Molecular mechanisms of multidrug resistance in *Mycobacterium tuberculosis* isolated in South Korea

Soro Gorgoh

Graduate School of Public Health

Yonsei University,

Department of Global Health Security,

Division of Global Health Security Detection Program

Molecular mechanisms of multidrug resistance in *Mycobacterium tuberculosis* isolated in South Korea

Soro Gorgoh

Graduate School of Public Health

Yonsei University,

Department of Global Health Security,

Division of Global Health Security Detection Program

Molecular mechanisms of multidrug resistance in *Mycobacterium tuberculosis* isolated in South Korea

Directed by: Seok Hoon JEONG

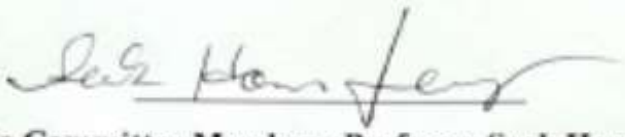
A Master's Thesis
Submitted to the Department of Global Health Security,
Division of Global Health Security Detection Program
and the Graduate School Public Health of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Public Health

Soro Gorgoh

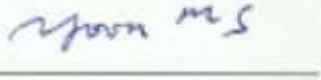
December 2019

This certifies that the Master's Thesis

of Soro Gorgoh is approved.



Thesis Committee Member : Professor Seok Hoon Jeong



Thesis Committee Member : Professor Moonsoo Yoon

Thesis Committee Member : Dr Eun Jeong Yoon

Graduate School of Public Health

Yonsei University

December 2019

ACKNOWLEDGMENT

I am grateful to Prof. Seok Hoon Jeong and Dr. Eun-Jeong Yoon for their scientific support and assistance.

I thank the Gangnam administration allowed me to use their facility for my research project.

I like to express my deep appreciation to Prof. Myung Ken Lee, for his wonderful academic guidance and support.

My sincere thanks go to Graduate School of Public Health, particularly the Department of Global Health Security, Yonsei University, South Korea.

Also, I want to thank KOICA for their financial support granted through the Master scholarship.

Finally, I must express my profound gratitude to my family and colleagues for providing continuous support and encouragement.

Contents

ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CDC	Centers for Disease Control and Prevention
DST	Drug susceptibility test
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
INH	Isoniazid
LAM	Latino-American and Mediterranean
MTB	*Mycobacterium tuberculosis*
MAS-PCR	Multi allele-specific PCR
MDDR	Molecular Detection of Drug-Resistant
MDR/RR-TB	Multidrug resistance Rifampicin Resistance TB
MTBDR	Drug-Resistant

PCR	Polymerase Chain Reaction
RIF	Rifampicin
RR-TB	Rifampicin-resistant TB
RRDR	rifampicin-resistance-determining region
TB	Tuberculosis
WHO	World Health Organization
XDR-TB	Extensively drug-resistant TB

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Background: Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB). Multidrug-resistant tuberculosis (MDR-TB) caused by MTB resistant to the first-line anti-TB drugs, rifampicin, and Isoniazid poses a major challenge to treat TB. The World Health Organization (WHO) reported in 2018 that 10 million people developed TB and at the same time, the global burden of MDR-TB was estimated to be 558,000 cases leading to 240,000 deaths. The objective of this study is to gain firm scientific evidence for a diagnostic strategy for MDR-TB reflecting the current situation. This study assessed the molecular mechanism of resistance to rifampicin and Isoniazid for the latest MTB isolates.

Method: A total of 54 MTB isolates from pulmonary specimens (n = 42), and other (n = 12) was collected from Gangnam Severance Hospital, South Korea from September 2018 to February 2019. DNA was extracted from liquid culture by boiling method and used for PCR. Partial and katG *genes* were amplified by using two pairs of gene-specific primers and directly sequenced . The translated nucleotide sequence was aligned by using the Basic Local Alignment Search Tool and the alterations were determined. The results were compared with the susceptibility testing results of the two drugs.

