DIRECT DETECTION OF *rpoB* MUTATION IN SPUTUM SMEAR AS A MARKER OF RIFAMPICIN RESISTANCE UNDER DOTS IN SIKKIM, INDIA

A THESIS

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GLOSSARY

The following are some of the commonly used terms in this thesis:

Cured	Initially sputum smear-positive patient who completed treatment and had negative sputum smears, on two occasions, one of which was at the end of treatment.
Defaulted	A patient who has not taken anti-tubercular drugs for two months or more consecutively after starting treatment.
False negative	Sick people incorrectly identified as healthy.
False positive	Healthy people incorrectly identified as sick.
Hot spot region	It is the 81bp region in *rpoB* gene.
INNO LiPA Rif.TB assay	It is a line probe assay based on reverse hybridization principle for in vitro use, allowing detection of MTBC and its resistance to RIF.
MDR-TB (Multi-drug resistance)	Defined as tuberculosis resistant to at least rifampicin and isoniazid.
Negative predictive value	It is the percentage of patients with a negative test who do not have the disease. It tells us how many of the negative tests are actually true negative.
Phenotype	The observable morphological, biochemical and physiological characteristics of an individual, either in whole or with respect to a single or a few traits, as determined by a combination of the genotype and the environment.

Positive predictive value	It is the percentage of patients with a positive test who actually have the disease. It tells us how many of test positives are actually true positive.
Prevalence	The number people with the disease in a given population who are alive during a specified period of time.
Proportion method	It is the standard method for detecting drug resistance in *M. tuberculosis* using solid culture (Loweinstein Jenson medium).
***rpoB* gene**	Ribosomal polymerase beta-sub-unit of ribosomal gene.
Sensitivity	Sensitivity is the ability of a test to correctly classify an individual as 'diseased'.
Specificity	The ability of a test to correctly classify an individual as disease-free is called the test's specificity.
TDR-TB (Totally drug resistant TB)	Those *M. tuberculosis* strains which are resistant to all known anti-tubercular drugs available at present.
True negative	Healthy people correctly identified as healthy.
True positive	Sick people correctly diagnosed as sick.
Validity	It is the extent to which a test measures what it is supposed to measure, in other words, it is the accuracy of the test. Validity is measured by sensitivity and specificity.
XDR-TB (Extensively drug resistant TB)	TB in a person whose *M. tuberculosis* isolates are resistant to isoniazid and rifampicin plus resistant to any fluoroquinolone and atleast one of the three injectable second-line drugs (amikacin, kanamycin, capreomycin).

ABBREVIATIONS

AFB	Acid fast bacilli.
AIDS	Acquired immuno deficiency syndrome.
CHC	Community health centre.
CPC	Cetyl pyridinium chloride.
DMC	Designated sputum microscopy center.
DOTS	Directly observed treatment shortcourse.
DRS	Drug resistance surveillance.
DST	Drug susceptibility test.
E	Ethambutol.
EP-TB	Extra-pulmonary tuberculosis.
EQA	External quality assessment.
GLC	Green Light Committee.
INH/H	Isoniazid.
IRL	Intermediate reference laboratory.
IUATLD	International Union Against Tuberculosis and Lung Diseases.
JALMA	National JALMA Institute for Leprosy and other Mycobacterial Diseases.
LJ	Loweinstein Jensen medium.
LRS	Lala Ram Swarup Institute of tuberculosis and respiratory diseases, Delhi.
MDG	Millennium development goal.
MIC	Minimum inhibitory concentration.
MTBC	*M. tuberculosis* complex.
NRL	National reference laboratories.

NTI	National tuberculosis institute, Bangalore.
NTP	National tuberculosis programme.
PHC	Primary health centres.
PHSC	Primary health sub centres.
RIF/R	Rifampicin.
RNTCP	Revised National Tuberculosis Control Programme.
RRDR	Rifampicin resistance determining region in 81bp region of *rpoB* gene.
S	Streptomycin.
SEAR	South-East-Asia-Region.
SLD	Second line drugs.
SRL	Supra National Reference Laboratory.
STDC	State tuberculosis training and demonstration centre.
TDR-TB	Totally drug resistant tuberculosis.
TRC	Tuberculosis research centre, Chennai.
UNOPS	United nations office for project services.
WHO	World Health Organization.
Z	Pyrazinamide.

