Molecular analysis of drug resistance associating gene mutations in *Mycobacterium tuberculosis* clinical isolates from Nepal

(ネパールで分離された結核菌の薬剤耐性関連遺伝子変異の解析**)**

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CONTENTS

CHAPTER II

Characterizations of pyrazinamide resistance associating *pncA* gene mutations in *Mycobacterium tuberculosis* isolates from Nepal

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CAS	Central and Middle Eastern Asian
DNA	Deoxyribonucleic acid
DOTS	Direct Observed Therapy, Short-course
DRS	Drug resistance surveillance
DR-TB	Drug resistant tuberculosis
DS-TB	Drug susceptible tuberculosis
DST	Drug Susceptibility Testing
EAI	East African Indian
EMB	Ethambutol
FLDs	First-line drugs
GENETUP	German Nepal Tuberculosis Project
gidB	Gene encoding m7G methyl transferase
HRE^r	Phenotypic resistance to isoniazid, rifampicin and ethambutol
HR^r	Phenotypic resistance to isoniazid and rifampicin
$HRSE^r$	Phenotypic resistance to isoniazid, rifampicin, streptomycin and ethambutol
$HRSE^s$	Phenotypic susceptibe to isoniazid, rifampicin, streptomycin and ethambutol
HRS^r	Phenotypic resistance to isoniazid, rifampicin and streptomycin
Indel	Insertion or deletion of nucleotide
INH	Isoniazid
inhA	Gene encoding enoyl-acyl carrier protein reductase
katG	Gene encoding catalase-peroxidase
LAM	Latin American and Mediterranean
LPA	Line Probe Assay
LSP	Large Sequence Polymorphism
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MTB	*Mycobacterium tuberculosis*
NTP	National Tuberculosis Program
NTRL	National TB Reference Laboratory
PCR	Polymerase chain reaction
pncA	Gene encoding pyrazinamidase
POA	Pyrazinoic acid
Pre-XDR TB	Pre-extensively drug resistant tuberculosis
PZA	Pyrazinamide

PZase	Pyrazinamidase
PZA-R	Pyrazinamide resistance
PZA-S	Pyrazinamide susceptible
RIF	Rifampicin
rpoB	Gene encoding RNA polymerase β-subunit
rpsL	Gene encoding ribosomal protein S12
RR-TB	Rifampicin resistant tuberculosis
rrs	Gene encoding 16S ribosomal RNA
SIRE	A combination of STR, INH, RIF and EMB
STR	Streptomycin
TB	Tuberculosis
URR	Upstream regulatory region
WHO	World Health Organization
WT	Wild type
XDR-TB	Extensively drug resistant tuberculosis

PREFACE

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (MTB), is a major cause of public health problems, one of the top ten causes of death worldwide, and the leading cause of death from a single infectious agent. According to the Global Tuberculosis Report, World Health Organization (WHO) estimated 10.0 million (range, 8.8-11.0 million) new cases in 2019. There were 1.2 million (range, 1.1-1.3 million) TB deaths among HIV-negative people and an additional ~0.2 million deaths (range, 0.18-0.24 million) among HIV-positive people [1]. TB affects people of both sexes and all age groups, but the highest burden is in adult men, who accounted for 56% of all TB cases in 2019; by comparison, adult women accounted for 32% and children for 12%. Among all TB cases, 8.2% were among people living with HIV [1].
Geographically, in 2019, most TB cases were in the WHO regions of South-East Asia (44%), Africa (25%), and the Western Pacific (18%), with smaller shares in the Eastern Mediterranean (8.2%), the Americas (2.9%) and Europe (2.5%). The 30 high TB burden countries accounted for 86% of all estimated incident cases worldwide, and eight of these countries accounted for two-thirds of the global total: India (26%), Indonesia (8.5%), China (8.4%), the Philippines (6.0%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%) and South Africa (3.6%) (Figure 1). The severity of national TB epidemics in terms of the annual number of incident TB cases relative to population size (the incidence rate), varied widely among countries in 2019 (Figure 2).

For the effective treatment of TB, first-line drugs (FLDs) including isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA) are used [2]. Treatment of drug susceptible TB (DS-TB) consists of a 2-months ‘intensive’ phase with INH, RIF, EMB, and PZA, followed by a 4-months ‘continuation’ phase with INH and RIF [1],[3].

With an increasing number of drug-resistant TB (DR-TB) each year, effective treatment of TB possesses challenges. Multi-drug resistant TB (MDR-TB), which is defined as TB resistant to at least two potent anti-TB drugs, isoniazid (INH) and rifampicin (RIF), is a serious threat to fight against TB [2],[3]. Extensively drug-resistant TB (XDR-TB) which is MDR-TB strains that have developed resistance to any fluoroquinolones and one of the injectable aminoglycosides used in anti-TB treatment, compromise the control of TB globally. The WHO estimated that, worldwide, in 2019, there were an estimated ~0.46 million (range, 0.40-0.50 million) incident cases of rifampicin-resistant TB (RR-TB), and of this, 78% had MDR-TB. The largest burdens of these cases were in India (27%), China (14%), and the Russian Federation (8%). As stated by WHO, an estimated 3.3% of new TB cases and 18% of previously treated cases had MDR/RR-TB (Figure 3). Furthermore, about 8.5% of MDR-TB cases were extensively drug-resistant tuberculosis (XDR-TB) [1],[2].

Nepal lies in between the high TB burden countries China and India. It is responsible for ill-health among thousands of people each year and is ranked as the seventh leading cause of death in Nepal [4]. Nepal's National TB Program (NTP) registered 32,043 cases of TB with an estimated annual case notification rate of 112/100,000 [4]. However, WHO estimated an incidence rate of 238/100,000 and a death rate of 58.77/100,000 for Nepal, indicating that there are missing cases in the community [1],[4].

Nepal has set an ambitious vision of ending TB in Nepal by 2050 in accordance with the National Health Policy 2014 and under the strategic direction of the worldwide initiative to end TB – The End TB Strategy. Despite the best efforts of the program is trying to find and cure more TB cases in Nepal, the TB case notification, as well as estimated incidence, has been stagnant for more than decades now in Nepal [4]. MDR-TB is another challenge for the country. The proportion of MDR-TB was 2.2% among new cases and 15.4% among retreatment cases based on DRS survey carries out in 2011/12. The routine surveillance showed a much higher proportion of drug-resistant pattern among second-line drugs used for the treatment of MDR patients in Nepal. Despite all the challenges, Nepal sustained treatment success rates of 90% or more for DS-TB and 70% or more for DR-TB cases. Significant challenges still lie in finding those missing cases and managing TB through quality and equitable services.

Early detection of the pathogen is extremely crucial to diagnose TB. The acid-fast bacilli (AFB) smear microscopy is the foremost frequently used microbiological test for detection TB in developing countries. However, it is not very helpful for follow up patients because it does not differentiate between live and dead bacilli. In this method, the sputum specimens are smeared directly on the clean glass slides which is subjected to Ziehl-Neelsen (ZN) staining. Although it is the fast, less expensive and simple method, it detects roughly 50% of all active cases of TB. A definitive diagnosis of TB can only be made by culturing MTB organisms from a specimen taken from the patients. MTB organisms can be isolated from sputum (most often), pus, cerebrospinal fluid (CSF), biopsied tissue, etc. Conventional culture-based solid media referred to as Löwenstein-Jensen (LJ) media remains the gold standard method for identification of MTB and is the most sensitive diagnostic techniques. However, conventional culture with solid LJ media might take up to eight weeks for mycobacterial growth because of slow growth of mycobacteria. In addition, it might take about six weeks for the drug susceptibility testing (DST) results. Conventional method using LJ media for culture and DST is a time-consuming, tedious and require skilled technician to perform [3],[4].

For the rapid treatment and interruption of transmission of DR-TB, rapid detection is essential before the implementation of appropriate drugs. The time consuming conventional proportional method using LJ medium is the only available DST in Nepal. Recently, NTP has adopted the latest diagnostic tools such as Xpert MTB/RIF, Line probe assay (LPA), and

Mycobacterium Growth Indicator Tube (MGIT). Rapid DST with Xpert MTB/RIF testing is expanding through GeneXpert centers (at-least 1 center per district by 2021). Nepal also planned at-least 1 Lab with LPA and culture services per province by 2021 which will be managed and supported through 2 National Reference Laboratories [4].

The molecular techniques for DST might be more effective for the rapid determination of drug-susceptibility in MTB, but it is currently unavailable in a resource-limited laboratory in Nepal. DNA sequencing could predict/provide/detect the drug resistance-associating gene mutations in a short duration of time, which can be used for effective and early identification of drug-resistance TB patients. In addition, poor management of TB results in death and creates DR-TB which is very difficult and costly to treat resulting in often poorer outcomes [4]. Therefore, it is important to establish easy, accessible, reliable, and rapid diagnostic tools for drug-resistant TB that can improve TB-control and interrupt DR-TB transmission in the communities. Molecular analysis of the target drug resistance-associating genes has been performed in order to determine the DR-TB rapidly for the proper implementation of appropriate drugs for the DR-TB treatment. Detection and determination of phenotypic and genotypic MDR-TB isolates have limited studies in Nepal and rises the gaps between detection and treatment. Gaps between the estimated number of new cases and the number reported are due to a mixture of underreporting of detected cases and underdiagnoses which is still a major programmatic gap of the TB program [4]. A gap could be averted through early diagnosis and treatment. This study was focused on frequency and patterns in drug-resistance-associating gene mutations which can contribute to policymaking on proper management of DR-TB treatment through early diagnosis and appropriate DR-TB treatment in Nepal.

Research on phenotypic and genotypic DST for FLDs (STR, INH, RIF, EMB, and PZA) have been studied worldwide. However, limited studies have been performed on phenotypic DST for STR in resource-limited laboratory of Nepal. According to our knowledge, studies on genotypic DST for STR; phenotypic and genotypic DST for PZA have not been studied yet in Nepal. Therefore, I have chosen two drugs STR and PZA for my Ph.D. study in order to collect the information regarding genotypic studies on STR resistance and PZA resistance in MTB isolates from Nepal.

Streptomycin (STR) has been an important drug for the treatment of pulmonary TB [5]. For the retreatment TB patients in Nepal, STR has been included in the intensive phase for two months with other first-line anti-TB drugs (FLDs) [6]. However, NTP no longer recommends STR to be used in retreatment regimens in Nepal [4]. According to the WHO-recommended regimen as published in "WHO treatment guidelines for MDR and RR-TB, 2018 Pre-final test" [7] and updated guidelines in "WHO consolidated guidelines on drug-resistant tuberculosis treatment, 2019" [3], STR is categorized into Group C agents in which it may be included in the treatment of

MDR/RR-TB patients aged 18 years or more on longer regimens when susceptibility has been demonstrated and adequate measures to monitor for adverse reaction can be ensured if amikacin is not available [4]. To move into the new WHO recommendation, NTP will develop a comprehensive transition plan [4]. With an increment of MDR-TB cases, and in addition of MDR-TB isolates resistant to second-line drugs and newly discovered drugs, STR should be used in combination with other drugs for MDR-TB treatment regimen. Despite its high rate of side effects and risk of developing drug resistance in TB of its single-use in a regimen [5], it is crucial to understand the molecular mechanism of resistance to STR in MTB from Nepal.

STR impairs translational proofreading, leading to the blockade of protein synthesis. Resistance to STR is associated with mutations in *rpsL* and *rrs* genes, encoding for ribosomal protein S12 and 16S ribosomal RNA, respectively. The most frequent mutations localize around two regions of *rrs*, namely the 530 loop and the 912 region, or at codons 43 or 88 within *rpsL* [8],[9]. Amino acid substitutions in the S12 protein affect the STR binding to 16S rRNA by distorting the stabilization of the high conserved structure of 16S rRNA. Alterations in the 16S rRNA cause lower affinity for STR. In particular, *rrs* mutation in 530 loop or 912 region and mutation in *rpsL* codon 43 or 88 have been reported to confer high-level or intermediate STR resistance in MTB [8],[9]. Another gene *gidB* encodes a conserved 7-methylguanosine methyltransferase or GidB, which methylates the nucleoside position 518 of the 16S rRNA to which STR directly interacts and also located 530 stem-loop pseudoknot region of the 16S rRNA site. Mutations within the *gidB* may likely alter GidB's methyltransferase function which causes changes in the methylation status of the 16S rRNA at position 518G, leading to low affinity of STR binds to the position 518G, and consequently present with STR resistance phenotype in clinical isolates. Mutations in *gidB* have been reported to confer low-level STR-resistance in MTB [10].

In addition, the type and frequency of STR resistance-associated gene mutations have been varied according to the geographical region [8-24]. For example, mutations in *rpsL* were relatively higher in Asia [8-11],[25], in which K43R mutation was found to be linked with the MTB Beijing genotype [22],[26],[27]. Similarly, mutations in *rrs* were relatively higher in Europe and America [28-30]. However, most of the *gidB* alterations have been found to be linked with either STR resistance or specific genotypes of MTB [9],[27]. Since STR resistance-associated genes vary geographically, it is important to perform molecular studies which will detect the common mutations in target genes conferring STR resistance among MTB strains [10],[11].

