MOLECULAR ANALYSIS OF *rpoB*, *katG* AND *embB* GENE MUTATION IN *Mycobacterium tuberculosis* ISOLATES FROM TERENGGANU MALAYSIA

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DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PATHOLOGY (MEDICAL MICROBIOLOGY)

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ABBREVIATIONS

AFB	Acid-fast bacilli
AIDS	Acquired immune deficiency syndrome
Bp	Base pair
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
EQA	External quality assurance
EPTB	Extrapulmonary tuberculosis
HIV	Human immunodeficiency virus
HIS	Hospital Information System
HPA	Hybridization Protection Assay
HSNZ	Hospital Sultanah Nur Zahirah
HUSM	Hospital Universiti Sains Malaysia
ICA	Immunochromatographic assay
JKNT	Jabatan Kesihatan Negeri Terengganu
LIS	Laboratory Information System
LPA	Line probe assay
MDR	Multi drug resistant
MKA	Makmal Kesihatan Awan
MOTT	Mycobacterium other than tuberculosis
MTB	Mycobacterium tuberculosis
NAD	Nicotinamide adenine dinucleotide
PAS	Para-aminosalicylic

PCR	Polymerase chain reaction
RIF	Rifampicin
RNA	Ribonucleic acid
RRDR	Rifampicin-resistance determining region
RR-TB	Rifampicin-resistance tuberculosis
SNP	Single nucleotide polymorphism
Taq	*Thermus aquaticus*
TB	Tuberculosis
WHO	World Health Organization
XDR	Extended drug resistant
Xpert MTB/RIF	Cartridge-based fully automated molecular diagnostic assay that can identify MTB complex DNA and the mutations associated with rifampicin resistance
μm	Micrometer

ABSTRAK

Tajuk: Analisis Molekular bagi mutasi gen *rpoB*, *katG*, dan *embB* untuk isolat *Mycobacterium tuberculosis* di negeri Terengganu, Malaysia.

Pengenalan: Penyakit tuberculosis (TB) di Malaysia semakin meningkat dan hampir menyamai kadar negara yang mempunyai beban penyakit TB yang tinggi. Di samping kadar penyakit TB yang semakin meningkat, kadar kerintangan ubat anti-TB juga kelihatan meningkat. Kefahaman berkenaan ciri-ciri molekular kerintangan anti-TB masih kurang di negara ini.

Kaedah: Dalam usaha untuk memahami taburan kerintangan dan kekerapan jenis mutasi terhadap ubat anti-TB di Terengganu, kami menganalisa gen *rpoB*, *katG*, dan *embB* untuk ubat rifampicin, isoninazid dan ethambutol. Isolat *Mycobacterium tuberculosis* dikumpul daripada makmal mikrobiologi HSNZ selama enam bulan, keputusan kerintangan ubat anti-TB didapati daripada MKA Kota Bharu dan *Mycobacterium tuberculosis* yang mempunyai kerintangan terhadap ubat anti-TB dilakukan ujian PCR and penjujukan DNA. Analisis genotipik dilakukan untuk mengesan mutasi dalam urutan gen sasaran.

Keputusan: Sebanyak 92 *Mycobacterium tuberculosis* complex isolat telah dikumpulkan. 84 (91.3%) mempunyai keputusan ujian kerintangan ubat anti-TB. 13 (15.5%) daripadanya mempunyai sekurang-kurangnya satu kerintangan terhadap ubat anti-TB. Daripada 13 isolat yang tersebut, hanya satu sampel mempunyai kerintangan terhadap rifampicin, tiga sampel mempunyai kerintangan terhadap isoniazid dan satu sampel mempunyai kerintangan terhadap ethambutol. Isolat yang mempunyai kerintangan terhadap rifampicin menunjukkan mutasi pada gen *rpoB* kodon S513T. Satu daripada tiga isolat yang mempunyai kerintangan terhadap isoniazid menunjukkan mutasi pada kodon S315T gen *katG* dan dua yang lain tidak menunjukkan mutasi

pada gen *katG* yang diuji. Isolat yang mempunyai kerintangan terhadap ethambutol tidak menunjukkan sebarang mutasi pada gen embB yang diuji. Lain-lain isolat tidak menunjukkan mutasi pada gen-gen yang diuji.

