Original Article

Genotypic Detection of *rpo*B and *kat*G Gene Mutations Associated with Rifampicin and Isoniazid Resistance in *Mycobacterium Tuberculosis* Isolates: A Local Scenario (Kelantan)

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Abstract

Background: **Drug resistant tuberculosis (DR-TB) remains a public health issue that is of major concern on a global scale. The characterisation of clinical isolates may provide key information regarding the underlying mechanisms of drug resistance, and helps to augment therapeutic options. This study aims to evaluate the frequency of gene mutations associated with Rifampicin (RIF) and Isoniazid (INH) resistance among nine clinical isolates.**

Methods: **A total of nine drug resistant *Mycobacterium tuberculosis* clinical isolates were screened for genetic mutations in *rpo*B and *kat*G using polymerase chain reaction (PCR) amplification and DNA sequencing. Genotypic analysis was performed to detect the mutations in the sequence of the target genes.**

Results: **Our findings reveal that 80% of the isolates possess mutations at codon 119 (His119Tyr) and 135 (Arg135Trp and Ser135Leu) within the *rpo*B gene; and 70% possess mutations in the *kat*G gene at codon 238 with amino acid change (Leu238Arg).**

Conclusion: **Findings from this study provide an overview of the current situation of RIF and INH resistance in a hospital Universiti Sains Malaysia (HUSM) located in Kelantan, Malaysia, which could facilitate molecular-based detection methods of drug-resistant strains. Further information regarding the molecular mechanisms involved in resistance in RR-/MDR-TB should be addressed in the near future.**

Keywords: *M. tuberculosis, isoniazid, rifampicin, rpoB, katG, mutation*

Introduction

Tuberculosis (TB) is among the major public health concerns, and is the leading cause of morbidity and mortality related with infectious diseases worldwide. The aetiological agent is Mycobacterium tuberculosis, which is transmitted by airborne particles. According to World Health Organization (WHO) reports, there have been a total of 9 million TB incidents worldwide, with 480 000 cases of multi-drug resistant tuberculosis (MDR-TB) in 2013 alone (1). Needless to say, the economic impact of TB is substantial, posing immense challenges to society.

The treatment of active TB requires the administration of Isoniazid (INH) in combination with one or more first line drugs such as Rifampicin (RIF), Ethambutol (EMB), Pyrazinamide (PZA) or Streptomycin (STR). Drug resistance and MDR-TB strains of *Mycobacterium tuberculosis* have been recognized in many parts of the world (2,3,4), which hinders the success of TB control programs. MDR-TB is defined as the resistance to at least two main first line TB drugs: RIF and INH (1). Drug resistance is generally an inevitable outcome of the inadequate, incomplete or prolonged usage of antibiotics (5). Genetic analysis of drug-resistant strains revealed that airborne transmission of undetected and untreated strains have played a key role in several outbreaks (6). In Malaysia, 277 cases of Rifampicin Resistance-/MDR-TB (RR-/MDR-TB) have been reported in 2013, compared to 141 cases in 2011, following drug sensitivity tests by the National Public Health Laboratory (NPHL) in Sg. Buloh (7). Although the number of cases is relatively low compared to other Asian

countries, the prevalence of RR-/MDR-TB is increasing. This is attributable to the increase of immigrants, resulting in endemicity (5).

Resistance is often associated with mutations in target-encoding, or related, genes. Mutations in the hypervariable region of the RNA polymerase beta-subunit encoding gene (*rpo*B), which encodes the β subunit of the DNA-dependent RNA polymerase (RNAP), are likely to impact its functions (8, 9), as observed in 95% of RIF resistant strains. In addition, RIF resistance may be regarded as a surrogate marker for MDR-TB, since 83% of the strains in the UK are also resistant to INH (10). The detection of a variety of mutations that involve one or several genes in INH resistant strains is highly complicated. About 60–70% of INH resistance is associated with mutations in the catalase-peroxidase encoding (*kat*G) gene (11,12,13), which is the only enzyme in TB that is capable of activating INH from pro-drug to active form (8). This study aims to determine the frequencies of mutations among drug resistant strains in TB cases at Hospital Universiti Sains Malaysia (HUSM), Kelantan.