The BACTEC MGIT 960 method has been widely validated for reliable and rapid testing of the susceptibility of MTB isolates to FLDs such as STR, INH, RIF, and EMB (a combination known as SIRE). Although the WHO has endorsed the BACTEC MGIT 960 (SIRE) for rapid diagnose of STR resistance, a high contamination rate has been reported [3]. The time consuming

conventional proportional method using Löwenstein-Jensen medium is the only available drug susceptibility testing (DST) for STR in Nepal. Recently, NTP has adopted the latest diagnostic tools such as Xpert MTB/RIF, Line probe assay (LPA), and Mycobacteria Growth Indicator Tube (MGIT) [3],[4]. Despite the high number of MDR-TB cases with STR-resistant MTB strains, no genetic information regarding the molecular mechanism of STR-resistant MTB in Nepal is currently available. Therefore, it is important to understand the underlying molecular mechanism of STR resistance for the detection of STR susceptibility through the development of a rapid diagnostic kit. As some of the specific mutations in *rpsL*, *rrs*, and *gidB* associated with STR resistance have been familiarized as high-specific predictive markers of STR resistance, it is important to elucidate and analyze the prevalence of each specific mutation among MTB isolates.

Pyrazinamide (PZA) is an important first-line anti-TB drug that has a preferred sterilizing activity against non-replicating persistent bacilli. It is used in the initial intensive phase of chemotherapy, in combination with other FLDs isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) for both drug-susceptible and drug-resistant TB. It shortens the treatment period of susceptible, MDR, and pre-extensively drug-resistant TB (Pre-XDR-TB)/XDR-TB [2]. Despite the important role of PZA in TB treatment, PZA-resistance in MTB has increased in both susceptible and MDR-TB. The estimated global burden of new PZA-resistant TB cases annually is 1.4 million cases, of which 270,000 cases occur in MDR-TB patients [31]. PZA is recommended in the initial phase of the TB treatment regimen for new cases and retreatment cases of all TB categories in Nepal [4].

PZA being an indispensable drug for all TB-treatment regimen, it is important to understand the PZA susceptibility accurately on time and monitor the spread of drug-resistance strains. A conventional method of DST using solid LJ media takes a long turnaround time of four to six weeks, besides culture. Another method of determining phenotypic DST is using liquid media by the modified BACTEC 460TB (B460) system or the BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system, which has the advantage of having results in two to three weeks. The modified B460 system is a radiometric, automated system and has been widely validated for reliable and rapid testing of the susceptibility of MTB isolates to FLDs front-line drugs such as SIRE and PZA. However, the MGIT 960 system is a nonradiometric, fully automated, continuous-monitoring system, and has been used as an alternative to the radiometric B460 system. Studies have reported the performance of the automated MGIT 960 system for testing of susceptibility to FLDs: SIRE and PZA [32-36]. The MGIT system uses a glass tube containing 7H9 broth and fluorescence quenching oxygen. The MGIT 960 instrument detects an increase in fluorescence caused by growing bacterial consumption of oxygen [2],[4]. Since PZA requires an acidic medium or environment for its function, the phenotypic PZA susceptibility testing is technically difficult to perform because the bactericidal activity of this drug is optimal

only in an acid environment that inhibits the growth of most of the MTB isolates [37]. For testing PZA susceptibility, the WHO recommends the performance of the assay in liquid medium at pH 5.9 in the BACTEC MGIT 960 system (Becton, Dickinson, Sparks, MD, USA) [38]. Although TB reference lab at the German Nepal Tuberculosis Project (GENETUP) has a BACTEC MGIT 960 system, a conventional culture-based PZA susceptibility testing is not routinely performed in Nepal due to the lack of reliability and cost of culture medium. There is no previous report on PZA susceptibility testing studies in Nepal. Despite the lack of testing, PZA is inappropriately being used to treat patients without confirming the PZA susceptibility.

PZA, an analog of nicotinamide, is a pro-drug which enters the bacteria through passive diffusion and is converted into its active form pyrazinoic acid (POA), by the non-essential enzyme pyrazinamidase (PZase), encoded by the *pncA* gene [39],[40]. It especially affects on semi-dormant tubercle bacilli which survive in an acidic environment of inflammatory sites that cannot be affected by other anti-TB drugs. The detailed mechanism of action of PZA is not well known and it is believed that an acidic medium is needed to activate the action of PZA (Figure 4). POA has functioned in the neutral pH in the cytoplasm which has no activity against MTB. By the efflux pump, POA is pumped out of the cell which has acidic environments and then protonated to HPOA which re-enters the cell, and accumulation of HPOA can eventually cause rupture of membrane. POA disrupts the bacterial membrane energetics and inhibits the membrane transport function which finally ends with bacterial death [41],[42].

Mutations in *pncA* gene, leading to a loss of PZase activity, are the major mechanism for PZA resistance in MTB. Therefore, the PZase activity assay or Wayne test has been performed as the alternative method to determine PZA susceptibility of MTB. However, the assay gives inaccurate results of PZA susceptibility due to different *pncA* mutations imparting a variety of enzyme activity. The mutations in *pncA* and its upstream regulatory region (URR) were observed in 45 to 85% of PZA-resistant MTB isolates [43],[44],[45]. Some degree of clustering of *pncA* mutations have been reported in the catalytic residues in the active site (Asp8Gly/Ala/Glu/Asn, His51Gln/Tyr, His71Arg, Asp49Glu/Asn/Ala, His57Arg/Tyr/Gln/Pro, Trp68Arg/Gly/Cys/Stop/Leu, Gln10Pro/Arg, and His137Pro/Arg/Asp) and metal-binding site (Ile6Thr, Val44Gly, Val139Gly/Leu, Met175Thr/Val, and Phe94Cys/Ser/Leu) of the PncA protein among PZA-resistant MTB strains [41],[44],[46]. Thus, sequencing of *pncA* can be rapidly and effectively determine the PZA susceptibility than by the phenotypic DST, indicating its usefulness in the development of rapid molecular tools to detect PZA susceptibility in MTB. However, the diverse and scattered nature of *pncA* mutations along the whole length of *pncA* among PZA susceptible and resistant MTB, highlight that there is no such major hot spot region in *pncA* [44],[47], which causes difficulty in the development of rapid diagnostic tools. In addition, the 3D structure analysis of *pncA* mutants revealed variation in structure, stability, and abundance of PncA

protein based on *pncA* mutations [48]. Molecular detection of drug resistance-associated gene mutations plays a pivotal role in the rapid diagnosis of drug susceptibility. Therefore, it is important to analyze the type, frequency, and location of each *pncA* mutation among MTB strains.

Since phenotypic PZA DST and molecular PZA DST are currently unavailable, the prevalence of PZA-resistant in MTB is unknown in Nepal. Without the proper information of PZA susceptibility of MTB, PZA is inappropriately recommended for chemotherapy among both susceptible and MDR-TB. Therefore, it is important to characterize the phenotypic and genotypic PZA DST of MTB isolates for the effective control of DR-TB in the community.

This thesis consisted of two chapters. In chapter I, I have described the characterization of the molecular mechanism of STR resistant MTB isolates from Nepal. I explored the first insight on the variation and frequency of mutations in genes conferring STR resistance in MTB from Nepal, analyzed the correlation between gene mutations with STR-resistant MTB, compared them with those in isolates from other countries, and studied their association with different MTB genotypes. In chapter II, I attempted the first insight to collect information regarding PZA susceptibility in MTB isolates from Nepal by analyzing mutations in *pncA*. I investigated the frequency and patterns of *pncA* mutations among MTB isolates. I analyzed the *pncA* mutations by comparing with previously reported PZA resistance-associated *pncA* mutations and *in vivo*/*in vitro* study. Also, I described the *pncA* mutations by analyzing the single nucleotide substitution of *pncA* with the webserver predictive tool (SUSPECT-PZA). Moreover, the association of *pncA* mutations among non-MDR, MDR, and Pre-XDR-TB isolates were also analyzed as well as the patterns of *pncA* mutation according to *M. tuberculosis* genotypes were observed.

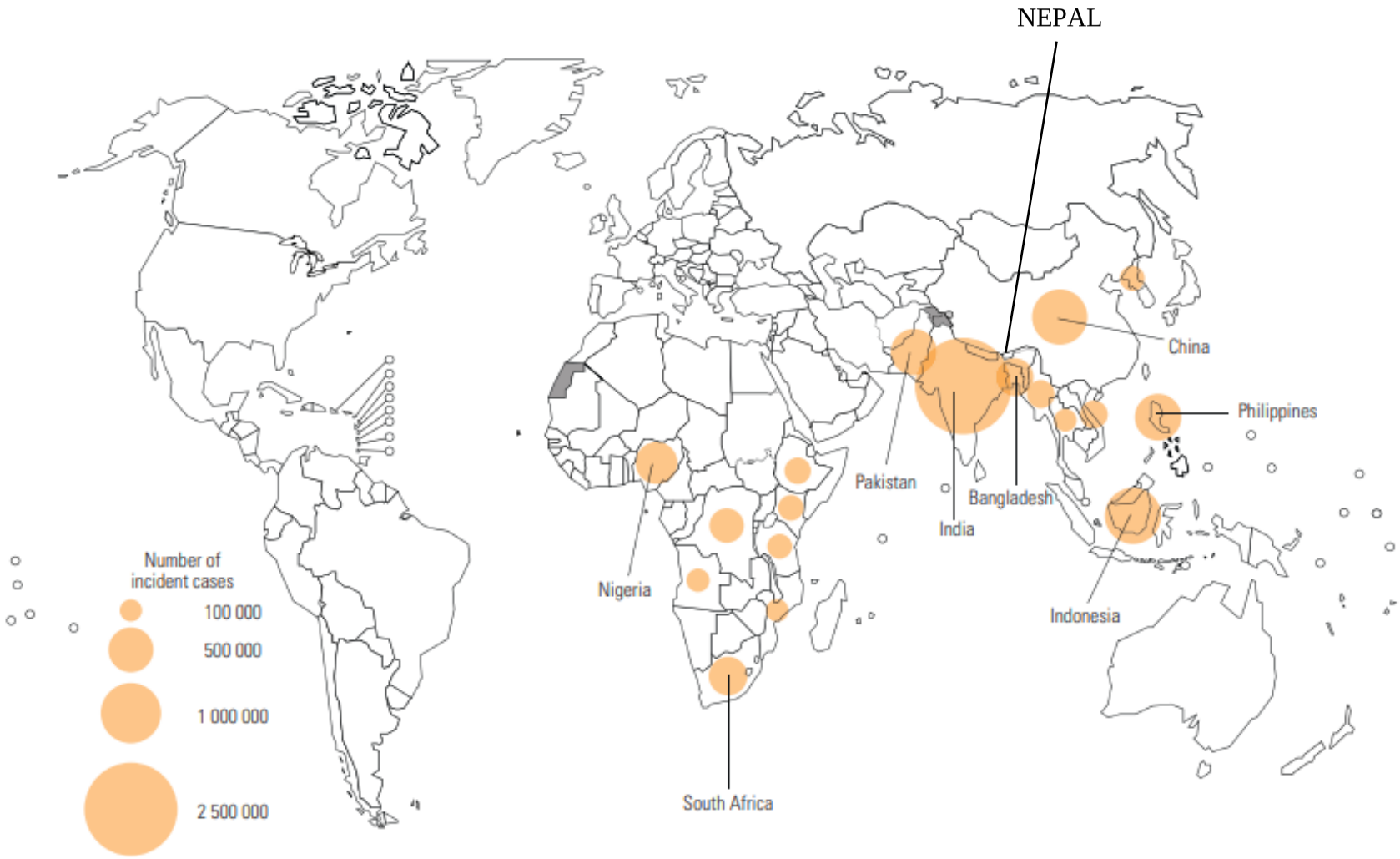

Figure 1. Estimated TB incidence in 2019, for countries with at least 100,000 incident [1]

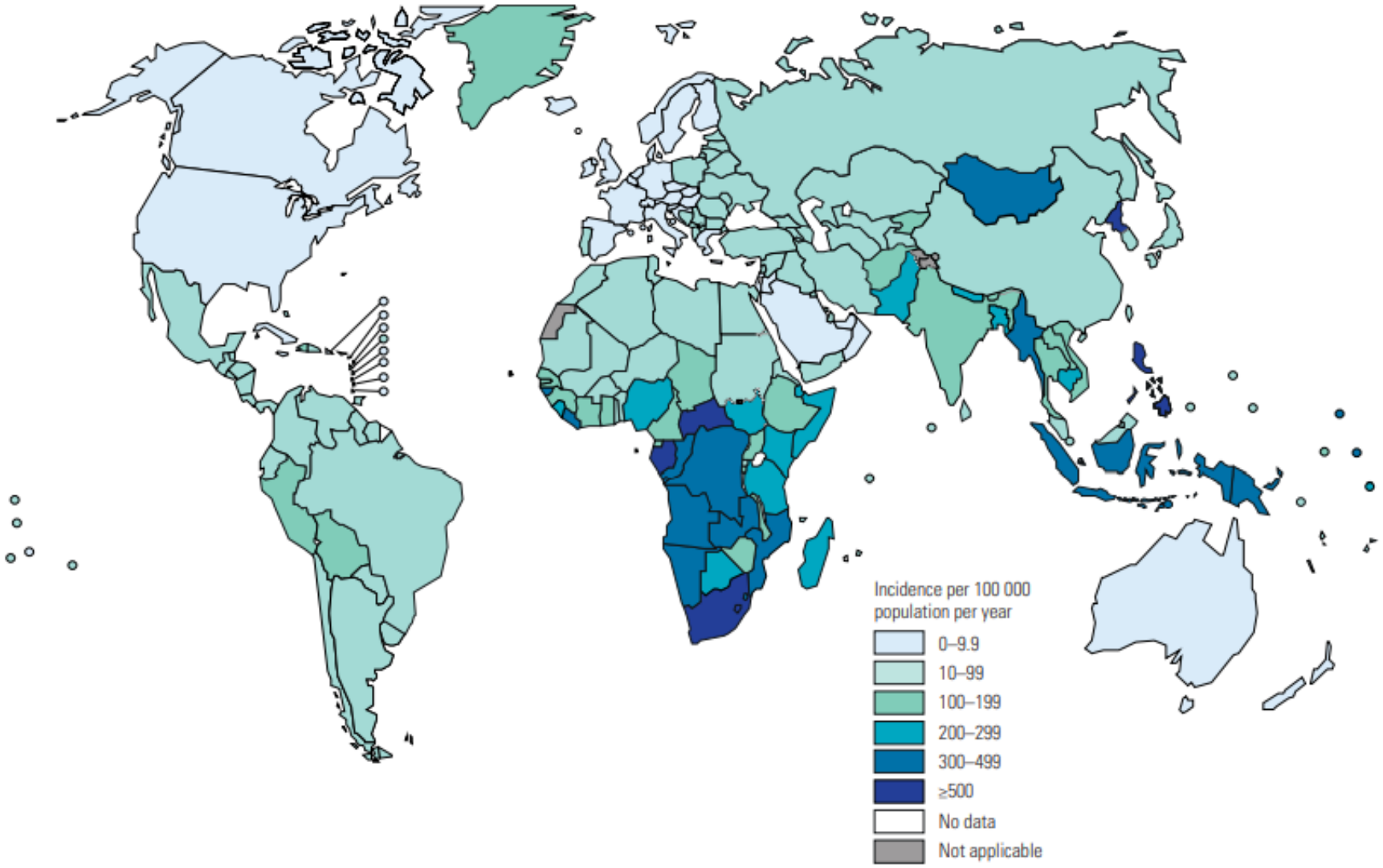

Figure 2. Estimated TB incidence rates, 2019 [1]

