SUPLEMEN
Volume 15, Suplemen, 2023
https://myjurnal.poltekkes-kdi.ac.id/index.php/hijp

HIJP : HEALTH INFORMATION JURNAL PENELITIAN

The Whole Genome Sequencing Of Mycobacterium Tuberculosis For Drug Resistance Prediction

Melya Puspitasari[1*], Andriansjah[2], Linda Erlina[3]

[1]Master Program of Biomedical Science, Faculty of Medicine, University of Indonesia; *puspitasarimelya8@gmail.com*
[2]Department of Microbiology, Faculty of Medicine, University of Indonesia; andriansjahrukmana@gmail.com
[3]Departement of Bioinformatics, Faculty of Medicine, University of Indonesia; lindaerlina22@gmail.com

**Corresponding Author Email: puspitasarimelya8@gmail.com*

Abstract

Whole-genome sequencing (WGS) has shown tremendous potential in rapid diagnosis of drug-resistant tuberculosis (TB). In the current study, we performed WGS on drug-resistant Mycobacterium tuberculosis isolates obtained from Shanghai (n = 137) and Russia (n = 78). We aimed to characterise the underlying and high-frequency novel drug-resistance-conferring mutations, and also create valuable combinations of resistance mutations with high predictive sensitivity to predict multidrug- and extensively drug-resistant tuberculosis (MDR/XDR-TB) phenotype using a bootstrap method. Most strains belonged to L2.2, L4.2, L4.4, L4.5 and L4.8 lineages. We found that WGS could predict 82.07% of phenotypically drug-resistant domestic strains. The prediction sensitivity for rifampicin (RIF), isoniazid (INH), ethambutol (EMB), streptomycin (STR), ofloxacin (OFL), amikacin (AMK) and capreomycin (CAP). The mutation combination with the highest sensitivity for MDR prediction was rpoB S450L + rpoB H445A/P + katG S315T + inhA I21T + inhA S94A, with a sensitivity of 92.17%, and the mutation combination with highest sensitivity for XDR prediction was rpoB S450L + katG S315T + gyrA D94G + rrs A1401G, with a sensitivity of 92.86%. The molecular information presented here will be of particular value for the rapid clinical detection of MDR- and XDR-TB isolates through laboratory diagnosis.

Keywords: Drug Resistance, Prediction, Sensitivity, Specificity, Tuberculosis, Whole-Genome Sequencing

INTRODUCTION

Drug-resistant tuberculosis has always been a significant problem to the global tuberculosis control program. The patients of TB drug-resistant might be affected by exposure to drug-resistant MDR-TB strains or may develop due to other clinical factors, including delayed diagnosis, inappropriate treatment and so on[1]. Controlling the high prevalence of drug-resistant TB mostly relies on timely laboratory diagnosis. Traditional TB drug susceptibility tests (DST) rely on solid or liquid cultures, which may take weeks or months to produce results.

A number of rapid molecular biology based on diagnostic methods have recently been implemented in the clinical environment, including the Xpert MTB/ Rif and the MTBDRplus[2-4] GenoType. Although this method is quick and simple, its extension to undeveloped world regions is limited by high cost and availability. Whole Genome Sequencing as a molecular diagnostic tool has been widely developed in TB research since the first full genome sequence for H37Rv was announced in 1998. The reported sensitivity and specificity for predicting anti-TB drugs has been more than 80% [5,6].

Consequently, Next Generation Sequencing was tested on drug-resistant Mycobacterium tuberculosis in this study. One of the aims of this study was to detect MDR and XDR based on a limited number of mutational sites, and establish several combinations of loci affiliated with drug resistance to predict MDR/ XDR with higher sensitivity and specificity at the same time. Another goal was to characterize drug resistance mutations.

METHODOLOGY

Collection and Processing of Samples

The sample employed in this study included 105 clinical isolates of Mycobacterium tuberculosis randomly. This sample involved 35 XDR cases, 35 MDR cases and 35 pre-susceptible cases. The MDR-TB shows that TB was resistant to at least isoniazid (INH) and rifampicin (RIF), whereas XDR-TB shows the TB was resistant not only to INH and RIF, but also to fluoroquinolone and at least one of the three injectable drugs. All experimental methods were in accordance with the standard of clinical laboratory procedures. The DNA was extracted by using the Mag-MK Bacterial Genomic DNA extraction kit according to the instructions given. Moreover, several genome sequences were downloaded from the National Center for Biotechnology Information for several foreign strains in fastq' file format[7].

