Molecular Analysis of phenotypic Multidrug Resistant *Mycobacterium tuberculosis* isolates

M.Sc. Thesis
2016

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

For the partial fulfillment of the requirement for the
Master of Science in Biotechnology

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Roll No.: BT 106/069
T.U.Regd No.: 5-2-37-547-2007

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ABSTRACT

The emergence of strains of *Mycobacterium tuberculosis* resistant to drugs is becoming a serious public health problem. MDR-TB refers to the diseased condition whereby *Mycobacterium tuberculosis* becomes resistant to the first line of drug treatment i.e. rifampin and isoniazid. Treatment regimens for MDR-TB are more complex, less potent, more toxic, and more expensive than first-line regimens, which will be too costly for resource- poor nations like Nepal. Rifampicin (RIF) and isoniazid (INH) which are the most important primary drugs used in treatment of tuberculosis patients. Resistance to rifampicin (RIF) and isoniazid (INH) is mainly achieved through mutations in the rpoB gene and katG gene respectively. DNA samples from phenotypic MDR- TB were collected from Global hospital. A Total of 34 DNA samples were subjected to the Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS)PCR using three different codon specific primers (516, 526 and 531).These three codons occupy large portion of total mutation responsible for rifampin resistance. Total DNA samples were bearing mutation in any of the three codons mentioned. In our study, the highest number of samples had mutation in codon 531 (96.97%) followed by codon 516(18.18%) and codon 526 (12.12%) respectively. And Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) assay was carried out on same above mentioned samples for detection of Ser315Thr mutation. The katG Ser315Thr mutation was observed in 25 (73.52%) out of 34 samples. . This result shows that 72.72% of Rif^r isolates are also isoniazid resistant. Thus ARMS PCR can be used as an important molecular technique for detection of rifampin resistance in Mycobacterium tuberculosis strains as well as surrogate marker for MDR TB and PCR-RFLP technique can be used for detection of isoniazid resistance in MTB in developing country like Nepal.

Keywords: Multiple drug resistance, Mycobacterium tuberculosis, Rifampin, rpoB, ARMS-PCR,PCR-RFLP

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Glossary Acronyms

%	Percentage
µl	Microlitre
µm	Micromolar
AFB	Acid Fast Bacilli
ARMS	Amplification Refractory Mutation System
DNA	Deoxyribonucleic Acid
DOTS	Direct Observation Treatment Short
DST	Drug Susceptibility Test
EDTA	Ethylene Diamine Tetra Acetic Acid
EMB	Ethambutol
Et.Br.	Ethidium Bromide
HA	Hetero duplex Assay
HIV	Human Immunodeficiency
INH	Isonicotinic and Hydrazide(Isoniazid)
katG	Catalase peroxidase Gene
LJ media	Lowenstein Jansen Medium
MDR	Multiple Drug Resistant
MDR	Multiple Drug Resistant
MIC	Minimum inhibitory Concentration
mM	Millimolar
MTB	Mycobacterium Tuberculosis
NFW	Nuclease Free Water
Pas	Para Amino Salicylic Acid
PCR	Polymerase Chain Reaction

PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
Rif	Rifampicin
rpoB	RNA Polymerase β subunit gene
RRDR	Rifampicin Resistance Determining Region
SNPs	Single Nucleotide polymorphisms
TB	Tuberculosis
TDR	Totally Drug – Resistant
UV	Ultraviolet
WHO	World Health Organization
XDR	Extensively Drug – Resistant

Chapter 1

INTRODUCTION

1.1 *Mycobacterium tuberculosis*

Mycobacteria entails a certain degree of interest as well as curiosity among the scientists since they possess a tantalizing complexity in their cell wall make up when compared to the standard setup of the gram positive and gram negative bacterium. The cell walls are regarded to be an efficient barrier with selective permeability conferring the organism the ability to gain drug resistance as well as survive under unfavorable conditions (Brennan and Nikaido, 1995). Taxonomically, Mycobacteria belong to the genus Mycobacterium, which is the single genus within the family of Mycobacteriaceae, in the order Actinomycetales (Rastogi *et al.,* 2001).

The Mycobacteria are unique in their cell wall composition in that they possess lipids such as mycolic acids that makes up about 30 to 40 % of the cell envelope mass. Such features capacitate the organism with higher virulence resulting in better survival and significant drug resistance (Neiderweis *et al.,* 2010), hence, accounting for the fact that Mycobacterium are one of the leading cause of infection in both humans as well as animals (Rastogi *et al.,* 2001).

Among the distinguished members, *Mycobacterium tuberculosis* holds a particular interest due to its clinical significance in causing various types of tuberculosis. Tuberculosis, a malady as old as the humans itself still holds an icy grip upon its victim despite the advances in its treatment with vaccines and antibiotics (Smith, 2003).

1.2 Tuberculosis (TB)

Tuberculosis (TB) is regarded as a historical disease, which has a long and continuing record of causing worldwide morbidity and mortality especially in developing countries, including Nepal (Chadha, 2009). Infection by the bacillus *Mycobacterium tuberculosis* leads to the chronic disease with aerial mode of transfer between people (Zaman, 2010). The bacterium usually attacks lung called pulmonary tuberculosis but it can also attack other parts of the body such as the kidney, spine, and brain called extra pulmonary tuberculosis (Farer *et al.,* 1979). The nature of symptoms depends upon the site where the bacterium inhabits. In the cases of pulmonary TB, it may cause symptoms, such as chronic cough, pain in the chest, haemoptysis, weakness or fatigue, weight loss, fever, and night-sweats (Zaman, 2010).

For the purposes of public health surveillance and prevention, tuberculosis is verified by given set of parameters:

1) Isolation of M. tuberculosis in a culture grown from a clinical specimen,

2) Finding of acid-fast bacilli in clinical specimens for which a culture has not been or cannot be obtained, and/or

3) A clinical presentation consistent with tuberculosis (e.g., a positive tuberculin skin test, symptoms compatible with tuberculosis, and an abnormal or unstable chest radiograph) in a patient who is started on two or more antituberculosis drugs (Wharton *et al.,* 1990).

1.3 Multidrug Resistant *Mycobacterium tuberculosis*

When the *M. tuberculosis* organism develops resistance to at least Isoniazid and Rifampin, considered as the most effective medication against tuberculosis, they are referred to as Multidrug-resistant tuberculosis. The emergence of primary drug resistance in a patient who has never before been treated for tuberculosis would be due to infection by drug-resistant bacilli. On the contrary, secondary (acquired) drug resistance arises in a patient, who initially had drug-susceptible *M. tuberculosis*, through inappropriate or inadequate treatment, noncompliance to a prescribed regimen or poor absorbance of prescribed medication (Gangadharam, 1993).

1.3.1 Resistance to Rifampicin (RIF)

It is mainly caused by the mutations in the *rpoB* gene coding the β *s*ubunit of RNA polymerase**.** Rifampicin is the most important drug available for TB treatment. It inhibits gene transcription by binding to the β subunit of the DNA dependent RNA polymerase, encoded by the rpoB gene. Most rpoB mutations occur through point mutations within a 81 bp rifampicin resistance- determining region (RRDR), located between codons 507 and 533 with the most common changes being observed in codons Ser531Leu, His526Tyr and Asp516Val (O'Sullivan *et al.,* 2005; Sekiguchi *et al.,* 2007). Furthermore, more than 90% of RIF-resistant isolates are also resistant to isoniazid; therefore, rifampin resistance can be assumed to be a surrogate marker for MDR TB, which identifies MDR strains (Drobniewski and Wilson, 1998).

1.3.2 Resistance to Isoniazid (INH)

Numerous genes can be involved in INH resistance (katG, inhA, KasA, ndh, the oxyR-ahpC intergenic region, fabG, fadE24, inhA promoter, iniA and the mabA-inhA operon) (Muller B et al., 2013; Siu GK et al., 2011). *KatG* gene is the most commonly targeted region with majority of mutations occurring in codon 315 in 30-90% of INH resistant M. tuberculosis clinical isolates depending on geographical distribution. The katG gene encodes the catalase-peroxidase enzyme that is involved in the activation of the pro-drug INH. The

isoniazid enter the bacterial cell wall and subsequently affects intracellular targets such as mycolic acid biosynthesis, an important component of the cell wall, which eventually results in loss of cellular integrity and the bacterial cell death. It produces free radicals that in turn are toxic to different bacterial molecules (Sacchettini & Blanchard, 1996). The loss or reduction of enzyme activity by mutations prevents this process, allowing survival in the presence of INH (Rouse et al., 1996; Gonzalez *et al.,* 1999; Slayden *et al.,* 2000).

1.4 Amplification Refractory Mutation System – Polymerase Chain Reaction (ARMS – PCR)

The amplification-refractory mutation system (ARMS) is a simple, rapid and reliable method for detecting any mutation involving single base changes or small deletions (Ferrie *et al*., 1992). ARMS – PCR is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele (Newton *et al.,* 1989).

The working principle behind ARMS- PCR is that oligonucleotides which are complementary to a wild type DNA sequence will only function as primers in PCR under optimized conditions, however, the mutated DNA sequence won't get amplified (Fan *et al.,* 2003). The protocol has been followed for the detection of several genetic polymorphisms including 1-antitrypsin deficiency (Newton et al., 1989), CFTR gene mutation (Ferrie et al., 1992), *apolipoprotein E* genotypes (Donohoe et al., 1999), and K-*ras* mutation (Carpenter et al., 1996). With respect to its limitation, ARMS PCR can be used for the detection of the mutation only but not its nature, hence cannot substitute for the results obtained through DNA sequencing (Fan et al., 2003).

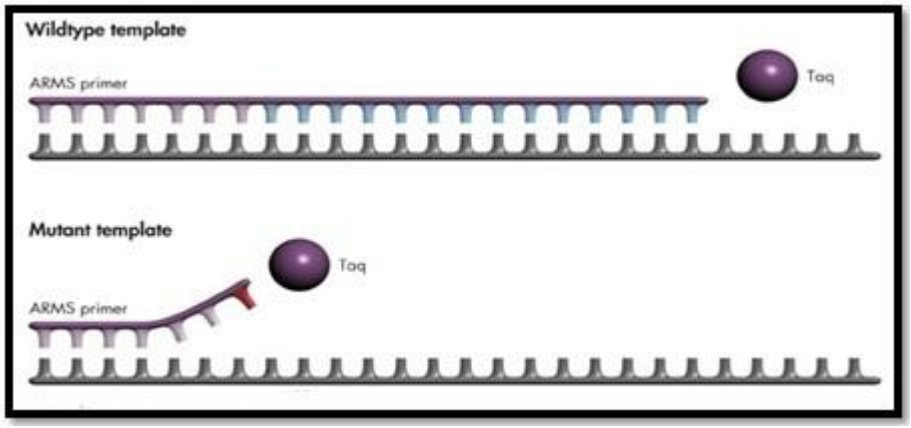

Fig. 1.1 Basic Principle of ARMS – PCR

1.5 Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR – RFLP)

Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (RFLP)-based analysis, also known as Cleaved Amplified Polymorphic Sequence (CAPS), is a popular technique for genetic analysis that is useful for the detection of intraspecies as well as interspecies variation. The technique exploits that Single Nucelotide Polymorphisms (SNPs), Multi - Nucleotide Polymorphisms (MNPs) and microindels that are often associated with the creation or abolishment of a restriction enzyme recognition site (Narayanan, 1991). The first step in a PCR-RFLP analysis is amplification of a fragment containing the variation. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic separation of the fragments.

1.5.1 Advantages of PCR – RFLP

1. Inexpensive
2. Easy to design
3. Applicable to analysis of single nucleotide polymorphisms as well as microindels
4. No requirement for expensive instruments
5. No requirement for extensive training of laboratory staff
6. Miniaturisable.

1.5.2 Disadvantages of PCR – RFLP

1. Requires that a variation generates or abolishes a restriction enzyme recognition site
2. Some restriction enzymes are expensive
3. Exact genotyping cannot be achieved in the event that there is more than one nucleotide variation in a restriction enzyme recognition site
4. Requires relatively large amounts of hand-on-time
5. Long time from start to completion of the analysis
6. Not suitable for high-throughput analysis (Rasmussen, 2012).

1.6 Present scenario of TB in Nepal

In Nepal, the incidence of all forms of TB was estimated to be 136/100,000 population, while the incidence of new smear-positive cases was at 57/100,000 in 2013/2014. The proportion of new cases with multidrug-resistant TB (MDR-TB) was 2.2% among new cases and 15.4% among retreatment cases based on survey carried out in 2011/12 in Nepal. In

2014, total of 349 MDR – TB patients were confirmed. Nepal is a landlocked country in Southeast Asia, bounded to the north by China and to the south by India, sharing an open border with India. India and China had the largest numbers of TB cases (23%, and 10% of the global total, respectively). Every year, a large number of people of Nepal and India cross the border for various purposes, such as work, study, trade, pilgrimage, cultural visits, and so on. Since drug resistance rates on one side of the border can impact the other side of the border, a high proportion of MDR-TB in Nepal may reflect the possible dissemination of infection from surrounding two countries, mainly from India (NTP Annual Report, 2014; WHO Report, 2015).

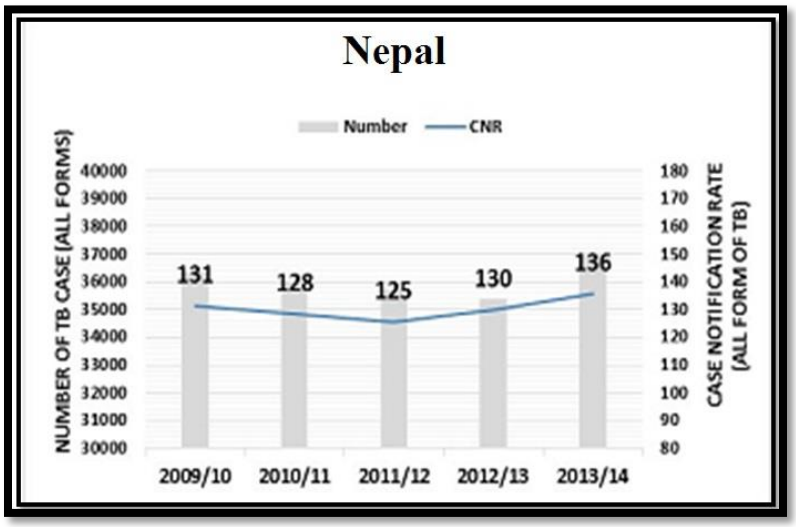

Fig. 1.2 Time-series of national TB notification numbers and rates in Nepal, 2009/10 - 2013/14 [Adapted from NTP Annual Report, 2014]

1.7 Statement of Problem:

The disease burden of the tuberculosis has been rising as can be seen through the annual report; this can create a negative impact on the financial and economic status of an entire nation. The disease, often considered to affect the poor nations severely when compared to the developed nations, demonstrates similar pattern in the context of Nepal as well where treatment is sometimes given more priority than prevention. Hence, the number of patients undertaking Anti- tuberculosis drugs is increasing by the year and equivalent situation is present for the incidence of TB among general population of Nepal. The major problem lies in the failure of early detection of MDR – TB which is generally recognized only when the patient is no longer responding to the drugs being administered resulting in high economic loss as well as health deterioration. So, keeping with state of affairs of Nepal, It would be

very much judicial to develop rapid and efficient methods for detection of MDR TB and prevent the major outbreaks.