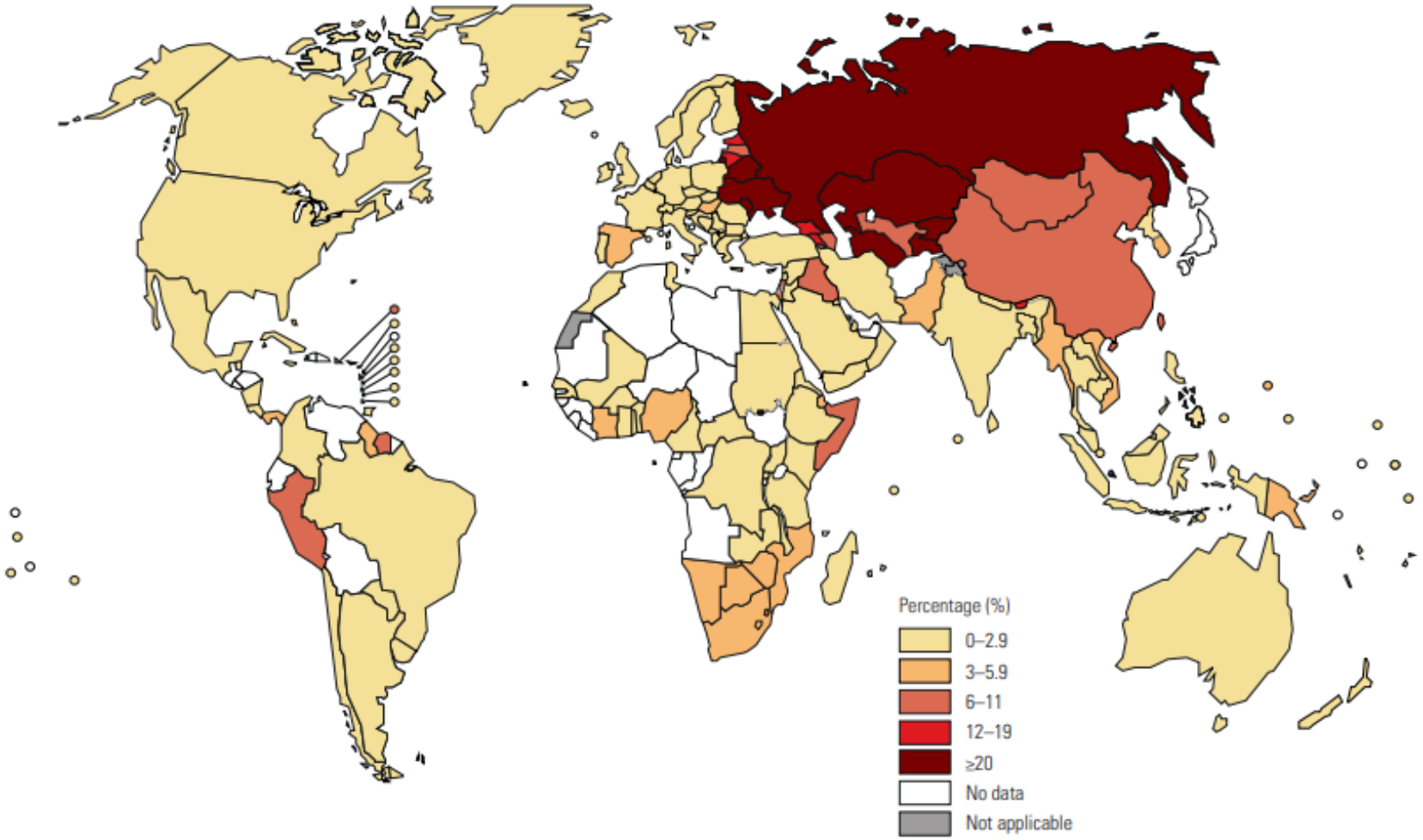

Figure 3. Percentage of new TB cases with MDR/RR-TB [1]

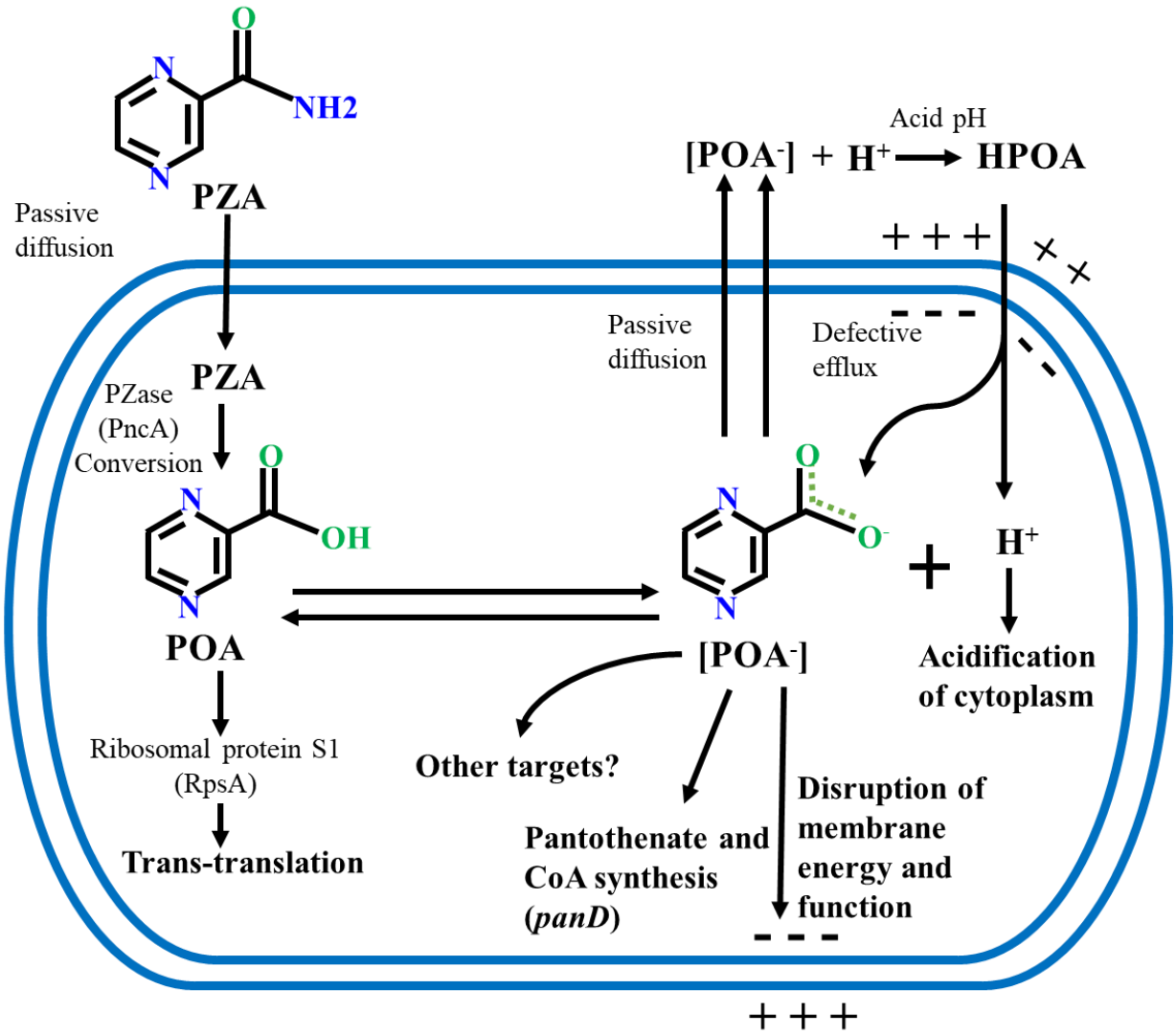

Figure 4. Mechanisms of pyrazinamide (PZA)/ pyrazinoic acid (POA) [42]

CHAPTER I

Molecular analysis of streptomycin resistance associating genes in *Mycobacterium tuberculosis* isolates from Nepal

Introduction

For the retreatment TB patients in Nepal, STR has been included in the intensive phase for two months with other first-line anti-TB drugs (FLDs) [6]. However, NTP no longer recommends STR to be used in retreatment regimens in Nepal [4]. According to the WHO-recommended regimen as published in "WHO treatment guidelines for MDR and RR-TB, 2018 Pre-final test" [7] and updated guidelines in "WHO consolidated guidelines on drug-resistant tuberculosis treatment, 2019" [3], STR is categorized into Group C agents in which it may be included in the treatment of MDR/RR-TB patients aged 18 years or more on longer regimens when susceptibility has been demonstrated and adequate measures to monitor for adverse reaction can be ensured if amikacin is not available [4]. Despite its high rate of side effects and risk of developing drug resistance in TB of its single use in a regimen [5], it is crucial to understand the molecular mechanism of resistance to STR in MTB from Nepal.

STR impairs translational proofreading, leading to the blockade of protein synthesis. Resistance to STR is associated with mutations in *rpsL* and *rrs* genes, encoding for ribosomal protein S12 and 16S ribosomal RNA, respectively. The most frequent mutations localize around two regions of *rrs*, namely the 530 loop and the 912 region, or at codons 43 or 88 within *rpsL* [8],[9]. Amino acid substitutions in the S12 protein affect the STR binding to 16S rRNA by distorting the stabilization of the high conserved structure of 16S rRNA. Alterations in the 16S rRNA cause lower affinity for STR. In particular, *rrs* mutation in 530 loop or 912 region and mutation in *rpsL* codon 43 or 88 have been reported to confer high-level or intermediate STR resistance in MTB [8],[9]. Another gene *gidB* encodes a conserved 7-methylguanosine methyltransferase or GidB, which methylates the nucleoside position 518 of the 16S rRNA to which STR directly interacts and also located 530 stem-loop pseudoknot region of the 16S rRNA site. Mutations within the *gidB* may likely alter GidB's methyltransferase function which causes changes in the methylation status of the 16S rRNA at position 518G, leading to low affinity of STR binds to the position 518G, and consequently present with STR resistance phenotype in clinical isolates. Mutations in *gidB* have been reported to confer low-level STR-resistance in MTB [10].

In addition, the type and frequency of STR resistance-associated gene mutations have been varied according to the geographical region [8-24]. For example, mutations in *rpsL* were relatively higher in Asia [8-11],[25], in which K43R mutation was found to be linked with the MTB Beijing

genotype [22],[26],[27]. Similarly, mutations in *rrs* were relatively higher in Europe and America [28],[29],[30]. However, most of the *gidB* alterations have been found to be linked with either STR resistance or specific genotypes of MTB [9],[27]. Since STR resistance-associated genes vary geographically, it is important to perform molecular studies which will detect the common mutations in target genes conferring STR resistance among MTB strains [10],[11].

The time consuming conventional proportional method using LJ medium is the only available drug susceptibility testing (DST) for STR in Nepal. Despite the high number of MDR-TB cases with STR-resistant MTB strains, no genetic information regarding the molecular mechanism of STR-resistant MTB in Nepal is currently available. Therefore, it is important to understand the underlying molecular mechanism of STR resistance for the detection of STR susceptibility through the development of a rapid diagnostic kit. As some of the specific mutations in *rpsL*, *rrs*, and *gidB* associated with STR resistance have been familiarized as high-specific predictive markers of STR resistance, it is important to elucidate and analyze the prevalence of each specific mutation among MTB isolates.

Spoligotyping and Large Sequence Polymorphisms (LSPs) are the genotyping methods that have been widely used to identify family or lineage. Spoligotyping is used to detect the presence or absence of spacer sequences by the PCR-based reverse-hybridization blotting technique and can differentiate MTB strains into distinct families such as Beijing, East African-Indian (EAI), Haarlem, Latin American and Mediterranean (LAM), Central and Middle Eastern Asian (CAS), European family X, and family T. In contrast, LSP is a PCR based method and used to detect genomic polymorphism based on large sequence deletions and known as regions of difference (RDs). Depend on the region of deletion, Beijing, EAI, CAS, and LAM family can be differentiated [17].

