

The Susceptibility of *katG* Ser315Thr *Mycobacterium tuberculosis* mutant Against Anti Tuberculosis Drugs

Ning Rintiswati^{1*}, Praseno¹

¹Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Mycobacterium tuberculosis resistant strains is a matter of great concern for TB control Program since no cure for some multi drug resistance TB (MDR-TB) cases. These strains could spread around the world, make a great challenges for control measures. *Mycobacterium tuberculosis* strains resistance to anti tuberculosis drugs is mainly caused by the alteration in several genes encoding the molecular targets. Mutation of *katG* at codon 315 especially Ser315Thr are responsible for INH resistance in a large proportion of TB cases. The aim of this study is to know the frequency of of *katG* Ser315Thr of *M. tuberculosis* mutant and the pattern of resistance to Anti TB drugs. The study design was observational laboratoric. Eighty five *M. tuberculosis* INH resistant clinical isolates were screened for mutation of *katG*Ser315Thr by using PCR-RFLP and DNA sequence analysis. The results showed that *katG* Ser 315Thr were identified in 23 (27,05%) of INH resistance isolates. Frequency of MDR among the *katG* Ser315Thr mutants almost double than the non mutant. The result suggested that the the *katG* Ser315Thr mutation may play important role in the development of MDR-TB.

Keywords: *katG* Ser315Thr *Mycobacterium tuberculosis* mutant, frequency, anti TB drugs, Multi Drug Resistant, pattern of resistance.

Introduction

Despite the declaration of tuberculosis (TB) as a “global emergency” by the World Health Organization almost 20 years ago, TB still remain as a global problem in the world (Zhang *et al.*,2005). The latest estimates included in this report are that there were 9.0 million new TB cases in 2013 and 1.5 million TB deaths (WHO, 2014). The increased of HIV cases and low socioeconomic status greatly influenced the development of TB (Rattan *et al.*, 1999; Dye, 2006). Another obstacle in overcoming TB problem is the emergence of *M.tuberculosis* mutant that is resistant to anti TB drugs. The emergence of resistant strain is caused by lengthy therapy of at least six months makes patient uncompliance, so have inadequate treatment, then induce

changes in bacterial properties which in turn become resistant to anti TB drugs. The WHO reported the frequency of drug-resistant strains *M.tuberculosis* ranging from 3 to 41% among 72 countries. The current problems are the emerge of multi drug resistant TB (MDR-TB), which is resistant to isoniazid (INH) and rifampicin, and extreme drug resistant TB (XDR-TB). The XDR-TB strain is MDR with additional resistant to one of fluoroquinolones, and one or more of injectable drugs, such as kanamycin, amikacin, and capreomycin. MDR-TB and XDR-TB caused several fatal outbreaks worldwide (WHO, 2009). INH is one of effective anti TB drugs with minimal inhibitory concentration (MIC) of 0.2-0.06 mg/L, nevertheless the rate of resistance to this drug is increasing (WHO & IUATLD, 2004). INH pro-drug which is highly dependent on KatG to become reactive radical that attack target in *M.tuberculosis* cell.

Until date, INH along with rifampicin are backbones of TB treatment. INH had been used as first line anti TB medicine since

* Corresponding author:

Ning Rintiswati
Department of Microbiology, Faculty of Medicine,
Universitas Gadjah Mada, Yogyakarta, Indonesia
Email : rintiswati@gmail.com

1952 to combat TB. There was a postulate stated that INH was an active *pro drug* after transforming by catalase peroxidase (KatG) became isonicotinate acid, and this change produced radicals that could interact with a main target within a mycobacterial cell (Musser, 1995; Heym *et al.*, 1996; Bardou *et al.*, 1998).

M.tuberculosis primary resistance towards INH emerged in the laboratory with estimated frequency of 10^{-5} - 10^{-7} that was higher than rifampicin. It was known that *M.tuberculosis* resistance towards INH was mostly related with mutation that involved 1 or 2 genes namely *katG*, *inhA* and *ahpC* (Heym *et al.*, 1997; Talenti *et al.*, 1997; Morlock *et al.*, 2003).

katG encodes catalase peroxide enzyme (KatG). This enzyme had a multiple function as a catalase and peroxidase with a wide specification. Along with a catalyst reaction and enzyme peroxide, it functioned to free peroxide hydrogen from a biological system to protect bacterial cells from damages by reactive peroxides that produced by macrophage. Peroxidase has important roles in INH activation.