Kesimpulan: Keadaan semasa menunjukkan kadar kerintangan anti-TB di Terengganu masih rendah. Karakteristik molecular pada kerintangan anti-TB menunjukkan persamaan dengan kawasan lain di dunia. Kajian yang lebih besar diperlukan untuk menunjukkan keadaan yang sebenar bagi negara ini.

Kata kunci: Tuberkulosis, *Mycobacterium tuberculosis*, kerintangan, mutasi

ABSTRACT

Title: Molecular Analysis of *rpoB, katG* and *embB* gene mutation in *Mycobacterium tuberculosis* isolates from Terengganu Malaysia.

Introduction: The incidence of TB in Malaysia is increasing, and our country is reaching the status of TB high burden countries. Besides the number of TB infection, drug resistance is an emerging too. Molecular characteristics of drug resistance are still limited in the country.

Methods: In an attempt to understand the distribution of drug-resistance TB, types and frequency of mutation in Terengganu, we analysed *rpoB*, *katG* and *embB* genes for rifampicin, isoniazid, ethambutol, respectively. *Mycobacterium tuberculosis* isolates collected from microbiology laboratory HSNZ for six months, drug susceptibility result traces from MKA Kota Bharu, and drug-resistant isolates and some drug-susceptible isolates proceed for PCR amplification and DNA sequencing. Genotypic analysis performed to detect the mutations in the sequence of the target genes.

Results: 92 *Mycobacterium tuberculosis* complex isolates collected. 84 (91.3%) drug susceptibility results were available. 13 (15.5%) isolates have drug resistance to at least one drug. Of 13 isolates, only one has rifampicin resistance, three isoniazid resistance and one ethambutol resistant. Isolate with rifampicin-resistant showed a mutation in *rpoB* S513T. 1 of 3 isoniazid-resistant has a mutation in S315T *katG*, while the other 2 has no mutation at *katG* gene. The ethambutol resistant isolate has no *embB* gene mutation. The other isolate has no mutation in tested genes.

Conclusion: Current drug resistance TB cases in Terengganu are low. Molecular characterization of drug resistance shows a common mutation, as reported in various regions of the world. A more extensive study is needed to show more representative data for the country.

Keywords: Tuberculosis, Mycobacterium tuberculosis, drug resistance, mutation

CHAPTER ONE

INTRODUCTION

1.1 Literature review

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (1). TB typically affects the lungs; however, it can cause a broad range of clinical illness and can affect virtually every organ. It can mimic other diseases such as pneumonia, fungal infection, or neoplasm. Almost all TB infections are due to the inhalation of droplet nuclei, and common presenting symptoms include prolonged cough in pulmonary tuberculosis, fever, night sweat, loss of weight, anorexia, fatigue, and lymphadenopathy. TB transmission occurs when a patient with pulmonary TB coughs, sneeze, talk, laugh, sing, or spit droplet containing bacteria in the air (2). It also can be transmitted via aerosol production due to lesion manipulation or specimen processing in the clinical laboratory. With the availability of effective anti-TB drug regime TB is curable and can be preventable with immunization and prevention programs (1).

Mycobacterium tuberculosis is an acid-fast bacillus, aerobic, with high cell wall content of high molecular weight lipids (2). *M. tuberculosis* is a slow-growing bacterium with the generation time is 15 to 20 hours. The growth is usually visible from 3 to 8 weeks on solid media. The organism appears as slightly bent, beaded rods 2 to 4 μm long and 0.2 to 5 μm wide. Broad-spectrum of laboratory techniques is available to diagnosed active TB, but no single test is perfect (2).