Results: One isolate (1.85%) was resistant to both rifampicin and Isoniazid and the other 4 isolates (7.40%) were resistant to only Isoniazid. Aligning the nucleotide sequences obtained from the PCR amplicons of 397-bp rpoB and 727-bp katG is assessed. Among the 54 isolates, only 5 isolates were resistant to rifampicin or Isoniazid or both. Of those, mutation in RpoB was identified in one isolate carried mutation in 508Leu to Gly and 515Ser to Leu. Mutations in KatG were identified in 5 (9.25%) resistant isolates, of which 3 carried a mutation in 335Ile to Ser, Pro, or Leu and 2 remaining carried mutations in 315Ser to Thr.

Conclusion: This study shows the importance of new diagnostic methods covering up the news mutations and alterations of amino acid of *KatG* and *RpoB* gene in South Korea along with the continued amplifications-based diagnostic for rapid detection of Isoniazid and rifampicin isolates resistance.

CHAPTER I

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB). The multidrug-resistant MTB (MDR-TB) refers to MTB resistant to the first-line anti-TB drugs, rifampicin, and Isoniazid, infections caused by the MDR-TB poses a major challenge to treat patients with TB. The World Health Organization (WHO) reported that 10 million people developed TB. About 1.7 billion people, 23% of the world's population, are estimated to have a latent TB infection and are thus at risk of developing active TB disease during their lifetime [1].

Drug-resistant TB continues to be a public health crisis. The global burden of rifampicin-resistant TB (RR-TB) was estimated to be 558,000 cases (ranged as 483,000 – 639,000) leading 240,000 deaths in 2017, of these, 82% MDR-TB. India (24%), China (13%) and the Russian Federation (10%) are notorious to have a high rate of RR-TB. Globally, 3.5% are new TB cases and 18% of previously treated cases had MDR/RR-TB. The highest proportions (>50% in previously treated cases) are in countries of the former Soviet Union. Among cases of MDR-TB in 2017, 8.5% (95% confidence interval, 6.2–11) were estimated to have extensively drug-resistant TB (XDR-TB), which is MTB resistant to one or more quinolone drugs and one or more of three injectable drugs, such as capreomycin, kanamycin and amikacin [1].

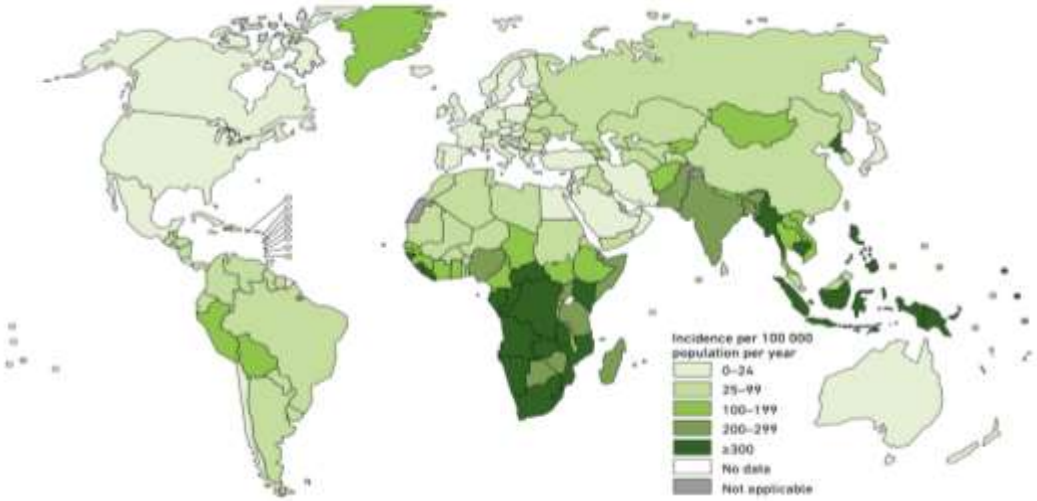


Figure 1. Estimated TB incidences in 2017 [1]

Numbers of incidences per 100 000 populations are indicated as heatmap

Africa 237; Southeast Asia 226; Eastern Mediterranean 113; Western Pacific 94; Europe 30; and The Americas 28.