It was reported that 20-80% of INH resistant *M.tuberculosis* associated with *katG* mutation, and the frequency was related to geographical region of clinical isolate origin. Among *katG* mutation, Ser315Thr mutation contributes to 50-93% of INH resistant *M. tuberculosis* clinical isolates (Zhang *et al.*, 2005).

Mutation of *katG* is the main resistance mechanism of INH, and mutation on codon 315 (Ser315Thr) is the most frequently documented. This mutation responsible to the increasing of MIC as of 200 times compared to that of H37Rv *M.tuberculosis*. This mutation is strongly associated with MDR mutant and transmitted easily among *M. tuberculosis* strain. Mutation may result in decrease activity of catalase-peroxidase and deletion of this gene may cause loss of the enzyme activity (Mokrousov, 2002; Xue-qiong *et al.*, 2006; Hu *et al.*, 2010).

It is important to analyze the pattern of resistance of *katG* Ser315Thr *M.tuberculosis* mutant to first line anti TB drugs. This study aimed to describe the pattern of anti TB drugs resistant among *katG* Ser315Thr *M.tuberculosis* mutant.

Material and Methods

M. tuberculosis isolates.

M. tuberculosis clinical isolates are archive strains of Microbiology Laboratory, Faculty of Medicine, Universitas Gadjah Mada. *M.tuberculosis* were cultured, identified, and continued by *katG* mutation detection. *katG* Ser315Thr mutant of *M.tuberculosis* were tested for its sensitivity towards anti TB drugs.

M. tuberculosis identification.

M.tuberculosis were cultured on Lowenstein Jensen (LJ) medium, and incubated at 37°C for 3-4 weeks, with weekly observation. *M. tuberculosis* was identified based on the colony morphology, Ziehl Nielsen staining, Niacin test and PNB test. *M.tuberculosis* on LJ medium developed a creamy colored colony with dry and rough consistence, grew slowly, did not grow on PNB supplemented LJ Medium, and positively reacted to Niacin. (Vestal, 1981)

Antimicrobial susceptibility test.

Antimicrobial susceptibility test was performed by using modified indirect proportional on LJ medium. Each sample was cultured on a series of LJ medium containing first line anti tuberculosis drugs; Streptomycin (4mg/L), INH (0.2 mg/L and 1.0 mg/L), Ethambutol (2 mg/L), and Rifampicin (40 mg/L). Specimen was also cultured on LJ medium without drug and served as control. Inoculated media were incubated at 37 C for 3 weeks. Confluent growth on control was considered as 100 % growth. Sensitive or resistant determination was based on comparison between growth on medium with and without drug. An strain was considered resistant if the colonies

reach 1% of colony count on control medium (Vestal, 1981).

DNA isolation

M. tuberculosis DNA isolation was carried out with boiling method according to Herrera *et al.* (2004). *M. tuberculosis* were scrapped from LJ and placed in tube containing 500 μ L of sterile aquadest and heated at 95 C for 5 minutes. Samples were then spinned at 12.000 X g for 5 minutes, supernatants were saved and used as DNA template.

Mutation analysis of *katG* Ser315Thr (AGC > ACC)

Mutation detection was performed by using PCR-RFLP method. One set of primers *katG* IF (5'-AGCTCGTATGGCACCGGAAC) and *katG* 4R (5'-AACGGGTCCGGGATGGTG-3') were used for *katG* fragment amplification (position 904 to 1103 in H37Rv) (Mokrousov, 2002). Amplified fragments were digested with *MspI*. Briefly, 15 μ L PCR product was added with 10 μ L of buffer and 2.7 μ L of the enzyme. The mixtures were incubated in waterbath at 37°C for 8-16 hours. Digested fragments and marker V (Roche) were subjected to 4 %

agarose gel electrophoresis and results were visualized with UV light. Mutated codon 315 will be digested by *MspI*, resulting in 7 fragments ; 14, 6, 21, 6, 11, 10, and 132 bps. Digestion of non mutant *katG* Ser315Thr resulted in 6 fragments; 14, 6, 6, 11, 10, and 153 bps (adapted from Mokrousov *et al.*, 2002). The PCR-RFLP analysis was confirmed with DNA sequencing analysis.