1.8 Rationale of the study

Early diagnosis of TB and rapid detection of rifampin resistance and isoniazid resistance i.e. multiple drug resistant (MDR) are essential for efficient treatment and control of *M. tuberculosis* which would help in survival of TB patient. It can also be helpful in early initiation of appropriate treatment therapy and improvement of treatment outcomes, even in resource-poor settings. Timely detection of drug resistance TB is very important for controlling the development and spread of MDR-TB as it facilitates the correct and judicious delivery of anti-TB therapy to the patients reducing overall cost required for treatment and transmission of resistant cases. The current study can also help in the understanding of existing state of MDR TB cases in Nepal**.**

1.9 Objectives:

1.9.1 General objective:

- To identify KatG mutation associated with isoniazid resistance and rpoB mutations associated with rifampin resistance in a panel of phenotypic Multidrug resistant *Mycobacterium tuberculosis* strains collected from Global hospital by PCR-RFLP, ARMS PCR techniques and sequencing.

1.9.2 Specific objective:

- To detect the mutation present in the codon number 516, 526 and 531 of rpoB gene of MDR – TB strains, collected from Global hospital, by ARMS PCR technique.
- To verify ARMS PCR technique as potential diagnostic tool for detection of mutation by sequencing of rifampicin resistance- determining region (RRDR).
- To screen Ser315Thr mutation of *Kat*G gene of INH resistant MTB strain by PCR-RFLP technique.
- To perform gel electrophoresis for the analysis of mutations associated with the codons under investigation.

1.10 Research Hypothesis:

Null Hypothesis: The phenotypic MDR TB strains do not contain mutation in the rpoB gene and katG gene.

Alternative Hypothesis: The phenotypic MDR TB strains contain mutation in the rpoB gene and katG gene.

Chapter 2

LITERATURE REVIEW

2.1 Tuberculosis (Past, Present and Future)

"The struggle [against tuberculosis] has caught hold along the whole line and enthusiasm for the lofty aim runs so high that a slackening is no longer to be feared. If the work goes on in this powerful way, then the victory must be won." – Robert Koch.

Tuberculosis is an age old plague that has haunted humankind throughout known history and human prehistory but is yet to become a disease of the past (Leinhardt *et al.,* 2012). Hard tissues like bone can be preserved for thousands of years, allowing the almost certain identification of individuals with bone TB who died more than 4,000 years ago. The frequency of unearthed skeletons with apparent tubercular deformities in ancient Egypt suggests that the disease was common among that population. The discovery of similarly deformed bones in various Neolithic sites in Italy, Denmark, and countries in the Middle East also indicates that TB was found throughout the world up to 4,000 years ago (Smith, 2003). It has surged in great epidemics and then receded, thus behaving like other infectious diseases, but with a time scale that challenges accepted explanations for epidemic cycles. *Mycobacterium tuberculosis* may be the most fatal pathogen than any other microbial pathogen in terms of its incidence rate and prevalence (Daniel, 2006).

With the advent of chemotherapy during 1940s and establishment of standardized short course treatment regimen in 1970s and 1980s, TB was predicted to be eradicated soon. Decrease in incidence rate was observed in most of the developed countries from 1950s to 1980s. While no such declines were seen in most developing countries, instead the trend showed increment in the developed world during late 1980s (Chadha, 2009). Despite disappearing from the world public health agenda in the 1960s and 1970s, havoc created due to HIV epidemic and evolution of multi-drug resistant (MDR) TB resulted in declaration of the disease as a global emergency by the World Health Assembly (WHA) in 1991 (Leinhardt *et al.,* 2012). A frame work for TB control was developed in the form of DOTS (the internationally recommended strategy for TB control) which aimed for annual targets of 70% case detection of new smear positive Pulmonary TB (PTB) cases and 85% treatment success were set to be achieved by the year 2000, only to be revised to 2005. The principle target of United Nations' Millennium Development Goals (MDGs) for TB control adopted in the year 2000 is to ensure that the incidence rate of TB is declining by 2015. The supplementary targets are to halve the prevalence of TB and TB mortality rates by 2015 as

compared to 1990. The ultimate goal is to eliminate TB by 2050, when the annual incidence should be less than one case per million populations (Chadha, 2009). Several new vaccines against TB are being developed. These vaccines are now being field-tested in different countries in different phases (Abel, 2010).

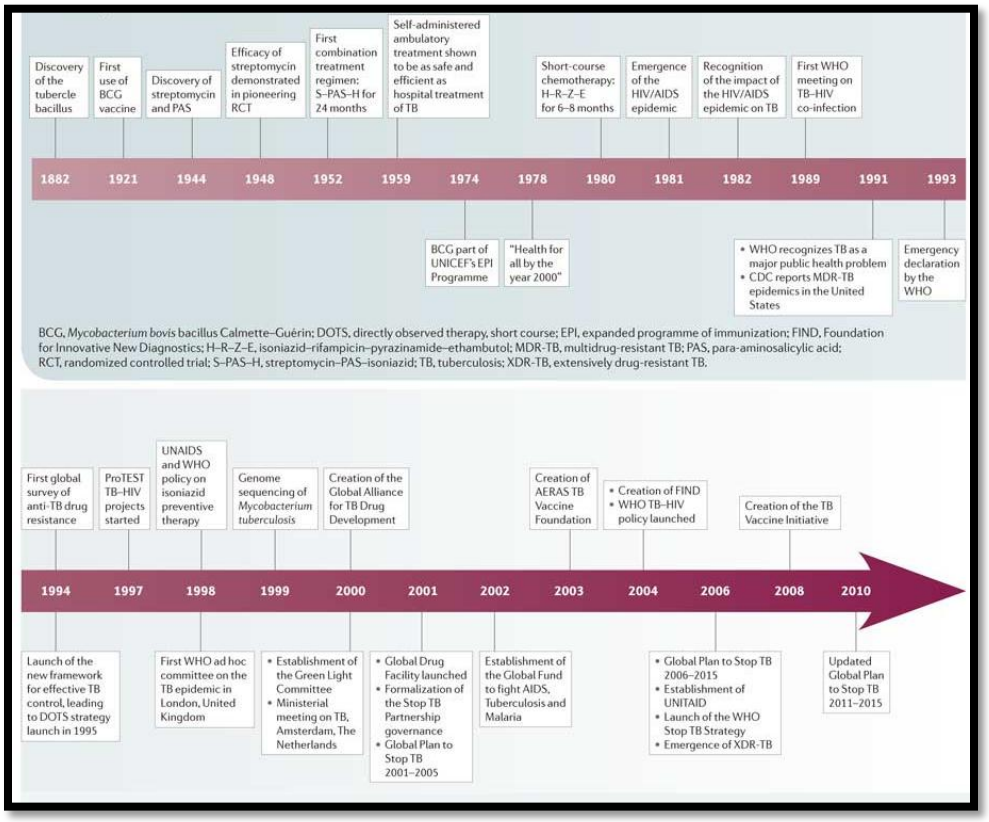

Fig. 2.1 Landmarks of TB Control. [Adapted from Leinhardt *et al.,*2012]

2.2 Causative Agent

"White Plague" of the 17th and 18th centuries in Europe which had the prevalence rate of nearly 100 percent and was the cause of fatality in approximately 25 percent of adult death was due to *Mycobacterium tuberculosis*. The bacterium is a fairly large non motile rod-shaped obligate aerobe, often 1-10 µm in length (usually 3-5 µm), and 0.2 -0.6 µm width. However, the size and shape of the bacilli may vary from coccobacilli to long rods depending on the growth conditions and age of the culture. *M. tuberculosis* complexes are always found in the well-aerated upper lobes of the lungs due to their aerobic nature. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence (Todar, 2005).

Tuberculosis infection depends on exposure to an active case while progression from infection to disease depends on the immune status of the infected person (Rieder *et al.,* 1989). Most tuberculosis infections result from inhalation of airborne droplets containing the bacillus *Mycobacterium tuberculosis* (Nardell, 1993). These droplets are emitted most efficiently by patients with laryngeal tuberculosis, cavitary disease on chest radiographs, and sputum samples which show acid-fast bacilli on smear.

Mycobacteria are Gram-positive, rod-shaped bacteria of the Actinomycete family, and are therefore most closely related to the nocardia, corynebacteria, and Streptomyces. Their most characteristic feature is their complex cell envelope, containing a high percentage of lipids, which include the mycolic acids (Parish and Stoker, 1999). Mycolic acids are exceptionally long fatty acids that account for 30% to 40% of the cell envelope mass. They are covalently linked to peptidoglycan via an arabinogalactan polymer, a polysaccharide composed of arabinose and galactose subunits. In a typical arrangement, the peptidoglycan network is substituted by linear galactan molecules, which bear several branched arabinose chains. These branches end in four arabinose dimers, each forming the head group for two mycolic acid molecules (Brennan and Nikaido 1995; Minnikin, 1982; Barry, 2001). This envelope constitutes an efficient permeability barrier and plays a crucial role in the intrinsic drug resistance and in survival under harsh conditions (Neiderweis *et al.,* 2010). Hence, My*cobacterium* species are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. Ziehl-Neelsen stain is often used for staining *Mycobacterium tuberculosis*. When this method is used, the *M. tuberculosis* Smear is fixed, stained with carbol- fuchsin (a pink dye), and decolorized with acid-alcohol. The smear is counterstained with methylene-blue or certain other dyes resulting in acid-fast bacilli appearing pink in a contrasting background (Todar, 2005).

M. tuberculosis was discovered in 1882 by Robert Koch (Koch, 1882); of the four species in the *M. tuberculosis* complex (*M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, and *Mycobacterium microti*) (Grosset, 1990), it is the one primarily responsible for tuberculosis in humans. An undiagnosed and untreated smear-positive patient has been estimated to infect 10-14 persons per year. The likelihood of infection following exposure to infectious droplets increases with increasing concentration of *M. tuberculosis* droplet nuclei in the air and increasing length of exposure (Houk *et al.,* 1968). The efficacy of the Bacille Calmette Guerin (BCG) vaccine in protecting against tuberculosis is variable and partial, although the vaccine does appear to offer protection against disseminated and meningeal tuberculosis in children. The diagnosis of TB among children is difficult. Moreover, young

children cannot produce sputum. Estimates indicate that children constitute about 10% of all new cases in high-burden areas (Dye, 2006).

The origin of *M. tuberculosis* has been the subject of much recent investigation, and it is thought that the bacteria in the genus *Mycobacterium*, like other actimomycetes, were initially found in soil and that some species evolved to live in mammals. The domestication of cattle, thought to have occurred between 10,000 and 25,000 years ago, would have allowed the passage of a mycobacterial pathogen from domesticated livestock to humans, and in this adaptation to a new host, the bacterium would have evolved to the closely related *M. tuberculosis* (Stead, 1997). However, recent studies provide strong evidence for the independent evolution of both *M. tuberculosis* and *M. bovis* from another precursor species, possibly related to *M. canetti* (Brosch *et al.,* 2002)

Other human pathogens belonging to the *Mycobacterium* genus include *Mycobacterium avium* which causes a TB-like disease especially prevalent in AIDS patients, and *Mycobacterium leprae***,** the causative agent of leprosy.

Two media are used to grow *M. tuberculosis:* one is Middlebrook's medium which is an agar based medium and another is Lowenstein-Jensen medium which is an egg based medium. *M. tuberculosis* colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing *M. tuberculosis*. It takes 4-6 weeks to get visual colonies on either type of media (Todar, 2005).

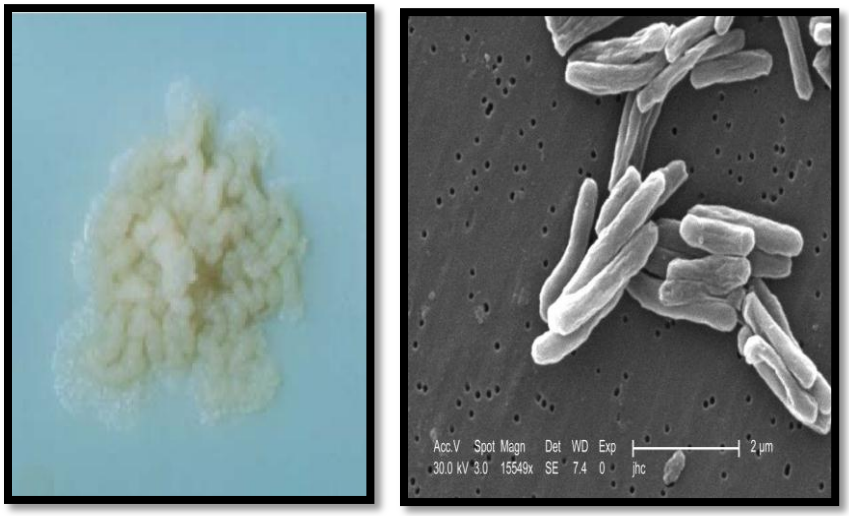

Fig. 2.2 A. Colony of *Mycobacterium tuberculosis* in Lowenstein Jensen Medium. (Todar, 2006); **B.** Scanning electron micrograph (SEM) Gram-positive *Mycobacterium tuberculosis* bacteria.

2.3 Scientific classification (Lehman and Neuman, 1896)

Kingdom: Bacteria

Phylum: Actinobacteria

Order: Actinomycetales

Suborder: Corynibacterineae

Family: Mycobacteriaceae

Genus: ***Mycobacterium***

Species: ***tuberculosis***

2.4 Global epidemiology of Tuberculosis

Tuberculosis (TB) is a major public health concern worldwide causing sickness among millions of people each year. The ailment ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide.

TB morbidity and mortality rates due to TB steadily dropped during the 20th century in the developed world, aided by better public health practices and widespread use of the *M. bovis* BCG vaccine (discussed below), as well as the development of antibiotics in the 1950s. This downward trend ended and the numbers of new cases started increasing in the mid-1980s. The major causes of this were increased homelessness and poverty in the developed world and the emergence of AIDS, with its destruction of the cell-mediated immune response in coinfected persons. Only by massive expenditures of funds and human resources, mainly by directly monitored antibiotic delivery, has this “miniepidemic” of new TB cases been reversed in Europe and the United States (Freiden *et al.,* 1995).

Although a declining trend in TB incidence, prevalence and mortality has been observed over the last decade, elimination of the disease at global level is still out of reach, and massive resource investment is still required. TB is a poverty-related disease which disproportionately affects the poorest, the most vulnerable and marginalized population groups wherever it occurs. Improving access to diagnosis and care, the basic requirements in the fight against TB, are particularly challenging in these persons (Sulis *et al.,* 2014).