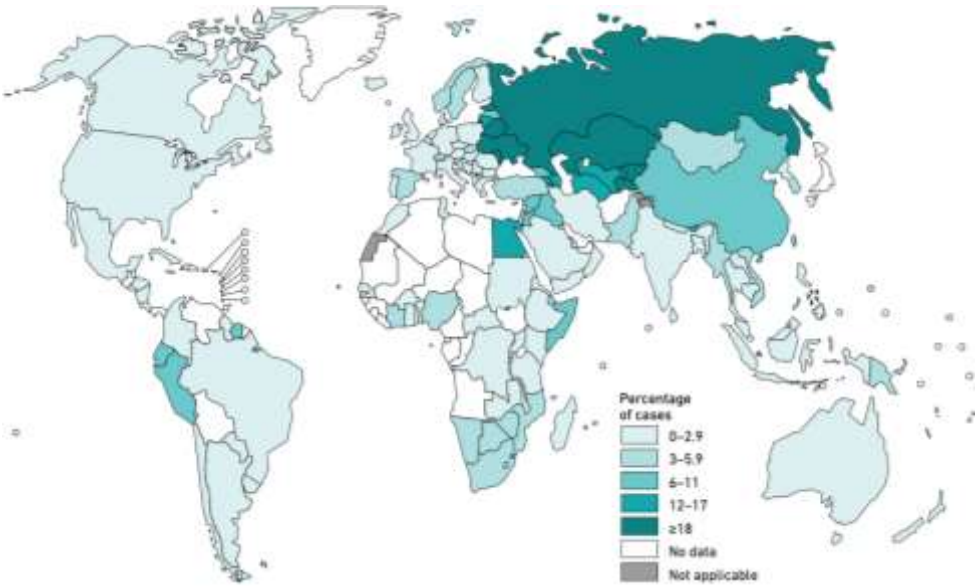


Figure 2. Percentage of MDR/RR-TB among the new TB cases in 2017 [1]

Percentages of the resistant TB are indicated as a heatmap

Europe 7.1%; Eastern Mediterranean 3.8 %; Southeast Asia 3.4%; Africa 2.4 %; Western Pacific 2.1%; and The Americas 1.9 %.