Materials and Methods

Clinical isolates and drug sensitivity testing

HUSM provided a total of nine clinical isolates of M. tuberculosis, as well as the H37Rv strain as the positive control. All isolates were cultured on Lowenstein-Jensen slants, and then subjected to drug sensitivity testing, which were classified as MDR-TB by the NPHL in Sg. Buloh, Malaysia. The data has been obtained from NPHL for molecular testing.

DNA extraction and PCR amplification

Genomic DNA was isolated using the boiling method according to standard procedures. Its concentration (ng/µL) was estimated spectrophotometrically at 260 nm. DNA fragments of the *rpo*B and *kat*G genes were amplified using the primer sets described by Zenteno-Cuevas et al. (3). PCR reactions were conducted in 25 µL reaction volumes containing 300 ng/µL genomic DNA. The PCR cocktail contained 1× PCR buffer, 200µM dNTPs (each), 1.25 units Top Taq DNA polymerase, 1.5 mM MgCl2, and 0.2 µM of *rpo*B and *kat*G primers. Each PCR consisted of 35 cycles of denaturation at 94 °C for 3 minutes; annealing at 60 °C for 30 seconds; with an extension at 72 °C for 1 minute; and a final extension step at 72 °C for 10 minutes. PCR products were subjected to electrophoretic separation using 1.5% (w/v) agarose gels containing SYBR® Safe DNA stain, visualized under UV light.

DNA sequencing

PCR products were purified using the GeneJET PCR Purification kit (Thermo Scientific, MA, USA). The H37Rv strain and a total of nine RIF and INH resistant samples were sequenced directly using the Big Dye® Terminator v.3.1 Cycle Sequencing Kit and an automated 3130xl Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The obtained sequences were directly compared to previously published *rpo*B and *kat*G gene sequences using the MEGABLAST search tool in the National Center for Biotechnology Information (NCBI) database. Sequence files were imported into Chromas Lite, and then assembled using DNA Baser Sequence Assembly v.3.5.4.

Mutational analysis

Genotypic analysis to detect mutations responsible for resistance was performed using the Mutation Surveyor® DNA Variant Analysis software version 4.0.9 (SoftGenetics, State College, PA).

Results

PCR and sequencing

Genotypic analysis was performed for RIF and INH resistant specimens to detect mutations responsible for drug resistance by PCR amplification of the *rpo*B gene (280bp) and *kat*G gene (580bp) respectively. Nine isolates (USM 01, 31, 43, 53, 54, 60, 98, 100 and 117) with known resistance patterns to first-line drugs from NPHL were then selected for sequencing, which revealed 99% similarities to reference strains in the NCBI database.

Mutation analysis

Mutations within the *rpo*B and *kat*G genes were analyzed by comparing the sequence of clinical isolates to the H37Rv strain, the result of which can be observed in table 1. The USM 98 and USM 100 isolates with RIF resistance revealed mutations at codon 119 (His119Tyr); USM 31 and USM 43 at codon 135 (Arg135Trp and Ser135Leu); and USM 01 at codon 90 (Asp90Val); accounting for 80% of mutations in the *rpo*B gene. No mutation was detected in two among the five RIF-resistant isolates. In contrast, 70% of mutations in the *kat*G gene of isolates with INH resistance showed missense mutation at codon 238 (Leu238Arg). USM 117 revealed changes at codon 61 (Val61Gly); USM 01 at codon 247 (Gln247His); and USM 53 at codon 62 (Ala62Thr).

The nucleotide and amino acid changes observed in the RIF/INH drug-resistant isolates are shown in figure 1.