In 2014, there were an estimated 9.6 million new TB cases: 5.4 million men, 3.2 million women and 1.0 million children. There were also 1.5 million TB deaths (1.1 million among HIV-negative people and 0.4 million among HIV-positive people), of which approximately

890 000 were men, 480 000 were women and 140 000 were children (Global Tuberculosis Report 2015, WHO).

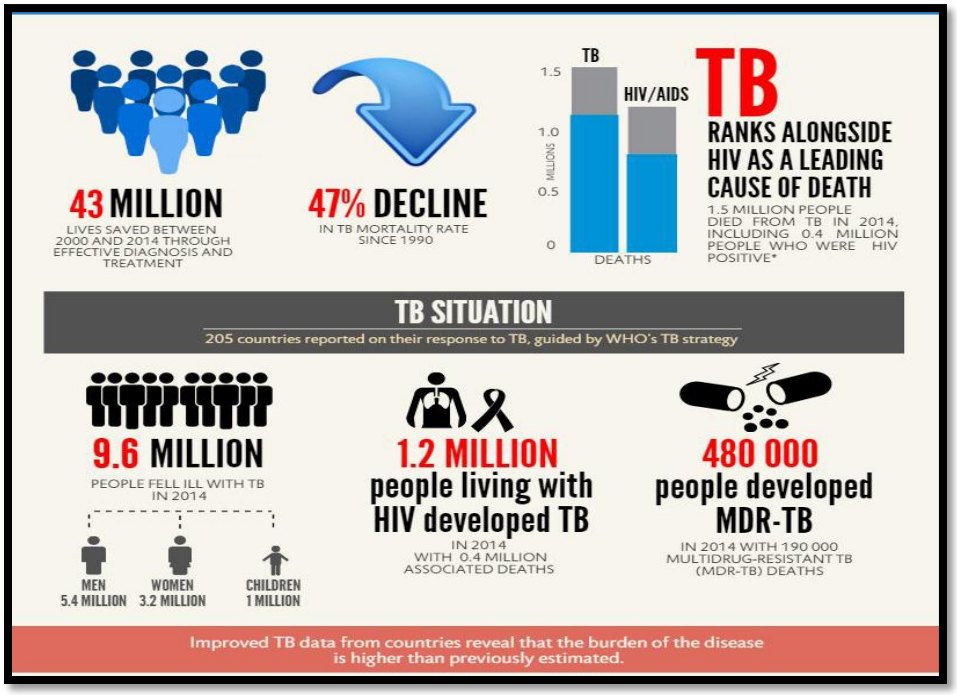

Fig. 2.3 Status of global TB epidemic. [Adapted from Global Tuberculosis Report 2015, WHO]

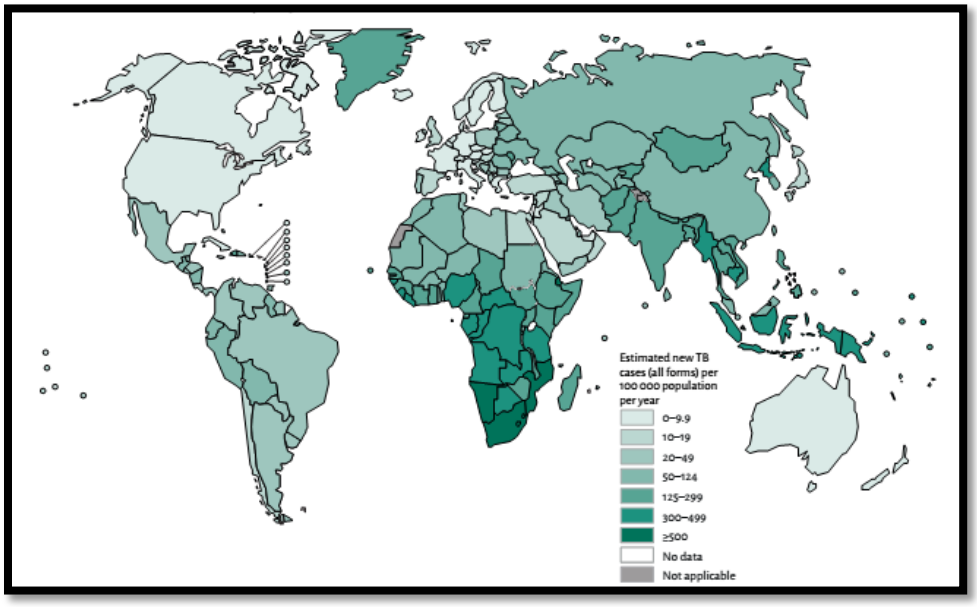

Fig. 2.4 Estimated TB incidence rates 2014. [Adapted from Global Tuberculosis Report 2015, WHO]

2.5 Risk Factors for Tuberculosis

Fig. 2.5 summarizes various risk factors for the progression of tuberculosis upon exposure to diseased condition.

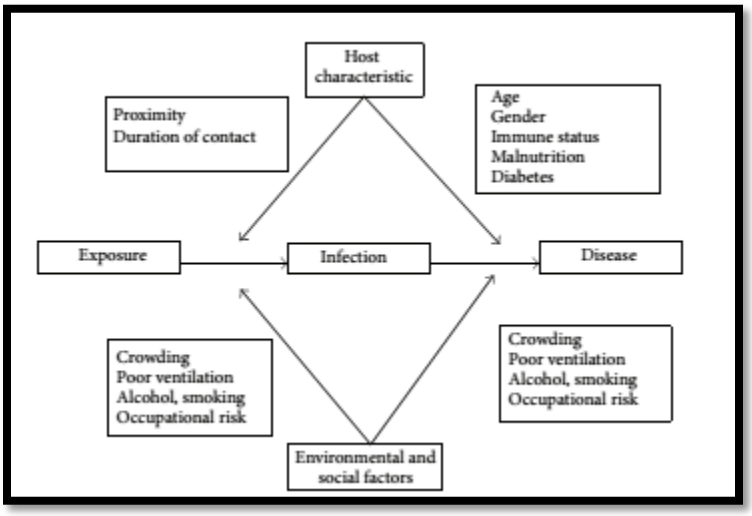

Fig. 2.5 Illustrative diagram depicting risk factors correlated with infection by *Mycobacterium tuberculosis.* [Adapted from Narasimhan *et al.,* 2013].

2.6 Clinical Manifestations of tuberculosis

The clinical manifestations of tuberculosis are dependent on a number of aspects: age, immune status, co-existing diseases, immunization status to the bacillus Calmette-Guerin (BCG); virulence of the infecting organism and host-microbe interaction. Before the beginning of the HIV epidemic, approximately 85% of reported tuberculosis cases were pulmonary only, with the remaining 15% being extra-pulmonary or both pulmonary and extra-pulmonary sites. Extrapulmonary involvement tends to increase in frequency with worsening immune compromise (Farer *et al.,* 1979).

2.6.1 Systemic Effects of Tuberculosis

Tuberculosis involving any site may produce symptoms and findings that are systemic in nature. The frequency of fever can range from 37 to 80%. Loss of appetite, weight loss, weakness, night sweats, and malaise are also common (Arango *et al.,* 1973).

The most common haematologic manifestations are increases in the peripheral blood polymophonuclear leukocyte count and anaemia in some instances, anaemia or pancytopenia may follow direct involvement of the bone marrow (Cameron, 1974).

Tuberculosis is associated often with other serious disorders including:

- HIV infection,
- alcoholism,
- drug abuse
- chronic renal failure,
- diabetes mellitus,
- Neoplastic diseases.

The clinical features of these diseases and complications may modify those of tuberculosis and so hinder diagnosis especially in HIV patients (Kramer *et al.,* 1990).

2.6.2 Various Stages of Tuberculosis

As the cellular processes occur, tuberculosis may develop differently in each patient, according to the status of the patient's immune system. Stages include latency, primary disease, primary progressive disease, and extra pulmonary disease. Each stage has different clinical manifestations (Knechel, 2009).

2.6.2.1 Latent Tuberculosis

Mycobacterium tuberculosis organisms can lie dormant in the necrotic material for a long period as people with latent tuberculosis show no clinical symptoms, remain healthy, and are not infectious. However, time dependent degradation of the immune system can result in reactivation of the disease (Guyot – Reval *et al.,* 2006). Although coinfection with human immunodeficiency virus is the major cause for progression to active disease, other factors, such as uncontrolled diabetes mellitus, sepsis, renal failure, malnutrition, smoking, chemotherapy, organ transplantation, and long-term corticosteroid usage that can trigger reactivation of a remote infection are more common in the critical care setting (Freiden *et al.,* 2003). Moreover, persons 65 years or older have a higher rate of disease than any other age group due to reactivation of disease ,often because of diminishing immunity (Thrupp *et al.,* 2004).

2.6.2.2 Primary Tuberculosis

Primary tuberculosis (TB) is a term that describes new tuberculosis infection or active disease in a previously naïve host. Primary TB was considered to be mainly a disease of childhood until the introduction of effective chemotherapy with isoniazid in the 1950s (Stead *et al.,* 1968). Many studies since that time have shown an increased frequency in the acquisition of TB in adolescents and adults (Buckner *et al.,* 1990). It is often asymptomatic, so that the results of diagnostic tests are the only evidence of the disease. Although primary

disease essentially exists subclinically, some self-limiting findings might be noticed in an assessment. Associated paratracheal lymphadenopathy may occur because the bacilli spread from the lungs through the lymphatic system. If the primary lesion enlarges, pleural effusion is a distinguishing finding. This effusion develops because the bacilli infiltrate the pleural space from an adjacent area. The effusion may remain small and resolve spontaneously, or it may become large enough to induce symptoms such as fever, pleuritic chest pain, and dyspnea (Murray *et al.,* 1978).

2.6.2.3 Primary Progressive Tuberculosis

Active tuberculosis develops in only 5% to 10% of persons exposed to *M. tuberculosis*. When a patient progresses to active tuberculosis, early signs and symptoms are often nonspecific. Manifestations often include progressive fatigue, malaise, weight loss, and a low-grade fever accompanied by chills and night sweats Wasting, a classic feature of tuberculosis, is due to the lack of appetite and the altered metabolism associated with the inflammatory and immune responses. Wasting involves the loss of both fat and lean tissue; the decreased muscle mass contributes to the fatigue (Paton *et al.,* 2004). Finger clubbing, a late sign of poor oxygenation, may occur; however, it does not indicate the extent of disease (Ddungu *et al.,* 2006).

A cough eventually develops in most patients which may initially be nonproductive, however advances to a productive cough of purulent sputum in the later stages. The sputum may also be streaked with blood which could be due to destruction of a patent vessel located in the wall of the cavity, the rupture of a dilated vessel in a cavity, or the formation of an aspergilloma in an old cavity. The inflamed parenchyma may cause pleuritic chest pain. Extensive disease may lead to dyspnea or orthopnea because the increased interstitial volume leads to a decrease in lung diffusion capacity. Although many patients with active disease have few physical findings, rales may be detected over involved areas during inspiration, particularly after a cough. Hematologic studies might reveal anemia, which is the cause of the weakness and fatigue. Leukocytosis may also occur because of the large increase in the number of leukocytes, or white blood cells, in response to the infection (Huseby and Hudson, 1976).

2.6.2.3 Extrapulmonary Tuberculosis

Although the pulmonary system is the most common location for tuberculosis, extrapulmonary disease occurs in more than 20% of immunocompetent patients, and the risk for extrapulmonary disease increases with immunosuppression (Knechel, 2003). The most serious location is the central nervous system, where infection may result in

meningitis or space-occupying tuberculomas. If not treated, tubercular meningitis is fatal in most cases, making rapid detection of the mycobacteria essential. Headaches and change in mental status after possible exposure to tuberculosis or in high risk groups should prompt consideration of this disease as a differential diagnosis. Another fatal form of extrapulmonary tuberculosis is infection of the bloodstream by mycobacteria; this form of the disease is called disseminated or miliary tuberculosis. The bacilli can then spread throughout the body, leading to multiorgan involvement. Miliary tuberculosis progresses rapidly and can be difficult to diagnose because of its systemic and nonspecific signs and symptoms, such as fever, weight loss, and weakness. Lymphatic tuberculosis is the most common extrapulmonary tuberculosis, and cervical adenopathy occurs most often. Other possible locations include bones, joints, pleura, and genitourinary system (Alvarez and McCabe, 1984; Weir and Thorton, 1985).

Early infection	Early primary progressive (active)	Late primary progressive (active)	Latent
Immune system fights infection	Immune system does not control initial infection	Cough becomes productive	Mycobacteria persist in the body
Infection generally proceeds without signs or symptoms	Inflammation of tissues ensues	More signs and symptoms as disease progresses	No signs or symptoms occur
Patients may have fever, paratracheal lymphadenopathy, or dyspnea	Patients often have nonspecific signs or symptoms (eg, fatigue, weight loss, fever)	Patients experience progressive weight loss, rales, anemia	Patients do not feel sick
Infection may be only subclinical and may not advance to active disease	Nonproductive cough develops	Findings on chest radiograph are normal	Patients are susceptible to reactivation of disease
	Diagnosis can be difficult: findings on chest radiographs may be normal and sputum smears may be negative for mycobacteria	Diagnosis is via cultures of sputum	Granulomatous lesions calcify and become fibrotic, become apparent on chest radiographs
			Infection can reappear when immunosuppression occurs

Fig. 2.6 Differences in the various stages of Tuberculosis. [Adapted from Knechel, 2003].

2.7 Diagnostic Tests for Tuberculosis

Variable	Sputum smear	Sputum culture	Polymerase chain reaction	Tuberculin skin test	QuantiFERON-TB test	Chest radiography
Purpose of test or study	Detect acid-fast bacilli	Identify *Mycobacterium tuberculosis*	Identify *M tuberculosis*	Detect exposure to mycobacteria	Measure immune reactivity to *M tuberculosis*	Visualize lobar infiltrates with cavitation
Time required for results	<24 hours	3-6 weeks with solid media, 4-14 days with high-pressure liquid chromatography	Hours	48-72 hours	12-24 hours	Minutes

Fig. 2.7 Diagnostic Tests for identifying tuberculosis. [Adapted from Knechel, 2003].

2.8 Pathogenesis of *Mycobacterium tuberculosis*

Tuberculosis pathogenesis is driven by a complex interplay between the host immune system and the survival strategies of the bacterium. The inflammatory response to *M. tuberculosis* infection is tightly regulated by both the host and the bacterium.

The infection process of airborne pathogen, *M. tuberculosis* can be divided into three different but interrelated stages (Sasindran and Torelles, 2011).