For chapter I, I had collected 197 STR-resistant MTB isolates to characterize the molecular mechanism of STR-resistance in *M. tuberculosis* isolates in Nepal. I explored the first insight into the variation and frequency of mutations in the genes conferring STR resistance; analyzed the correlation between mutations in the *rpsL*, *rrs*, and *gidB* with the STR-resistant MTB; compared them with those in isolates from other countries and studied their association with different MTB genotypes.

Materials and Methods

Study sample

MDR-TB isolates with resistance to all four FLDs INH, RIF, STR, and EMB have been preserved at the German Nepal Tuberculosis Project (GENETUP), Nepal. A total of 197 representative STR-resistant MTB isolates from April 2009 to March 2012 were selected in this study. These samples were subjected to genotyping and mutation analysis.

Drug susceptibility test (DST)

DST for INH, RIF, EMB (FatolArzneimittel GmbH, Schiffweiler, Germany) and STR (Sigma-Aldrich, St. Louis, MO) were performed by the indirect proportional method on LJ medium with the critical concentrations of 0.2, 40, 2, and 4 μg/ml, respectively [12],[13]. Briefly, a mycobacterial colony was freshly grown on LJ media, and a 1.0 McFarland standard mycobacterial suspension was prepared from a freshly grown colony by a serial dilution method (10^{-1} to 10^{-4}). Then, the suspension was inoculated onto LJ slants with and without drugs and incubated at 37ºC for 28 to 42 days. Then, the interpretation of results was done according to the growth of two slants. An isolate was determined to be resistant to a drug if it had ≥1% colony growth on a drug-containing medium when compared with a control isolate.

DNA extraction

Extraction of DNA from MTB colonies was done for PCR following the manufacturer's protocol using the Genotype MTBDR*plus*Ver2 (HainLifescienceNehren, Germany) [14]. Purified DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), quantified by a Nanodrop-1000 spectrophotometer (Thermo Scientific, USA) and stored at -20ºC until further use.

Spoligotyping

The genotypes of the MTB isolates were identified using spoligotyping which was performed as described previously [15],[16]. Briefly, a primer set was used to amplify the direct repeat region, and the resulting PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes on the membrane. Hybridization patterns were converted into binary and octal formats to determine the Spoligo-international types (SITs). The resulting SITs were compared with the patterns previously reported in the SpolDB4 database provided by the Institute Pasteur de Guadeloupe (http://www.pasteurguadeloupe.fr:8081/SITVIT_ONLINE/) [26]. For those isolates considered to be undersigned or new spoligotypes, the genotype was determined by LSP using specific primers for the expected region of difference.

PCR amplification and sequencing

Amplification of the 437 bp, 590 bp, and 870 bp fragments of *rpsL*, *rrs*, and *gidB*, respectively, were performed using primers shown in Table 1. PCRs were carried out in a thermal cycler (BIO-Rad Laboratories, Inc., Hercules, CA) under the condition previously described [17]. The 20 µl reaction mixture consisted of 5X Green GoTaq® Reaction Buffer (Promega, CA, USA), 0.5 M betaine, 0.25 mM $MgCl_2$, dNTPs (0.25 mM each), primers (0.5 µM each), 1.25 U GoTaq® DNA Polymerase (Promega, CA, USA) and 1 µl of template DNA (approximately 10 ng/µl). The PCR was conducted with an initial denaturation at 95ºC for 60 sec, followed by 35 cycles of amplification at 95ºC for 10 sec, annealing at 50ºC (*rpsL* and *rrs*) or 58ºC (*gidB*) for 10 sec, an extension at 72ºC for 30 sec, and a final extension at 72ºC for 5 min. The presence of PCR products was confirmed by agarose gel electrophoresis. Purified PCR products were sequenced using a BigDye ver. 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) in a DNA sequencer, 3500 Genetic Analyzer (Life Technologies Corp.) using both forward and reverse primers same as for amplification. The obtained sequences were aligned with the corresponding sequences from the wild-type *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9) [18].

Statistical analysis

All data were analyzed using R version 3.6. Pearson's Chi-square test was used to compare different variances and to determine the association between MTB lineages, drug resistance profile and mutations in the genes examined. A *p*-value of less than 0.05 was considered statistically significant.

Ethical approval

This study proposal was submitted to Nepal Health Research Council (NHRC) with register no 136/2013 on 20th Sep 2013 and approved by Ethical Review Board on 29th Nov 2013.

Results

Mycobacterium tuberculosis genotypes

Through spoligotyping, the genotypes of 197 STR-resistant MDR-MTB isolates were detected as 11 lineage 1, 116 lineage 2, 47 lineage 3, and 23 lineage 4 (Figure 5).

Mutation in *rpsL*, *rrs*, and *gidB*

Mutations in *rpsL* and *rrs* were harbored by 80.7% (159/197) of isolates. Mutations in only *rpsL* were harbored by 65.9% (130/197) of isolates. Mutations conferring amino acid substitution at *rpsL* codon 43 and 88 were found in 58.8% (116/197) and 7.1% (14/197) of isolates, respectively (Table 2). The most frequent mutation K43R specific to Beijing lineage was found in 78.6% (92/117) of isolates. Two novel mutations in *rpsL* codon 71 and 118 were found in two isolates (Table 2).

There were seven different substitutions found at seven different positions in the *rrs* gene. Mutations in only *rrs* were harbored by 13.2% (26/197) of isolates (Table 2). Mutations at positions A514C and C517T of *rrs* were found in 5.6% (11/197) and 2.5% (5/197) of isolates, respectively. Mutations around 912 loop (A906G, A907C, A908C, or A912C) of *rrs* were found in 5.1% (10/197) of isolates. Mutation at position A644G of *rrs* was found in one isolate which was not previously reported. A co-mutation in *rpsL* and *rrs* (*rpsL* K43R, *rrs* A907C; *rpsL* K88R, *rrs* A644G; and *rpsL* G118D, *rrs* A514C) was found in 1.5% (3/197) of isolates.

Polymorphisms in *gidB* were found to be very common, with 35 different polymorphisms among 86.8% (171/197) of isolates (Table 3). Of the 35 polymorphisms, 22, 5, 6 and 1 types of mutations were missense, non-sense, deletion, and insertion, respectively. In our study, *gidB* mutants showed more than one mutation in *gidB*. The number of isolates with one to three mutations in *gidB* was 29 (14.7%), 22 (11.1%) and 1 (0.5%), respectively. Mutations in *gidB* were harbored by 25.8% (51/197) of isolates (Table 4). The *gidB* mutants showing mutations in *rpsL* and/or *rrs* were found in 12.7% (25/197) of STR-resistant isolates (Table 5). About 13.2% (26/197) of *gidB* mutants do not show any mutation in *rpsL* and *rrs* (Table 5). No mutation in three genes examined in this study was found in 5.6% (11/197) of isolates (Table 5).

Determining STR resistance with results from DNA sequencing

The sensitivity for detecting STR resistance from DNA sequencing was determined by analyzing the association between phenotypic STR resistance and the DNA sequencing (Table 6). Mutations in *rpsL* alone showed a prediction of STR resistance with a sensitivity of 65.9%. Mutations in *rrs* alone and *gidB* alone showed a sensitivity of 13.2% and 26.9%, respectively. Two genes mutations analysis (*rpsL* and/or *rrs*) had higher predictability to detect STR resistance with

a sensitivity of 80.7%. Moreover, the predictability to detect STR resistance increases with three gene mutations analysis (*rpsL* and *rrs* and *gidB*) with a sensitivity of 94.4%.

Discussion

According to our knowledge, this is the first study on the molecular characterization conferring STR resistance-associated genes in MTB isolates from Nepal. It has been determined that mutations in *rpsL* and *rrs* genes are associated with STR resistance in MTB [20]. In MDR-TB isolates, about 80.7% (159/197) of STR-resistant MTB isolates harbored mutations in either *rpsL* or *rrs*. This is within the range, reported from Asian countries, 40% to 98% [19],[20],[21],[22],[23]. Mutations in *rpsL* were harbored by 65.9% (130/197) of isolates (Table 2), which suggest that these mutations strongly correlate to STR-resistance in MTB. The most common mutation in *rpsL* codon K43R was harbored by 58.8% of *rpsL* mutants in this study. The detection rate of mutations in *rpsL* codon K43R varies considerably between geographical areas (Table 3). The high detection rate of mutation in *rpsL* K43R in Nepal might be due to its non-restrictive mutation leading to no fitness cost in MTB [24] which can be widely transmitted; and the bad practice of STR mono-therapy usage for the treatment of TB and other bacterial diseases in the past in Nepal [49]. The predominant mutation *rpsL* K43R was found in 80% (92/117) of Beijing isolates. There was a significant association between Beijing strains and mutation K43R compared with the group of STR-resistant non-Beijing isolates ($P<0.001$). Sun et al reported that mutation in *rpsL* causing K43R showed a strong association with the Beijing genotype in STR-resistance isolates [22]. However, in India and Mexico, where the Beijing genotype was not frequent, mutations in these two loci had no significant contribution to the Beijing genotype [19],[21]. The Beijing genotype has been reported as more virulent than other MTB genotypes, and has a propensity to acquire the *rpsL* K43R mutation [22]. The high mutation rate in *rpsL* K43R represents the geographical areas predominate by Beijing genotype family. In contrast, mutation *rpsL* K88R was not significantly associated with the Beijing family ($P=0.34$). Mutations in *rpsL* K43R and G71D were found in one STR-resistant non-Beijing isolate. As mutation K43R of *rpsL* strongly contributes to STR resistance in MTB, it is doubtful that whether mutation G71D of *rpsL* contributes to STR resistance in MTB or not. A mutation conferring G71D found in this study was reported by study [50] with an isolate with a mutation in *rpsL* G71S (GGC→AGC), compounded with a mutation in *rpsL* K43R; and might be of only minor importance.

About 13.2% (26/197) of isolates harbored *rrs* mutations (except co-mutation in *rpsL*) in our study, which showed a frequency range of 0 to 12.5% (Table 3) in the Mideast and Southeast Asian countries [25],[26],[51],[52],[53]. Mutations in *rrs* were found in two highly mutable regions of *rrs*, the 530 loop and the 912 region, which suggest these mutations contributed to STR resistance in MTB as reported [8],[10],[19],[20],[21],[27],[51],[54],[55],[56]. As the high mutation rate of *rpsL* overweight the mutation rate of *rrs* in STR-resistant MTB, the low rate of mutation in *rrs* in these geographical areas might be the possible outcome.

Three strains showed co-mutations in both *rpsL* and *rrs*. Among them, one strain showed mutations in *rpsL* causing K43R and *rrs* 912 region A907C. Meier et al had reported similar findings of co-mutations in *rpsL* and *rrs*, which described that the *rrs* mutation (A→G at position 904) presumably conferred as yet ill-defined partial resistance to STR *in vivo*, thereby increasing the number of cells which could acquire a second mutation in the *rpsL* (alteration of K88), conferring resistance to STR-induced misreading [55]. Similar co-mutations *rpsL* L81L/*rrs* A905G [37]; *rpsL* K43R/*rrs* C789T [28]; and *rpsL* K88Q/*rrs* A905G and *rpsL* K43R/*rrs* A513C [29] have been reported. In contrast, Jagielski *et al*., had reported that mutations in *rrs* and *rpsL* were mutually exclusive, that is isolates with *rrs* mutations had no *rpsL* mutations and *vice versa* [30]. The discrepancy in our results might be due to the presence of a mixed population of *rpsL* K43R mutants and *rrs* A907C mutants. This was confirmed to be 1:1 mixture of A and G at nucleotide position 128 in *rpsL* by the electropherogram obtained. One strain harbored mutations in *rpsL* causing K88R and *rrs* A644G. One studies had reported an isolate with a mutation in *rpsL* K43R and a second mutation in *rrs* nucleotide 645 A deletion [50]. A mutation in *rrs* A644G is a novel mutation and this mutation is located far away from hot spot mutation regions of *rrs*, the 530 loop and 912 region, which is associated with the STR-resistance in MTB. As mutation in *rpsL* causing K88R is enough to cause STR-resistance in MTB, a mutation in *rrs* A644G is not associated with the STR resistance in MTB. It is very likely that A/G variation in position 644 of the *rrs* gene is a natural polymorphism that is not responsible for STR resistance. Other strains harbored mutations in *rpsL* causing G118D and *rrs* A514C. As MTB has a single rRNA operon [58], a single mutation in *rrs* A514C is enough to cause STR-resistance in MTB. Mutation G118D have not been previously reported and this mutation is located far away from frequent mutations in *rpsL*, K43R and K88R, which cause STR resistance in MTB. It is unclear that the mutation in *rrs* A644G and mutation in *rpsL* causing G118D may affect the mechanism of STR-resistance in MTB. So, further studies should be carried out to find out the exact role of these mutations in causing STR-resistance in MTB.

I observed that 13.7% (27/197) of STR-resistant isolates showed many missense mutations in *gidB* that were close to active or part of the interacting amino acid residues between the GidB protein and S-adenosylmethionine (SAM) which are critical for methylation at the position G517 of 16S rRNA [51]. These alterations may have an impact on the function of GidB which leads to the low affinity of STR to 16S rRNA and hence contribute to STR-resistance in MTB. The missense mutations in *gidB* codons causing amino acid substitutions A19G, R20P, A27P, A27V, V36L, A82P, L101E, V112G, V124G, A133V, M218V.

The *gidB* mutants harboring co-mutations in *rpsL*/*rrs* genes were found in 12.7% (25/197) of isolates. These co-mutations of in *rpsL*/*rrs* of *gidB* mutants might suggest the evolution of *rpsL*/*rrs* mutations for its existence as compensation for losing methylation function of GidB

caused by a distortion of its structure and consequently results in high-level resistance to STR [10],[57].