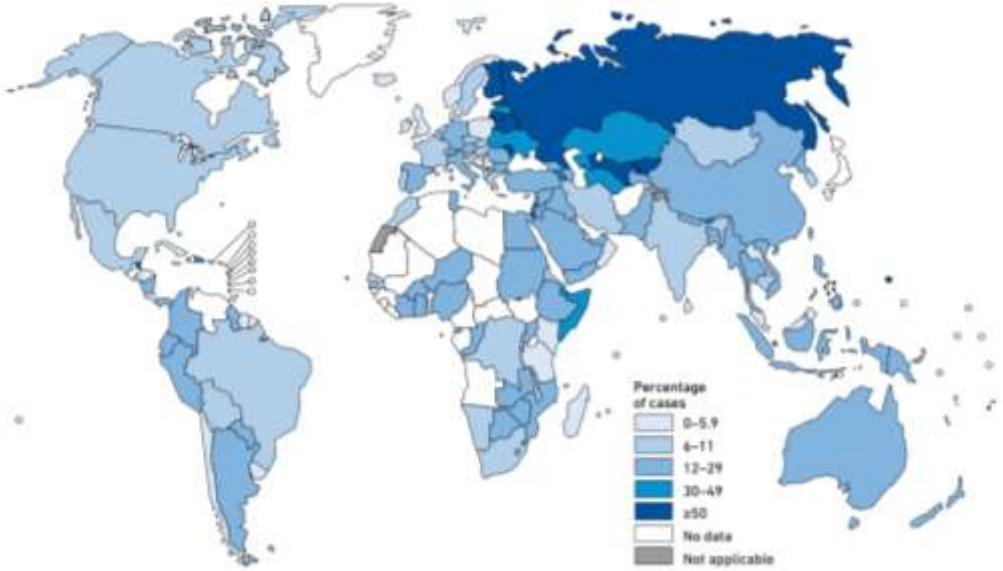


Figure 3. Percentage of MDR/RR-TB among the previously treated TB cases in 2017 [1]

Percentages of the resistant TB are indicated as a heatmap

Europe 21 %; Eastern Mediterranean 21 %; Southeast Asia 19 %; Africa 15%; Western Pacific 14 % and The Americas 14 %.

Drug susceptibility test (DST) is required for the definitive diagnosis of MDR-TB or XDR-TB, which is the conventional way to do this through culture-based methods [2].

MTB is isolated from patient sputum and then tested for growth in the presence of anti-TB drugs. Culture-based methods take weeks to months. They are also expensive and difficult to master, making them mostly unavailable in resource-limited settings. Molecular methods mostly the polymerase chain reaction (PCR) techniques, detect the genetic mutations conferring resistance to drugs. Commercially available systems include Gene Xpert System (Xpert MTB/RIF, Cepheid, USA), Genotype MTBDR plus and MTBDRsl assays (Hain Life science GmbH, Germany), and INNO-LiPA Rif.TB line probe assay (Inno-genetics Inc., Belgium), but there are other systems in development. Molecular methods of DST give results much faster than culture-based methods. Some commercially available systems are almost fully automated and require little training. For these reasons, these systems are increasingly the method of choice for DST in resource-limited settings [2].

Molecular tests can detect mutations conferring low levels of resistance that are not recognized by culture-based assays but are still clinically significant. Molecular tests also fail to detect all mutations known to confer resistance to a drug. Nevertheless, it is not necessary to systematically confirm the results of molecular DST with culture-based DST, although clinicians may choose to do so if the clinical picture warrants it. Specifically, a positive molecular test for rifampicin resistance may be considered a diagnosis of MDR-TB, as, in most countries, more than 90% of rifampicin-resistant strains are also resistant to isoniazid [2].

The GeneXpert system for sputum nucleic acid amplification assays and molecular detection of rifampin resistance was approved by the Food and Drug Administration (FDA) in 2013 and is available in many public health laboratories. Besides, the United States Tuberculosis Control Laboratory (CDC) conducts rapid molecular drug resistance testing for first-line drugs and numerous second-line drugs on sputum sediment and MTB isolates [3].

For many years, it was thought that isoniazid (INH) kills the largest subpopulation of exponentially growing bacilli during the first three days of treatment and that, once depleted, INH does not is more effective. An in vitro infection model showed that, although the earliest bactericidal activity decreased by 72 hours, the result is better explained by the emergence of drug-resistant isolates, which experienced exponential phase growth. Resistance to INH can be conferred by modifications of the katG and / or *inhA* genes. The katG and / or inhA mutations account for 85-90% of the INH resistance reported by the CDC Molecular Drug Resistance Detection (MDDR) service [3].

It was observed in the 1950s that MTB strains that do not produce catalase are generally resistant to INH. This discovery led to the hypothesis that INH is a prodrug requiring modification of the catalase in its functional form. Subsequently, suppression or mutation of the catalase gene (katG) is responsible for 10 to 25% of cases of INH resistance. Such a mutation would also reduce the survival of the organism, without the compensatory hyper expression of alkyl hydroxyl peroxidase to protect against the toxic effect of organic peroxides [3].