2.8.1 Aerosol transmission of droplets containing *M. tuberculosis*

The infection starts with aerosol transmission of droplets containing *M. tuberculosis* from an infected individual to a healthy individual. Once within the lungs, *M. tuberculosis* enters and resides within alveolar macrophages (AMs) and dendritic cells (Cooper, 2009). Though the AM ingests bacilli and often kills them, the bactericidal capacity of the AM is still not very well defined. In a given *M. tuberculosis* infection, the initial containment of the infection depends partially on the genetics of the human population (i.e., defined by the intrinsic killing capacity of host phagocytes) and *M. tuberculosis* strain (i.e., defined by innate virulent factors in each *M. tuberculosis* strain). In the primary infection *M. tuberculosis* multiplies in the lungs and causes mild inflammation. Although AMs are thought to be an effective barrier to contain pathogens, *M. tuberculosis* has evolved various mechanisms to evade the host immune response and survive in these cells. These survival mechanisms include triggering an anti-inflammatory response, blocking reactive oxygen and nitrogen intermediate (ROIs and RNIs, respectively) production, and reducing the acidification of the *M. tuberculosis* -containing phagosome (Flynn and Chan, 2001; Fenton *et al.,*2005; Cooper, 2009).

2.8.2 Emergence of cell-mediated immunity and the formation of granulomas

The emergence of cell-mediated immunity and the formation of granulomas follow post-infection. *M. tuberculosis* bacilli that escape the bactericidal effects of the AM, will multiply and result in destruction of AMs. This will in turn attract blood monocytes and other inflammatory cells (i.e., neutrophils) to the site of infection. Monocytes mature to become antigen presenting AMs and DCs and ingest, but not effectively kill the bacteria. At this stage, *M. tuberculosis* grows under limited tissue damage. By 6–8 weeks post-infection, antigen presenting DCs have traveled to lymph nodes where T lymphocytes are activated and recruited. Activated T lymphocytes that migrate to the site of infection proliferate forming an early stage granuloma, where macrophages become activated to kill intracellular *M. tuberculosis* (Ulrichs and Kaufmann, 2006). However, continuing T cell activation leads to formation of granulomas that mark the persistence stage of the infection (latency), where the growth and spread of bacteria into additional tissue sites are limited. At this stage more than 90% of infected people remain asymptomatic, but *M. tuberculosis* may survive within AMs.

2.8.3 Reactivation of latent and controlled *M. tuberculosis* infection

The third and final stage is when latent and controlled *M. tuberculosis* infection is reactivated. There are two main reasons described for a reactivation event to occur, a decline in the host's immunity due to genetic or environmental cause; and a failure to develop and maintain immune signals. Under these circumstances, the granuloma structure disrupts and results in lung cavitation and pulmonary disease (Kaplan *et al.,*2003; Dheda *et al.,* 2005; Ulrichs and Kaufmann, 2006; Russell, 2007). Among the genetic causes described that make a subject susceptible to TB are mutations in specific host C-type lectins, cytokines, chemokines, and their specific receptors disrupting critical signaling pathways involved in the immune response against *M. tuberculosis*. Compromised immune surveillance for reasons such as co-infection with HIV, where a host becomes immunocompromised especially for CD4 T cells (the cell target for HIV), is the most important environmental or exogenous cause of susceptibility to TB (Geldmacher *et al.,* 2010). The reactivation of *M. tuberculosis* infection can also be due to changes in host cytokine/chemokine networks, implicated in the inflammatory response against *M. tuberculosis* infection, that are a consequence of stress and/or old age (Turner, 2011). Earlier studies have also suggested that exogenous re-infection with another strain of *M.*

tuberculosis is an additional factor leading to active disease (Sonnenberg *et al.,* 2001; Behr, 2004).

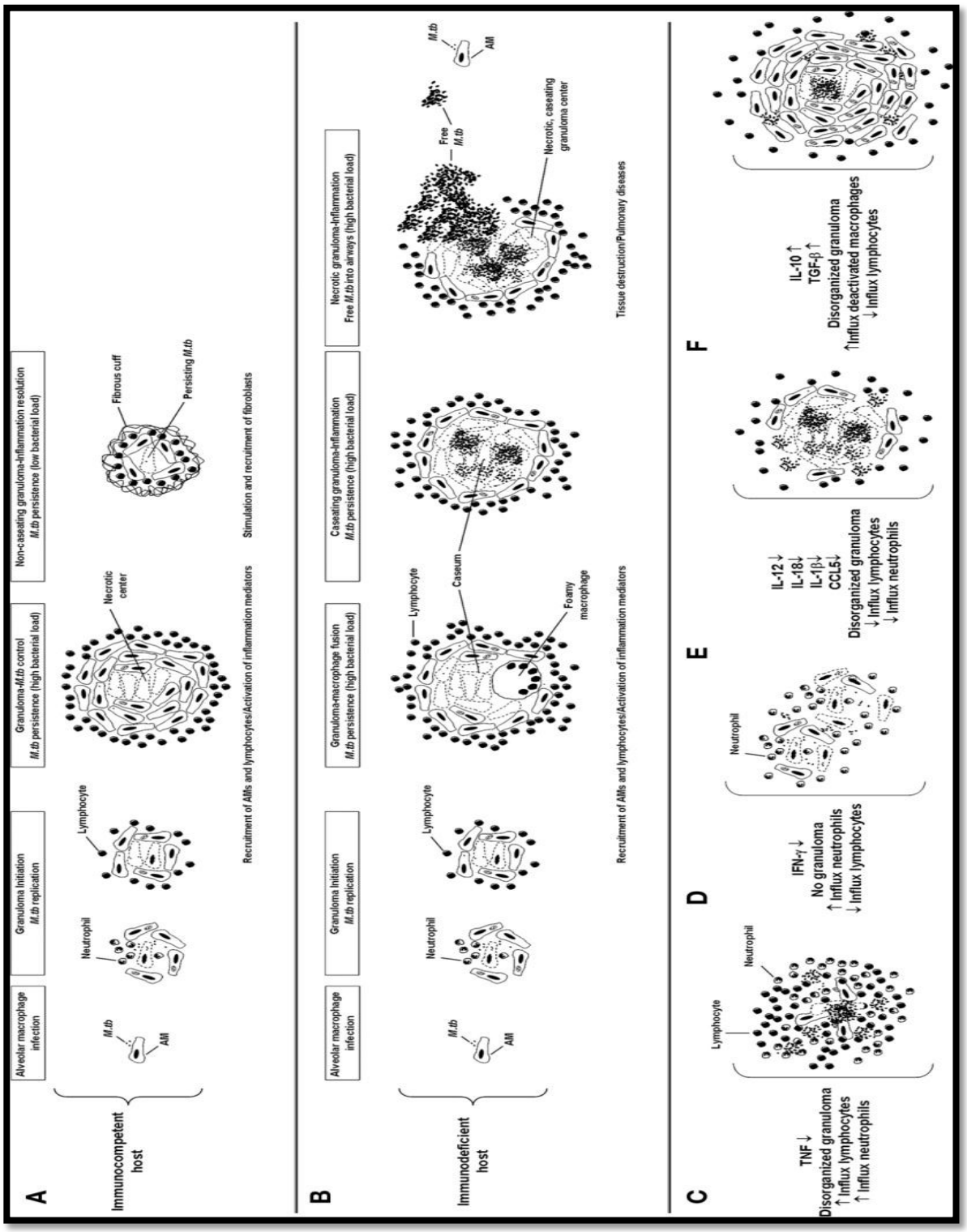

Fig. 2.8 Pathogenesis of *Mycobacterium tuberculosis.* [Adapted from Sasindran and Torelles, 2011].

2.9 Treatment for *Mycobacterium tuberculosis*

The major historical landmarks of tuberculosis (TB) therapy started with discovery of effective medications (streptomycin and para-aminosalicylic acid) in 1944; the revelation of "triple therapy" (streptomycin, para-aminosalicylic acid and isoniazid) in 1952, which made cure possible followed with discovery in the 1970s that isoniazid and rifampin could reduce the duration of treatment from 18 to 9 months. As recent as the 1980s, it was observed that adding pyrazinamide to these drugs allowed cures in only 6 months (Iseman, 2002).

Agent	Description	Adverse effects
Isoniazid	Highly active against replicating but not dormant or near-dormant tubercle bacilli. No cross-resistance with other anti-TB agents. Converted to an inactive form by acetylation, the rate of which is genetically determined; people are either rapid or slow acetylators. The rate of acetylation does not affect the efficiency of standard short-course anti-TB therapy, but slow acetylators are more prone to adverse drug reactions and interactions.	Uncommon and mostly involve the nervous system, manifesting as restlessness, insomnia, muscle twitching and psychiatric disorders. Risk is greatly reduced by prescription of pyridoxine (vitamin B6) at 10 mg/day. Occasionally causes hypersensitivity skin reactions, notably in patients with disease caused by HIV.
Rifampicin (rifampin)	A member of the rifamycin group of antibiotics and the most powerful anti-TB agent currently available; effective concentrations are obtained in all tissues. No cross-resistance with other anti-TB agents, except for other rifamycins. Its red colour is imparted to urine, tears and sweat.	Include mild itching and erythema (usually self-limiting), gastrointestinal upsets and impaired liver function in alcoholics and those with liver diseases. A few patients develop the "flu syndrome", with fever, chills, headache, bone pain and, rarely, a mild thrombocytopenic purpura. The flu syndrome occurs more often in those on intermittent than on daily treatment. Rare serious complications, also more frequent in those on intermittent therapy, include respiratory collapse, low platelet counts leading to purpura and haemorrhages, haemolytic anaemia and renal failure.
Ethambutol	Active during the early, intensive, phase of treatment and may enhance the activity of other anti-TB agents by increasing the permeability of the mycobacterial cell wall.	Include peripheral neuritis, joint pain, low platelet counts, jaundice and optic neuritis. The latter is very uncommon (especially if the maximum recommended dose is not exceeded and the drug is only given in the 2-month intensive phase), but is very serious as it may cause irreversible blindness. National codes of practice for detection and prevention of this adverse effect should therefore be strictly adhered to.
Pyrazinamide	Active against tubercle bacilli in acidic inflammatory lesions but not in the neutral or slightly alkaline cavity wall.	Uncommon and include gastrointestinal upsets and anorexia, photosensitization of the skin, arthralgia and gout. Hepatic toxicity is rare, except in patients with pre-existing liver disease.
Streptomycin	A member of the aminoglycoside group of antibiotics. In contrast to pyrazinamide, it is active in neutral or alkaline conditions. It must be given by intramuscular injection, with the risk of transmission of HIV and other viruses.	Include renal and inner ear damage, the latter leading to vertigo and deafness which may be permanent if treatment is not stopped.

Fig. 2.9 Properties of first line anti – TB agents. [Adapted from Onyebujoh *et al.,*2005].

Agent	Description	Adverse effects
Fluoroquinolones	Include ofloxacin, moxifloxacin, gatifloxacin and levofloxacin, which are being evaluated for the treatment of drug-resistant TB.	
Ethionamide and prothionamide	Closely related to each other and also to isoniazid, although there is no cross-resistance with the latter. They are weak drugs and are bacteriostatic rather than bactericidal agents.	Distressing gastric irritation is common.
Other aminoglycosides, e.g. kanamycin and amikacin		Like streptomycin, they are given by intramuscular injection and they have similar adverse effects.
Capreomycin and viomycin	Of restricted availability and rarely used. Although unrelated structurally to the aminoglycosides, partial cross-resistance occurs.	Must be given by intramuscular injection and their adverse effects are similar to those of the aminoglycosides.
para-Aminosalicylic acid (PAS)	Of limited availability and very rarely used nowadays. Has only limited bacteriostatic activity.	Gastrointestinal upsets are common.
Thiacetazone	A weak drug that should be dropped from the list of agents used to treat TB.	A cause of an unacceptably high incidence of severe and life-threatening skin reactions, particularly in those with disease caused by HIV.
Cycloserine	A weak bacteriostatic drug.	Use is limited by the high incidence of neurological and psychiatric adverse effects including headache, dizziness, depression and confusion.
Other agents	Other members of the rifamycin group of antibiotics, notably rifabutin and rifapentine, are used as alternatives to rifampicin under some circumstances. Rifabutin is used as an alternative to rifampicin in HIV-infected patients receiving antiretroviral therapy, as adverse drug interactions are less frequent. Other agents, with some evidence of efficacy, include newer macrolides (azithromycin, clarithromycin, the antileprosy drug clofazimine (Lamprene) and β-lactam/β-lactamase inhibitors.	

Fig. 2.10 Properties of second line anti – TB drugs. [Adapted from Onyebujoh *et al.,*2005].

TB diagnostic category	TB patients	Initial phase[b] (daily or, in some regimens, three times per week)	Continuation phase[b]
I	New smear-positive patients; new smear-negative PTB[c] with extensive parenchymal involvement; concomitant HIV disease or severe forms of extrapulmonary TB	**Preferred** 2 HRZE[d, e]	**Preferred** 4 HR 4 (HR)[3] 6 HE
		Optional 2 (HRZE)[3] or 2 HRZE	**Optional** 4 (HR)[3] or 6 HE
II	Previously treated sputum smear-positive PTB: - relapse; - treatment after default	**Preferred** 2 HRZES[f] / 1 HRZE[g]	**Preferred** 5 HRE[g]
		Optional 2 (HRZES)[3] / 1 HRZE[3]	**Optional** 5 (HRE)[3]
	Treatment failure of category I in settings with: - adequate programme performance; - representative DRS[h] data showing high rates of MDR-TB and/or capacity for DST[i] of cases, and - availability of category IV regimens	Specially designed standardized or individualized regimens are often needed for these patients.	
	In settings where - representative DRS data show low rates of MDR-TB or individualized DST shows drug-susceptible disease	**Preferred** 2 HRZES / 1 HRZE	**Preferred** 5 HRE
	or	**Optional** 2 (HRZES)[3] / 1 HRZE[3]	**Optional** 5 (HRE)[3]
	in settings of - poor programme performance, - absence of representative DRS data, - insufficient resources to implement category IV treatment		
III	New smear-negative PTB (other than in category I) and less severe forms of extra-pulmonary TB	**Preferred** 2 HRZE[j]	**Preferred** 4 HR 4 (HR)[3]
		Optional 2 (HRZE)[3] or 2 HRZE	**Optional** 4 (HR)[3] or 6 HE
IV	Chronic (still sputum-positive after supervised re-treatment); proven or susceptability MDR-TB cases[k]	Specially designed standardized or individualized regimens	

Fig. 2.11 Short Course anti – TB drug regimens recommended by WHO for different categories of patients. TB – Tuberculosis; PTB – Pulmonary Tuberculosis; H – Isoniazid; R – Rifampicin; Z – Pyrazinamide; E – Ethambutol; S – Streptomycin. [Adapted from Onyebujoh *et al.,*2005].

2.9.1 Major problems associated with currently available TB treatment.

- The duration and complexity of treatment results in nonadherence to treatment. This leads to suboptimal response (failure and relapse), the emergence of resistance, and continuous spread of the disease (Volmink and Garner, 2007).
- Adverse events in response to anti-TB drugs are common and contribute to the problem of nonadherence (Chan and Iseman, 2002; Volmink and Garner, 2007).
- The increasing incidence of multidrug-resistant (MDR; resistance to at least rifampin and isoniazid) and extensively drug-resistant (XDR; MDR resistance plus resistance to a fluoroquinolone and an aminoglycoside) TB is a serious concern. Resistant TB occurs in the presence of partially suppressive drug concentrations that enable replication of bacteria, the formation of mutants, and overgrowth of wild-type strains by mutants (WHO, 2008).
- Co – infection of TB and HIV is a problem by itself. Combined treatment of TB and HIV involves a high pill count with associated adherence problems, overlapping toxicity profiles

of the antiretroviral and anti-TB drugs, drug interactions between rifampin and the antiretroviral protease inhibitors, and the risk of immune reconstitution syndrome (Narita *et al.,*1998).