It was found that 13.2% (26/197) of isolates showed mutations in *gidB* without those in *rpsL* and *rrs* genes. All 13 missense mutations had an exclusive *gidB* mutation without mutations in *rpsL* and *rrs* conferring these *gidB* mutations contribute to STR-resistance in MTB. Deletion mutations in *gidB* nucleotide without those in *rpsL* and *rrs* (112delC, 254delA, 347delG, and 372delG) and insertion mutations in *gidB* nucleotide (243insGC) causing frameshift in *gidB* in STR-resistant isolates found in this study which alter the GidB protein and consequently leading to low-level STR resistance in MTB. Deletions with variable sizes at different positions in *gidB* were also reported [8],[10],[25]. Frameshift mutations (112delC, 254delA, 347delG, 372delG, 384delG and 243insGC) were previously reported. The deletion mutation in *gidB* delG at nucleotide 98 has also been reported in both STR-susceptible and STR-resistant isolates [30],[59]. Mutations in *gidB* found exclusively in STR-resistant strains play a role in STR-resistance in MTB. These 13 missense mutations and 5 frameshift mutations in *gidB* were highly associated with STR-resistance in MTB.

There were four *gidB* polymorphisms phylogenetically specific to MTB lineages which were found in 75.6% (149/197) of isolates. The missense mutation E92D conferred by 100% (116/116) of lineage 2 isolates was significantly associated with the Beijing family of lineage 2 ($P<0.001$). Similar indications of high specificity to Beijing strains have been reported in previous studies [27],[56],[57],[60]. The silent mutation in *gidB* codon at V110 was significantly associated with EAI strains ($P<0.001$), which is also supported by previous findings [27],[60]. Another silent mutation in *gidB* at codon A205 was present in 100% (23/23) of the Euro-American strains of lineage 4 which were significant ($p<0.001$). In contrast, polymorphisms in *gidB* codon at A205 was absent from almost all of the Euro-American strains in which this allele may be used as a specific marker for differentiating Euro-American strains from non-Euro-American strains [27],[60]. Finally, all LAM strains conferred the allele mutation in *gidB* codon at L16 was significant ($p<0.01$) which is also confirmed by the previous findings [57],[61],[62].

As mutation in *rpsL* alone was detected in most of STR resistant isolates, detection of *rspL* mutation can be proposed as a satisfactory predictor of STR resistance. In total, 94.4% (186/197) of STR-resistant isolates harbored mutations in either *rpsL* or *rrs* or *gidB* alone or combinations which showed a high predictive value for resistance. This suggests that mutations in *rpsL*, *rrs*, and *gidB* play a significant role in the mechanism of STR-resistance in MTB. In this study, we found 11 isolates (one lineage 1 strains, two lineage 2 strains, seven lineage 3 strains, and one lineage 4 strains) do not harbor mutation in any of the genes studied, accounting for 5.6% of the STR-resistant strains. This suggests that there might be involvement of other genes in the mechanism

of STR resistance such as the efflux system, cell membrane permeability barrier, or aminoglycosides converting enzymes.

Finally, the mutations in *rpsL* and *rrs*; and some exclusive mutations in *gidB* identified in this study are responsible for STR-resistance in MTB. The sequence analysis of mutations in *rpsL*, *rrs*, and *gidB* could be used for the establishment of rapid and effective molecular diagnostic tools to detect STR-resistant MTB strains in Nepal. This study can be further implicated for designing rapid diagnostic tools considering geographic specificity in which MDR-TB transmission can be interrupted worldwide.

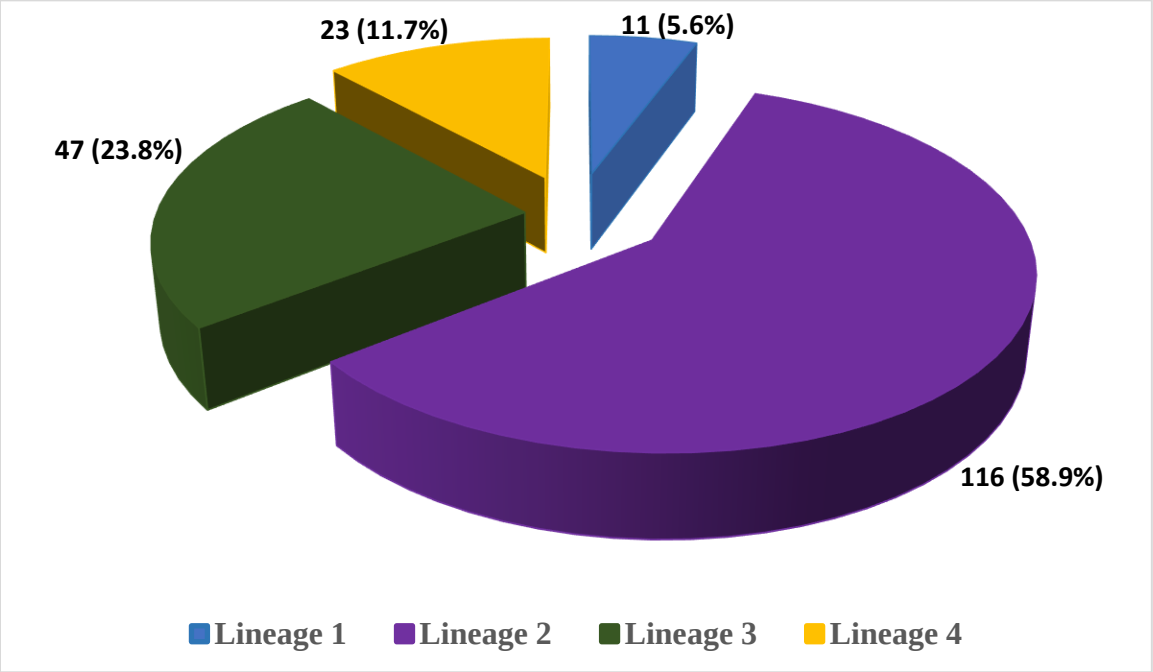

Figure 5. Distribution of 197 streptomycin resistant *Mycobacterium tuberculosis* isolates into different lineages

Table 1. Primers used for PCR amplification and sequencing of streptomycin resistance-associated genes in *M. tuberculosis*

Locus	Primers	Nucleotide sequence (5'-3')	Product size (bp)
rpsL	Forward (ON-1505)	GGCCGACAAACAGAACGT	437
	Reverse (ON-1506)	CTTCACCAACTGGGTGAC	
rrs	Forward (ON-1507)	GATGACGGCCTTCGGGTTGT	590
	Reverse (ON-1508)	AGGCCACAAGGGAACGCCTA	
gidB	Forward (ON-1509)	CGCCGAGTCGTTGTGCT	870
	Reverse (ON-1510)	AGCCTGGCCCGACCGTTA	

bp represents base pair

Table 2. Distribution of mutations in *rpsL* and *rrs* genes among different genotypes of 197 streptomycin resistant *Mycobacterium tuberculosis* isolates

***rpsL* nucleotide (amino acid substitution)**	***rrs* nucleotide**	**Lineage 1**	**Lineage 2**	**Lineage 3**	**Lineage 4**	**No. of isolates**
A128G (K43R)	-	3	91	12	9	115
A128G (K43R)	A907C		1			1
A128G (K43R), G212A (G71D) *	-			1		1
A263G (K88R)	-		12	2		14
A263G (K88R)	A644G *		1			1
G353A (G118D) *	A514C				1	1
-	A514C	1	5	2	3	11
-	A517T	1	1	2	1	5
-	A906C			1		1
-	A907C		3			3
-	A908C			3	2	5
-	A912G			1		1
-	-	6	2	23	7	38
Total		**11**	**116**	**47**	**23**	**197**

Fs denotes frameshift mutation

No. represents number

* denotes mutation previously not reported

- denotes no mutation

Table 3. Detection rate of mutations in *rpsL*, *rrs* and *gidB* between countries

Countries Mutations	This study (N=197)	India (N=52)	Pakistan (N=32)	China (N=227)	Myanmar (N=141)	Thailand (N=46)
***rpsL* codon K43R**	58.8%	32.6%	34.3%	47.5%	64.0%	69.5%
***rpsL* codon K88R**	7.1%	19.2%	3.1%	12.3%	6.0%	2.1%
rrs	13.2%	5.7%	12.5%	5.7%	3.5%	0%
gidB	13.2%	25.0%	21.8%	12.7%	2.7%	2.1%
Reference	This study	[51]	[52]	[26]	[25]	[53]

Table 4. Distribution of mutations in *gidB* among different genotypes of 197 streptomycin resistant *Mycobacterium tuberculosis* isolates

***gidB* nucleotide (amino acid substitution)**	**Lineage 1**	**Lineage 2**	**Lineage 3**	**Lineage 4**	**Number of isolates**
G28C (A10P)			2		2
G28C (A10P), A119G (E40G)	1				1
C56G (A19G)*, γ, θ				1	1
G59C (A20P)*, β	1				1
G79C (A27P)*			1		1
C80T (A27V)*, α		1			1
G106C (V36L)*			1		1
C142A (H48N), γ				2	2
T236G (L79Y)			1		1
G244C (A82P)*			1		1
T302G (L101E)*			1		1
T335G (V112G)*			1		1
T371G (V124G)*			1		1
C398T (A133V)*, α		1			1
C413T (A138V), γ				1	1
G471A (G157G)*, α		2			2
A487G (K163E)			1		1
C492T (G164G)*			1		1
T583C (Y195H), α		7			7
G630A (G210G)*, α		1			1
A652G (M218V)*, γ				2	2
243insGC (Fs)*, γ				1	1
98delG (R33Fs)			6		6
98delG (R33Fs), β	3				3
98delG (R33Fs), γ				3	3
112delC (P38Fs)*, β	1				1
112delC (P38Fs)*, γ				1	1
254delA (D85Fs)*, γ				1	1
347delG (R116Fs)			1		1
372delG (V124Fs)*			1		1
384delG (L128Fs)			2		2

α	1	104			105
β	4				4
γ				8	8
γ, θ				3	3
-			26		26
Total	**11**	**116**	**47**	**23**	**197**

A276C (E92D)=α, G330T (V110V)=β, G615A (A205A)=γ and T47G (L16R)=θ

Fs denotes frameshift mutation

* denotes mutation previously not reported

- denotes no mutation

del denote deletion mutation

Table 5. Distribution of mutations in *rpsL*, *rrs* and *gidB* genes among different genotypes of 197 streptomycin (STR) resistant *Mycobacterium tuberculosis* isolates

***rpsL* nucleotide (codon)**	***rrs***	***gidB* nucleotide (codon)**	**Lineage 1**	**Lineage 2**	**Lineage 3**	**Lineage 4**	**No. of isolates**
A128C(K43R)	-	α	2	77		1	80
A128C(K43R)	-	α, C398T(A133V)*		1			1
A128C(K43R)	-	α, T583C(Y195H)		7	1		8
A128C(K43R)	-	A445C(S149R), γ		1			1
A128C(K43R)	-	α, G630A(G210G)*		1			1
A128C(K43R)	-	112delC (P38Fs)*, β	1				1
A128C(K43R)	-	C286T (R96C)*		1			1
A128C(K43R)	-	G106C (V36L)*			1		1
A128C(K43R)	-	G28C (A10P)			2		2
A128C(K43R)	-	β		1			1
A128C(K43R)	-	γ		1		6	7
A128C(K43R)	-	A652G (M218V)*, γ				1	1
A128C(K43R)	-	γ, θ				1	1
A128C(K43R)	-	-		1	9		10
A128C(K43R)	A907C	α		1			1
A128C(K43R), G212A(G71D)*	-	-			1		1
A263G(K88R)	-	α		10			10
A263G(K88R)	-	G471A (G157G)*, α		2			2
A263G(K88R)	-	C492T (G164G)*			1		1
A263G(K88R)	-	-			1		1
A263G(K88R)	A644G*	α		1			1
G353A(G118D)*	A514C	98delG (R33Fs), γ				1	1
-	A514C	α		4			4
-	A514C	C80T (A27V)*, α		1			1
-	A514C	98delG (R33Fs), γ				2	2
-	A514C	β	1				1
-	A514C	γ, θ				1	1
-	A514C	-			1		1
-	A517T	α		1			1
-	A517T	β	1				1

-	A517T	γ, θ				1	1
-	A517T	-			2		2
-	A906G	-			1		1
-	A907C	α		3			3
-	A908C	C142A (H48N), γ				2	2
-	A908C	384delG (L128Fs)			1		1
-	A908C	-			2		2
-	A912G	384delG (L128Fs)			1		1
-	-	243insGC (Fs)*, γ				1	1
-	-	254delA (D85Fs)*, γ				1	1
-	-	α		2			2
-	-	A487G (K163E)			1		1
-	-	C413T (A138V), γ				1	1
-	-	112delC (P38Fs)*, γ				1	1
-	-	G244C (A82P)*			1		1
-	-	347delG (R116Fs)			1		1
-	-	372delG (V124Fs)*			1		1
-	-	G59C (A20P)*, β	1				1
-	-	γ				1	1
-	-	A652G (M218V)*, γ				1	1
-	-	G79C (A27P)*			1		1
-	-	98delG (R33Fs)			6		6
-	-	98delG (R33Fs), β	3				3
-	-	G28C(A10P), A119G(E40G)			1		1
-	-	β	1				1
-	-	T236G (L79Y)			1		1
-	-	T302G (L101E)*			1		1
-	-	T335G (V112G)*			1		1
-	-	T371G (V124G)*			1		1
-	-	C56G (A19G)*, γ, θ				1	1
-	-	-	1		7		8
Total			**11**	**116**	**47**	**23**	**197**

A276C (E92D)=α, G330T (V110V)=β, G615A (A205A)=γ and T47G (L16R)=θ

EAI, East-African Indian; CAS, Central Asian strain; and other, orphan and X2.