Next Generation Sequencing (WGS)

In line with the fast technology development, the methods of sequencing work to sequence the DNA of organisms are also advanced. One sequencing technique that is being developed and popularly used today is the Next Generation Sequencing. The NGS is a sequencing method developed after the Sanger method with the aim of deep, high-throughput and parallel sequencing. There are three general stages in working on NGS, including library preparation then library amplification and sequencing using a different approach. These days, NGS applications have been popularly used, including for research in the health and diagnostic fields, detection of variations and so on.

1. **The first stage**, during the NGS processing, is to perform **DNA extraction**. The procedure for DNA extraction is according to the type of sample to be used, for example blood and tissue, and body fluids. After the DNA extraction procedure is completed, the results must be evaluated for its quality and concentration.
2. **The second stage** is to carry out a **preparation library** containing all the extracted DNA parts, then followed by carrying out specific enrichment in the desired target area. The library was prepared by randomly cutting genomic DNA (gDNA).

The DNA fragments cutting generally use nebulization or sonication of the gDNA that has been cut, and it will go through a series of enzymatic reactions to make adenosine overhangs which will be bound by adapters using DNA ligase[9]. After the adapter ligation, samples will be selected according to their size by using gel electrophoresis, gel extraction and purification. During this process, the genomic library has been created and can be used for sequencing or specific regions can be extracted for downstream sequencing.

3. **The third stage**, the sequencing reaction can be carried out by a number of existing methods. In this case, the method largely used is the sequencing by synthesis which requires a DNA library.

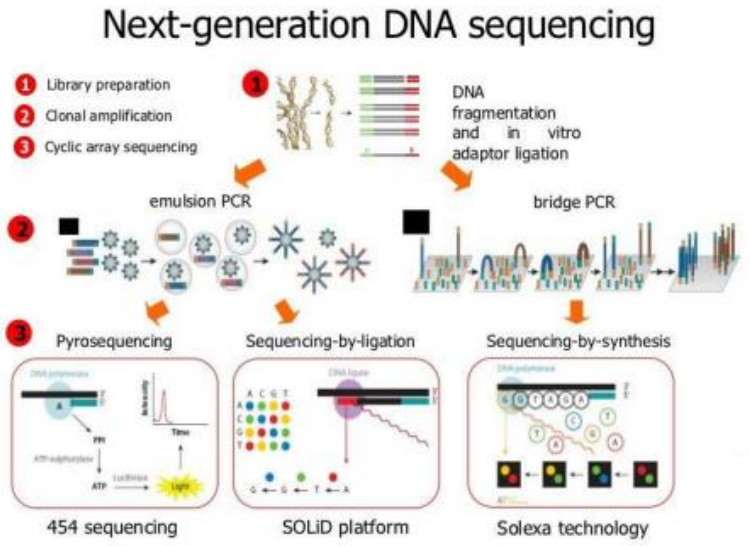

Figure 1. Stages of NGS
Source :http://ueb.ir.vhebron.net/NGS

Sequencing

Next Generation Sequencing was carried out on the Illumina HiSeq 2000 platform. Stable (http://github.com/ucdavis-bioinformatics/ sickle) was used to cut the readings, with a minimum of Q20 baseline quality. Bowtie 2[10] version 2.3.3 was used to map the genom by reference Mycobacterium tuberculosis H37Rv (NC_0009623) with the minimum mapping quality Samtools Q30[11], version 1.6 was used to call single nucleotide polymorphisms (SNP) and VarScan (version 2.3.9)[12] was used to call mutation variants.