Discussion

Genotypic methods are often deemed superior to those of traditional phenotypic in terms of both sensitivity and specificity. Given the slow growth rate and fastidious nature of Mycobacterium tuberculosis, identification and drug susceptibility testing of TB is time consuming. TB diagnosis using standard culture techniques, even in the presence of automated fluid culture methods, takes an average of 14 days to complete (14). This presents a major hindrance for any TB control program, and complicates therapeutic options. The use of molecular based methods, based on the detection of mutations in specific genes associated with resistance, is more efficient and effective. Its utilization in clinical microbiology laboratories could minimize the

Table 1: Characteristics of nine RIF/ INH-resistant clinical isolates of *M. tuberculosis* isolates

Isolate	*rpo*B gene			*kat*G gene			Drug resistance profile
	Codon	Amino acid change	Nucleotide substitution	Codon	Amino acid change	Nucleotide substitution	
USM 01	90	D →V	GAC →GTC	238 247	L →R Q →H	CTC →CGC CAG →CAC	RIF INH
USM 31	135	R →W	CGG →TGG	-	-	-	RIF
USM 43	135	S →L	TCG →TTG	238	L →R	CTC →CGC	RIF
USM 53	-	-	-	62 238	A →T L →R	GCG →ACG CTC →CGC	INH
USM 54	-	-	-	-	-	-	INH
USM 60	-	-	-	238	L →R	CTC →CGC	RIF
USM 98	119	H →Y	CAC →TAC	238	L →R	CTC →CGC	RIF
USM 100	119	H →Y	CAC →TAC	238	L →R	CTC →CGC	RIF
USM 117	-	-	-	61 238	V →G S →A	GTG →GGA TCG →GCG	INH

[-] No mutation detected.

Abbreviations: USM = Universiti Sains Malaysia; RIF = Rifampicin; INF = Isoniazid.

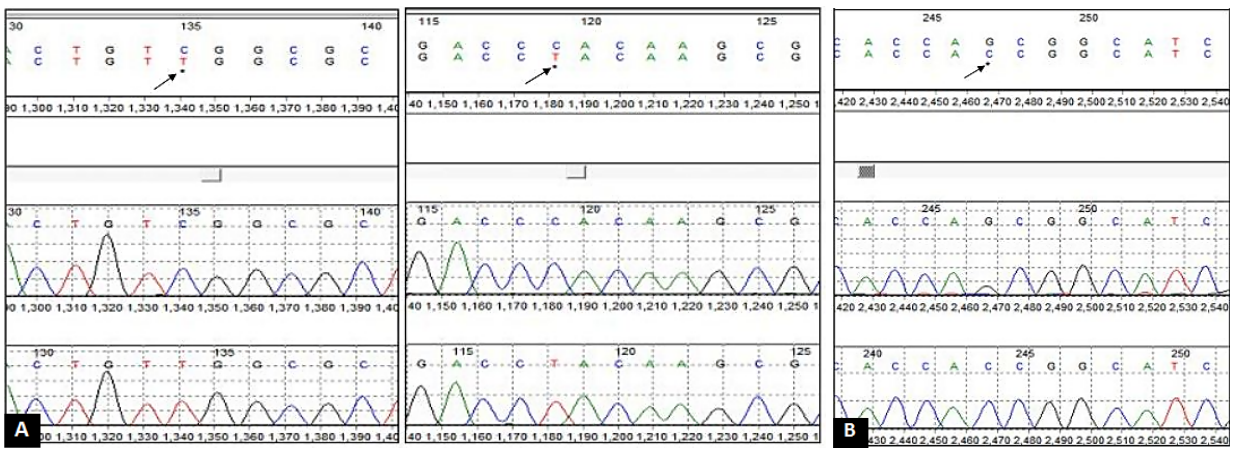

Figure 1: Chromatograms of nucleotide substitutions detected in TB positive samples. Star (*) represents the mutation site revealed from the samples. (A) *rpo*B mutations detected in RIF resistance cases. (B) Mutation found in *kat*G gene INH resistance case.

turnaround time required to diagnose cases from weeks to hours, hence, its use in this study.