- Prophylactic therapy of latent TB (TB infection without symptoms) with isoniazid is also associated with problems of nonadherence (Zhang, 2004).

Table 2.1 Required properties of new anti – TB drugs. [Adapted from Boogaard *et al.,* 2009]

What a new drug should do	Characteristic(s) required
Simplify treatment or reduce treatment duration	Strong (early) bactericidal and sterilizing activity
	Low pill count, fixed-dose combinations
	Allow for intermittent therapy
Have an acceptable toxicity profile	Low incidence of treatment-limiting adverse events
	No overlapping toxicity profile with other TB drugs
Be active against MDR/XDR TB	No cross-resistance with first-line drugs
Be useful in HIV-infected patients with TB	Minimal interactions with antiretroviral drugs
	No overlapping toxicity profile with antiretroviral drugs
Be active against latent TB	Activity against dormant bacilli
	Favorable toxicity profile

2.9.2 Challenges of developing new anti-TB drugs (Boogaard *et al.,* 2009)

- The TB drug market is associated with insufficient profit opportunity or investment return to motivate pharmaceutical industries to develop new drugs.
- The difficulty to identify new compounds with activity against *M. tuberculosis*. Regimens against TB should kill both the rapidly growing mycobacteria (bactericidal activity) and the persisting mycobacteria in lesions (sterilizing activity). The molecular mechanisms responsible for mycobacterial dormancy (mycobacteria in a state of low metabolic activity and not forming colonies), persistence (drug-susceptible mycobacteria that manage to survive despite continuous exposure to TB drugs), and drug resistance are not yet fully understood.
- A next challenge rises with the evaluation of new compounds, as there are currently no animal models available that predict with accuracy the required treatment duration with newly identified compounds. The guinea pig model is being explored as an alternative for the mouse model since it resembles TB pathology in humans more closely.

- Another challenge is the scarcity of trial sites with sufficient research capacity to conduct clinical trials with large sample sizes. Trials should be performed in countries where the TB burden is highest, but the human and infrastructural capacity for performing large, high-quality phase III clinical trials is usually limited in these settings.

Table 2.2 Overview of anti-TB drugs in the clinical pipeline (Adapted from Boogaard *et al.,* 2009)

Drug	Trial phase	Potential to shorten treatment	Acceptable toxicity profile	Active against MDR TB	Useful in HIV-infected patients with TB	Active against latent TB	Interaction with rifampin
High-dose rifampin	II	Yes	To be established	Limited	Yes, but not co – administered with protease inhibitors	Yes, but not first choice	NA
High-dose rifapentine	II	Yes	To be established	Limited	To be established	Yes	NA
Moxifloxacin	III	Yes	Yes	Yes	Yes	Yes	Yes; reduced AUC of moxifloxacin by 30%
Gatifloxacin	III	Yes	Yes (caution: dysglycemia in elderly)	Yes	Yes	Unkown	Possible
TMC207	II	Yes	To be established	Yes	Unknown	Unknown	Yes; reduced serum TMC207 concn by 50%
PA-824	II	Doubtful	Yes (moderate increase in creatinine observed)	Yes	Unknown	Yes	No
OPC-67683	I/II	Yes	To be established	Yes	Unknown	Unknown	No
SQ109	I/II	Yes	To be established	Yes	Unknown	Unknown	Synergism in vitro
LL3858	I	Yes	Unknown	Yes	Unknown	Unknown	Synergism in vitro

2.10 Multi drug resistance *Mycobacterium tuberculosis*:

Several intrinsic and acquired drug resistance factor of *M. tuberculosis* attributes to the drug resistance against antitubercular agents. Lowering permeability of cell wall of *M. tuberculosis* for antibiotic and chemotherapeutic agents due to unusual structure of its mycolic acid containing cell wall attributes to the intrinsic drug resistance. The role of efflux mechanisms is one of the important factors in the natural resistance of mycobacteria against antibiotics such as tetracycline, fluoroquinolones and aminoglycosides, among others. Acquired drug resistance in *M. tuberculosis* is generally mediated through horizontal transfer by mobile genetic elements, such as plasmids, transposons or integrons. Acquired drug resistance in *M. tuberculosis* is mainly caused by spontaneous mutations in chromosomal genes, producing the selection of resistant strains during sub-optimal drug therapy (Silva and Palomino, 2011).

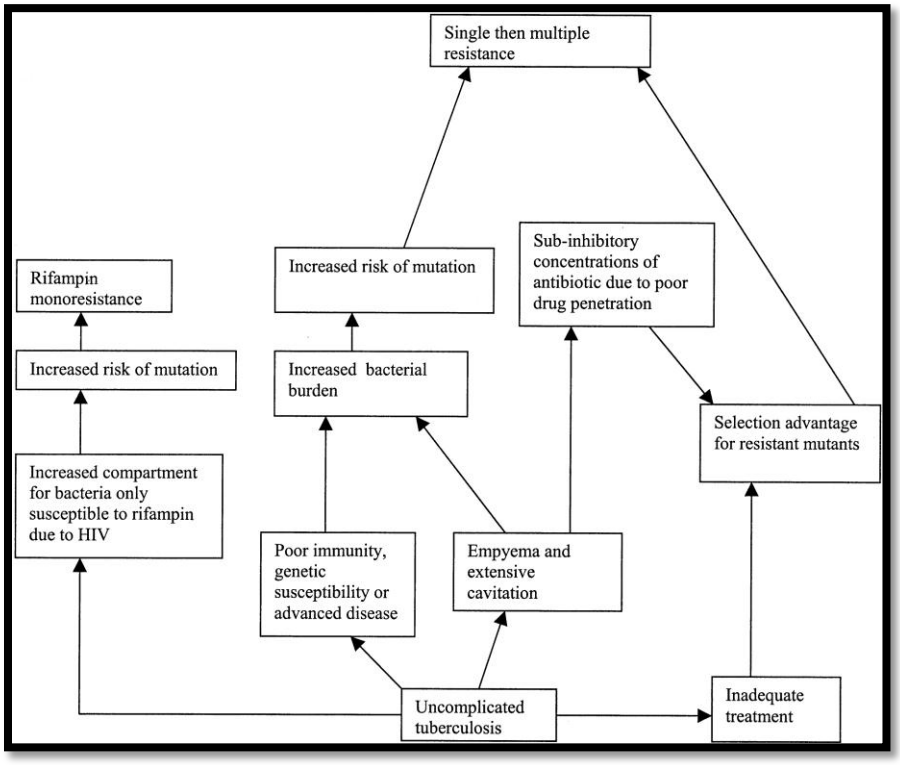

Fig. 2.12 Diagrammatic representation of the link between mutation rate, population size, and clinical complication in the emergence of resistance in M. tuberculosis infection. [Adapted from Gillespie, 2002].

M. tuberculosis organism resistant to at least Isoniazid and Rifampin are referred to as Multidrug-resistant tuberculosis (Gangadharam, 1993).

Drug (year of discovery)	MIC µg/ml	Gene(s) involved in resistance	Gene function	Role	Mechanism of action	Mutation frequency %
Isoniazid (1952)	0.02–0.2	*kat*G *inh*A	Catalase-peroxidase Enoyl ACP reductase	Pro-drug conversion Drug target	Inhibition of mycolic acid biosynthesis and other multiple effects	50–95 8–43
Rifampicin (1966)	0.05–1	*rpo*B	β subunit of RNA polymerase	Drug target	Inhibition of RNA synthesis	95
Pyrazinamide (1952)	16–50 (pH 5.5)	*pnc*A	Nicotinamidase/pyrazinamidase	Pro-drug conversion	Depletion of membrane energy	72–97
Ethambutol (1961)	1–5	*emb*B	Arabinosyl transferase	Drug target	Inhibition of arabinogalactan synthesis	47–65
Streptomycin (1944)	2–8	*rps*L *rrs* *gid*B	S12 ribosomal protein 16S rRNA rRNA methyltransferase (G527 in 530 loop)	Drug target Drug target Drug target	Inhibition of protein synthesis	52–59 8–21 ?
Amikacin/kanamycin (1957)	2–4	*rrs*	16S rRNA 16S rRNA	Drug target	Inhibition of protein synthesis	76
Capreomycin (1960)		*tly*A	2′-O-methyltransferase			
Quinolones (1963)	0.5–2.5	*gyr*A *gyr*B	DNA gyrase subunit A DNA gyrase subunit B	Drug target	Inhibition of DNA gyrase	75–94
Ethionamide (1956)	2.5–10	*eta*A/*eth*A *inh*A	Flavin monooxygenase	Prodrug conversion Drug target	Inhibition of mycolic acid synthesis	37 56
PAS (1946)	1–8	*thy*A	Thymidylate synthase	Drug activation?	Inhibition of folic acid and iron metabolism?	36

Fig. 2.13 Mechanism of Drug Resistance in *M. tuberculosis.* MIC – Minimum Inhibitory Concentration; ACP – Acyl Carrier Protein; PAS – Para- Aminosalicylic Acid. [Adapted from Zhang and Yew, 2009].

2.10.1 Rifampicin

Rifampicin is a lipophylic ansamycin introduced in 1972. It was derived by fermentation from *Streptomyces mediteranei*, renamed as *Amycolatopeus rifamycinica* (Mitchison and Davies, 2012). Due to its efficient antimicrobial action, it is considered, together with isoniazid, to be the basis of the short-course treatment regimen for TB (Rattan *et al.,* 1998).

2.10.1.1 Mode of action

The target of rifampicin in *M. tuberculosis* is the β-subunit of RNA polymerase, where it binds and inhibits the elongation of messenger RNA. An important characteristic of rifampicin is that it is active against actively growing and slowly metabolizing (non-growing) bacilli (Silva and Palomino, 2011). It is highly bactericidal against *M. tuberculosis* throughout treatment, with an MIC of 0.5 µg/mL, but its therapeutic margin is only 4; the standard dose of 450–600 mg (10 mg/kg) is therefore marginal. Nevertheless, it is responsible for the major part of the bactericidal activity of the regimen. It has been suggested that the main reason why it has such a small therapeutic margin is that only the 15% of the circulating drug that is unbound to plasma proteins is available in lesions (Mitchison and Davies, 2012).

2.10.1.2 Mechanism of resistance for Rifampicin

Resistance to rifampin arises due to mutations in the beta subunit of RNA polymerase encoded by the gene rpoB (Heep *et al.,* 2000). This includes point mutations, deletions, and insertions (Ramaswamy and Musser, 1998). This results in conformational changes that determine a low affinity for the drug and consequently the development of resistance (Telenti *et al.,* 1993). Most mutations were determined to be restricted to an 81-bp core region and are dominated by single nucleotide changes, resulting in single amino acid substitutions, although inframe deletions and insertions also occur at lower frequencies. Changes in the codons Ser531 and His526 have been documented in more than 70% of the RIF-resistant isolates (Rattan *et al.,* 1998).

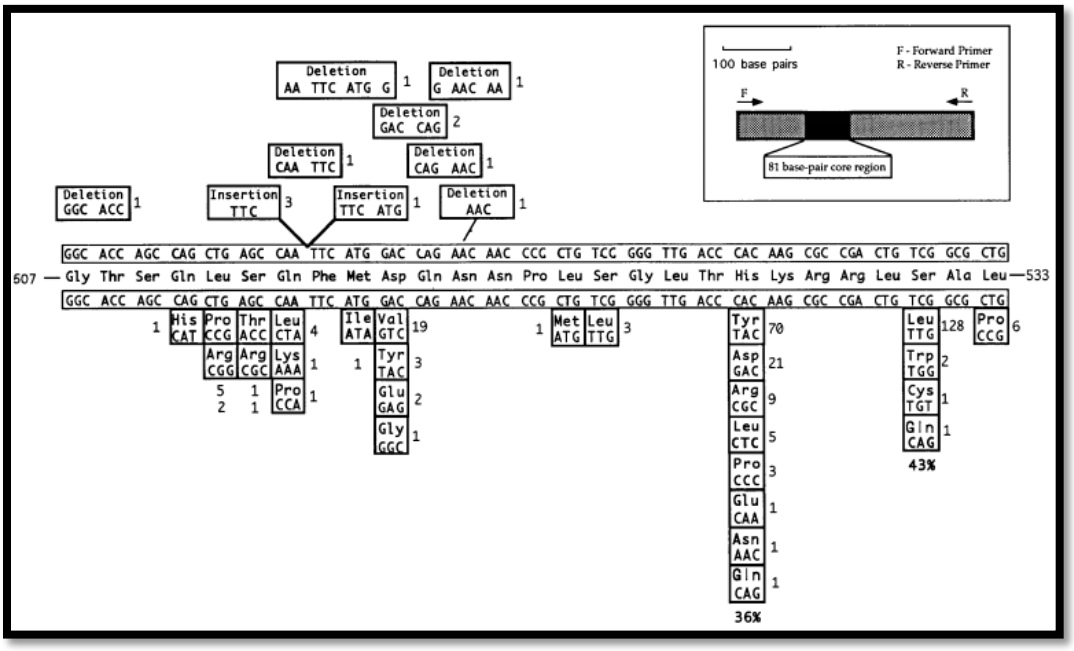

Fig. 2.14 Mutations occurring in codons 507 through 533 of the *M. tuberculosis* rpoB gene. [Adapted from Musser, 1995]

2.10.1.3 ARMS PCR for detection of rpoB mutation

The rationale of ARMS PCR is that a single nucleotide mismatch at the 3'-OH extremity of the annealed forward primer renders Taq DNA polymerase unable to extend the primer in the PCR under appropriate conditions. Thus, the absence of the specific PCR product, with a positive result for the internal control, reveals a deviation from the wild-type DNA sequence. An additional deliberate mismatch adjacent to the 3'-OH terminus of the ARMS primer introduced would enhance discrimination between normal and mutant alleles. In each PCR, one ARMS primer and the common reverse primer would be used for mutation

detection, which generates a short PCR product from the wild-type gene but fails to amplify from a mutant allele with a corresponding mutation at the location covered by the mismatch positions on the ARMS primer. A control forward primer that is expected to anneal efficiently to all alleles would be used in conjunction with the common reverse primer to generate a longer PCR product as an internal control. Following this principle, Fan et al. were able to detect mutations in the rpoB gene of the multidrug-resistant *M. tuberculosis* isolated from Shangai and later confirmed the results by sequencing (Fan *et al.,* 2003).