Identification of the mutations that leads to drug resistance

Common drug resistance granting mutant genes and their mutation frequencies are varying between isolates. A total of 18 candidate genes were chosen for the process of drug resistance mutation analysis by considering genes that were reported more than once[13]. Then, the resistance mutations were found in only eight of the genes, possibly due to the limited sample size and/or lower mutation frequency. For all of the gene candidates, the mutations that took place in at least one phenotypically resistant isolate were defined as determinants of resistance[13]. Additionally, the sensitivity was calculated by dividing the number of strains with phenotypic drug resistance mutations found by Next Generation Sequencing in phenotypic resistant strains by the total number of phenotypic resistant strains. Also, Fisher's exact test was applied to find out the statistical differences in resistance mutations for each drug between the different groups performed in R (v4.0.2).

Bootstrap Analysis

First, a perl script was used to merge the SNP sites of all types. Second, resistance sites with high-frequency for each drug were screened and combined in R (v4.0.2). Finally, a bootstrap analysis was carried out in R (v4.0.2) on selected combinations to predict MDR and XDR isolates, calculating the sensitivity and specificity.

RESULTS AND DISCUSSION

Results

Characteristics of Samples

There were a total of 215 samples used for the genetic mutation analysis. Four first-line drugs, rifampicin (RIF), isoniazid (INH), streptomycin (STR) and ethambutol (EMB) and three second- line drugs, amikacin (AMK), capreomycin (CAP) and ofloaxin (OFL) were found to be the ones considered for analysis in this study. Then a phylogenetic tree was constructed from a total of 215 Mycobacterium tuberculosis strains resulting in 162 935 high quality SNPs (Figure 1). Alternative alleles identified with less than 25% reads were screened and SNPs in the PE/ PPE region, insertion elements and repeating regions were not included in the analysis[14]

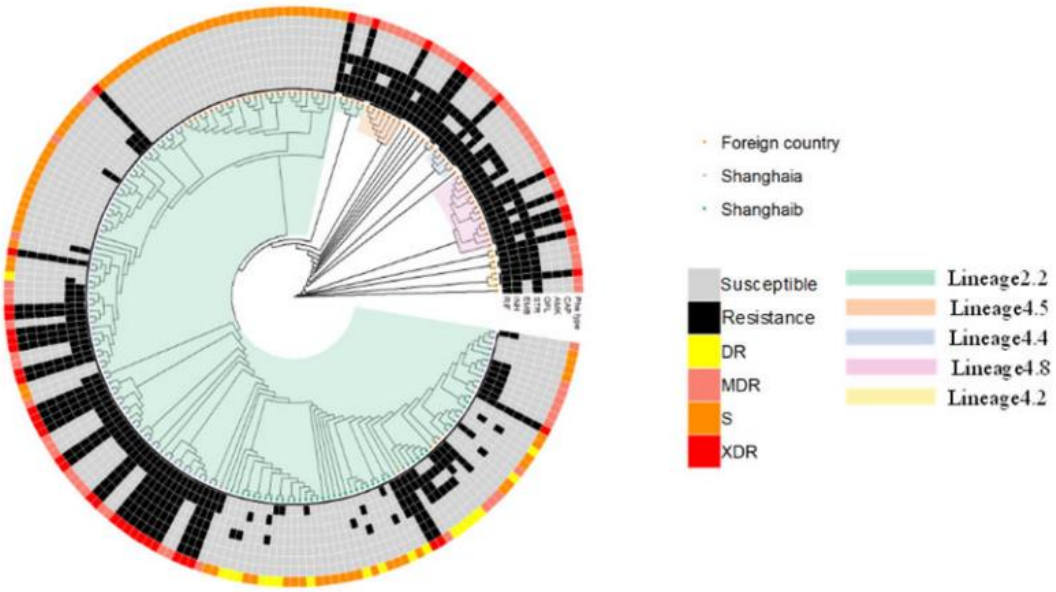

Figure 1. Phylogenetic tree of 215 strains of Mycobacterium tuberculosis

A total of 191 strains have lineage of 2.2 ('ancient' Beijing), then 24 strains have four sublineages of L4.2, L4.4, L4.5 and L4.8 which are called 'Euro- American' lineages. Then, it was also found a total of 30 markers of drug resistance in eight candidate genes among 215 strains of Mycobacterium tuberculosis as summarized in Table 1.

