The influence of mutation gene rpoB of *Mycobacterium tuberculosis* cluster I (507-534) on the elimination 25-desacetyl rifampicin in urine of tuberculosis subjects

Lily[1*], B Y M Sinaga[2], D Ardinata[3] and Y Siregar[4]

[1]Master of Biomedical Sciences, Faculty of Medicine, Universitas Sumatera Utara, Medan 20155, Indonesia
[2]Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Sumatera Utara, RSUP. Haji Adam Malik, Medan 20136, Indonesia
[3]Department of Physiology, Faculty of Medicine, Universitas Sumatera Utara, Medan 20155, Indonesia
[4]Department of Biomedical Sciences, Faculty of Medicine, Universitas Sumatera Utara, Medan 20155, Indonesia
[*]Corresponding author: lucky_chocho@yahoo.com

Abstract. The study aimed to evaluate the influence of mutation gene rpoB of Mycobacterium (M.) tuberculosis cluster I (507-534) on the elimination of 25-Desacetyl Rifampicin (25-DR) in the urine of tuberculosis (TB) subjects. Early morning sputum took from patient TB before treatment. Urine collected after 2 hours taken Fixed-Dose Combination (FDC) at days 7th treatment. All sputum were sequencing at Macrogen Korea Laboratory. Urine was analyzed by high-Performance Liquid Chromatography (HPLC) using the method of Lily et al. Mean (standard deviation) for mutation and non-mutation of rpoB M. tuberculosis group were 7.6147 (4.4478) and 4.5772 (1.7532) µg/ml, respectively. Shapiro-Wilk test showed normally distributed data, with significance 0.3. Independent t-test performed p-value 0.167 and confidence interval (CI) from -1.648 to 7.723. The mutation gene rpoB of M. tuberculosis cluster, I (507-534) in this study, did not affect elimination 25-DR in theurine of TB subjects statistically and clinically.

1. Introduction

Drug-resistant TB continues to threaten global TB control and remains a major public health concern in many countries.[1] Rifampicin (R) resistance (r) is a reliable marker of Multi-Drug Resistance (MDR)-TB since by far the majority of Rr strains are also isoniazid (INH) resistance. Rr in M. tuberculosis associated with amino acid changes in the subunit of RNA polymerase, encoded by rpoB.The majority (90 to 95%) of mutations responsible for resistance are located in the 81-base pair [2] of cluster I—the so-called Rr determining theregion (RRDR) of rpoB [3], which includes codons 507to 534, encoding 27 amino acids. DNA sequencing of this region could use as a clinical marker for probe assay of mutation and treatment.[4]

TBtreatment requires multiple drugs [5], the advance in pharmacology and pharmaceutics have contributed to creating a tablet comprising a combination of several kinds of anti-TB medicines without disturbing the bioavailability of those drugs in one fixed dose, known as FDC. One of the FDC types available in the market is the combination of R, INH, pyrazinamide (PYR), and ethambutol

(ETM), commonly called as a4-FDC tablet. The combination of a 4-FDC has potent bactericide and has a low toxicity; therefore, it can use as the first choice for the treatment of TB.[6]

The bioavailability of R is important in FDC.[7] R has been shown to undergo a process of deacetylation in animals and man; thediacetyl derivative showed an increased polarity as compared to the parent compound. The diacetyl derivatives are present in bile, serum,and urine after several hours of antibiotics administration.[8] R is excreted about 20% of the administered dose as R plus DR over a 24 h period.[9] WHO recommended the use of HPLC for analysis of TB 4-FDC tablet using two separate systems in which R is set apart due to its stability consideration.[6] The present study was undertaken to assess the influence of mutation gene rpoB of M. tuberculosis cluster (507-534) on the elimination 25-DR in the urine of pulmonary tuberculosis subjects who had FDC.

2. Material and Methods

2.1. Study design

Type of study was aquasi-experimental method.The protocol study was approved by the Health Research Ethical Committee of North Sumatera c/o Medical School, Universitas Sumatera Utara, Jl. Dr. Mansyur No. 5, Medan-20155, Indonesia.

2.2. Study Subjects

Subjects were taken using purposive sampling technique. The criteria inclusions were pulmonary TB male or female patients diagnosed by a pulmonary physician at Polyclinic Directly Observed Treatment Short Course (DOTS) RSUP. Haji Adam Malik Medan, age >18 years, no antituberculosis treatment drug for at least 2 weeks. Criteria exclusion were pregnancy, HIV infection, stage renal or hepatic disease.Those subjects were willing to participate and gave written informed consent.