2.10.2 Isoniazid

Isoniazid is also known as isonicotinic acid hydrazide. It was synthesized in the early 1900s but its anti-TB action was frst detected in 1951 (Rattan et al., 1998). Since its discovery in 1952, INH has been the cornerstone of all effective regimens for the treatment of TB disease and latent infection. *M. tuberculosis* is highly susceptible to INH (minimum inhibitory concentration [MIC] 0.02–0.2 μg/ml) (Youatt, 1969). INH is only active against growing tubercle bacilli, and is not active against non-replicating bacilli or under anaerobic conditions (Zhang and Yew, 2009).

2.10.2.1 Mode of action

Isoniazid is a pro-drug that is activated by the catalase peroxidase enzyme encoded by the katG gene to generate highly reactive species, is capable of attacking multiple targets in *M. tuberculosis*, the primary one being the InhA enzyme (enoyl acyl carrier protein reductase). The active species (isonicotinic acyl radical or anion) reacts with nicotinamide adenine dinucleotide (H), forming INH-NAD adduct, which then inhibits InhA, causing inhibition of cell wall mycolic acid synthesis (Zhang and Yew, 2015).

2.10.2.2 Mechanism of resistance

INH is active only against growing *M. tuberculosis* but not against non-growing bacilli (persisters). INH tolerance in non-growing organisms may be caused by mycobacterial DNA-binding protein 1 (MDP1), a histone-like protein, which downregulates katG transcription and could lead to tolerance to INH (Niki et al., 2012).

Mutations in katG are the major mechanism of INH resistance. The KatG S315 mutation is the most common mutation in INH resistant strains, accounting for 50–95% of INH resistant clinical isolates. KatG S315 mutations usually do not completely eliminate catalase activity, and such strains may still retain fitness and virulence, which may explain its frequent occurrence among clinical isolates. Mutations in the katG promoter region furA – katG

intergenic region that affect KatG expression were occasionally found to cause INH resistance in some strains. Resistance to INH can also occur due to mutations in the promoter region of mabA (fabG1)/inhA operon causing overexpression of InhA or by mutations at the InhA active site. In contrast to katG mutations, which usually cause high-level resistance, mutations in inhA or its promoter region are usually associated with low level resistance (minimum inhibitory concentration [MIC] 0.2–1 lg/ml) and are less frequent than katG mutations. Mutations in inhA not only cause INH resistance, they also confer cross-resistance to the structurally related drug ethionamide (ETH). A small percentage of low-level INH resistant strains do not have mutations in katG or inhA, which may be due to new mechanism(s) of resistance (Zhang and Yew, 2015).

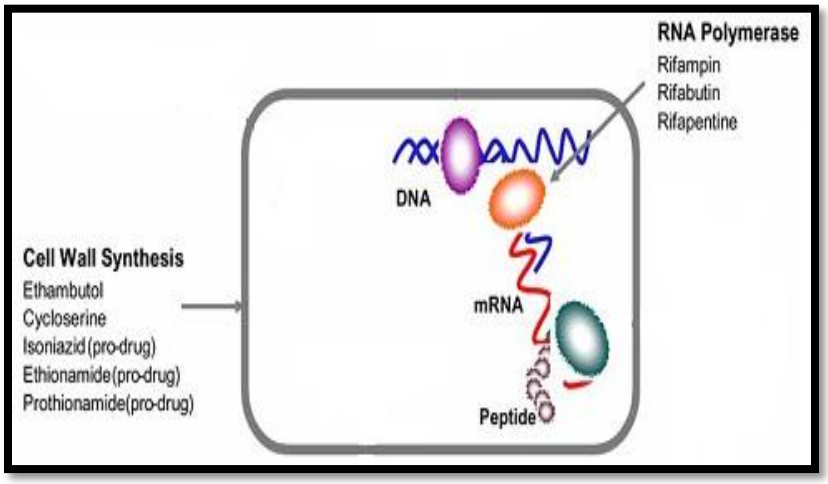

Fig. 2.15 Mode of action of various anti – TB drugs. [Modified from Laurenzi e al., 2007].

2.10.2.3 PCR-RFLP for detection of KatG mutation

Mutations associated with resistance can be identified by digestion of amplified PCR products with a restriction enzyme that cuts at the specific polymorphic DNA sequence followed by gel electrophoresis. Since not all mutations result in the gain or loss of a restriction site, general use of RFLP to screen for mutations associated with drug resistance is limited (Victor *et al.*, 2002; Johnson et al., 2009). It has been used for identification of point mutations in the *katG* gene of *M. tuberculosis* that correspond to various levels of resistance to isoniazid by examination of RFLP patterns of the amplified gene (Cockerill *et al.*, 1995). By this method, the limited region of the genome can be examined like detection of S315T mutation in katG gene by generation of a restriction site for MspI (Marahatta *et al.*, 2011).

2.11 Prevalence and Incidence of *M. tuberculosis* in Nepal

Tuberculosis (TB) remains one of the major public health problems in Nepal. TB ranks as the sixth leading cause of death among top 20 causes of death in Nepal. In 2014, total of 37,025 cases of TB were registered. Among them, 51 % were pulmonary TB. Most cases were reported among the middle aged group with the highest among 15-24 year of age (20%). The childhood TB is low at 2%. The Case Notification Rate (CNR) (all forms) was 136 / 100,000 which was stable without any significant changes for last 5 years. At regional level, the CNR change can be appreciated but with less significance. Whereas in eco-terrain, CNRs were highest in Terai zones followed by hill then mountain zones with rates of 156, 121 and 72 per 100,000 populations in 2013/14 respectively.

TB program In Nepal was able to save 31,187 lives this year nationally, but still 1049 deaths were reported among general TB cases. The overall treatment success rates (all forms) nationally of drug susceptible TB was 91% with 1.1% failure rates, 2% defaulted rates and 3.3% death rates. The treatment success rates of New Smear Positive (NSP) were 91% compared to 83% in retreatment cases (relapse + Lost to follow up+ failure), 95% in New Smear Negative (NSN) and 95% EP cases. Failure rate in NSP was 1% compared to 3% in retreatment cases, 0.3% in NSN cases and 1% in Extra Pulmonary (EP) cases. Regional trend of treatment outcome was not significantly different among each other, whereas eco-terrain trend showed that mountain region had more negative outcome in terms of death, failure and lost to follow up rates, especially for retreatment cases, whereas, hilly region had more decline in TSR for EP cases with increased lost to follow up. Nationwide, the proportion of new cases with multidrug-resistant TB (MDR-TB) was 2.2% among new cases and 15.4% among retreatment cases based on survey carried out in 2011/12, and new surveillance on MDR TB has not been done in recent years. In 2014, total of 349 MDR TB and 25 XDR TB were enrolled for treatment. Treatment Success Rate (TSR) of MDR patients was 76.6%; however the TSR of XDR is low at 11%. Total of 28 deaths among MDR and 10 deaths in XDR were reported in 2014. The drug resistant pattern in Nepal showed much higher levels of resistance to fluoroquinolones (36%). Among the MDR patients, 8% further develop XDR (NTP Annual Report, 2014).

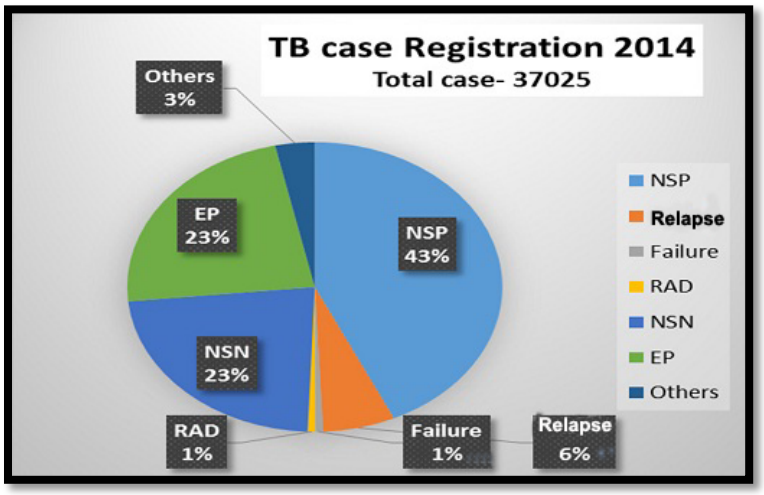

Fig. 2.16 TB case registration in 2014. NSP – New Smear Positve; RAD – Return After Defaulter; NSN – New Smear Negative; EP – Extra Pulmonary. [Adapted from NTP Annual Report, 2014].

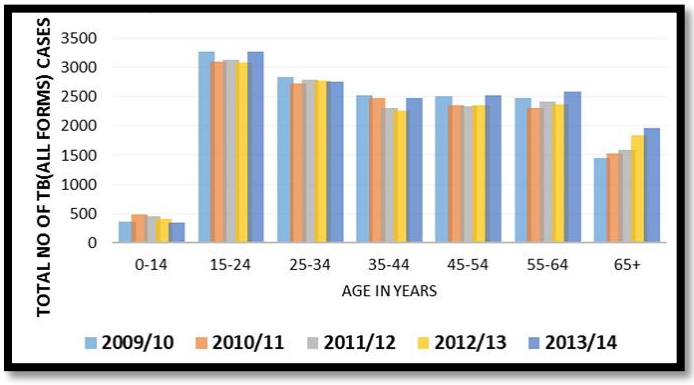

Fig. 2.17 Age distribution trend for New Smear Positive cases. [Adapted from NTP Annual Report, 2014].

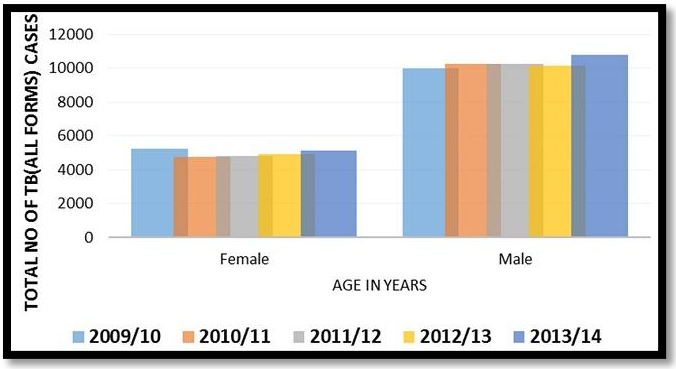

Fig. 2.18 Sex distribution trend for all forms of tuberculosis. [Adapted from NTP Annual Report, 2014].

Chapter 3

MATERIALS AND METHODOLOGY

3.1 DNA Preparation

Mycobacterium tuberculosis samples which were phenotypically determined as multiple drugs resistant by Drug Susceptibility Test were collected from Global hospital. Concerned with biosafety, live cultures were not brought to the laboratory. DNA isolation from the lysate was performed by following the protocol and reagents provided by Accuprep DNA Purification Kit™. Hence, purified DNA samples which was late used as template for further work. The DNA integrity of the provided samples was checked by running on 1 % Agarose gel supplemented with 0.5 µg/ml of Ethidium bromide and visualized under ultra-violet (UV) light on a transilluminator.

3.2 Confirmation of *Mycobacterium tuberculosis*

To confirm the presence *Mycobacterium tuberculosis* DNA in the provided samples, PCR was performed using MPB64 primers on a total of 44 samples. The amplified products were then run on 2 % Agarose gel for visualization under ultra-violet (UV) light on a transilluminator. The components for PCR amplification and its condition have been described in Table 3.2 and 3.3.

3.2.1 Primer Design

The primers were designed according to the information provided by Aryal *et al.* and ordered after confirmation by BLAST and primer3 (Aryal *et al.,* 2013).

Table 3.1 PCR primer for MPB64

Gene	Primer	Sequence
MPB64	Forward Primer	5’-TCCGCTGCCAGTCGTCTTCC-3’
	Reverse Primer	5’-GTCCTCGCGAGTCTAGGCCA-3’

Table 3.2 Composition of PCR reaction mixture using MPB64 primers

S. No.	Reagents	Volume (µL)
1.	10X PCR buffer with 15mM Mgcl2	2.5
2.	2.5mM dNTPs	2
3.	Nuclease free water	17.2
4.	Forward Primer(10 µM)	1
5.	Reverse Primer(10 µM)	1
6.	Template (50 ng/ µl)	1
7.	Tag DNA polymerase(3U/µL)	0.3
Total		25

Table 3.3 PCR condition for MPB64 gene

S. No.	Steps	No. of cycles	Temperature	Time
1.	Initial denaturation	1	95°C	5 min
2.	Denaturation	35	94°C	45 sec
	Annealing		55°C	45 sec
	Extension		72°C	30 sec
3.	Final Extension	1	68°C	10 min
	Hold	-	4°C	∞

3.3 ARMS – PCR for detection of rpoB mutation

After PCR with MPB64 primers, the confirmed Mycobacterial samples were subjected to PCR using ARMS primer. Upon completion of PCR, the amplicon were then run on 2 % Agarose gel supplemented with 0.5 µg/ml of Ethidium bromide, alongside 100 base pair ladder (Cat. N3231S) and visualized under ultra-violet (UV) light on a transilluminator. The components for PCR amplification and its condition have been described in Table 3.5 and 3.6.

3.3.1 Primer Design

The primers used in this study were designed based upon the article by Fan *et al.* The rationale of primer designing for ARMS PCR is that a single nucleotide mismatch at the 3'-OH extremity of the annealed forward primer renders Taq DNA polymerase unable to extend the primer in the PCR under appropriate conditions. Thus, the absence of the specific PCR product, with a positive result for the internal control, reveals a deviation from the wild-type DNA sequence. An additional deliberate mismatch (shown in the Table 3.4 as bold and underlined base) adjacent to the 3'-OH terminus of the ARMS primer was introduced in order to enhance discrimination between normal and mutant alleles (Fan *et al.,* 2003).

Table 3.4 PCR primer for ARMS PCR

Gene	Primer	Sequence
rpoB	Control Forward Primer	5' – CGAATATCTGGTCCGCTTGC – 3'
	Common Reverse Primer	5' – GTCGACCACCTTGCGGTACG – 3'
	ARMS – 516 primer	5' – CAGCTGAGCCAATTCA**C**GGA – 3'
	ARMS – 526 primer	5' – CGCTGTCGGGGTTG**T**CCC – 3'
	ARMS – 531 primer	5' – ACCCACAAGCGCCGAC**A**GTC – 3'

Table 3.5 Composition of PCR reaction mixture using ARMS primers

S.N	Reagents	Volume(µl)
1.	10X PCR buffer with 15mM $Mgcl_2$	2.5
2.	2.5mM dNTPs	2
3.	Nuclease free water	15.2
4.	Forward primer(10 µM)	1
5.	Reverse primer(10 µM)	1
6.	ARMS primer(10 µM)	1
7.	Template DNA(50 ng/ µl)	1.5
8.	Tag DNA polymerase(3U/µl)	0.3
Total		25

Table 3.6 PCR condition for detection of mutation in rpoB gene by ARMS PCR

S. No.	Steps	No. of cycles	Temperature	Time
1.	Initial denaturation	1	95°C	5 min
2.	Denaturation	35	94°C	35 sec
	Annealing		56°C	35 sec
	Extension		72°C	35 sec
3.	Final Extension	1	72°C	10 min
4.	Hold	-	4°C	∞

3.3.2 Validation of ARMS PCR Result for Mutation Detected in rpoB gene through sequencing

The results obtained after ARMS PCR were further validated by sequencing. For sequencing purposes, control forward and common reverse primers were used and the products were sent for sequencing to Xcelris Labs Limited, Ahmedabad, India. Preliminary analysis of the sequence obtained was done using Chromas Lite 2.1.1 followed by sequence alignment in the software MEGA 6.06 by Clustal W and finally sequence was compared with the RNA polymerase beta-subunit (rpoB) gene of the *Mycobacterium tuberculosis* (Accession No. L27989.1).