Table 1. Mutations identified in loci associated with drug resistance

			Isolat Domestik (137)				Rusia terisolasi (78)			
Obat	Gen nama	Mutasi	Jumlah (%) dari isolat diidentifikasi pada 35 strain MDR	Jumlah (%) dari isolat diidentifikasi pada 30 strain XDR	Jumlah (%) dari isolat diidentifikasi pada 16 galur DR	Jumlah (%) dari isolat diidentifikasi pada 56 Sastrain	Jumlah (%) dari isolat diidentifikasi pada 39 strain MDR	Jumlah (%) dari isolat diidentifikasi pada 12 strain XDR	Tidak.(%) dari isolat diidentifikasi pada 27 S strain	Pnilai
RIF	rpoB	S450L	17 (48.57)	22 (73.33)	3 (75.00)	–	37 (94,87)	10 (83.33)	–	> 0,05
RIF	rpoB	D435V	2 (5.71)	2 (6.67)	–	–	–	–	–	
RIF	rpoB	H445D/P	5 (14.29)	–	–	–	1 (2,56)	–	–	
RIF	rpoB	Q172R	–	1 (3.33)	–	–	–	–	–	
RIF	rpoB	Q432P/K	1 (2,86)	2 (6.67)	–	–	–	–	–	
INH	katG	S315T	26 (74.29)	21 (70.00)	5 (62,50)	–	37 (94,87)	10 (83.33)	–	> 0,05
INH	katG	A144V	1 (2,86)	1 (3.33)	–	–	–	–	–	
INH	katG	H97R	–	1 (3.33)	–	–	–	–	–	
INH	katG	E607K	2 (5.71)	–	–	–	–	–	–	
INH	katG	S17G	1 (2,86)	–	–	–	–	–	–	
INH	menghirup	I21T	1 (2,86)	2 (6.67)	–	–	–	–	–	
INH	menghirup	S94A	–	1 (3.33)	–	–	–	–	–	
INH	ndh	A352S	–	1 (3.33)	–	–	–	–	–	
STR	rpsL	K43R	2 (5.88)	17 (56.7)	8 (47,06)	–	8 (20.51)	6 (50,00)	–	> 0,05
STR	rpsL	K88R	–	5 (16,67)	2 (11,76)	–	1 (2,56)	–	–	
STR	rrs	C517T	–	4 (13.33)	–	–	–	3 (25.00)	–	
penyelenggara pemilu	embB	M306V	–	12 (40.00)	1 (5.88)		6 (28,57)	3 (25.00)	–	<0,05
penyelenggara pemilu	embB	M306L	–	–	1 (5.88)	–	–	–	–	
penyelenggara pemilu	embB	D354A	–	–	–	–	16 (76.19)	3 (25.00)	–	
penyelenggara pemilu	embB	M306I	–	5 (16,67)	–	–	–	–	–	
STR	rrs	C517T	–	4 (13.33)	–	–	–	3 (25.00)	–	
penyelenggara pemilu	embB	M306V	–	12 (40.00)	1 (5.88)		6 (28,57)	3 (25.00)	–	<0,05
penyelenggara pemilu	embB	M306L	–	–	1 (5.88)	–	–	–	–	
penyelenggara pemilu	embB	D354A	–	–	–	–	16 (76.19)	3 (25.00)	–	
penyelenggara pemilu	embB	M306I	–	5 (16,67)	–	–	–	–	–	
penyelenggara pemilu	embB	Q497R	–	2 (6.67)	–	–	–	1 (8.33)	–	
penyelenggara pemilu	embB	G406A	–	3 (10.00)	–	–	–	–	–	
penyelenggara pemilu	embB	G406S	–	2 (6.67)	–	–	–	–	–	
OFL	gyrA	D94G	–	10 (33.33)	–	–	–	2 (16,67)	–	> 0,05
OFL	gyrA	A90V	–	9 (30.00)	–	–	–	–	–	
OFL	gyrA	D94A	–	3 (10.00)	–	–	–	–	–	
OFL	gyrA	D94N	–	3 (10.00)	–	–	–	1 (8.33)	–	
AMK/CAP	rrs	A1401G	–	19 (63.33)	–	–	–	3 (25.00)	–	> 0,05
AMK/CAP	rrs	C1402T	–	1 (3.33)	–	–	–	1 (8.33)	–	
AMK/CAP	rrs	G1484T	–	1 (3.33)	–	–	–	–	–	