Results and Discussion

Ser315Thr mutant of *M. tuberculosis* isolates

In this study 85 INH-resistant *M. tuberculosis* isolates were obtained. Ten INH-sensitive isolates, which were culture collection from Laboratory of Microbiology, Faculty of Medicine Gadjah Mada university, were also included in the study. PCR-RFLP procedures were performed for 85 *M. tuberculosis* clinical isolates. The representative electrophoresis of PCR-RFLP procedure was showed in Figure 1.

DNA sequence analysis was performed to confirm the PCR-RFLP results for randomly selected eight *M. tuberculosis* isolates (7 mutants and 1 standard strain of H37Rv) (Fig 2).

Overall mutation on *katG* Ser315Thr of *M. tuberculosis* among INH resistant

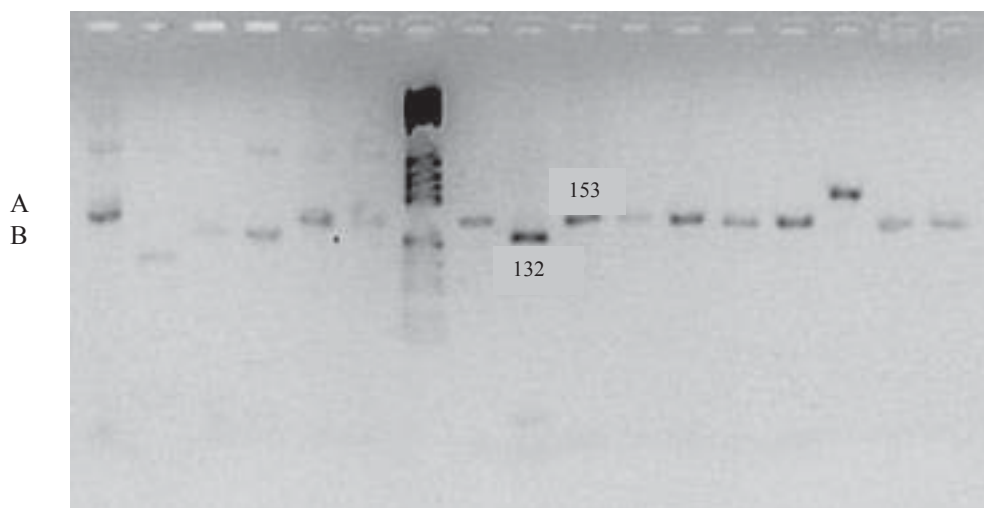
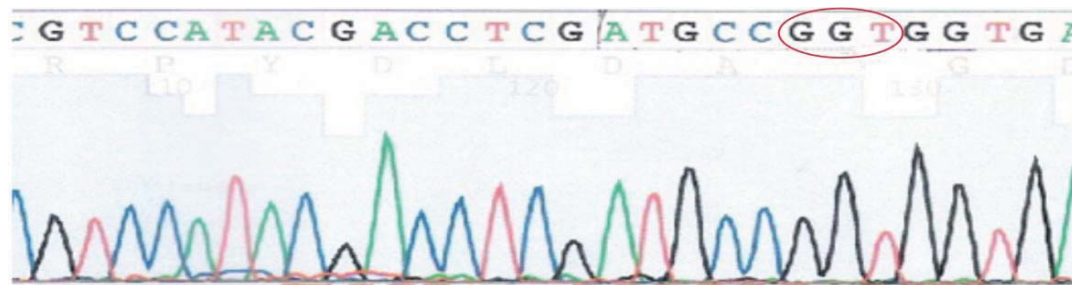


Figure 1. PCR-RFLP analysis result. PCR amplification of *katG* gene segment produced 199 bp bands. PCR products were digested by using *MspI* restriction enzyme. After digestion, mutant *katG* Ser315 Thr produced 132bp fragment while non mutant produced 153bp fragment.