In 1993 the World Health Organization (WHO) declared TB as a global public health emergency, and it continues to be a global public health problem (3). WHO has set a strategy to end TB and developed a specific goal for 2030 with 90% reduction in the total number of TB death and 80% reduction of TB incidence in new cases per 100 000 population per year which compare with levels in 2015 (3). TB remains in the top 10 causes of worldwide death of single infectious agents even surpass HIV/AIDS, and millions of people suffered from TB each year (3).

In 2017, WHO estimated 10 million new cases of TB occur worldwide. About 6.4 million of the new cases were notified to national authorities and reported to WHO. Differences between the estimated and reported cases are due to underdiagnosis and underreporting. Underestimation or overestimation of the total number of new cases is also possible. Out of 10 million estimated, 5.8 million are men, 3.2 million are women, and 1.0 million are children. Estimated death from TB is about 1.3 million (range, 1.2–1.4 million) among HIV-negative people and an additional 300 000 (range, 266 000–335 000) deaths among HIV-positive people. There was a case in all age groups, but 90% were adults (aged ≥15 years). It involved 9% of people with HIV (72% in Africa). All countries reported having TB, but two-third were in eight countries. This two-third includes India (27%), China (9%), Indonesia (8%), Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). These and 22 other countries in WHO's list of 30 high TB burden countries accounted for 87% of the world's cases (3).

Malaysia classified as a country with an intermediate TB burden. The notification rate for TB was 93 cases per 100,000 population in 2017 (3). TB in Malaysia declines by 1.3% in 2018, recording a total of 25 837 cases compared to 26 168 cases in 2017. Selangor and Sabah recorded the highest claims in 2018 with 5071 and 5008, respectively. TB death rate in 2018 was 6.6 per 100 000 people, an increase from 6.5 per 100 000 people in 2017. Sabah recorded the highest number of deaths in 2018 cases, followed by Selangor with 376 and 375 cases, respectively (4). In unpublished data from Terengganu Health Department, the number of TB cases in Terengganu almost static in 2017 and 2018 with 771 and 777 cases, respectively. Meanwhile, TB deaths are a decline in Terengganu from 140 in 2017 to 95 cases in 2018.

The anti-tuberculosis drug was initially discovered and started in the 1940s with para-aminosalicylic (PAS) and streptomycin. Initially, streptomycin used as monotherapy; however, resistance then rapidly developed. After that, it combined with PAS, and the resistance significantly reduced. In the 1950s, isoniazid used in combination with both. Isoniazid opened the modern era of treatment of TB, which was inexpensive, well-tolerated and safe. In the 1960s, ethambutol shown to be effective and well-tolerated than PAS, which replaces it (7).

In the 1970s, rifampicin was found its place as a keystone in tuberculosis chemotherapy regimens (7). A combination of rifampicin, isoniazid and ethambutol enabled the treatment course to be shortened from 18 months to 9 months and led to improves cure rates. The observation in the 1980s that adding pyrazinamide to the regimens permitted cures in only six months (7). The short course includes four drugs for two months, then followed by two drugs for four months. Four drugs were isoniazid, rifampicin, pyrazinamide, and ethambutol, and two drugs include isoniazid and rifampicin. To improve compliance, intermittent regimes, twice or three times weekly, have been established to cure TB in about 62-78 meetings over 26 weeks (7). Though, these regimens are not good enough to facilitate effective treatment, especially in resource-poor countries. Therefore, drug-resistant strains have emerged to threatened TB control in various parts of the world. In the early 1990s', the emergence of multiple drug-resistant tuberculosis known to be resistant to isoniazid and rifampicin (7).

MDR-TB defined as the resistance to at least two main first-line TB drugs rifampicin and isoniazid (5). Drug resistance is generally an unavoidable consequence of the inadequate, incomplete, or prolonged usage of antibiotics (6). Early detection and correct treatment regime of MDR-TB could overcome the spread of multidrug-resistant pathogens in the community, and these measures are the top priorities in TB control.