Fs denotes frameshift mutation.

* denotes frameshift mutation.

- denotes no mutation.

del denote deletion mutation.

Ins denote insertion mutation.

α, β, γ and θ represent A276C (E92D), G330T (V110V), G615A (A205A) and T47G (L16R), respectively.

Table 6. Proportion of mutations in *rpsL*, *rrs*, and *gidB* in STR resistant *Mycobacterium tuberculosis* isolates.

Gene mutations	No. of MDR-isolates		Sensitivity (%)
	With mutation	Without mutation	
rpsL	130	67	65.9%
rrs	26	171	13.2%
gidB	53	144	26.9%
rpsL and/or *rrs*	159	38	80.7%
rpsL and/or *gidB*[a]	166	31	84.3%
rrs and/or *gidB*[a]	74	123	37.5%
rpsL and *rrs* and *gidB*[a]	186	11	94.4%

[a]= Lineage-specific polymorphisms and mutations not likely to cause STR resistance were excluded

Summary

Mutation in *rpsL* (encoding ribosomal protein S12), *rrs* (encoding 16S ribosomal RNA), and *gidB* (encoding 7-methylguanosine methyltransferase) are associated with resistance to streptomycin (STR), which is used for the treatment of multi-drug resistant tuberculosis (MDR-TB) in Nepal. The aim of our study is to analyze the correlation between mutations in the target genes and STR-resistance in 197 *Mycobacterium tuberculosis* (MTB) isolates from Nepal. Mutations in *rpsL* were harbored by 65.9% of isolates, in which the most common mutation in *rpsL* is caused by K43R (58.8%) and was significantly associated with the Beijing genotype ($P<0.001$). About 13.2% of isolates harbored mutations in two highly mutable regions of *rrs*, the 530 loop and the 912 region. About 13.2% of *gidB* mutants do not show any mutation in *rpsL* and *rrs*, which might suggest the role of *gidB* mutations in STR-resistance in MTB. Also, 5.6% of isolates do not show any mutations in the three genes examined, suggesting the involvement of other mechanisms in STR resistance in MTB. Our findings can be implemented for the establishment of molecular STR-susceptibility testing, in which tuberculosis can be treated with appropriate drugs and can improve control strategies for DR-TB.

CHAPTER II

Characterizations of pyrazinamide resistance associating *pncA* gene mutations in *Mycobacterium tuberculosis* isolates from Nepal.

Introduction

PZA has a preferred sterilizing activity against non-replicating persistent bacilli and is used in the initial intensive phase of chemotherapy, in combination with other FLDs INH, RIF, EMB for both drug-susceptible and drug-resistant TB. Despite the important role of PZA in shortening the TB treatment period of susceptible, MDR, and pre-extensively drug-resistant TB (Pre-XDR-TB)/XDR-TB [2], PZA resistance in MTB has increased in both susceptible and MDR-TB. PZA is recommended in the initial phase of the TB treatment regimen for new cases and retreatment cases of all TB categories in Nepal [4].

PZA being an indispensable drug for all TB-treatment regimen, it is important to understand the PZA susceptibility accurately on time and monitor the spread of drug-resistance strains. Phenotypic PZA drug susceptibility testing (DST) is difficult to perform, not cost-effective for a developing country like Nepal and has a long turnaround time. Since PZA requires an acidic medium (pH) for its function, there is a possibility for causing false resistance rates, making phenotypic PZA DST unreliable [63],[64]. The phenotypic PZA DST is routinely not performed in most of the laboratories worldwide including Nepal.

PZA is a pro-drug which enters the bacteria through passive diffusion and is converted into its active form POA, by the non-essential enzyme PZase, encoded by the *pncA* gene [39],[40]. POA has functioned in the neutral pH in the cytoplasm which has no activity against MTB. By the efflux pump, POA is pumped out of the cell which has acidic environments and then protonated to HPOA which re-enters the cell, and accumulation of HPOA can eventually cause rupture of membrane. POA disrupts the bacterial membrane energetics and inhibits the membrane transport function which finally ends with bacterial death [41],[42].

Mutations in *pncA* gene, leading to a loss of PZase activity, are the major mechanism for PZA resistance in MTB. Therefore, the PZase activity assay or Wayne test has been performed as the alternative method to determine PZA susceptibility of MTB. However, the assay gives inaccurate results of PZA susceptibility due to different *pncA* mutations imparting a variety of enzyme activity. The mutations in *pncA* and its upstream regulatory region were observed in 45 to 85% of PZA-resistant MTB isolates [43],[44],[45]. Some degree of clustering of *pncA* mutations have been reported in the catalytic residues in the active site (Asp8Gly/Ala/Glu/Asn,

His51Gln/Tyr, His71Arg, Asp49Glu/Asn/Ala, His57Arg/Tyr/Gln/Pro, Trp68Arg/Gly/Cys/Stop/Leu, Gln10Pro/Arg, and His137Pro/Arg/Asp) and metal-binding site (Ile6Thr, Val44Gly, Val139Gly/Leu, Met175Thr/Val, and Phe94Cys/Ser/Leu) of the PncA protein among PZA-resistant MTB strains [41],[44],[46]. Thus, sequencing of *pncA* can be rapidly and effectively determine the PZA susceptibility than by the phenotypic DST, indicating its usefulness in the development of rapid molecular tools to detect PZA susceptibility in MTB. However, the diverse and scattered nature of *pncA* mutations along the whole length of *pncA* among PZA susceptible and resistant MTB, highlight that there is no such major hot spot region in *pncA* [44],[47], which causes difficulty in the development of rapid diagnostic tools. In addition, the 3D structure analysis of *pncA* mutants revealed variation in structure, stability, and abundance of PncA protein based on *pncA* mutations [48]. Molecular detection of drug resistance-associated gene mutations plays a pivotal role in the rapid diagnosis of drug susceptibility. Therefore, it is important to analyze the type, frequency, and location of each *pncA* mutation among MTB strains.

The prevalence rates of 49 to 61% of PZA-resistance were observed in MDR-TB isolates [43],[45],[65]. The occurrence of *pncA* mutation could be higher among MDR-TB isolates. Genetic changes in *rpoB* have been associated with RIF resistance, *katG* or *inhA* with INH resistance, *gyrA* or *gyrB* with a fluoroquinolone (levofloxacin)-resistance, and *rrs* with second-line injectable agents (amikacin, or capreomycin, or kanamycin, or streptomycin) [66],[67]. Therefore, I sequenced the MDR conferring genes, in addition to *pncA,* to detect the association of *pncA* mutations with the mutations in *rpoB*, *katG*, *inhA*, *gyrA*, *gyrB,* and *rrs.*

Since phenotypic PZA DST and molecular PZA DST are currently unavailable, the prevalence of PZA-resistant in MTB is unknown in Nepal. Without the proper information of PZA susceptibility of MTB, PZA is inappropriately recommended for chemotherapy among both susceptible and MDR-TB. Therefore, I aimed to collect information regarding PZA-susceptibility in MTB isolates from Nepal by analyzing mutations in *pncA.*

In chapter II, I attempted the first insight to collect information regarding PZA susceptibility in MTB isolates from Nepal by analyzing mutations in *pncA.* I investigated the frequency and patterns of *pncA* mutation among MTB isolates. I analyzed the *pncA* mutations by comparing with previously reported PZA resistance associated *pncA* mutations and *in vivo*/*in vitro* study. In addition, I described the *pncA* mutations by analyzing the single nucleotide substitution of *pncA* with the webserver predictive tool (SUSPECT-PZA). Moreover, the association of *pncA* mutations among non-MDR, MDR, and Pre-XDR TB isolates were also analyzed as well as the patterns of *pncA* mutation according to *M. tuberculosis* genotypes were also observed.

Materials and Methods

Sample collection and study sample

A total of 211 samples collected at the German Nepal Tuberculosis Project (GENETUP) National Reference Lab, Nepal from August 2008 to February 2011 were included in this study. These samples were collected from 155 males and 56 females, with an age range from 12-70 years.

Drug susceptibility testing (DST)

DST for INH, RIF, EMB (FatolArzneimittel GmbH, Schiffweiler, Germany), and STR (Sigma-Aldrich, St. Louis, MO) were performed by the indirect proportion method on Lowenstein-Jensen medium with the critical concentration of 0.2, 40, 2, and 4 μg/ml, respectively [13],[68].

DNA extraction

DNA were extracted for PCR amplification and sequencing following the manufacturer's protocol using the Genotype MTBDRplusVer2 [14] as in chapter I.

Spoligotyping

The genotypes of the MTB isolates were identified using spoligotyping, as described earlier [16] as in chapter I.

PCR amplification and DNA sequencing

The targeted gene fragments (*rpoB*, *katG*, *inhA* regulatory region, *gyrA*, *gyrB*, *rrs*, and *pncA* with the upstream regulatory region) were amplified and sequenced using primers as shown in Table 7. I followed the standard protocol of PCRs amplification in a thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) [69]. PCR was performed in 20 μl reaction mixture consisted of 5X Green GoTaq® Reachtion Buffer (Promega), 0.5M betaine, 0.25 mM $MgCl_2$, dNTPs (0.25 mM each), primers (0.5 μM each), 1.25 U GoTaq® DNA Polymerase (Promega) and 1 μl of template DNA (approximately 10 ng/μl). The following PCR condition were used. One cycle of initial denaturation 95ºC for 10 sec, annealing at 50 or 55ºC for 10 sec, an extension at 72ºC for 30 sec, and a final extension at 72ºC for 5 min. Upon amplification, PCR products of target genes were confirmed by agarose gel electrophoresis. The PCR products were purified by the ExoSAP method and sequenced using a DNA sequencer, 3500 Genetic Analyzer (Thermo Fisher Scientific, Inc., Waltham, MA) using both forward and reverse primers. The obtained sequences were aligned with the corresponding sequences from the wild-type *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9) [18],[68].

SUSPECT-PZA

The SUSPECT-PZA is a web-server predictive tool (http://biosig.unimelb.edu.au/suspect_pza/) to rapidly detect the PZA susceptibility by computational assessment of the single nucleotide substitution. This web tool only uses the single nucleotide substitution at the putative *pncA* region to predict PZA susceptibility of MTB. The 3D structure of *pncA* as an output of the single nucleotide substitution from this web tool could accurately predict mutations associated with PZA resistance [48].

Statistical analysis

All data were analyzed using the IBM SPSS Statistics for Windows software version 22.0 (IBM Corp., Armonk, NY, USA). Two-tailed Fisher's exact test was used to compare the PZA resistance-associated mutations according to the resistance conferring mutations of other FLDs (SIRE). The odds ratio was calculated to detect the association between the frequency of *pncA* mutations and that of *rpoB* or *katG* or *inhA* mutations, *gyrA* or *gyrB* or *rrs* mutations; and *pncA* mutations among different drug-resistant profile and MTB genotypes. A *p*-value of less than 0.05 was considered statistically significant.

Ethical approval

This study proposal was submitted to Nepal Health Research Council (NHRC) with register no 136/2013 on 20th Sep 2013 and approved by the Ethical Review Board on 29th Nov 2013.

Results

Mycobacterium tuberculosis genotypes and drug resistance patterns

Through spoligotyping, the genotypes of 211 MTB isolates were identified as lineage 1 (n=13), lineage 2 (n=113), lineage 3 (n=56), and lineage 4 (n=29) (Table 8).

The DST results showed that a majority of isolates were MDR (108, 51.2%), followed by Pre-XDR isolates (57, 27%), and non-MDR (46, 21.8%) (Table 8). Eight different first-line drug susceptibility patterns among MTB isolates were observed (Table 9). The drug susceptibility patterns for all FLDs INH, RIF, EMB, and STR were available for 201 isolates, but 10 isolates had only H and R susceptibility patterns (Table 9). Non-MDR isolates include mono-resistant and pan-susceptible isolates.

pncA mutation profile

The sequencing results revealed that 125 (59.2%) of isolates harbored alterations in *pncA* and its upstream regulatory region (URR). The detected 57 different types of mutations (46 reported and 11 novels) were scattered through the whole length of the *pncA* gene.

Among 57 distinct *pncA* mutations profiles, six insertions, four deletions, 45 single nucleotide substitutions, one double mutation (single nucleotide substitution + deletion), and one entire *pncA* gene deletion were identified (Table 10, and Table 11). I detected three nucleotide changes in the URR of *pncA*, two novel single nucleotide substitutions (A-40C and A-40G) in three isolates, and one insertion of C between -2/-3 in one isolate. Among 66 isolates with single nucleotide substitutions, 38 types of mutations were associated with phenotypic PZA resistance (63 isolates). Insertion and deletion of nucleotides in the putative gene causing frameshift mutations were associated with PZA resistance and were observed in 11 isolates. An entire *pncA* gene deletion was found in four isolates that were associated with PZA resistance. The most frequent *pncA* mutation observed was Leu182Ser in 12 isolates followed by Leu4Ser in 6 isolates. Silent mutation Ser65Ser was significantly associated with the CAS genotype (48/56). Mutations Thr100Pro and Val157Gly do not show any concomitant results between SUSPECT-PZA and the literature review (Table 10).

I identified that 87 (41.2%) isolates had mutations in *pncA* and its URR (excluding silent mutations) (Table 10). The *pncA* mutations were found in 1 (2.1%) non-MDR, 48 (44.4%) MDR, and 38 (66.6%) XDR-TB isolates (Table 12). There was a significant association of *pncA* alterations and MDR/Pre-XDR-TB than non-MDR-TB ($p<0.005$). Among 87 isolates with *pncA* mutations, the majority were observed in the Beijing genotype (47, 54%), followed by, Euro-American (18, 20.6%), CAS (15, 17.2%), and EAI (7, 8%). (Table 13).