Resistance to First-line of Anti-tuberculosis Drugs

The strain of Mycobacterium tuberculosis that showed resistance to RIF generally shows mutations in the 81bp rpoB resistance-determining region RIF15. The most common drug resistance mutation includes rpoB Ser450 Leu. Among the domestic isolates, 69 strains were resistant to RIF, of which 42 strains had mutations in the rpoB missense Ser450 Leu. Among the 56 pan-susceptible isolates, no resistance mutations were characterized. The sensitivity and specificity of the Ser450 Leu rpoB in predicting phenotypic resistance to rifampicin were 60.87% and 100%. Among the Russian isolates, 47 resistant strains carried the Ser450 Leu rpoB mutation. The sensitivity and specificity of rpoB Ser450 Leu in predicting phenotypic resistance to rifampicin were 92.16% and 100%, respectively. Other strains that did not have this mutation showed a lower frequency of the other missense mutations. In total, Next Generation Sequencing was able to enumerate 79.71% of domestic strains that were phenotypically resistant to rifampicin and 94.12% of Russian strains that were phenotypically resistant to rifampicin.

Isoniazid is a produg activated by the catalase-peroxidase enzyme KatG and encoded by the KatG16 gene. Among 215 strains of Mycobacterium tuberculosis, a total of 124 of them showed resistance to isoniazid. The most common drug resistance mutation is KatG Ser315 Thr, where the codon change may be GC.

Among the domestic isolates, 52 strains carried the KatG Ser315Thr mutation, the sensitivity and specificity of KatGSer315Thr in predicting phenotypic resistance to isoniazid were 71.23% and 100%, respectively. Among Russian isolates, 10 XDR and 37 MDR strains carried the KatG Ser315Thr mutation. The frequency of the KatG Arg463Leu mutation used in the phylogenetic markers was higher than the other mutations among the selected genes, but because it did not confer isoniazid resistance, hence it was excluded. The Next Generation Sequencing was able to predict 86.30% of the domestic strains which were phenotypically resistant to INH and as much as 92.16% of the Russian strains were phenotypically resistant to INH.

The mutations in the rpsL gene, which encoded the 30S ribosomal protein associated with the first step of RNA translation, were found to cause approximately 80% STR resistance. Therefore, the rpsL gene was chosen as a candidate gene to assess STR resistance in different strains. The most frequently detected mutation was rpsL Lys43Arg, and phenotypically, 41 strains were detected. Additionally, the sensitivity and specificity of the rpsL Lys43Arg mutation in predicting phenotypic STR resistance were 45.56% and 100%, respectively.

The drug resistance mutations correlated with EMB mostly occured in the embB¹8 gene. The most frequent mutation in EMB resistant strains was the embB Met306val, and among 67 isolates that were phenotypically resistant, 22 strains carried this mutation. Out of the domestic isolates, there were 12 XDR strains with the Met306Val embB mutation and among the Russian isolates, six MDR strains and three XDR strains carried the Met306Val embB mutation. The Next Generation Sequencing was able to predict 76.47% of domestic lines that were phenotypically resistant to EMB and 87.88% of Russian lines that were phenotypically resistant to EMB.

Mutations associated with Second-line Drugs

The mutations in the rss gene encoding 16rRNA were the markers associated with AMK and CAP resistance, especially mutations that occured at nucleotide positions 1401, 1402 and 148419. Among the 30 domestic XDR strains, the most common mutation that gave AMK/CAP resistance was rrsA1401G which was found in 19 phenotypically resistant strains. The sensitivity and specificity of the rrs A1401G mutation in predicting phenotypic resistance to AMK/ CAP were 63.33% and 100%, respectively.

Other mutations detected among the domestic XDR lines were rrs C1402T and rrs G1484T, each of which was found in one strain. Among the 12 Russian XDR isolates, there were three with the rrs A1401G mutation, and one with the rrs C1402T mutation. The Next Generation Sequencing was able to phenotypically predict 70% of domestic lines that were resistant to AMK/ CAP and 33.33% of Russian lines that were phenotypically resistant to AMK/ CAP.