Drug-resistant TB remains to be a multinational public health problem. Globally, a small increment of multidrug-resistant (MDR)/rifampicin resistant (RR) TB was detected and notified in 2017 compared to 2016 with 160 684 and 153 116, respectively (3). Of these, 139 114 people in 2017 and 129 689 in 2016 received second-line treatment regimen, and this was only 25% of the estimated 558 000 people who developed MDR/RR-TB in 2017. Globally, 3.5% of new TB cases and 18% of previously treated cases had MDR/RR-TB (3). Extensively drug-resistant TB (XDR-TB) were an estimated 8.5% of MDR-TB in 2017 (3). Urgent action is essential to improve the quality of diagnosis, coverage of treatment, and care for people with drug-resistant TB.

In Malaysia, about 101 cases of MDR-TB reported in 2015. Estimated MDR/RR-TB cases among notified TB pulmonary cases in Malaysia for new cases was 1.5%, and for previously treated cases was 3.1% for the year 2015 (3). WHO estimates MDR/RR-TB for Malaysia in 2017 is about 570 (range, 420–730) cases, and estimated MDR/RR-TB cases among notified pulmonary TB cases is 370 (range, 290-460) (3). In unpublished data from Terengganu Health Department, MDR-TB for Terengganu in 2017 and 2018 were 2 cases and 1 case, respectively.

A few factors that can cause drug-resistant TB include inappropriate treatment, inadequate or poor quality of drugs, and the most frequent cause is insufficient drug intake by patients (6). Besides that, it also can be caused by direct contact with MDR-TB infected person. There are a few primary ways to prevent drug-resistant TB. First early detection of TB and provide high-quality treatment of drug-susceptible TB, then early detection and high-quality treatment of drug-resistant TB. The next effective action of infection control measures, strengthening and regulation of health systems and addressing underlying risk factors and social determinants (5).

Resistant mutation of rifampicin is hard to occur when compared with any other anti TB drugs. Rifampicin resistance rate is increasing due to the extensive usage of the drug (8). Twelve

amino acids were surrounding the rifampicin binding pocket, where the location of the RNA polymerase active site (8). The mutation results in a change of the one amino acid and causes modification of active site. The mutation is associated with the replacement of an amino acid having a small side chain by an amino acid with a large side chain, and the consequence is the inactivation of RNA polymerase resulting in rifampicin resistance (8). The action of rifampicin is on the *rpoB* gene encoding RNA polymerase B subunit; because of that, more than 95% of rifampicin resistance mutations associated with mutations in the *rpoB* gene (9). Most of the mutations in the *rpoB* gene are present within 81 bp rifampicin-resistance determining region (RRDR), a mutation hot spot region from codon 507 and 533. Among the different types of mutations, non-synonymous mutations are more common than insertion, deletion, and frameshift mutations (10). Rifampicin resistance is taken as a surrogate marker of drug-resistant TB because the emergence of rifampicin-resistance was relatively slow when compared to other antibiotics (11). Various regions in the world have shown to have a different mutation, and the common mutation is at the *rpoB* gene includes codon 531, 526, and 516. Mutation outside the RRDR has been reported in <5% of rifampicin-resistant isolates (12). A few studies from various parts of the world have shown its existence and significance, including a study from Malaysia.

A study in India show, of 90 isolates the most frequent mutations observe at codons 531(54.4%) and codon 526 (18.9%). Other mutation inside RRDR region observes in codon 516 (5.6%), codon 511 (12.2%) and codon 521 (15.6%). Mutations in other regions observed at codons 413 (11.1%), codon 435 (6.7%), and codon 451 (8.9%) of the *rpoB* gene. Fifteen isolates were observed with a double mutation at codon 531 and 526, while four isolates with triple mutations at codon 531, 526, and 516 (12).

