensitive, I1 - I7: Isoniazid resistant.

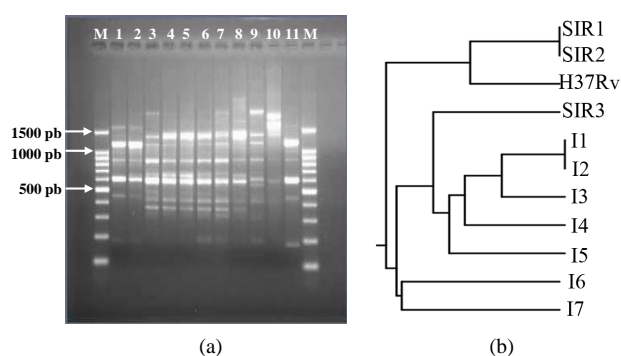


Figure 5. (a). RAPD Profiles of *M. tuberculosis* with primer N-9. M = 100 bp DNA marker, 1 - 3: Isoniazid and Rifampicin sensitive, 4 - 10: Isoniazid resistant, 11: H37Rv. (b). Dendrogram *M. tuberculosis* Isoniazid resistant isolates, SIR1 - SIR3: Isoniazid and Rifampicin sensitive, I1 - I7: Isoniazid resistant.

and resistant to Isoniazid. The larger cluster consisted of two isolates namely I1, I2, and SIR1, SIR2, and the smaller cluster consisted of one isolate (**Figure 5(b)**). It was found specific DNA fragment at Isoniazid resistant isolate using primer N 9 is 1450 bp, there are 5 from the 7 isolates resistant to Isoniazid (**Figure 5(a)**) that are sample I1, I2, I3, I4, and I5, on location 4 - 8.

4. DISCUSSION

RAPD is faster and technically less demanding than most other molecular typing methods and furthermore, no DNA sequence information is necessary. Also, much smaller amounts of purified DNA are required than for methods such as *Restricted Fragment Length Polymorphism* (RFLP). Although RAPD is relatively simple and useful for epidemiological analysis, standardization of the PCR mixture and conditions are very important for reproducibility [12]. Finally, even with this standardization, reproducible profiles were difficult to obtain, and it was necessary to perform duplicate analysis for the true profile differences to be differentiated from experimental variation.

Figures 1-5 show band of fragment DNA with different thickness. The thicker fragment represents higher DNA concentration as compared to the thinner bands. Variation of DNA profile is present which can be seen by the difference of fragment number and difference of fragment size. The relatedness of isolates was recovered from different patients, and multi varieting isolates were recovered from the patients from similar location.

The dendrogram of *M. tuberculosis* Isoniazid resistant isolates showed a high degree of polymorphism with RAPD analysis (**Figures 1(b)-5(b)**). The dendrogram analysis *M. tuberculosis* Isoniazid resistant isolates with primer OPN 02, OPN 09, OPN 20, BG 65, and N 9, that have genetic diversity. The highest is primer N 9; and the

lowest is primer OPN 20.

I found specific DNA fragment at Isoniazid resistant isolates using primer N 9 is 1450 bp as a marker. This study gives information about RAPD marker of *M. tuberculosis* strain to Isoniazid resistant.

5. ETHICAL CLEARANCE AND INFORM CONSENT

This study has been ethical clearance before being carried out, with number 1464/H4.8.4.5.31/PP36-KOMETIK/2012 under the approval of the head of Ethical clearance Hasanuddin University, Makassar, Indonesia. Notifications were concerned not be done because those samples used are isolate and taken from Provincial Health Laboratory of South Sulawesi, Indonesia and they have got the permission from the head of Provincial Health Laboratory of South Sulawesi, Indonesia with number PL00.03.3.2.749.

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