Resistance to OFL is correlated with mutations in the gryA and gyrB genes, encoding subunits that were heterotetrameric, DNA gyrase, these mutations occurred in codons 88-9420. Among 30 domestic XDR strains, the most common drug resistance mutation was gyrA Asp94Gly

which was present in 10 phenotypic OFL resistant strains. The sensitivity and specificity of the Asp94Gly gyrA mutation in predicting phenotypic resistance to OFL were 33.33% and 100%, respectively. Among the 12 Russian strains carrying the gyrA Asp94Asn mutation. The Next Generation Sequencing was able to phenotypically predict 83.33% OFL- resistant domestic lines and 25% of OFL-resistant Russian strains phenotypically.

phenotypic.

Locus Combination Predictive Value with Bootstrap

In order to generate a highly prevalent combination of drug resistance sites to predict XDR/ MDR resistant phenotypes, a bootstrap approach (1000 times) was implemented. The sensitivity and specificity of the combinations performed are summarized in Table 2.

Table 2. Bootstrap approach to validate MDR/ XDR

	Obat	Mutasi	Sensitivitas 95% CI	Spesifisitas 95% CI	Ramalan jenis
Kombinasi 1	RIF+INH	rpoB S450L + katG S315T	87,83% (0,8069–0,9274)	92,00% (0,8514–0,9604)	MDR
Kombinasi 2	RIF+INH	rpoB S450L + rpoB H445A + rpoBH445P + katG S315T	90,43% (0,8375–0,9478)	92,00% (0,8585–0,9612)	MDR
Kombinasi 3	RIF+INH	rpoB S450L + rpoB H445A + rpoBH445P + katG S315T + inhA I21T + inhAS94A	92,17% (0,8615–0,9646)	92,00% (0,8556–0,9596)	MDR
Kombinasi 4	RIF+INH	rpoBS450L + rpoBH445A + rpoBH445P + rpoB A435V katG S315T + inhA I21T + inhAS94A + katG G607L	92,17% (0,8626–0,9633)	92,00% (0,8493–0,9623)	MDR
Kombinasi 5	RIF + INH + AMK/CAP	rpoB S450L + katG S315T + rrs A1401G	92,86% (0,7950–0,9787)	58,38% (0,5110–0,6629)	XDR
Kombinasi 6	RIF + INH + OFL	rpoB S450L + katG S315T + gyrA D94G	90,48% (0,7841–0,9744)	58,38% (0,5058–0,6519)	XDR
Kombinasi 7	RIF + INH + STR + EMB	rpoB S450L + katG S315T + rpsL L43A + embB M306V	90,47% (0,7935–0,9744)	56,65% (0,4910–0,6407)	XDR
Kombinasi 8	RIF + INH + AMK/CAP + OFL	rpoB S450L + katG S315T + gyrA D94G + rrs A1401G	92,86% (0,8158–0,9796)	58,38% (0,4967–0,6543)	XDR
Kombinasi 9	RIF + INH + STR + EMB + AMK/CAP + OFL	rpoB S450L + katG S315T + rpsL L43A + embB M306V + rpoBH445A + rpoBH445P + inhA I21T + inhAS94A + gyrA D94G + rrs A1401G + gyrAA90V + rrsC1402T	92,86% (0,7930–0,9787)	54,91% (0,4671–0,6181)	XDR

The highest sensitivity regarding MDR to rfiampin and isoniazid was 92.17% conferred by the combination of rpoB S450L + rpoB H445A + rpoB H445P+ katG S315T+ inhA 121T + inhA S94A mutation. The highest sensitivity regarding XDR prediction against RIF, INH, AMK/ CAP and OFL was 92.86% which was given by the combination of rpoB S450L + katG S315T + gyrA D94G+mutations rrs A1401G.