3.5 PCR – RFLP for detection of mutation in KatG gene

Similarly, the Mycobacterium DNA templates were used for detection of mutation in KatG gene through PCR – RFLP. The PCR condition and its composition have been described in Table 3.8 and 3.9. Following PCR, the amplified product was subjected to restriction fragmentation using *Msp*I restriction enzyme. The composition for RFLP has been given in Table 3.10. The reaction mixture was incubated at 37°C for 3 hours. The restriction fragments obtained were electrophoresed in a 2% agarose gel and were visualized under ultra-violet (UV) light on a transilluminator.

3.5.1 Primer Design

Primers were developed based upon the previous information as described by Marahatta et al. (Marahatta et al., 2011).

Table 3.7 PCR primer for PCR – RFLP

Gene	Primer	Sequence
KatG	Forward Primer	5' – AGCTCGTATGGCACCGGAAC – 3'
	Reverse Primer	5' – GTCCTCGCGAGTCTAGGCCA – 3'

Table 3.8 Composition of PCR reaction mixture using KatG primers

S.N.	Reagents	Volume(µl)
1.	10X PCR buffer with 15mM	2.5
2.	2.5 mM dNTPs	2
3.	$Mgcl_2$Nuclease Free Water	17.2
4.	Forward Primer(15 µM)	1
5.	Reverse Primer(15 µM)	1
6.	Template DNA(50 ng/µl)	1
7.	Tag DNA Polymerase(3U/µl)	0.3
	Total	25

Table 3.9 PCR condition for KatG gene

S.N.	Steps	No of cycles	Temperature	Time
1.	Initial denaturation	1	95°C	10min
2.	Denaturation	35	94°C	1min
	Annealing		55°C	1min
	Extension		72°C	1min
3.	Final Extension	1	72°C	4min
4.	Hold	-	4°C	∞

Table 3.10 Restriction digestion setup for RFLP of KatG

S.No	**Components**	**Volume (µL)**
1.	10X Tango buffer	3
2.	Nuclease free water	16
3.	PCR product	10
4.	*Msp*I (10 U/µL)	1
Total		30

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Confirmation of *Mycobacterium tuberculosis* by PCR

The samples obtained were confirmed as that of *Mycobacterium tuberculosis* by performing PCR with MPB64 primers. The confirmation was done as evident from presence of PCR products comparative to the band of approximately 240 bp visualized under UV transilluminator as shown in Fig 4.1. Out of 44 samples subjected to PCR, 34 samples showed positive results which were then further analyzed downstream. Although all 44 samples were identified as phenotypic MDR TB, the absence of bands in 10 samples could be due to loss of DNA during purification step resulting in false negative results. This is though hard to be determined, as many samples despite of lacking visible band in the gel gave positive result during the PCR amplification. So, the next probable reason would be the degradation of DNA during storage causing loss of amplification. This could be due to improper storage condition. In conclusion, out of 44 samples gathered for this study, only 34 samples could be utilized for further downstreaming processes.

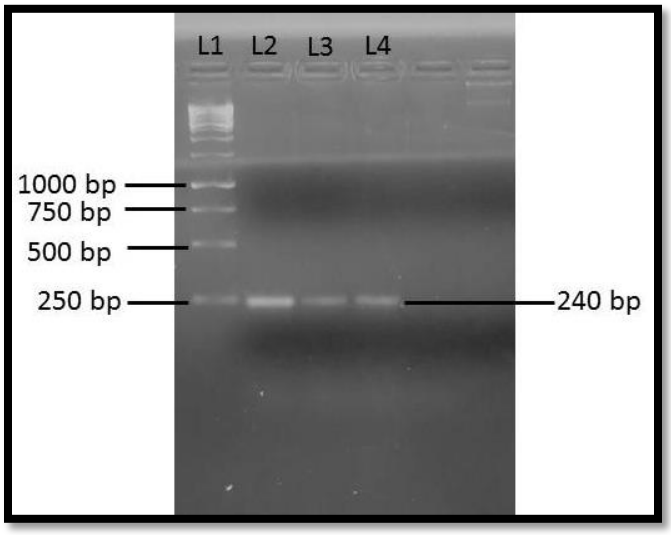

Fig. 4.1 Agarose gel (2 %) electrophoresis for PCR products amplified using MPB64 primers for confirmation of Mycobacterium samples. L1 – Ladder; L2 – Sample No. 4; L3 – Sample No. 17; L4 – Sample No. 21.

4.2 Detection of rpoB mutation by ARMS PCR

For detection of mutation in rpoB gene, the ARMS primers employed was complementary to the corresponding sequence of the wild-type gene except for one additional deliberate mismatch at the fourth nucleotide from the 3′OH terminus of the primer. This, in turn would create two mismatched nucleotides at the 3' end between the ARMS primer and the mutated codon. A single mismatch at the fourth nucleotide from the 3' end of the ARMS primer would have little influence on the yield of PCR products, whereas the mismatch at the 3_-OH extremity of the primer is obstinate to extension by the *Taq* DNA polymerase so that amplification from the mutant allele does not occur(Fan et al.,2003).

A total of 34 DNA samples of phenotypic MDR *Mycobacterium tuberculosis* were subjected to ARMS PCR assay. Each isolate had mutation in at least one of the codons namely 516, 526 or 531, with several samples showing cross mutations. Quantitatively, 33 samples had mutation at codon 531(97.05 % of total confirmed samples), 6 samples were found to bear mutation at codon 516 (17.64 % of total mutated samples) and the codon 526 was found to have been mutated in the least number of samples, being detected only among 4 samples (11.76% of total mutated samples) (Fig.4.2). Hence, among the samples used in this study, the maximum number of mutation were detected to be in codon 531 while the least mutation was observed in the codon 526. Among 34 isolates considered in our study, the magnitude for frequency of mutation in the codons were found in following order 531>516>526.

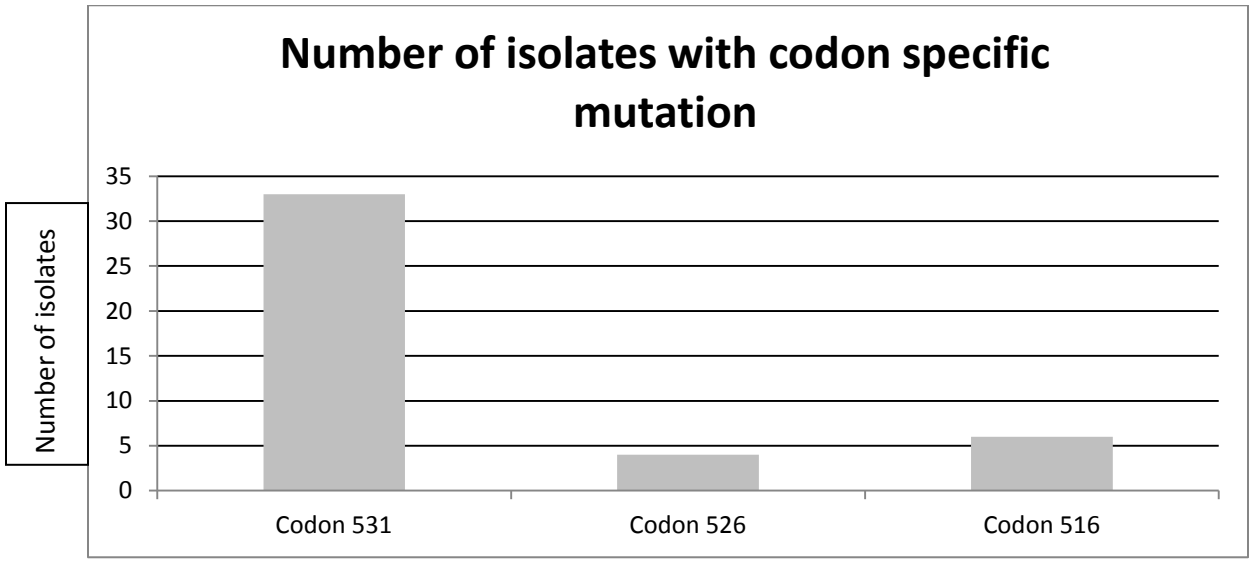

Fig. 4.2 Graph representing the number of phenotypic MTB samples with mutations in specific codons.

ARMS PCR products of some strains with typical mutations in the codons being studied from the *rpoB* gene are shown in Fig. 4.3. Control forward primer and common reverse primer amplified 537 bp of rpoB gene containing RRDR region which contained three codons i.e. codon 516, 526 and 531. Three codon specific primers were used in each samples separately for detection of mutation in respective codons. Control forward primer and common reverse primer were used in eachof the samples, along with codon specific primer, for amplication of 537 bp region of rpoB gene serving as an internal control to avoid false negative results. Double bands were observed in the samples with non - mutated codons corresponding to a band of approximately 261 bp and 537 bp for the codon specific primer of 516, approximately 230 bp and 537 bp for for the codon specific primer belonging to 526 codon, and the band length of 261 bp and 537 bp was visible for the codon 531. Single band representative of the amplification by control forward and common reverse corresponding to a band length of 537 bp was observed in case of samples with mutated codon.

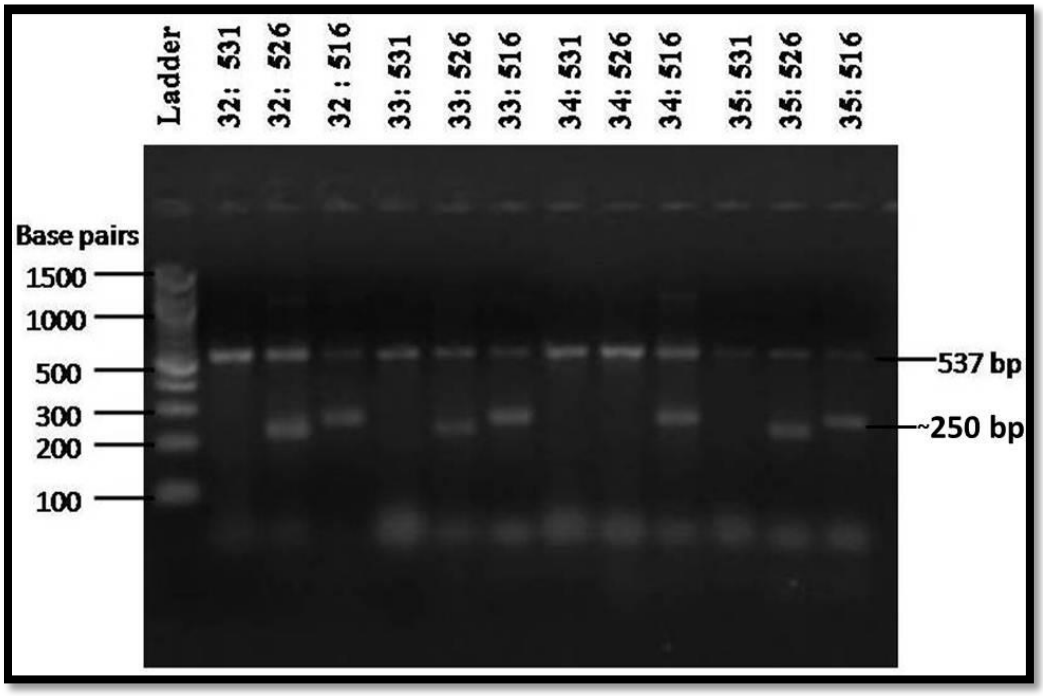

Fig. 4.3 Agarose Gel Electrophoresis (2 %) for amplicons obtained after PCR with control forward, common reverse and ARMS primers (531, 526 and 516). 100 base pair Ladder (Cat. No. N3231S). The code 32 : 531 signifies that the PCR amplification for sample number 32 has been performed with 531 codon specific ARMS primer, control forward primer and common reverse primer.

Summary of the analyzed data showing the number of isolates with codon specific as well as multi codon mutation as gathered from the ARMS PCR assay has been presented in the Table 4.1.

Table 4.1 Table showing the isolates with mutation in the specific codons as detected by the ARMS PCR assay.

Samples	Mutation		
	Codon531	Codon526	Codon516
02r	√	-	-
03r	√	-	-
04r	√	-	-
05r	√	-	-
06r	√	-	-
07r	√	-	-
08r	√	-	√
10r	√	√	-
11r	√	√	-
12r	√	-	-
13r	√	-	-
14r	√	-	-
16r	√	√	-
17r	√	-	-
18r	√	-	-
21r	√	-	√
24r	√	-	√
26r	√	-	√
29r	√	-	-
31r	√	-	-
32r	√	-	-
33r	√	-	-
34r	√	√	-
35r	√	-	-
36r	√	-	-
37r	√	-	-
38r	√	-	-
40r	-	-	√
42r	√	-	-
43r	√	-	-
44r	√	-	-
45r	√	-	-
46r	√	-	-
49r	√	-	√

4.3 Validation of result obtained from ARMS PCR by sequencing

Sequencing was performed on the previously analyzed samples using control forward and common reverse primer utilized in the ARMS assay. The data obtained from the sequencing could be associated with that obtained from the ARMS assay. Although all 34 samples were sent for sequencing, the data was sent back for only 19 of the samples. Of the 19 sampes, all of them showed only single mutation despite ARMS PCR showing presence of multi codon mutation in the samples. This anomaly could be due to error in the PCR amplification step, whereby, the codon specific primers might have made error during the amplification causing false positive results to occur. However, this incidence of false positive result is observable only in some of the samples. Due to small size of the samples followed by absence of sequencing data for 15 samples, the result obtained by this study could be taken as a pilot scale study at most and would require further inquiry before making any final conclusions regarding the efficiency of the ARMS PCR against sequencing, which is a gold standard in terms of detecting the mutations in the nucleotide sequence.

The results obtained were comparable to that done by Fan *et al.,* whereby the authors observed that among the samples they procured, mutation was highly probable in the codon 531 (Fan *et al.,* 2003). In our study, mutations were perceived only in the positions 516, 526 and 531 with highest percentage of mutation being observed at the position 531 (57.89 %) followed by that in codon number 526 (21.05 %) and 516 (15.78 %). One sample showed mutation in neither of the aforementioned codons nor any other sequenced region. Hence the phenotypic resistance of that sampe could be attributed to the mutation outside of the analyzed region for this study. All of the mutations witnessed in this study comes within the Rifampicin Resistance Determining Region (RRDR) of the rpoB gene as has been observed in several of the studies previously done (Cavusoglu et al., 2002; Kim et al., 1997).