A



B

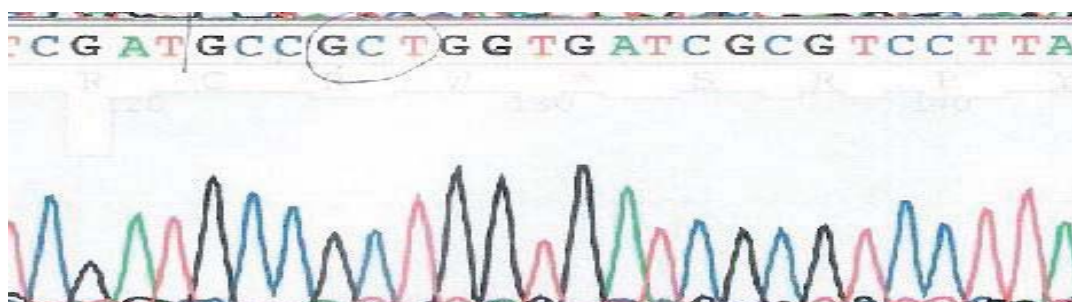


Figure 2. DNA Sequencing result of codon 315 *katG* *M.tuberculosis* (reverse primer) A : DNA fragment of representative mutant *M. tuberculosis* with ACC at codon 315, B : DNA fragment of H37Rv strain at the same position showed AGC

Table 1. The Frequency of *katG* Ser315Thr *M.tuberculosis* mutant among the samples and the resistency to INH

<i>M. tuberculosis</i>	Resistant to INH	Sensitive to INH	No	Sensitivity, Specificity PPV, NPV (%)
Mutant	23 (27,06%)	0 (0%)	23	Sensitivity : 27,05 Specificity : 100,0
Non mutant	62 (72,94%)	10 (100%)	72	PPV : 100,0
Total	85 (100,00%)	10 (100%)	95	NPV : 13,88

PPV= Positive Predictive Value, NPV =Negative Predictive Value

strain were relatively low (27,05%). It was reported that mutation on *katG* 315 codon in *M. tuberculosis* clinical isolates was most frequently found (50%-90%), the remaining mutations occurred in other loci (Musser, 1995; van Solingen *et al.*, 2000; Mokrousov *et al.*, 2004) It was suggested that mutation in this codon is beneficial for the bacteria, since it can reduce INH activity without removing catalase peroxidase activity that serve as virulence factor.

The results indicated that mutation *katG*315T may responsible for the phenotypic

of INH resistance in index strains. The mutation only occur in INH resistant isolates, but not in INH sensitive isolates.

Susceptibility of *katG*Ser315Thr mutants to anti TB drugs

Table 2 showed that the highest proportion of resistance in *katG*Ser315Thr mutant was to three drugs (INH, rifampicin, and streptomycin). In non mutant isolates the highest frequency found was single drug resistance against INH. Among 23 mutant isolates, 14 (60.1 %) isolates were resistant

Table 2. Susceptibility of *katG* Ser315Thr *M.tuberculosis* mutant and non mutant against anti tuberculosis drugs.

<i>M. tuberculosis</i>	n	Resistant to Anti Tuberculosis Drugs					
		I	I + R	I + E	I + R + S	I + S + E	I + R + S + E
<i>katG</i> Ser315Thr Non mutant	62	30	13	13	5	0	1
<i>katG</i> Ser315Thr Mutant	23	4	4	4	10	1	0

Note: I; INH R: rifampicin; S: streptomycin; E: ethambutol

Table. 3 Susceptibility of *katG* Ser315Thr mutant and *katG* Ser315Thr non mutant against INH

<i>M. tuberculosis</i>	n	Resistant to more than one drug (%)	Resistant to INH (%)
<i>katG</i> Ser315Thr non mutant	62	32 (51.61)	30 (48.39)
<i>katG</i> Ser315Thr mutant	23	19 (82.61)	4 (17.40)

to at least INH and rifampin, which was categorized as MDR isolates. Of 62 non mutant isolates, 19 (36.5 %) were MDR (data not shown).