Point mutations in several key genes of *Mycobacterium tuberculosis* is believed to be the major basis governing resistance. The catalase-peroxidase enzyme of TB is encoded by the *kat*G gene, which is responsible for converting isoniazid to its active form, as well as for the detoxifying activity against reactive oxidative stress (15). The mutation of this gene causes loss of, or reduced, activity of the catalase-peroxidase activity required to maintain the level of oxidative protection against host antibacterial radicals (16). Together with INH, RIF is another vanguard antimicrobial drug that is commonly used in the therapy of TB since the 1980s (17). Mutations in the *rpo*B gene generally confer resistance to RIF, commonly through amino acid changes involving a compact side chain with one that is larger (e.g., Ser Leu), thus, preventing RIF access to RNAP (9). Mutations in the *rpo*B gene have a negative impact on key physiological functions often associated with treatment failure and fatal clinical outcomes (18). Both genes were analyzed concurrently using clinical isolates from local settings.

There has been little information on the molecular characterization of drug resistant TB clinical isolates in Malaysia, which is alarming, given its endemicity. In general, our findings support prior hypotheses that link the *rpo*B and *kat*G genes to RIF and INH drug resistance. All of the RIF and INH mutations were characterized as single nucleotide changes. Nevertheless, the site of mutation and type of changes differ from findings reported in prior work. The most frequent mutations in the *rpo*B gene of RIF resistant strains were at codons 135 and 119; this has been observed in 80% of the isolates. We did not observe mutation 531 (Ser531Leu), which is a common documented site of *rpo*B mutation (19,14). Furthermore, while most studies report mutation at Ser315Thr (20,19,14), our findings detected changes at codon 238 (Leu238Arg), accountable for 70% of the *kat*G mutation. However, this change did not contribute to INH phenotypic resistance, since it is possibly considered the most common polymorphism or low frequency mutation in the *kat*G gene. We also observed changes at codon 247 (Gln247His), codon 61 (Val61Gly), and codon 62 (Ala62Thr). Two isolates (USM 31 and USM 54) did not exhibit any mutation, despite being phenotypically resistant.

Our findings are unique compared to other reports in other parts of the world, reflecting the difference of gene mutations in different geographical locations. Discrepancies observed could be attributed to the limited number of samples, presence of other mutations which have not been documented to date, or its presence outside selected target regions in the *rpo*B and *kat*G genes. For instance, INH resistance involves alterations of multiple genes other than the *kat*G gene, such as inhA, kasA and ahpC (13), which were beyond the scope of this work. In addition, newly undefined genes may also be implicated, and many underlying mechanisms remain unexplored. Not all mutations are known, some which are of low frequency; many vary according to geographical regions, all of which convolute the task of detection based on single genes. Although molecular-based methods offer many advantages, these limitations should be taken in consideration when designing a diagnostic assay for resistant strains.

Conclusion

The detection of mutations in resistance determining regions plays a crucial role for the rapid detection of anti-TB resistance, and could aid strategies to further explore the mechanisms of resistance. The high percentage of mutations in the *rpo*B and *kat*G genes highlights their importance, and should be further investigated in order to gain a more comprehensive of the geographical distribution of resistant strains, as well as of the mechanisms underlying them.

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Conflict of Interest

None.

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None.

Authors' Contributions

Conception and design: NAI, SSNM, SNC
Analysis and interpretation of the data: NAI
Drafting of the article: NAI, SNC

Critical revision of the article for important intellectual content, statistical expertise: SNC
Final approval of the article: SSNM, SNC
Provision of study materials or patients: SSNM
Administrative, technical, or logistic support: NAI, MFI, SSNM
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