Tallying the overall results obtained, it can be appreciated that in a resource constrained environments where sequencing is not always a valid option, ARMS PCR can be recommended as an alternative. ARMS PCR can act as a substitute for the discrimination of mutants from the wild at relatively efficient and cost effective manner.

Table 4.2 Table showing the isolates with mutation in the specific codons as validated by the sequencing. The highlighted codons represent the mutation in the codons of the sample when compared with the standard sequence of the strain L27989.1.

Sample No.	L27989.1 516 Codon/AA	Sequenced 516 Codon/AA	L27989.1 526 Codon/AA	Sequenced 526 Codon/AA	L27989.1 531 Codon/AA	Sequenced 531 Codon/AA
06r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
07r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
10r	GAC/ Asp	GAC/ Asp	CAC/His	**GAC/Asp**	TCG/Ser	TCG/Ser
11r	GAC/ Asp	GAC/ Asp	CAC/His	**GAC/Asp**	TCG/Ser	TCG/Ser
12r	GAC/ Asp	**TAC/Tyr**	CAC/His	CAC/His	TCG/Ser	TCG/Ser
14r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
16r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	TCG/Ser
21r	GAC/ Asp	**GTC/Val**	CAC/His	CAC/His	TCG/Ser	TCG/Ser
24r	GAC/ Asp	**TTC/Phe**	CAC/His	CAC/His	TCG/Ser	TCG/Ser
26r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
29r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
31r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
32r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
35r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TGG/Trp**
40r	GAC/ Asp	GAC/ Asp	CAC/His	**GGC/Gly**	TCG/Ser	TCG/Ser
43r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
45r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
46r	GAC/ Asp	GAC/ Asp	CAC/His	CAC/His	TCG/Ser	**TTG/Leu**
49r	GAC/ Asp	GAC/ Asp	CAC/His	**CGC/Arg**	TCG/Ser	TCG/Ser

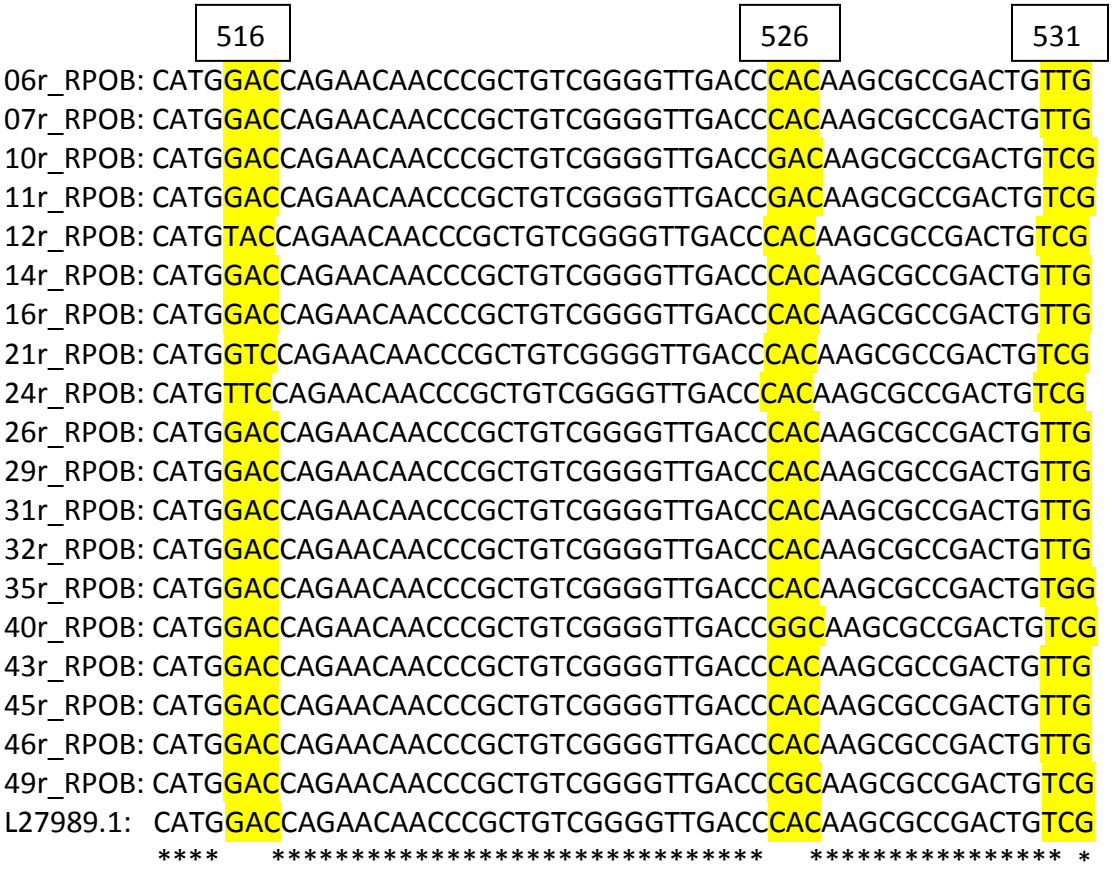

Fig. 4.4 Multiple Sequence Alignment for selected region of rpoB gene to display mutations in the codons 516, 526 and 531. The highlighted nucleotides show the changes in the bases when compared with the standard sequence of the strain L27989.1.

Samples	Mutation detected by ARMS – PCR			Mutation verified by sequencing		
	Codon531	Codon526	Codon516	Codon531	Codon526	Codon516
02r	√	-	-	NA		
03r	√	-	-	NA		
04r	√	-	-	NA		
05r	√	-	-	NA		
06r	√	-	-	D	ND	ND
07r	√	-	-	D	ND	ND
08r	√	-	√	NA		
10r	√	√	-	ND	D	ND
11r	√	√	-	ND	D	ND
12r	√	-	-	ND	ND	D
13r	√	-	-	NA		
14r	√	-	-	D	ND	ND
16r	√	√	-	NA		
17r	√	-	-	NA		
18r	√	-	-	NA		
21r	√	-	√	ND	ND	D
24r	√	-	√	ND	ND	D
26r	√	-	√	D	ND	ND
29r	√	-	-	D	ND	ND
31r	√	-	-	D	ND	ND
32r	√	-	-	D	ND	ND
33r	√	-	-	NA		
34r	√	√	-	NA		
35r	√	-	-	D	ND	ND
36r	√	-	-	NA		
37r	√	-	-	NA		
38r	√	-	-	NA		
40r	-	-	√	ND	D	ND
42r	√	-	-	NA		
43r	√	-	-	D	ND	ND
44r	√	-	-	NA		
45r	√	-	-	D	ND	ND
46r	√	-	-	D	ND	ND
49r	√	-	√	ND	D	ND

Table.4.3 Comparison between rpoB gene mutation of *Mycobacterium tuberculosis* isolates detected by ARMS-PCR and DNA sequencing results. Where D represents mutation detected, ND for not detected and NA for not applicable.

4.4 Detection of KatG mutation by PCR - RFLP

Of the 34 positive samples obtained for further downstream analysis, PCR was performed in all of the samples using the *kat*G primers. An amplicon of approximately 200 bp in size could be observed when visualized under UV transilluminator after Agarose gel electrophoresis (Fig. 4.5 L5). 34 isolates for which the Restriction Fragment Length Polymorphism was performed, 20(58.82%) showed Ser315Thr mutation in MTB strains as detected by presence of band length of 132 bp while 14 isolates were either wild type for *kat*G codon 315 or were differently mutated causing the loss of restriction site of MspI as shown in Fig. 4.5.

The most prevalent mutation AGC (Ser) to ACC(Thr) creates an additional *Msp*I site (CCGG) and thus can be detected by use of this Msp I restriction endonuclease (Marahatta *et al.,* 2011). Hence, the size of the RFLP product for Isoniazid resistant isolates with mutated 315 ACC allele would be 132bp and *kat*G codon 315 wild type or that of differently mutated allele would be 153 bp. The limitation of this procedure is that although the Ser315Thr mutation in the gene can be detected due to existence of the restriction site of MspI, however any other type of mutation renders the site useless making it improbable to determine whether the absence of 132 bp band length is due to presence of wild type or different type of mutation in the codon under study.

The prevalence of *kat*G S315T substitution in MTB strains vary all over the world especially with regard to TB prevalence. In places like Singapore and Madrid where prevalence of TB incidence is intermediate and low, mutation has been reported in 26-30% isolates (Lee et al., 1999; Piatek et al., 2000). In contrast the Ser315Thr mutation accounted for INH resistance in 52-64% of strains in Central Africa (Dobner et al., 1997). Several studies have revealed that mutation in *kat*G gene is responsible for 60-70% of INHr strains (Aragon et al., 2006). A study by Negi *et al.* in India revealed *kat*G S315T mutation in 74.19% of strains of MTB from Delhi (Negi *et al.,* 2006). Similarly, Wang *et al.* from China reported 68.6% of INH^r strains associated with mutation in *kat*G due to S315T substitution (Wang *et al.,* 2009). In Nepal, the *kat*G Ser315Thr mutation was reported in 81.4% of INH resistant isolates (*Poudel et al.,* 2012).

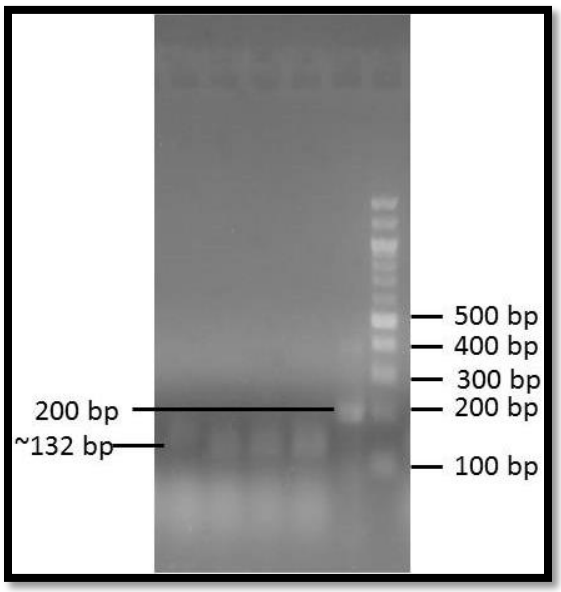

Fig. 4.5 Agarose Gel Electrophoresis (2 %) for PCR amplified KatG gene (L5) and its RFLP products (L1 – L4) run against 100 bp ladder(N3231S).

The data summarizing the type of mutation in the codon 315 of KatG gene has been presented in the Table 4.3.

Table. 4.4 Table showing the isolates with mutation in 315 codon of KatG gene as detected by PCR – RFLP.

S.N	Sample No.	Isoniazid resistant(132 bp)	Isoniazid wild type or differently mutated codon(153 bp)
1	02r	√	-
2	03r	√	-
3	04r	√	-
4	05r	√	-
5	06r	-	√
6	07r	-	√
7	08r	-	√
8	10r	√	-
9	11r	√	-
10	12r	√	-
11	13r	√	-
12	14r	√	-
13	16r	√	-
14	17r	-	√
15	18r	-	√
16	21r	-	√
17	24r	√	-
18	26r	√	-
19	29r	√	-
20	31r	√	-
21	32r	-	√
22	33r	√	-
23	34r	√	-
24	35r	√	-
25	36r	-	√
26	37r	√	-
27	38r	√	-
28	40r	√	-
29	42r	√	-
30	43r	√	-
31	44r	√	-
32	45r	√	-
33	46r	-	√
34	49r	√	-

After the evaluation of both of the genes, it was observed that out of 34 isolates that showed mutation in rpoB gene by ARMS PCR assay, 25 also showed mutation in KatG gene, as shown by PCR RFLP of the gene. This result could be the indication of amplifier effect defined as the increased rate of mutation in one gene due to mutation caused in another gene. This is specifically significant in the case of MDR TB cases as the mutation in one gene could cause the domino effect resulting in sequential mutation of several genes and hence could lead to rise of multi drug resistant or extensively drug resistant or even totally drug resistant strains.

This rise in resistant strains will lead to prolonged intake of drug which would put much economic burden as well as may give rise to several health related complications. Hence, early detection of drug resistant strains would not only relieve the patient of economic burden but also improve the quality of life for the patient with increased longevity. The techniques employed in this study despite of being 100 % efficient could still be utilized under the current resource strained circumstances with easier and cheaper method of detection of mutation. However, the results obtained should be analyzed only by the experts to avoid unnecessary complications arising due to wrong prescription of drugs.

CHAPTER 5

CONCLUSION

The present study was conducted on a pilot scale for molecular analysis of the genes responsible for generation of multi drug resistant *Mycobacterium tuberculosis*. The results showed that ARMS PCR for detection of mutation in rpoB can be used as an alternative to sequencing under resource constrained environments. PCR – RFLP carried out for the detection of mutation in KatG gene also showed its relative abundance among the Rifampicin resistant isolates. Hence, the analysis of rpoB gene can also be used as a benchmark for the detection of MDR TB as it may play role as a driver mutation following which other mutations occur. However, detection of rpoB gene mutation alone cannot be the surmise for confirmation of multiple drug resistance in the given organism. Furthermore, we analyzed only a certain portion of rpoB universally regarded to be mutation prone region, i.e. the mutation in other parts were left undetected. Hence further studies are required for analysis of mutations in the wholesome gene sequence using larger sample sets and also inclusive of several other genes that are responsible for multi drug resistance.

Recommendations

1. The present study was conducted using small sample set, hence for the statistical significance, a bigger data set should be employed to achieve the better picture regarding the status of MDR TB in the context of Nepal.
2. The analysis was done in a universally mutation prone region of rpoB gene, however, analysis could be done in the whole sequence of the rpoB gene.
3. Although ARMS PCR can be utilized as an alternative for sequencing, Sequencing can be considered as a gold standard for analyzing the mutations present in a given sequence. Hence, cheaper methods of sequencing could be explored in the future.
4. Only two genes were analyzed for this study, however, there are several genes that induce multi drug resistance in the *Mycobacterium tuberculosis*, hence; such genes should also be analyzed.
5. Technology as well as Social awareness should be developed in such a way as to not only detect the emergence of MDR TB but also prevent them.

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Appendix I

Composition of buffers

50 x Tris Acetate – EDTA (TAE) buffer

242 g Tris base

57.1 mL Glacial Acetic acid

100 mL 0.5 M EDTA (pH 8.0)

Final volume 1000 ml to be made with Distilled Water

6XDNA loading Dye

25 mg bromophenol blue

25 mg xylene cyanol FF

4 gm sucrose

Final volume 10 ml was made with deionized water

Tris - EDTA

10 mMTris (pH 8.0)

1 mM EDTA

Appendix II
Sequences of rpoB gene amplicon

>5_RPOB_S015139

CGAATATCTGGTCCGCTTGCACGAGGGTCAGACCACGATGACCGTTCCGGGCGGCGTCGAGGTGCCGG
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Sequence: L27989.1

Mycobacterium tuberculosis RNA polymerase beta-subunit (rpoB) gene, complete cds

>Mycobacterium tuberculosis H37Rv RPOB

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