Table 2 and 3 showed the difference of single drug resistant frequency among *katG* Ser315Thr mutant and non mutant. *katG* Ser315Thr mutant tend to resistant against more than 1 anti tuberculosis drugs. It was noted that in the mutant group the MDR-TB (at least resistant against INH and Rifampicin) frequency is higher (60.85%) compare to 31.6% in the non mutant group.

The *katG* Ser315Thr mutant *M. tuberculosis* predominantly resistant against 3 first line drugs, while *katG* Ser315Thr non mutant *M. tuberculosis* predominantly resistant against single drugs (INH). This observation was in accordance to the previous report, 60.1 % of *katG* Ser315Thr mutant were MDR-TB, whereas only 36.5% MDR-TB were reported in non mutant (Hasbon *et al.*, 2006).

There are two hypothetical theories concerning the association between mutation on *katG* and INH resistant mechanism. Mutation on *katG*315 plays important role in the emergence of secondary resistance after development of primary resistance. Mutation on *katG* occurs after the isolates become MDR. The other hypothesis stated that mutation on *katG*315 causes isolates become more resistant, more easily transmissible, and develop to MDR (Zhang *et al.*, 2005).

Hazbon suggested that resistance to INH and evolution of MDR strains is very complex and dynamic, involving various genes interaction and anti TB drug resistance phenotype (Hazbon *et al.*,2006; Bulatovic *et al.*, 2002; Van Solingen *et al.*, 2000). More extensive studies are needed to be able to explain mechanism of resistance to anti TB drugs. It is also important to develop new molecular-based diagnostic method (Zhang *et al.*, 2005).

Resistance to INH has been associated with deletion or point mutation on *katG* gene in which serine is replaced with threonine at position 315 (Ser315Thr). Substitution of ser-315 is one of the most frequent mode in clinical isolates with resistance to INH (Van Solingen *et al.*, 2000; Luo *et al.*, 2010; Jagielski *et al.*, 2013). Minimal inhibitory concentration of INH resistant isolates increase 20-200 times. With position of cytochrome oxidase super family peroxidase-catalase, it is suggested that Ser315 is close to active site of KatG so that Ser315Thr mutation influence enzyme activity of KatG (Heym *et al.*, 1998; Bulatovic *et al.*, 2002; Van Solingen *et al.*, 2000.; Luo *et al.*, 2013).

Our results showed that *katG*Ser315Thr mutation was frequently (27.06%) found in the INH resistant *M. tuberculosis* that may responsible for the multi resistant against anti tuberculosis drugs.