2.3. Processing Collection Specimen

Subjects were treated by a pulmonary physician, with therapeutic doses of the intensive phase FDC tablets, according to the DOTS strategy. Subjects were administered FDC orally with a glass of water (± 150 ml) after 8 hours an overnight fasting. No food or beverages permitted for two hours following the administration.

2.4. Sample collection

The eligible patients were asked to provide early morning expectorated sputum before treatment. Post urine samples were collected 2 hours after taking FDC tablets, at day 7^{th} treatment.

2.5. Bioanalysis

2.5.1. Sample Sputum

2.5.1.1. Decontamination Process. Samples sputum decontaminated with the procedure of.[10]

2.5.1.2. DNA M. tuberculosis Isolation Process. Genomic DNA extracted from the sputum samples by using TIANamp Genomic DNA Kit (TIANGEN, Hilden, Germany) procedure. The kit purchased from PT Sciencewerke, Jakarta-Indonesia.DNA was purified and collected for Polymerase chain reaction (PCR) detection.

2.5.1.3. Amplification DNA Process. The partial sequence 126-bp of the rpoB gene, including the RRDR (an 81-bp region of the rpoB gene that encodes residues 507 to 533), was amplified by PCR Applied Biosystem from all samples sputum with forward (5'-CGCCGCGATCAAGGAGTTCT-3') and reverse (5'- TCACGTGACAGACCGCCGGG-3') primers purchased from PT Sciencewerke, Jakarta-Indonesia. PCR reactions performed according to [11] described.

2.5.1.4. DNA Sequencing. The PCR product sequenced at Macrogen Korea Laboratory through PT. Biosains Medika Indonesia.

2.5.2. Sample Urine Post
The concentration 25-DR in urinary excretion post-treatment of subjects measured by HPLC according to the procedure method of.[12]

2.6. Data analysis
The Statistical Product and Service Solutions (SPSS) 15.0 for Windows Evaluation Version Software, used for data analysis. The datafirst tested for normality by using Shapiro-Wilk Test. Normally distributed data will be tested by Independent-Test, to identify differences in post urine metabolite levels of 25-DR from mutation and nonmutation of rpoB gene M. tuberculosis cluster I (507-534). Data found not normally distributed will be analyzed by the non-parametric test of Mann-Whitney.[13]

3. Results and Discussion

3.1. Results
3.1.1. Data Distribution. The quasi-experimental study is an analytic method. The parameter of ananalytic method to know data normally distributed with atotal of 12 subjects used Shapiro-Wilk test. The categorical data group of the mutation and nonmutationM. tuberculosis obtained from sputum specimen sequencing. Numerical data urine metabolite levels of 25-DR resulted from HPLC measurements of urine specimens. The normality Shapiro-Wilktest using SPSS 15.0, performed on data urine metabolite of 25-DR to agroupof mutation and nonmutation rpoB M. tuberculosis. The test yields probability (p)-values for the mutation and nonmutation group are 0.330 and 0.392, respectively (table1). p-value from this study >0.05 meansdata distributednormally.[13]

Table 1. Tests of normality Shapiro-wilk.

	M. tuberculosis	Shapiro-Wilk	
		N	Significance
Metabolite urine 25DR	Mutation	6	0,330
	Nonmutation	6	0,392

3.1.2. The presentation of numeric data distribution. The study data normally distributed, so that, the numerical data urine metabolite levels of 25-DR weredescribed in the concentration measure (mean), size of spread (standard deviation (SD)) and graph (error bar).[13] The mean values, as well as the standard deviation (SD) from the numerical data urine metabolite of 25-DR forgroups of the mutation and nonmutation M. tuberculosis, were 7.6147 and 4.5772, 4.4478 and 1.7532 µg/ml, respectively. The number (n) of samples per group of mutation and nonmutation M. tuberculosis, mean (SD) from data of urine metabolite 25-DR and error bar graph presented with amean (SD) showed in table 2 and figured 1.

Table 2. Independent t test unpairedresults.