Rifampicin is the cornerstone of short-course chemotherapy regimens, so rifampin resistance prolongs and complicates treatment. Rifampin is thought to act against MTB by binding to RNA polymerase, resulting in interference with transcription and RNA prolongation. Mutations in the *rpoB* gene, which encodes the beta chain of mycobacterial RNA polymerase, have been found to cause clinical rifampin resistance [3].

In one report, one RpoB mutation was identified in 64 of the 66 resistant organisms in various geographic areas, but not in any of the 56 susceptible organisms. Resistance to rifampicin can be detected as quickly as four hours after receiving a sputum sample from the laboratory using the GeneXpert system available in most public health research laboratories in the United States. CDC's MDDR service also detects rpoB mutations associated with rifampicin resistance [3].

Our understanding of the molecular basis for drug resistance in MTB is being improving. This knowledge is important for new drug design, development of new rapid diagnostic tools for case tailored therapy based on specific dug resistance in the individual patient and creating new therapeutic strategies against drug-resistant TB [3].

In this study,

We assessed the latest MTB regarding the molecular mechanism of resistance to rifampicin and Isoniazid for further development of powerful diagnostic methods of MDR-TB reflecting the current clone. The resistance rates to rifampicin and Isoniazid were determined and any amino acid alterations either in RpoB or in KatG associated with the drug resistance.

CHAPTER II

Materials and Methods

Bacterial strains used in the study

A total of 54 deduplicated MTB strains was collected from Gangnam Severance Hospital, Seoul, South Korea for six months between from September 2018 to February 2019 are indicated in the **Table 1.**

The strains were recovered mostly from pulmonary specimens, such as sputum (n = 36), bronchial lavage fluids (n = 6), and few from others (n = 12). Positive for liquid culture used BD Mycobacteria Growth Indicator Tube system (MGIT, BD BBL, USA).

Table 1. MTB clinical strains used in the study

Specimens	Numbers
Pulmonary samples	
Sputum	36
Bronchial lavage fluids	6
Others	12
Total	54

Antimicrobial susceptibility test

Susceptibility test to Isoniazid and rifampicin was conducted using the absolute concentration method on Lowenstein–Jensen medium. Critical concentrations for resistance were 0.2 μg/mL for isoniazid and 40 μg/mL for rifampin [4,5].

DNA manipulation and PCR

After mixing the growth bacteria with an equal amount of sodium hydroxide, the culture was centrifuged to harvest the bacterial cells. The bacterial pellets were washed with phosphate- buffered saline, homogenized with DNA extraction buffer (Kogen Biotech) boiling for five minutes. PCR was carried out using the extracted DNA with primer pairs targeting the partial *rpoB* gene between na-1318 and na-1715 and the partial *katG* gene between na-288 and na-1015, following the 35 cycles of 7 sec at 97 °C, 20 sec at 61 °C and 30 sec at 72 ° C. Nucleotide sequences of the primers are indicated in **Table 2**.

Amplicons obtained from PCR were purified by using MG PCR product purification SV (MGmed, Seoul, Korea) and direct sequencing was conducted using each forward primer and total DNA was extracted.

Table 2. Primers used in the study

Target gene	Primer	Location	Nucleotide sequence (5'-3')	Tm (ºC)
rpoB	MTB_rpoB-F1318-1337	1318-1337	CTG TCG GGG TTG ACC CAC AA	62.5
	MTB_rpoB-R1715-1696	1715-1696	TAG TCC ACC TCA GAC GAG GG	62.5
katG	MTB_katG-F288-307	288-307	CCA CTA CGG GCC GCT GTT TA	62.5
	MTB_katG-R1015-996	1015-996	AGC CGT ACA GGA TCT CGA GG	62.5

Observe alignment of amino acid sequences and identification of the substitutions

The obtained nucleotide sequences were aligned against the amino acid sequences of RpoB (GenBank accession CCP43410.1) and KatG (GenBank accession, CCP44675) extracted from wild type *Mycobacterium tuberculosis* H37Rv strain (GenBank accession, AL123456) by using BLASTX, and the nucleotide substitution resulting in amino acid sequence alteration was determined.