References

- Bardou, F., Raynaud, C., Ramos, C., Laneelle, M.A., Laneelle, G., 1998. Mechanism of isoniazid uptake in *M.tuberculosis*. *Microbiology* .144: 2539-2544.
- Bulatovic, V.M., Wangenack, N.L., Uhl, J.R., Halal, L., Roberts, G.D., Cockerill III, F.R., Rusnack, F., 2002. Oxidative stress increases susceptibility of *M.tuberculosis* to Isoniazid. *Antimicrob. Agent Chemother*, 46(9): 2765-2771.
- Dye, C., 2006. Global epidemiology of tuberculosis. *Lancet* 367: 938-40.
- Hazbon, M.H., Brimacombe, M., del Valle, M.B., Cavatore, M., Guerrero, M.I., Varma-Basil, M., *et al.*, 2006. Population Genetic Study of Isoniazid Resistance Mutation and Evolution of Multidrug-Resistant *Mycobacterium tuberculosis*. *Antimicrob. Agent Chemother*. 50 (8):2640-2649.
- Herrera, L., Valverde, A., Saiz, P., Saez-Nieto, J.A., Portero, J.L., Jimenez, M.S., 2004. Molecular characterization of isoniazid-resistant *M.tuberculosis* clinical strains isolated in Philippines. *Intern J.of Antimicrob. Agents*. 23: 572-576.
- Heym, B., Philipp, W., Cole, S.T., 1996. Mechanism of drug resistance in *Mycobacterium tuberculosis*. Dalam Shinnick, T.M (ed.), *Tuberculosis, Curr in Microb and Immunol*. Springer:49-70.
- Hu, Y., Hoffner, S., Jiang, W., Wang, W., Xu, B., 2010. Extensive transmission of isoniazid resistant *M.tuberculosis* and its association with increased multidrug-resistant TB in two rural counties of eastern China : A molecular epidemiological study. *BMC infect. dis*. 10(43).1471-2334.
- Jagielski T, Grzeszczuk M, Kamiński M, Roeske K, Napiórkowska A, Stachowiak R, Augustynowicz-Kopeć E, Zwolska Z, Bielecki J. 2013. Identification and analysis of mutations in the *katG* gene in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. *Pneumonol Alergol Pol*. 2;81(4):298-307.
- Luo, T., Zhao, M., Li, X., Xu, A.P., Gui, X., Pickerill, S., DeRiemer, K., Mei J., and Gao, Q., 2010. Selection of Mutations To Detect Multidrug-Resistant *Mycobacterium tuberculosis* Strains in Shanghai, China *Antimicrob Agent Chemother*, 54 (3), . 2010:. 1075-1081
- Mokrousov, I., Otten, T., Filipenko, M., Vyazovaya, A., Chrapov, E., Limeschenko, E., Steklova, L., Vyshnevskiy, B., Narvskaya, O., 2002. Detection of isoniazid -resistant *M.tuberculosis* strain by a multiplex allele-specific PCR assay targeting *katG* codon 315 variation. *J. Clin. Microbiol*, 46(5): 2509-2512.
- Morlock, G.P., Metchock, B., Sikes D, Craford JT, Cooksey RC., 2003. *ethA*, *inhA* and *katG* loci of ethionamide resistant clinical *M.tuberculosis* isolates. *Antimicrob. Agent Chemother*. 47(12): 3799-3805.
- Musser, J.M., 1995. Antimicrobial agent resistance in Mycobacteria: molecular genetic insight. *Clin. Microb. Rev.*, 8 (4) : 496-514.
- Rattan, A., Kalia, A., Ahmad, N., 1999. Multidrug resistant *Mycobacterium tuberculosis* molecular respectives. *Ind.J. Tub*. 46:51-63.
- Talenti, A., Honore, N., Bernasconi, B., , J, Ortega, A., Heyan, B., Takiff, H.E., and Cole, S.T., 1997. Genotypic assessment of isoniazid and rifampin resistance in *M.tuberculosis*: A blind study at reference lab level. *J. Clin. Microbiol*, 35(3):.719-723
- van Solingen, D., de Haas, P., van Doorn, H.R., Kuijper, E., Rinder, H., Borgdorff, M.W., 2000. Mutation at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance in The Netherlands. *J. Infect Dis*. 182: 1788-90.
- Vestal AL. ,1981. *Procedures for Isolation and Identification of Mycobacterium*. Center for Disease Control. US. Dep. of health and human services, Atlanta, Georgia.

- World Health Organization , 2014 *Global Tuberculosis Report 2014*. WHO, 10-13
- World Health Organization & IUATLD. 2004. *Anti Tuberculosis in the World*. Report no.3.The WHO/IUATLD Global Project on Anti-TB drug Resistance Surveillance 1999-2002 WHO :11-12.
- Xue-qiong, W., Yang, L., Jun-xian, Z., Jian-qin, L., Hong- Min, L., Guang- Yu, Z., Cui- Huan, L., Bei-Chuan, D., 2006. Detection of mutant *katG* 315 & *Inh A*-15 of *Mycobacterium tuberculosis* strain isolates from Chinese patients. *Clin. Med. J.* 119(3): 230-233.
- Zhang, Y., Vilcheze, C., Jacobs, W.R., 2005. Mechanisms of Drug Resistance in *Mycobacterium tuberculosis*. Dalam Cole, S.T. *Tuberculosis and tubercle bacillus*. ASM Press, Washington DC. 115-132.