	M. tuberculosis	Mean (SD)	p-value	Mean difference (CI 95%)
Metabolite urine 25-DR	Mutation (n=6)	7.6147 (4.4478)	0.167	3.0375 (-1.648-7.723)
	Nonmutation (n=6)	4.5772 (1.7532)		

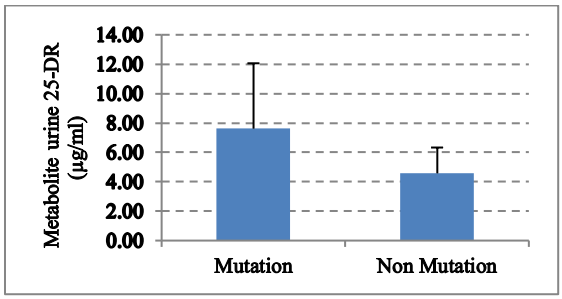

Figure 1. Graph error bar between mean (SD) metabolite urine 25-DR.

3.1.3. Hypothesis Test. Parametric independent T-test is a hypothesis test of numerical comparative, 2 group, unpaired, 1 measurement, normal distribution, different variance, used in this study to determine the influence group of mutation and nonmutation rpoB M. tuberculosis on urine metabolite of 25-DR of TBsubjects.The Levene's test from independent T-test presented-value=0.025 less than 0.05, the meaning of different variance data, then using"equal variances not assumed."[13]

3.1.3.1. Statistic Interpretation. Independent T-test table 1.2 showed data p-value is 0.167 more than α=0.05 and CI from -1.648 to 7.723 has 0 value, meaning there is statistically no significant difference in the group of mutation and nonmutation gene rpoB M. tuberculosis to metaboliteurine 25-DR.[13]

3.1.3.2. Clinical Interpretation. Independent T-test table 1.2 presented the mean difference value was 3.0375 less than 10 that clinically showed no significant difference between agroup of the mutation and nonmutation gene rpoB M. tuberculosisto metabolite urine 25-DR.[13]

3.2. Discussion

The ability of M. tuberculosis to harbor multiple drug resistance mutations while retaining the ability to infect, persist, and cause disease in human host raises fundamental questions.[3]In this study, sequencing results from Macrogen Korea Laboratory revealed mutation M.tuberculosis in one group (n=6) and nonmutation in another group (n=6). The impact of drug resistance on the host-pathogen interaction remains largely unexplored.The interaction between M. tuberculosis and human host is complex and dynamic.[3]

M. tuberculosis can potentially infect tissues and organs throughout the body.The pharmacokinetic (PK) of a whole-body framework may be of use in quantifying drug-bacteria interactions in organs such as the lungs and spleen as well as providing drug concentrations in locations relevant to drug-drug interactions and toxicity, such as the liver and kidneys. Determination of the clearance would be better using experimental measurements of the time of excretion from bile and elimination R in urine.[5]The recent study, metabolite urine 25-DR was measured by HPLC.The data obtained, processed and fed into each group of mutation and nonmutation M. tuberculosis. The mean (SD) metabolite urine 25-DR of the mutation group was obtained 7.6147 (4.4478) and nonmutation 4.5772 (1.7532) µg/ml.

The information about relationships PK-pharmacodynamic (PD) of drugs against M. tuberculosis still limited.[14] A particular dose does not lead to a specific concentration-time profile in all patients, but rather a distribution determined in part by alleles of genes encoding enzymes involved in xenobiotic metabolism.[15]R interfereswith transcription and elongation of RNA by binding to the DNA-dependent RNApolymerase M. tuberculosis.[16] The pathogen response to a particular drug concentration profile is itself related to several PK/PDfactors. Drug resistance in M. tuberculosis was shown to be related to PK/PD factors, and factors such as PK variability. The appearance of drug resistance depends on the exposure of the drug and in many situations the actual form of the concentration-time curve, which is often different from the PK/PD parameters associated with microbial killing. Poor PK/PD exposures due to non-optimize dose regimens, initiate a chain of

evolution, starting with induction of multi-drug efflux pumps, then the development of chromosomal mutations, which together lead to high-level MDR-TB and Extensively Drug Resistance (XDR)-TB.[15] Unexpectedly, the key finding of this study, field conditions real-life and not in the controlled environment of a dedicated PK unit, is that measured levels of metabolite urine 25-DR for agroup of mutation and nonmutation M. tuberculosis were highly variable with p = 0.167 and meant difference (CI 95%) 3.0375 (-1.648-7.723).