In Vietnam, out of 74 rifampicin-resistant isolates, 56 (76%) of which had mutations in the 81-bp RRDR region of the *rpoB* gene. The most common mutations observed in codons 531 (37.8%), 526 (23%), and 516 (9.46%) of the *rpoB* gene. Fifteen (20.3%) isolates had mutations at codon 490 was outside the 81-bp RRDR of *rpoB* gene. Three (4.05%) isolates did not show any mutation (13).

In Jiangxi province, China, of 157 phenotypic MDR isolates, 147 (93.6%) isolates have the mutation that harboured in 12 codons of RRDR *rpoB* gene, and ten (6.4%) showed no mutation. All mutation occurs within the 81-bp RRDR. The most frequent mutation of *rpoB* was codon 531 (90/157 [57.3%]) followed by codon 526 (20/157 [12.7%]) (14).

A study in Pakistan, of 24 MDR TB isolates that proceeded with genotypic analysis by PCR of 157 bp *rpoB* gene, 19 (79%) showed a mutation in the region tested. Eleven (58%) showed a mutation at codon 531, 4 (21%) exhibited a mutation at codon 516, 2 (11%) showed a mutation in codon 512 while 1 (5%) isolate showed a mutation at the codon 533. One isolate showed a silent mutation at codon 528. Five (20%) showed a lack of any mutation in the RRDR region of the *rpoB* gene. These could be the presence of mutation outside the RRDR region or another mechanism of resistance to rifampicin (15).

In Thailand, 39 of *M. tuberculosis* isolates, including three rifampicin susceptible isolates, were studied for mutations in the *rpoB* gene associated with rifampicin-resistance. Mutation in 21(53.8%) isolates was able to identify. Mutation in codon 531 accounting for 13 (61.9%) isolates, silent mutation at 536 for 4 (19%), deletion of 523 for 2 (9.5%), combination of 526 and novel 533 for 1 (4.8%), and a novel 538 for 2 (4.8%). This study shows a similar pattern to other previous studies from a different region of the world. Mutation analysis of the 81-bp fragment and five

codons beyond in *rpoB* provides a useful approach in predicting rifampicin phenotype allowing early diagnosis and appropriate drug therapy (11).

In Eastern Cape Province, South Africa, MDR TB cases isolated in 2012 and 2013 showed that rifampicin-resistant mutations were different from the previous studies in the world. Mutation observes at *rpoB* gene codon 42 in 30 (21.4%) isolates, codon 52 in 20 (14.3%) isolates, codon 87 in 10 (7.1%) isolates, codon 92 in 10 (7.1%) isolates, codon 441 in 10 (7.1%) isolates, codon 450 in 10 (7.1%) isolates, and codon 457 in 10 (7.1%) isolates. All mutations were outside the RRDR of *rpoB* gene and rarely reported in the previous studies (16).

In Malaysia, nine isolates of *M. tuberculosis* studied. One isolate has mutations to rifampicin and isoniazid, five isolates have resistant to rifampicin alone, and three isolates have a mutation to isoniazid only. Five rifampicin-resistant isolates showed mutations in the *rpoB* gene, and two had a mutation at codon 119, 2 at codon 135, and 1 at codon 90. All mutation occurs outside the RRDR region of *rpoB*. No isoniazid-resistant isolates had a mutation in the *rpoB* gene (17).

The studies discussed above show the characteristic of molecular changes in the geographic environment and genealogy. There has been little information on the molecular characterization of drug-resistant TB clinical isolates in Malaysia. This finding may not represent the actual mutation of tuberculosis in Malaysia. However, this finding is alarming since the molecular test for drug-resistant TB that endorse by WHO mainly detect the mutation in the RRDR regions of *rpoB*. If rifampicin-resistant TB in Malaysia harbours a mutation outside the RRDR region of *rpoB*, two molecular methods that endorse by WHO; Xpert MTB/RIF and MTBDR plus line probe assay (LPA) will miss to diagnosed rifampicin-resistant TB. Hence the diagnosis will depend on the phenotypic method, which is time-consuming.