NOTATION

≥	greater than equal to
≤	less than equal to
=	equal to
<	less than
>	greater than
P	Probability

CHAPTER 1

GENERAL INTRODUCTION

Tuberculosis (TB) is a global public health problem (1).The contagious nature of the disease was recognized by Aristotle (2) and cause of tuberculosis as *M. tuberculosis* was discovered in 1882 by Sir Robert Koch (3). TB is caused by MTBC (4) consisting of several species of genus *Mycobacterium*, the typical human-associated pathogens namely *M. tuberculosis, M. africanum, M. canettii* and other so called "smooth TB bacilli" (5), the actual host range of which remains unknown and several lineages adopted to different mammal species that include *M. microti, M. bovis, M. bovis* BCG *M. caprae, M. orygis, and M. pinnipedii* (6). TB is called by many common names such as: Captain of all these men of death (7), White Death (8), White Plague (9), Consumption (10) and Phthisis (11). TB was leading cause of death until early 20^{th} century, until it got controlled with advent of anti-tubercular drugs, improvement of hygiene and sanitation (12). TB resurged back in 1980s worldwide (13), spreading from AIDS patients to others in prisons, homeless shelters, and hospitals with enclosed settings and promoted spread of the disease causing death in many countries (14). Today one third of world's population is infected with TB (15) and 10% of the infected population manifests TB in their lifetime (16). Globally more than 1.3 million people die of TB every year and is presently the leading infectious cause of death more than AIDS, malaria, and other tropical diseases combined (7, 17). Efforts to control TB is getting hampered by expanding human immunodeficiency virus (HIV) infection and its association with active disease and increasing resistance of *M. tuberculosis* strains to most first-line anti-tubercular drugs (18). Worldwide, 9.2

million new TB cases (139 per 100000 population) and 1.7 million deaths from TB was reported to occur in 2006 (19). TB primarily affects lungs called pulmonary TB (20) and may disseminate to other body parts causing extra-pulmonary TB (21) incutaneous region (22), bones (23), urinary bladder (24), pleural region (25), kidney (26, 27), eye (28, 29), meningitis (30) etc.

TB was prevalent throughout the world since ancient times, as indicated by the discovery of deformed bones from various neolithic cities in Italy, Denmark, Middle East countries and also from the Egyptian mummies unearthed skeletons with apparent tubercular deformities dating from 3000 to 2000 B.C (11). *M. tuberculosis* and *M. bovis*, share greater than 99.9% similarity in their DNA sequence, but the rare synonymous single-nucleotide polymorphisms (sSNP) discriminates them and suggests, that they evolved at the same time independently from precursor species, related to *M. canetti* (11). TB can be transmitted to laboratory rabbits by inoculating them with tuberculous tissue from a cadaver and the course of the disease can be influenced by the environmental conditions in which the animals live (11, 31).TB usually is seen to be common amongst the poorer section of society with malnutrition, over-work and poor sanitation (11).

In today's world, TB is looked upon as a dreadful disease, because of the growing resistance to available anti-tubercular drugs, emerging as highly mutated TB bacilli making the TB as MDR-TB, XDR-TB and TDR-TB (18).In present days, India ranks first among the five countries in the world, in terms of having total numbers of cases worldwide, followed by China, Indonesia, South Africa and Nigeria (32).

In response to the killing effect of tuberculosis, world is facing challenge to combat this deadly disease by developing new genotypic methods for rapid identification of

MDR-TB strains to provide immediate and appropriate treatment. So, in order to make a successful technique to combat drug resistance, it is important to understand basic concept of drug resistance development in *M. tuberculosis* which is usually a acquired resistance either by alteration of the drug target by mutation or by titration of drug through over production of the target genes (33, 34). Most molecular diagnostics tools have technically demanding procedures and in some cases need specialised and costly equipment (34). A variety of diagnostics today are capitalised on recent elucidation of molecular mechanisms of drug resistance, especially to RIF, which is the marker for MDR-TB, due to its limited number of mutations responsible for causing RIF resistance, in a single *rpoB* gene (34, 35)**.**

Keeping in mind the importance of *rpoB* gene, several techniques have come up to detect RIF resistance, like PCR single-strand conformation polymorphism analysis (36, 37), heteroduplexing (38), dideoxy-fingerprinting (39), line probe assay (40, 41) and automated DNA sequencing (42, 43). The line probe assay has been commercially available for several years and is usually used on culture isolates and it has been used only in a limited way directly on the patients' samples (34, 44). However, aside then *rpoB* mutation which leads to RIF resistance, all resistance related genes for other anti-tubercular drugs have not been well-characterized.

Doing drug susceptibility by conventional method is not the best method for TB patients care, due to its complexity, labour-intensive, critical reading and longer time taken. In order to obtain clinically relevant and reliable results for case management, the probability of error of clinically susceptible or clinically resistant strains must be minimal and this is very critical (45). Probably Resistant and Probably Sensitive strains at 100% is, in fact, not feasible because the technical variations occurs even

with the skilled laboratory workers resulting from the physico-chemically labile environment of the test (45). Molecular methods are sensitive technique, and are quite promising for DST (Drug sensitivity testing) of MDR-TB, several of these tests are in a validation stage and many countries are already using these methods to identify MDR-TB patients. Tests for rapid identification of SLD resistance are not yet available. Several newer methods have been developed to document anti-TB drug resistance faster. Most of these methods are expansive and are not available routinely.