4. Conclusion

Based on the result of the study, it can conclude that there is no significant difference (p> 0.05) between agroup of mutation and nonmutation gene rpoB M. tuberculosis to urine metabolite 25-DR.

References

[1] World Health Organization 2015 *Global Tuberculosis Report* (France: WHO Press)
[2] Chen X Y, Wang B, Yang W, Kong F R, Li C Y, Sun Z G, Jelfs P and Gilbert G L 2014 Rolling circle amplification for direct detection of rpob gene mutations in Mycobacterium tuberculosis isolates from clinical specimens *J. Clin. Microbiol.* **52** 1540–8
[3] Koch A, Mizrahi V and Warner D F 2014 The impact of drug resistance on Mycobacterium tuberculosis physiology: what can we learn from rifampicin? *Emerg. Microbes Infect.* **3** 1–11
[4] Minh N N, Bac N V, Son N T, Lien V T K, Ha C H, Cuong N H, Mai C T N and Le T H 2011 Molecular characteristics of rifampin- and isoniazid-resistant Mycobacterium tuberculosis strains isolated in vietnam *J. Clin. Microbiol.* **50** 598–601
[5] Lyons M A, Reisfeld B, Yang R S H and Lenaerts A J 2013 A physiologically based pharmacokinetic model of rifampin in mice *Antimicrob. Agents Chemother.* **57** 1763–71
[6] Khoiria S, Martonoa S and Rohmana A 2015 Optimisation and validation of HPLC method for simultaneous quantification of rifampicin, isoniazid, pyrazinamide, and ethambutol hydrochloride in anti-tuberculosis 4-FDC tablet *Jurnal Teknologi (Sciences & Engineering)* **77** 171–6
[7] Zhu H, Guo S C, Hao L H, Liu C C, Wang B, Fu L, Chen M T, Zhou L, Chi J Y, Yang W, Nie W J and Lu Y 2015 Relative bioavailability of rifampicin in four Chinese fixed-dose combinations compared with rifampicin in free combinations *Chin. Med. J.* **128** 433–7
[8] Acocella G, Bonollo L, Garimoldi M, Mainardi M, Tenconi L T and Nicolis F B 1972 Kinetics of rifampicin and isoniazid administered alone and in combination to normal subjects and patients with liver disease *Gut* **13** 47–53
[9] Immanuel C, Gurumurthy P, Ramachandran G, Venkatesan P, Chandrasekaran V and Prabhakar R 2003 Bioavailability of rifampicin following concomitant administration of ethambutol or isoniazid or pyrazinamide or a combination of the three drugs *Indian J. Med. Res.* **118** 109–14
[10] Sekiguchi J-i, Akiyama T M, Kopec´ E A, Zwolska Z, Kirikae F, Toyota E, Kobayashi I, Morita K, Kudo K, Kato S, Kuratsuji T, Mori T and Kirikae T 2007 Detection of multidrug resistance in Mycobacterium tuberculosis *J. Clin. Microbiol.* **45** 179–92
[11] Li J, Xin J, Zhang L, Jiang L, Cao H and Li L 2012 Rapid detection of *rpoB* mutations in rifampin resistant *M. tuberculosis* from sputum samples by denaturing gradient gel electrophoresis *Int. J. Med. Sci.* **9** 148–56
[12] Lily, Laila L and Prasetyo B E 2017 Optimization and validation of high-performance liquid chromatography method for analyzing 25-desacetyl rifampicin in human urine *Accepted in ICTROMI ID 2593*
[13] Dahlan S M 2007 *Statistik untuk kedokteran dan kesehatan: deskriptif, bivariat dan multivariat* (Jakarta: Epidemiologi Indonesia)
[14] Jayaram R, Gaonkar S, Kaur P, Suresh B L, Mahesh B N, Jayashree R, Nandi V, Bharat S, Shandil R K, Kantharaj E and Balasubramanian V 2003 Pharmacokinetics-

pharmacodynamics of rifampin in an aerosol infection model of tuberculosis *Antimicrob. Agents Chemother.* **47** 2118–24

[15] Pasipanodya J G and Gumbo T 2011 A new evolutionary and pharmacokinetic-pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs *Curr. Opin. Pharmacol.* **11** 457–63

[16] Mani C, Selvakumar N, Narayanan S and Narayanan P R 2001 Mutations in the rpoB gene of multidrug-resistant Mycobacterium tuberculosis clinical isolates from India **39** 2987–90