Association of *pncA* mutations with mutations in *rpoB, katG, inhA, gyrA, gyrB,* and *rrs*

Among 108 MDR isolates, 45 (48.3%) *rpoB* or *katG* or *inhA* mutants had mutations in the regulatory or coding region of *pncA*, while 3 (20.0%) isolates without *rpoB* or *katG* or *inhA* mutation had *pncA* mutation. This study found that the odds of *pncA* mutations were 3.75 times more likely to occur in *rpoB* or *katG* or *inhA* mutants than *rpoB* or *katG* or *inhA* non-mutants (p=0.05) (Table 14).

Similarly, a higher frequency of *pncA* mutations was found in 34 (77.3%) *gyrA* or *gyrB* or *rrs* mutants among Pre-XDR isolates than isolates without *gyrA* or *gyrB* or *rrs* mutants (Odds ratio=7.65, p=0.003) (Table 15).

Discussion

To the best of our knowledge, this study is the first attempt to molecular analysis of PZA susceptibility of MTB isolates from Nepal. The genetic information of *pncA* mutations found in this study was analyzed by comparing with *in vitro*/*in vivo* susceptibility studies [71], previous studies, and the SUSPECT-PZA web tool. Alterations in *pncA* and its URR were found scattered throughout *pncA* gene with different mutations at codons (Table 8), as previously described [72]. In this study, about 41.2% of MTB isolates carry mutations in *pncA* and its URR. Other studies of PZA-resistant MDR-MTB isolates carry *pncA* mutations from low to high correlation as China (78%, 84.6% and 88.0%), Bangladesh (57.2%), South Africa (100%), Japan (79.6%), and India (97%) [73],[74],[75],[76],[77],[78],[79]. A low prevalence rate of *pncA* mutations in our study could be due to the unknown phenotypic PZA susceptibility of isolates.

The most frequent mutation T545C (Leu182Ser) was found in 12 (5.6%) isolates, followed by T11C (Leu4Ser) in 6 (2.8%) isolates, and shared similar mutations in *rpoB*, *katG*, *gyrA*, or *rrs* suggesting the spread of those mutant strains in an outbreak. The Leu182Ser and Leu4Ser mutants have been reported to cause PZA resistance [43],[80].

The mutation C169G (His57Asp) were found in two isolates. The amino acid change His57Asp is a unique change specific to *M. bovis* and is naturally resistant to PZA [81]. *M. bovis* has been used to distinguish from MTB by this mutation [81]. PZA-resistant MTB isolates harboring the same mutation have been reported earlier [40],[65],[66]. However, our isolates were confirmed to be MTB by spoligotyping.

In our study, 41 types of mutations (excluding silent mutation) were reported as PZA resistance-associated mutations. Mutations in *pncA* were dispersed throughout the gene, and some degree of clustering of mutations was found in three regions (codon 3-17, codon 61-85, codon 127-154) of the PncA protein among PZA resistance [46],[58],[70]. The diverse and scattered nature of *pncA* mutations at different locations, as reported by previous studies [45],[46],[47],[73],[74],[75],[76],[77],[78],[79] possess a challenge for developing a rapid molecular assay for the detection of PZA susceptibility. Isolates harboring mutations at the active site residues or metal ion binding site residues causes the loss of enzymatic activity or chelation of ion atom [65],[71],[80], and leads to the emergence of PZA resistance. This information could be used to predict PZA-resistance by molecular PZA DST. Mutations Ser59Phe, Val139Ala, Leu116Pro have been previously reported as PZA-susceptible and resistant strains. This discrepant result might be due to the inaccuracy of the phenotypic PZA susceptibility by the MGIT 960 method [63],[64], or due to the presence of hetero-resistance or a mixed bacterial population [79]. These mutations were compared with the PZA-susceptibility by SUSPECT-PZA and interpreted accordingly (Ser59Phe, Val139Ala, and Leu116Pro as resistant, resistant, and susceptible, respectively).

An entire *pncA* gene deletion was found in one non-MDR and three Pre-XDR MTB isolates, as in previous reports [66],[77],[82]. The deletion of an entire *pncA* causes PZase negative; leading to PZA resistance. These four *pncA* mutants show good PCR and sequencing results for *rpoB, katG, inhA, gyrA, gyrB,* and *rrs* genes. So, I could deny the possibility of failure of *pncA* PCR. However, to nullify the technical error during sequencing, sequencing of a polycistron should be performed to confirm the whole length of *pncA* deletion in MTB.

I did not find *pncA* mutation in non-MDR isolates in our study, except an isolate with an entire *pncA* deletion. Most of the *pncA* mutations associated with PZA resistance were found among MDR-TB (44.4%) and Pre-XDR-TB (66.6%). The statistical analysis shows that *pncA* alterations were more likely to occur among MDR and Pre-XDR-TB isolates than non-MDR-TB isolates. Other studies have reported the high prevalence of PZA resistance among MDR and Pre-XDR/XDR-TB isolates [72],[76],[83]. An explanation for this could be that the bacterial exposure to antibiotics (rifampicin, fluoroquinolones, and aminoglycosides) can generate reactive oxygen species that induce the SOS system, which increases mutation frequency and facilitates the development of additional drug resistance [71],[84]. The increasing rate of PZA resistance among DR-TB highlights the appropriate use of PZA for drug-resistant TB. In addition, the odds of *pncA* mutation were 3.75 and 7.65 times more likely to occur in *rpoB* or *katG* or *inhA* mutants and *gyrA* or *gyrB* or *rrs* mutants respectively. Our results support that the detection of *pncA* mutation could predict the prevalence of PZA resistance among MDR and Pre-XDR-TB isolates in Nepal. This information could provide the proportion of PZA-resistance among different groups of MTB isolates from Nepal.

The frequency of predicted PZA resistance among Beijing MTB isolates was slightly higher than other genotype isolates. Due to uneven sample size, PZA susceptibility did not correlate with the genotypic lineage of MTB, as previously reported [63],[79]. The silent mutation Ser65Ser is linked to the CAS genotype of PZA-susceptible MTB isolates [85].

In our study, 11 novel mutations were found in 12 isolates. The seven novel mutations (causing frameshift or sense or non-sense mutation), and the positions of novel mutations at active sites or metal ion binding sites were associated with PZA resistance. Three Pre-XDR isolates with single nucleotide substitution at URR -40 (A-40C and A-40G) were found in this study. Study have reported that *pncA* (*Rv2043c*) is co-transcribed as a polycistron with its surrounding gene, *Rv2044c* located 40 nucleotides upstream to *pncA*, and *Rv2042c* located downstream with a 1 nucleotide overlap with *pncA* [78]. As the nucleotide position at URR -40 is a part of a larger operon comprising of two additional genes, any changes in the upstream regulatory region might alter the transcription of *pncA*, causing in alteration of PncA and finally results in PZA-resistance. Therefore, two single nucleotide substitutions at URR A-40C and A-40G were associated with PZA resistance. Seven novel mutations causing frameshift in coding region were considered to

cause PZA resistance as a high likelihood of frameshift mutations resulting in resistance, as previously reported [77]. However, one novel mutation was predicted to be PZA-susceptible based on their silent mutation and one novel mutation as PZA-susceptible based on SUSPECT-PZA.

In total, 87 (41.2%) isolates with *pncA* mutations were associated with PZA resistance. PncA is the non-essential enzyme, and the mutations in *pncA* or deletion of an entire *pncA* have no fitness cost in bacteria [48],[71]. Since PZA is included in the treatment regimen of non-DR and DR-TB without the phenotypic DST in Nepal, this practice causes the selective pressure of PZA during infection of MTB. Our study shows a high variation of mutation frequency at *pncA* nucleotide in MTB. In-vivo and in-vitro studies have shown that selective pressure occurs in the presence of PZA, which causes the maximal variation in the average mutation frequency at *pncA* [71].

A limitation of our study is the lack of phenotypic PZA susceptibility testing data of the isolates. Nonetheless, most of the alterations in *pncA* was correlated with in-vitro/in-vivo susceptibility study, the phenotypic susceptibility in the literature as well as in the SUSPECT-PZA web tool, which enables us to estimate the potential prevalence of PZA resistance [48],[71],[83]. Even though SUSPECT-PZA can be only used for single nucleotide polymorphisms at putative *pncA*, it has a positive predictive value of 95.4%, so is a reliable and convenient alternative to the existing gold standard methods MGIT 960 method [48]. However, resistance to PZA has been associated with a mutation in other genes including *rpsA*, *panD*, *clpC1*, *NicT*, *ctpL*, *ctpE*, and the putative efflux pumps *Rv0191*, *Rv 3756c*, *Rv3008*, *Rv1667c*, *Rv2044c*, *Rv2783c*, *Rv0194*, *MppL11*, *MmpL3*, *MgtE*, *Rv0987*, *mmpL13a*, *MmpL10*, *Rv1348*, *Rv3239c*, *Mez*, *Rv2994*, *GpsL*, *PstC2*, and *Rv1285* [43],[86],[87]. Therefore, it is important to analyze these genes for development of a rapid diagnostic tool to predict the PZA resistance in MTB accurately.

In conclusion, the rate of *pncA* mutation was high in MDR-TB/Pre-XDR-TB in our study, and most of the *pncA* mutations resulted in PZA resistance in MTB. An increasing number of PZA resistance among DR-TB in Nepal alarms the necessity of the routine testing of PZA susceptibility. Considering the high cost and long turnaround time of phenotypic DST in developing countries including Nepal, the more feasible method of molecular *pncA* sequencing for the detection of PZA susceptibility is recommended. Our study highlights the importance of PZA susceptibility testing before the implementation of PZA in DR-TB treatment in Nepal.

Table 7. Primers used for PCR amplification and sequencing of targeted drug-resistance associating genes in *Mycobacterium tuberculosis*

Locus	Primers	Nucleotide sequences (5'-3')	Annealing temperature (ºC)	Product size (bp)
rpoB	Forward (ON-513)	CAGGACGTGGAGGCGATCAC	56.8	278
	Reverse (ON-914)	GAGCCGATCAGACCGATGTTGG		
katG	Forward (ON-114)	ATGGCCATGAACGACGTCGAAAC	57.9	392
	Reverse (ON-103R)	CGCAGCGAGAGGTCAGTGGCCAG		
***inhA* promoter region**	Forward (ON-097)	TCACACCGACAAACGTCACGAGC	59.3	231
	Reverse (ON-098)	AGCCAGCCGCTGTGCGATCGCCA		
gyrA	Forward (ON-747)	AGCGCAGCTACATCGACTATGCG	62.9	321
	Reverse (ON-748)	CTTCGGTGTACCTCATCGCCGCC		
gyrB	Forward (ON-1100)	GCGGCACGTAAGGCAGAGTTG	58.9	397
	Reverse (ON-1101)	CTTGGCCTTGTTGTTGCAGTTGTT		
rrs	Forward (ON-1066)	CGGATCGGGGTCTGCAACTCGAC	61.3	299
	Reverse (ON-1067)	CAAGAACCCCTCACGGCCTACG		
pncA	Forward (On-1464)	GGCGTCATACCCTATATC	50	843
	Reverse (On-1465)	CAACAGTTCATCCCGGTTC		

Table 8. Distribution of MTB isolates according to genotypes and drug resistance patterns

Lineages	Non-MDR	MDR	Pre-XDR	Total
1 (EAI)	3	8	2	13 (6.2%)
2 (Beijing)	15	62	36	113 (53.6%)
3 (CAS)	21	23	12	56 (26.5%)
4 (Euro-American)	7	15	7	29 (13.7%)
Total	**46 (21.8%)**	**108 (51.2%)**	**57 (27%)**	**211**

Table 9. Distribution of first-line drug-susceptibility patterns in *Mycobacterium tuberculosis* families.

Drug susceptibility pattern	Genotypes				
	Lineage 1 (EAI)	**Lineage 2 (Beijing)**	**Lineage 3 (CAS)**	**Lineage 4 (Euro-American)**	**Total**
HR^r	3	7	5	1	16
HRS^r	2	21	6	4	33
HRE^r	1	8	3	2	14
$HRSE^r$	4	56	19	13	92
HR^r *	0	6	2	2	10
H^r	0	1	1	0	2
S^r	0	1	1	0	2
E^r	0	1	0	0	1
$HRSE^s$	3	12	19	7	41
Total	**13**	**113**	**56**	**29**	**211**

HR^r = Phenotypic resistant to isoniazid and rifampicin

HRS^r = Phenotypic resistant to isoniazid, rifampicin, and streptomycin

HRE^r = Phenotypic resistant to isoniazid, rifampicin, and ethambutol

$HRSE^r$ = Phenotypic resistant to isoniazid, rifampicin, streptomycin, and ethambutol

H^r = Phenotypic resistant to isoniazid

S^r = Phenotypic resistant to streptomycin

E^r = Phenotypic resistant to ethambutol

$HRSE^s$ = Phenotypic susceptible to isoniazid, rifampicin, streptomycin, and ethambutol

* = Isolates having only isoniazid and rifampicin susceptibility patterns.

Table 10. Mutations found in *pncA* gene and its upstream regulatory region of the *Mycobacterium tuberculosis* isolates from Nepal. The diversity of *pncA* mutations (57 types) and their association with PZA susceptibility as suggested by in-vitro/in-vivo susceptibility, literature review and the webserver "SUSPECT-PZA".