Discussion

Referring to the analysis, it was found that Next Generation Sequencing was able to predict approximately as much as 82.07% and 72.33% of phenotypic drug resistance for Chinese and Russian strains, respectively. For domestic strains, the predictive sensitivity from highest to lowest includes STR (88.37%), INH (86.30%), OFL (83.33%), RIF (79.91%), EMB (76.47%)) and AMK/ CAP (25.00%). Geographical variation may also explain differences in mutant frequencies in predictive value. Then, the predictive sensitivity of STR resistance was found to be best in China by using Next Generation Sequencing, which might be due to the fact that mutations conferring STR resistance were mostly found in China. Furthermore, the sensitivity of Next Generation

Sequencing in detecting resistance to second-line drugs was not satisfactory, possibly due to the limited number of isolates.

The phylogenetic tree shows that lineage 2 and line 4 are spread across Russia and China, and L2.2 is the dominant line. Lineage 2 and lineage 4 are distributed worldwide, while the other lineages show geographic boundaries. In addition, susceptible, drug-resistant and MDR strains are present in lineage 2 and lineage 4 but the XDR strain is almost exclusively present in lineage 2.2 indicating that different lineages might play different roles in drug resistance outcome.

It was found that there were only eight resistance genes among the 18 gene candidates and a total of 30 mutations of these eight resistance genes were identified, possibly due to a lack of sufficient samples and low mutation rates. The limited number of samples did not have the ability to identify comprehensive mutations. In addition, this study also identified katG Arg463Leu, gyrA Glu21Gln, gyrA Ser95Thr and gyrA Gly668Asp as lineage-defining mutations. These mutations, which had been reported as natural polymorphisms, are actually useful for evolutionary characterization of genomes[21].

Although the specificity of representative genes in predicting phenotypes was 100%, it might be overestimated because it was assumed that strained without detecting the corresponding mutations were susceptible. The dominant mutation rates of the seven drugs in this study include INH (71.23%), RIF (60.87%), STR (62.79%), EMB (38.24%), AMK/ CAP (63.33%) and OFL (33.33%). In this study, for rifampicin resistance, mutations conferring other rifampicin resistance exclusively occurred in the rpoB gene region. Then the KatG gene was considered to have a high relationship with INH resistance, the most frequently reported being the KatG Ser 315Thr mutation. In this study, the most frequently reported EMB resistance mutation was the embB Met306Val. The resistance mutations in STR mainly occurred in the rpsL and rrs22 genes. According to the analysis, the most common mutation in the rpsL gene was the rpsL Lys43Arg. For the OFL-resistant strains, two high-frequency mutations were gyrA Ala90Val and gyrA Asp94Gly. And lastly, it was found that the mutations rrs A1401G, rrs C1402T and rrs G1484T were the most commonly detected in isolates that were resistant to AMK/ CAP.

In order to increase the predictive power of Next Generation Sequencing, a bootstrap approach was implemented to determine the combinations of high-frequency mutations that might prove to be more sensitive. To predict the MDR phenotype, it was found that the lowest predictive sensitivity combination was rpoBS450L + katGS315T. Additionally, predictive sensitivity increased by including more mutation sites, with the highest predictive sensitivity combining rpoBS450L + rpoBH445A/ P + katGs315T + inhA121T+inhAS94A. To predict the XDR phenotype, the mutation combination with the highest sensitivity was rpoBS450L + katGS315T + gyrAD94G + rrsA1401G.

Whole Genome Sequencing has been progressively used in these past few years to predict drug resistance and guide drug susceptibility testing patterns. Moreover, several automated software have also been developed for this, including CASTB, KvarQ, Mykrobe Predictor TB, PhyResSE and TB Profiler23.

CONCLUSION

The Next Generation Sequencing was found to have greater sensitivity and specificity in predicting resistance to first-line anti-TB drugs, compared to second-line drugs. Recent studies also identified several new mutations in the KatG and rpoB genes, but additional insights into drug resistance mechanisms are still required. These novel mutations, together with frequent mutations, can provide a reference point for clinical microbiology laboratory diagnostic methods to identify drug-resistant TB. Valuable combinations of mutations were identified using the bootstrap method to predict the MDR- TB phenotype.

REFERENCES

[1]Kerubo G et al. (2016) Drug susceptibility profiles of pulmonary Mycobacterium tuberculosis isolates from patients in informal urban settlements in Nairobi, Kenya. *BMC Infectious Diseases* 16, 583.

[2]Helb D et al. (2010) Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. *Journal of Clinical Microbiology* 48, 229–237.