CHAPTER III

RESULTS

1. Rates of resistance to rifampicin and Isoniazid

A total of 54 MTB was recovered mostly from pulmonary specimens (n = 42), such as sputum (n = 36), bronchial (n = 5), pleural (n = 1), and others (n = 12). Of those, one (1.85%) isolate was resistant to rifampicin and Isoniazid and the other four (7.40%) isolates were resistant only to Isoniazid.

2. Alterations occurred either in RpoB or in KatG

Aligning the nucleotide sequences obtained from the PCR amplicons of the 397-bp rpoB gene and the 727-bp katG gene are assessed. Among the 54 isolates, only 5 isolates were resistant to rifampicin or Isoniazid or both. Mutations in RpoB was identified in one isolate carried mutation both in 508Leu to Gly and 515Ser to Leu. Mutations in *KatG* were identified in 5 (9.25%) resistant isolates, of which 3 carried a mutation in 335Ile to Ser, Pro, or Leu and 2 remaining carried mutation in 315Ser to Thr (**Table 3**).

Table 3 – Alterations occurred in RpoB and KatG

Protein	Codon	Amino-acid replacement	Nucleotide replacement	N (total 54)
RpoB	508 Leu	Gly	CTG to GGG	1
	515 Ser	Leu	TGC to TTG	
KatG	335 Ile	Ser	ATC to TTC	3
		Pro	ATC to CCC	
		Leu	ATC to CTC	
	315 Ser	Thr	TGC to ACC	2

3. Correlation between the amino acid alterations and resistance to the drugs

Mutations conferring resistance to rifampin in rifampin-resistant clinical MTB isolates occur mostly in the 81 bp region (codons 507—533, 27amino acids) of the *rpoB* gene. The mutation was substitution in two codons 508 and 515 of the *rpoB* gene which was found in 1 (1.85%) of the 54 isolates. Of the 5 MTB isolates of Isoniazid or rifampicin resistance, 2 (3.70%) carried mutations in KatG amino acid alteration at KatG Ser315, which has been reported to confer high-level INH

resistance. Three (5.55%) had mutations in KatG at Ile335.

CHAPTER IV

DISCUSSION

Except for a few developed countries, most national TB programs worldwide do not routinely provide diagnostic services based on culture and DST. The laboratory is an essential component in TB control programs, and broader access to DST is a priority for most countries. The early choice of appropriate treatment is an essential determinant of a favorable outcome, and rapid determination of drug resistance can allow a customized approach to treatment early during the disease and can potentially reduce morbidity, mortality, and infectiousness. The diagnosis of MDR-TB and XDR-TB is hampered by the absence of effective and affordable rapid diagnostic techniques for drug sensitivity. Several approaches, phenotypic and molecular, have been explored to develop rapid, reliable and accurate methods for the rapid detection of drug resistance in MTB. These methods should also be evaluated and applied in high-incidence areas [6].

In this study, the molecular method has been used to document amino acid substitutions occurring at the rifampicin-resistance-determining region (RRDR) in the beta subunit of RNA polymerase associated with rifampicin resistance in MTB and those in the catalase-peroxidase (KatG) resulting in Isoniazid resistance.

Resistance to both Isoniazid and rifampicin were found in 5 (9.25%) of 54 MTB isolates. Resistance related to drug were identified to one isolate for rifampicin and Isoniazid and 4 (4.70%) isolates only for Isoniazid.

The results were not in accordance with the previous studies reported in Russia and Iran. This discordance of frequencies found in rifampicin and Isoniazid resistance could probably be explained by a biased-on isolates selection. Indeed, Lipin and the colleagues in Russia found resistance to both Isoniazid and rifampicin in 217(69.55 %) of 312 MTB isolates belonging to different spoligotype families such as Beijing or Latino-American and Mediterranean (LMA). Most of the MTB isolates were obtained from chronic patients 241(77.2%) and from different families of MTB which probably explains the high numbers of MTB resistance isolates for both rifampicin and isoniazid [7].