Nucleotide changes	Amino acid changes	No. of isolates	Non-MDR	MDR	Pre-XDR	Genotype	In vitro/in vivo susceptibility [71]	SUSPECT-PZA [48]	PZA susceptibility	Reported	Reference
A-40C	NA	1			1	L2	ND	NA	ND*	Novel	
A-40G	NA	1			1	L2	ND	NA	ND*	Novel	
A-40G, C195T	NA, Ser65Ser	1			1	L3	ND	NA	ND*	Novel	
ins of C at -2 and -3	NA	1		1		L1	ND	NA	S	Yes	[44]
ins of CG at 9/10	Ala3Fs	1		1		L4	ND	NA	ND*	Novel	
T11C	Leu4Ser	6		2	4	L2	R	R	R	Yes	[44]
T17C, C195T	Ile6Thr, Ser65Ser	1		1		L3	R	R	R	Yes	[44]
A29C, C195T	Gln10Pro, Ser65Ser	1		1		L3	ND	R	R	Yes	[70]
G34A	Asp12Asn	1		1		L4	R	R	R	Yes	[39]
A35G	Asp12Gly	1		1		L1	R	R	R	Yes	[44]
T80C	Leu27Pro	2		1	1	L2	R	R	R	Yes	[44]
A143C	Lys48Thr	1		1		L4	R	R	R	Yes	[44]
A152G	His51Arg	1			1	L4	R	R	R	Yes	[18]
C161T	Pro54Leu	1		1		L4	R	R	R	Yes	[44]
ins of G at 165/166	Gly55Fs	1			1	L2	ND	NA	ND*	Novel	
ins of G at 166/167	Gly56Fs	1		1		L2	ND	NA	R	Yes	[88]
C169G	His57Asp	2		1	1	L4	R	R	R	Yes	[44]
A170G	His57Arg	1			1	L3	R	R	R	Yes	[44]
C176T	Ser59Phe	2		2		L4	R	R	R/S*	Yes	[44]
C195T	Ser65Ser	37	20	15	2	L3	S	NA	R/S[a]	Yes	[44]
T202G	Try68Gly	1		1		L2	ND	R	R	Yes	[44]
A226C	Thr76Pro	1		1		L2	ND	R	R	Yes	[18]
G233T	Gly78Val	1			1	L2	R	R	R	Yes	[44]
T254G	Leu85Arg	3		2	1	L2	R	R	R	Yes	[44]
G290T	Gly97Val	1			1	L2	ND	R	R	Yes	[66]
A298C	Thr100Pro	1		1		L4	ND	S	R	Yes	[44]
C309G	Tyr103Stop	1		1		L4	R	NA	R	Yes	[44]
C312G	Ser104Arg	1		1		L2	R	R	R	Yes	[70]
G322C	Gly108Arg	2			2	L2	R	R	R	Yes	[44]
G357C	Trp119Cys	1			1	L4	S/ND	R	R	Yes	[44]
T359G	L120R	1		1		L2	ND	R	R	Yes	[44]
G362C	Arg121Pro	1		1		L2	ND	R	R	Yes	[40]
del of G at 370, del of C at 372, del of T at 374, del of	Arg123Fs	1			1	L4	ND	NA	ND*	Novel	

GATGAG from 376-381											
del of 12 nucleotide (TGAGGTCCATG T) from 378-389	Val125Fs	1		1		L1	ND	NA	ND*	Novel	
G395C	Gly132Ala	3		2	1	L2	R	R	R	Yes	[44]
C401T	Ala134Val	1		1		L4	R	R	R	Yes	[44]
G406A	Asp136Asn	1			1	L4	S/R	R	R	Yes	[44]
G406T	Asp136Tyr	1		1		L2	R	R	R	Yes	[44]
A407G	Asp136Gly	3		3		L2	ND	R	R	Yes	[44]
T416C	Val139Ala	2		1	1	L2	R	R	R/S*	Yes	[70]
A424G	Thr142Ala	1			1	L2	ND	R	R	Yes	[70]
T452C	Leu151Ser	1		1		L2	R	R	R	Yes	[44]
C459G	Thr153Thr	1		1		L2	S	NA	ND#	Novel	
T467C	Leu156Pro	1		1		L2	R	R	R	Yes	[44]
T470G	Val157Gly	1		1		L1	ND	S	R	Yes	[44]
ins of G at 485/486	Gly162Fs	1		1		L4	ND	NA	ND*	Novel	
C500T	Thr167Ile	1			1	L4	ND	S	ND#	Novel	
A502C	Thr168Pro	1			1	L2	S/R	R	R*	Yes	[44]
T545C	Leu182Ser	7		5	2	L2(6), L4(1)	R	R	R	Yes	[46]
C195T, G271T	Ser65Ser, Glu91Stop	1			1	L1	R	NA	R	Yes	[44]
C195T, A287C	Ser65Ser, Lys96Thr	2			2	L3	R	R	R	Yes	[39]
C195T, T347C	Ser65Ser, Leu116Pro	1		1		L3	S/R	S	R/S#	Yes	[44]
C195T, del of at 366	Ser65Ser, Arg121Fs	1		1		L3	ND	NA	R	Yes	[43]
C195T, del of GG at 391/392	Ser65Ser, Val130Fs	1		1		L3	ND	NA	R	Yes	[44]
C195T, ins of 524GC525	Ser65Ser, Glu174Fs	1			1	L3	ND	NA	ND*	Novel	
C195T, T545C	Ser65Ser, Leu182Ser	5		1	4	L3	R	R	R	Yes	[46]
C211T, del of A at 268	His73Tyr, Ile90Fs	2		2		L1(1), L2(1)	R	R	ND*	Novel	[44]
PCR negative		4	1		3	L1(1), L2(3)	ND	NA	R	Yes	[66]
WT		86	25	44	17	L1(6), L2(65), L3(4), L4(11)					
Total		211	46	108	57						

Fs= Frameshift mutation

NA= Not applicable

R= resistant

S= susceptible

ND= Not determined

*= Final interpretation as PZA-resistance by combining *in vitro*/*in vivo* susceptibility, SUSPECT-PZA and literature review

[#]= Final interpretation as PZA-susceptible by combining in-vitro/in-vivo susceptibility, SUSPECT-PZA and literature review

[a]= silent mutation associated with CAS genotype

Table 11. Distribution of polymorphisms of *pncA* among 211 *Mycobacterium tuberculosis* isolates from Nepal.

Types of mutations	Number of types of mutations (57)	No. of isolates (n=125, 59.2%)
Nucleotide substitution	45	109
Regulatory region	2	3
Amino acid substitution	39	66
Termination	2	2
Silent mutation	2	38
Nucleotide deletion	4	4
Nucleotide insertion	6	6
Regulatory region	1	1
Putative region	5	5
Nucleotide substitution + deletion	1	2
Entire gene deletion	1	4

Table 12. Mutation profile among different *Mycobacterium tuberculosis* isolates

	Non-MDR	**MDR**	**Pre-XDR**	**Total**
Mutations in *pncA* and its regulatory region	1	48	38	87
WT	45	60	19	124
Total	**46**	**108**	**57**	**211**

Table 13. Mutation profile among *Mycobacterium tuberculosis* genotypes

	EAI	**Beijing**	**CAS**	**Euro-American**	**Total**
Mutations in *pncA* and its regulatory region	7	47	15	18	87
WT	6	66	41	11	124
Total	**13**	**113**	**56**	**29**	**211**

Table 14. Comparison of *pncA* mutation frequencies with *rpoB*, *katG*, and *inhA* genes in 108 MDR-TB isolates.

Mutation patterns	**No. of isolates**		**Odd ratio, 95% CI**	**P-value**
	With *pncA* mutations	Without *pncA* mutations		
rpoB or *katG* or *inhA* mutation	45	48	3.75 (0.9 to 14.1)	0.05
No *rpoB* or *katG* or *inhA* mutation	3	12		

Table 15. Comparison of *pncA* mutation frequencies with *gyrA*, *gyrB*, and *rrs* genes in 57 Pre-XDR-TB isolates.

Mutation patterns	**No. of isolates**		**Odd ratio, 95% CI**	**P-value**
	With *pncA* mutations	Without *pncA* mutations		
gyrA or *gyrB* or *rrs* mutation	34	10	7.65 (1.9 to 30.18)	0.003
No *gyrA* or *gyrB* or *rrs* mutation	4	9		

Summary

Without the proper information of pyrazinamide (PZA) susceptibility of *Mycobacterium tuberculosis* (MTB), PZA is inappropriately recommended for the treatment of both susceptible and multi-drug resistant tuberculosis (MDR-TB) in Nepal. This study aimed to collect information regarding PZA susceptibility in MTB isolates from Nepal by analyzing mutations in *pncA*. A total of 211 MTB isolates collected from August 2008 to February 2011 were included in this study. Sequence analysis of *pncA* and its upstream regulatory region was performed to assess PZA resistance. First-line drug susceptibility testing, spoligotyping, and sequence analysis of *rpoB*, *katG*, *inhA* regulatory region, *gyrA*, *gyrB*, and *rrs* were performed to assess their association with pncA mutation. The sequencing results revealed that 125 (59.2%) isolates harbored alterations in *pncA* and its upstream regulatory region. I detected 57 different mutation types (46 reported and 11 novels) that were scattered throughout the whole length of the *pncA* gene. I identified 87 (41.2%) isolates harbored mutations in *pncA* causing PZA resistance in MTB. There was a significant association of *pncA* alterations among MDR/pre-extensively drug-resistant (Pre-XDR) TB than among non-MDR-TB ($p<0.005$). The rate of *pncA* mutation was high in MDR-TB/Pre-XDR-TB, and most of the *pncA* mutations resulted in PZA resistance in MTB. The increasing number of PZA resistance among DR-TB in Nepal highlights the importance of PZA susceptibility testing for DR-TB treatment. Considering the long turnaround time of phenotypic DST in Nepal, we recommend the more feasible method of molecular *pncA* sequencing for the detection of PZA susceptibility.

CONCLUSION

With an increasing multidrug-resistant TB in the world, Nepal is not an exception. Even though TB is ranked as the top seventh disease to cause death in Nepal, limited studies have been conducted to determine the phenotypic and genotypic drug-resistant TB in Nepal. For the effective treatment and control of DR-TB in Nepal, a rapid diagnostic tool for DST is required. For the development of a rapid DST tool, information regarding the frequency and patterns of drug resistance-associated gene mutations among DR-TB isolates is necessary.

In chapter I of this thesis, I have described the characterization of the molecular mechanism of STR resistance MTB isolates from Nepal. Mutation in *rpsL* (encoding ribosomal protein S12), *rrs* (encoding 16S ribosomal RNA), and *gidB* (encoding 7-methylguanosine methyltransferase) are associated with resistance to STR. Mutations in *rpsL* were harbored by 65.9% of isolates, in which the most common mutation in *rpsL* is caused by K43R (58.8%) and was significantly associated with the Beijing genotype ($p<0.001$). About 13.2% of isolates harbored mutations in two highly mutable regions of *rrs*, the 530 loop and the 912 region. About 13.2% of *gidB* mutants do not show any mutation in *rpsL* and *rrs*, which might suggest the role of *gidB* mutations in STR resistance in MTB. In addition, 5.6% of isolates do not show any mutations in the three genes examined, suggesting the involvement of other mechanisms in STR resistance in MTB. Our findings of mutations in *rpsL*, *rrs*, and *gidB* satisfactorily predict the STR-resistant MTB in Nepal and can be implemented for the establishment of molecular STR susceptibility testing, in which TB can be treated with appropriate drugs and can improve control strategies for DR-TB.

In chapter II, I aimed to collect information regarding pyrazinamide susceptibility in MTB isolates from Nepal by analyzing mutations in *pncA*. I investigated the frequency and patterns of *pncA* mutation among MTB isolates. I analyzed the *pncA* mutations by comparing with previously reported PZA resistance associating *pncA* mutations and *in vivo*/*in vitro* study. In addition, I described the *pncA* mutations by analyzing the single nucleotide substitution of *pncA* with the webserver predictive tool (SUSPECT-PZA). Sequence analysis of *pncA* and its upstream regulatory region was performed to assess PZA resistance. First-line drug susceptibility testing, spoligotyping, and sequence analysis of *rpoB, katG, inhA* regulatory region, *gyrA*, *gyrB*, and *rrs* were performed to assess their association with *pncA* mutations. The sequencing results revealed that 125 (59.2%) isolates harbored alterations in *pncA* and its upstream regulatory region. We detected 57 different mutation types (46 reported and 11 novels) that were scattered throughout the whole length of the *pncA* gene. I identified 87 (41.2%) isolates harbored mutations in *pncA* causing PZA resistance in MTB. There was a significant association of *pncA* alterations among MDR/pre-extensively drug-resistant (Pre-XDR) TB than among non-MDR-TB ($p<0.005$). The rate of *pncA* mutation was high in MDR-TB/Pre-XDR-TB, and most of the *pncA* mutations

resulted in PZA resistance in MTB. The increasing number of PZA-resistance among DR-TB in Nepal highlights the importance of PZA susceptibility testing for DR-TB treatment. Considering the long turnaround time of phenotypic DST in Nepal, we recommend the more feasible method of molecular *pncA* sequencing for the detection of PZA susceptibility.

The findings from this study can provide the elaborated knowledge on the genotypic drug resistance mechanism of STR and PZA. This study focused on the routine DST before the implementation of drugs for DR-TB treatment in Nepal. Moreover, this study was focused on frequency and patterns in drug resistance-associated gene mutations which can help in the development of easy, rapid, and accessible DST tools. The diagnostic tools could contribute to policymaking on proper management of DR-TB treatment through early diagnosis and appropriate DR-TB treatment in Nepal.

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