[3]Hillemann D, Rüsch-Gerdes S and Richter E (2007) Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of Mycobacterium tuberculosis strains and clinical specimens. *Journal of Clinical Microbiology* 45, 2635–2640.

[4]Hillemann D, Rüsch-Gerdes S and Richter E (2009) Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of Mycobacterium tuberculosis strains and clinical specimens. *Journal of Clinical Microbiology* 47, 1767–1772.

[5]Faksri K et al. (2019) Comparisons of whole-genome sequencing and phenotypic drug susceptibility testing for Mycobacterium tuberculosis causing MDR-TB and XDR-TB in Thailand. *International Journal of Antimicrobial Agents* 54, 109–116.

[6]Papaventsis D et al. (2017) Whole genome sequencing of Mycobacterium tuberculosis for detection of drug resistance: a systematic review. *Clinical Microbiology and Infection* 23, 61–68.

[7]Coll F et al. (2018) Genome-wide analysis of multi- and extensively drug-resistant Mycobacterium tuberculosis. *Nature Genetics* 50, 307–316.

[8]Bradford P. (2001). Extended Spectrum ß Lactamases in the 21st century: Characterization, Epidemiology, and Detection of this Important Resistance Threat. *Clinical Microbiology* Revisi; 14: 933–951.

[9]Chaudary U, Aggarwal R. (2004). Extended Spectrum β-Lactamases (ESBL), an emerging threat to clinical therapeutics. *Indian Journal of Medical Microbiology*;22(2):75- 80

[10]Langmead B and Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9, 357–359.

[11]Li H et al. (2009) The sequence alignment/map format and SAMtools. *Bioinformatics* (Oxford, England) 25, 2078–2079.

[12]Koboldt DC et al. (2012) VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Research* 22, 568–576.

[13]Walker TM et al. (2015) Whole-genome sequencing for prediction of Mycobacterium tuberculosis drug susceptibility and resistance: a retrospective cohort study. *Lancet Infectious Diseases* 15, 1193–1202.

[14]Stamatakis A, Hoover P and Rougemont J (2008) A rapid bootstrap algorithm for the RAxML Web servers. *Systematic Biology* 57, 758–771.

[15]Heep M et al. (2001) Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical Mycobacterium tuberculosis isolates. *Journal of Clinical Microbiology* 39, 107–110.

[16]Cade CE et al. (2010) Isoniazid-resistance conferring mutations in Mycobacterium tuberculosis KatG: catalase, peroxidase, and INH-NADH adduct formation activities. *Protein Science* 19, 458–474.

[17]Chakraborty S et al. (2013) Para-aminosalicylic acid acts as an alternative substrate of folate metabolism in Mycobacterium tuberculosis. *Science* (New York, N.Y.) 339, 88–91.

[18]Jagielski T et al. (2016) Methodological and clinical aspects of the molecular epidemiology of Mycobacterium tuberculosis and other mycobacteria. *Clinical Microbiology Reviews* 29, 239–290.

[19]Bhembe NL et al. (2014) Molecular detection and characterization of resistant genes in Mycobacterium tuberculosis complex from DNA isolated from tuberculosis patients in the Eastern Cape province South Africa. *BMC Infectious Diseases* 14, 479.

[20]Disratthakit A et al. (2016) Role of gyrB mutations in pre-extensively and extensively drug-resistant tuberculosis in Thai clinical isolates. *Antimicrobial Agents and Chemotherapy* 60, 5189–5197.

[21]Lau RW et al. (2011) Molecular characterization of fluoroquinolone resistance in Mycobacterium tuberculosis: functional analysis of gyrA mutation at position 74. *Antimicrobial Agents and Chemotherapy* 55, 608–614.

[22]Hlaing YM et al. (2017) Mutations in streptomycin resistance genes and their relationship to streptomycin resistance and lineage of Mycobacterium tuberculosis Thai isolates. *Tuberculosis and Respiratory Diseases* (Seoul) 80, 159–168.

[23]Schleusener V et al. (2017) Mycobacterium tuberculosis resistance prediction and lineage classification from genome sequencing: comparison of automated analysis tools. *Scientific Reports* 7, 46327.