In the worldwide setting, isoniazid resistance was highly prevalent and observed in one of every seven TB cases (18). Isoniazid is the prodrug, and catalase-peroxidase (katG) activates isoniazid, which reacts with NAD+. This compound inhibits InhA with the consequence of the stoppage of mycolic acid biosynthesis, which ends in mycobacterial cell death. Single nucleotide polymorphism (SNP) in the *katG* gene result in inactive katG with loss of activation of isoniazid (19). Multiple genes are involved in Isoniazid resistance. These include *katG, inhA, kasA, ahpC,* and *oxyR* (20). Among that, *katG* is more common, and *katG* 315 mutation is highly prevalent (20). This mutation site can be used as a molecular determinant of isoniazid resistance in most of the studies, although their frequency did not reach 100% in most of the studies. InhA promoter region -15 mutation was the most common mutations in the *inhA* gene, and it was common up to 19% together with other mutations in the promoter region of the gene (21). As with rifampicin mutation, a different part of the world recorded different percentages or frequency and various parts of mutation involved.

A study in Poland show out of 50 cases of MDR TB, 46 (92%) of MDR TB isolates had a mutation in the *katG* gene. Codon 315 of *katG* predominated in 36 (72%) isolates. Eight (16%) isolates (six with a mutated *katG* allele) had mutations in the *inhA* promoter region. Mutations also noted in the *oxyR-ahpC* intergenic region found in five (10%) isolates. One isolate had no mutations in any of the tested loci. Analysis of *katG* 315 and *inhA* –15 mutation enables the detection of isoniazid resistance in 84% of the MDR-TB sample in this study. Genetic mechanisms of isoniazid resistance in many TB isolates remain unknown. One isolate in this study shows no mutation in any of the nine genetic loci analyzed (18).

In Taiwan, Tseng ST et al. report that out of 41 isoniazid-resistant isolates, 51% of isolates have a mutation in codon 463, 38.8% isolates have a mutation in codon 315, and 22% in other loci.

Only 1 of 41 isolates has a mutation in the *inhA* gene. This study shows that codon 315 is not the most frequent mutation site for isoniazid resistance (22).

In Myanmar, of 14 MDR TB isolates, all the isolates were resistant to isoniazid with the mutations observed were *katG* 315 in 11 isolates, and one has a mutation in codon 299. One isolate has a double mutation, frameshift mutation in *katG* gene and, also *inhA* promoter mutation, and one isolate has a mutation in the *inhA* promoter region alone. This study showed that *katG* 315 is the most frequent mutation site for isoniazid resistance (23).

In Brazil, Oliveira LNC et al. 2016 reported that among the 43 strains studied for MDRTB, 18 (41.9%) isolates displayed mutation at the codon 315 *katG*, 11 (25.6%) isolates had the −15C/T *inhA* polymorphism (24).

In Uganda, out of 90 isolates of MTB, 61 isolates conferring mutation to isoniazid. Mutation at the codon 315 *katG* alone found in 44/61 (72.1%). Mutations in the *fabG1* promotor region were present in 9/61 (14.8%) of the isolates, and none had *inhA* mutations. One isoniazid-resistant patient had the *ahpC*-48 G/A mutation (25).

The study in HUSM, Kelantan of 9 drug-resistant isolates, only four isolates observed to have isoniazid-resistant phenotypes. Three of these four isolates have two mutation sites in codon 61 and 238, 62 and 238, and 247 and 238, respectively. One isolate has no mutation in the amplified region of *katG*. None of the tested isolates has a mutation in the *inhA* promoter region. Mutation in codon 315 not observed in this study. Besides that, a single mutation in codon 238 *katG* also found in 4 isolates with mono resistant to rifampicin. The most common mutation observed in codon 238, which involved seven isolates out of 9 (17).