Similarly, Azar Dokht Khosravia and colleagues in Iran reported that the most common mutations related to drug resistance were demonstrated as rifampicin 12 (15%) and isoniazid 11 (13.7%) among a total of 80 clinical MTB isolates from the TB reference laboratory of Khuzestan. Furthermore, the diagnostic techniques using LJ culture and PCR sequencing were like the present study. Certainly, the difference of results could be

essentially attributed to the numbers of MTB included in the study [8].

In a current study, mutation in KatG was identified in 5 (9.25%) isolates carried a mutation in codon 315 and 335. The mutation in RpoB was identified in one isolate, with mutations in codon 508 and 515.

Mutation in KatG at codon 315 and codon 335 have been described in many studies. The frequently detected mutation in clinical isolate at KatG at codon 315 (Ser to Thr) has been associated with resistance to Isoniazid therapy. As has been observed previously, the mutation in codon 315 of the KatG gene has been found in the most Isoniazid resistance isolates worldwide (Russia and Africa).

In Russia, Spoligotype, IS6110 restriction fragment length polymorphism typing, and sequencing of the KatG and RpoB genes performed for 217 consecutive MDR MTB isolates from patients, found mutations in the KatG in 207 (95%) isolates, all of which had mutations in codon 315 [7].

Similarly, the study from different geographical in Africa (South, Central, and West), on the detection of KatG gene mutations in MTB, 212 African strains of the MTB complex selected isolates. Of which, 124 INH resistant and 88 susceptible strains of Mycobacterium tuberculosis complex were analyzed by direct sequence analysis and PCR-restriction fragment length polymorphism analysis of their catalase-peroxidase (*katG*) genes. Point mutations at codon 315 were found in the genomes of 64% of INH-resistant strains [9].

Our results were similar to those reported in Russia and Africa showing the accuracy of the detection of mutation particularly at codon gene 315.

Mutation occurring in *katG* at 335, being outside of the region the sequencing region of KatG and thus having a low profile of resistance, has also been reported in some studies. Probably the finding was linked to Isoniazid resistance with not strong evidence.

The mutation identified in RpoB at amino acid 508 and 515 has been reported to be a less common mutation to rifampicin resistance. However, in different studies worldwide, on detection o*f* RpoB using either PCR sequencing or MAS-PCR, the RpoB 531, 526 and 516 have been reported to be the most frequently mutated. In Russia, Mutations in the RpoB gene were identified in 200 (92%) isolates; a mutation in codon 516 was carried in 75% of LAM isolates and 71% of Beijing isolates carried a mutation in codon 531 [7].

Like Iran, the reported mutations showed that MAS-PCR is on accordance with PCR-sequencing with high sensitivity and specificity for katG 315 and *RpoB* (531, 516, and 526**)** [8].

Alteration amino acid at the *KatG* gene at codon 315 from TGC (Ser) to ACC (Thr) has been described in many studies. Whereas, alterations at codon 335 ATC (Ile) to TTC (Ser), CCC (Pro) and CTC (Leu) have not been described in previous studies.

The frequently detected alteration in clinical isolate in KatG at codon 315 (Ser) to (Thr) has been associated with resistance to Isoniazid therapy [7,9,10, 11, 12].

Alteration of amino acid occurring in katG at codon 335 from ATC (Ile) to TTC (Ser), CCC (Pro) and CTC (Leu), which is outside the region the sequencing region of KatG has not been found in previous studies.

Probably these new findings were linked to Isoniazid resistance with not strong evidence, but the structural mutation missense has structural effects in katG protein.

In this study, new allele mutations have been reported from CTG (Leu) to GGG (Gly) at 508 and from TGC (Ser) to TTG (Leu) at 515. The evidence of new allele mutations in this study indicates that mutations continue to arise, probably due to the ability of MTB to adapt to drug exposure.

CHAPTER V

CONCLUSION

Overall, the current study detected the previously defined and novel mutations associated with rifampicin and Isoniazid, which are important for TB treatment. This study emphasized the importance of updating the molecular diagnostic methods covering up the new alterations in the *katG* and the *rpoB* genes in South Korea.

REFERENCES

1. World Health Organization. (2018) Global Tuberculosis Report 2018. World Health Organization, Geneva.
2. Seung, K. J., Keshavjee, S., & Rich, M. L. (2015). Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis. Cold Spring Harbor perspectives in medicine, 5(9), a017863. doi:10.1101/superset. a017863.
3. Neil W Schluger, C Fordham von Reyn, Elinor L Baron (2018). Epidemiology and molecular mechanisms of drug-resistant tuberculosis. Official reprint from UpToDate.www.uptodate.com ©2019 UpToDate, Inc. and/or its affiliates.
4. Canetti, G., Fox, W., Khomenko, A., Mahler, H. T., Menon, N. K., Mitchison, D. A., Smelev, N. A. (1969). Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programs. *Bulletin of the World Health Organization, 41*(1), 21–43.
5. World Health Organization. Companion Handbook to the WHO Guidelines for the Programmatic Management of Drug-resistant Tuberculosis. Geneva: World Health Organization; 2014.
6. Seung, K. J., Keshavjee, S., & Rich, M. L. (2015). Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis. Cold Spring Harbor perspectives in medicine, 5(9), a017863. doi:10.1101/cshperspect. a017863.

7. M. Y. Lipin; V. N. Stepanshina; I. G. Shemyakin; T. M. Shinnick (2007) Association of specific mutations in katG, rpoB, rpsL and rrs genes with spoligotypes of multidrug-resistant Mycobacterium tuberculosis isolates in Russia. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases, ISSN: 1198-743X, Vol: 13, Issue: 6, Page: 620-6. 2007.
8. Azar Dokht Khosravia,b, Hamed Goodarzia*, Seyed Mohammad Alavib, Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences (AJUMS), Ahvaz, Iran Infectious and Tropical Diseases Research Center, AJUMS, Ahvaz, Iran Infectious Disease Ward, Razi Teaching Hospital AJUMS, Ahvaz, Iran.
9. Haas, W. H., Schilke, K., Brand, J., Amthor, B., Weyer, K., Fourie, P. B., Bremer, H. J. (1997). Molecular analysis of katG gene mutations in strains of Mycobacterium tuberculosis complex from Africa. Antimicrobial agents and chemotherapy, 41(7), 1601–1603.
10. Jnawali, Hum Nath; Hwang, Sung Chul; Park, Young Kil; Kim, Hyejin; Lee, Yeong Seon; Chung, Gyung Tae; Choe, Kang Hyeon; Ryoo, Sungweon(2013) Characterization of mutations in multi- and extensive drug resistance among strains of *Mycobacterium tuberculosis* clinical isolates in Republic of Korea. Diagnostic microbiology and infectious disease, ISSN: 1879-0070, Vol: 76, Issue: 2, Page: 187- 96.2013.

11. Sajduda, A., Brzostek, A., Poplawska, M., Augustynowicz-Kopec, E., Zwolska, Z., Niemann, S. … Hillemann, D. (2004). Molecular characterization of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Poland. Journal of clinical microbiology, 42(6), 2425–2431. doi:10.1128/JCM.42.6.2425-2431.2004.
12. Takawira, F. T., Mandishora, R., Dhlamini, Z., Munemo, E., & Stray-Pedersen, B. (2017). Mutations in *rpoB* and *katG* genes of multidrug- resistant *mycobacterium tuberculosis* undetectable using genotyping diagnostic methods. The Pan African medical journal, 27, 145. doi:10.11604/pamj.2017.27.145.10883.