Fewer than 3% of patients with diagnosed TB worldwide have drug-susceptibility testing performed and only 10% of estimated half-million new cases of MDR-TB annually are treated appropriately (46). This leads towards ineffectively treated MDR-TB patients to acquire further resistance and spread drug resistant organisms. Though, sputum smear, is main stay diagnosis in TB health centers, it detects only 45% of TB infections as atleast not less than 5000 bacilli per ml should be present to give AFB positive upon microscopy (47). Hence MDR-TB and XDR-TB spreads to new people as the carrier patient is getting the wrong and late treatment.

Recent study from Chinese Centre for Disease Control and Prevention, showed that majority of MDR-TB in China was due to transmission of resistant strains to patients who had never been treated for tuberculosis, rather than mismanagement of previously treated patients (48). Thereby, data presented demanded a radical change in global approach to MDR tuberculosis, by understanding that though, testing patients with history of prior treatment seem more efficient, but in reality this approach misses most resistant cases of TB in community (48).

With advent of INNO LiPA Rif.TB assay, it seems possible to achieve rapid and reliable detection of RIF resistance directly on most clinical specimens to diagnose MDR-TB just a few days after sample collection. This offered a considerable improvement in management of MDR-TB as the vulnerable patients could commence their treatment with second-line drugs at a very early stage of tuberculosis. Transmission was further arrested by doing so. With the present cost of INNO LiPA Rif.TB, it seemed reasonable to restrict the analysis to smear-positive specimens, especially those patients originating from areas with high incidence of MDR-TB, immuno-compromised patients and patients previously treated for TB and failure to sputum conversion 2-3 months after program regimen (49-51). United Kingdom Health Protection Agency Mycobacterium Reference Unit detected RIF resistance by using the INNO LiPA Rif.TB assay on sample following culture growth of 1,997 clinical specimens in a non-trial, routine context. The overall unadjusted concordance, sensitivity, specificity, PPV, and NPV for detecting RIF resistance was 99.1%, 95.0%, 99.6%, 92.7%, and 99.7%, respectively. The assay enabled earlier diagnosis of MTBC and RIF resistance [15.2 days] compared with culture-based techniques of DST [30.7 days] (52).

In a comparison study between LiPA (using LiPA direct on clinical sample) and conventional method to detect rifampicin, LiPA correlated 97% (65 out of 67 cases) with other method using culture positive samples in the study (53). Twelve of 14 studies that applied LiPA to isolates had sensitivity greater than 95%, and 12 of 14 had specificity of 100%. Four studies that applied LiPA directly to clinical specimens had 100% specificity, and sensitivity that ranged between 80% and 100% (54). Though LiPA's study showed good sensitivity, specificity with culture and clinical

specimens, yet the method cannot be reliable until the correlation with clinical response is good (45).

By present study, the scope of INNO LiPA would be much better, if possibly the *rpoB* mutation be detected using the DOTS collected sputum positive slides, in order to reduce the turnaround time of rifampicin sensitivity test result availability by omitting culture of the MTBC isolates.

CHAPTER 2

MOTIVATION FOR THE INVESTIGATION

One third of world's population is infected with TB (15) and 10% of the infected population manifests TB in their lifetime (16). Globally more than 1.3 million people die of TB every year and is presently the leading infectious cause of death more than AIDS, malaria, and other tropical diseases combined (7, 17). India is among the twenty-two high burden countries in the world. Sikkim do not have any facility for culture and drug-sensitivity testing and the growing MDR-TB in the state undiagnosed, getting inappropriate DOTS treatment, provoked me to take up this study to find out a method which can be rapid and done direct on smears prepared using sputum positive samples, targeting 81bp region of *rpoB* gene to detect rifampicin resistance, marker of MDR-TB within short period of time. Also with the findings, to achieve research degree along with the benefits discovered by the study.

The factors enumerating the importance of this pathogen are:

1. In 1993 WHO declared TB as a global emergency. Today the drug resistance in *M. tuberculosis* has emerged from MDR-TB to XDR-TB to TDR-TB.

2. Efforts to control TB is getting hampered by expanding HIV infection and its association with increasing resistance of *M. tuberculosis* strains to most effective first-line anti-tubercular drugs (18).

3. Limited treatment options: Most of the anti-tubercular-drugs available have side effects on patient's health. MDR-TB drugs causes toxic effects. None of

the anti-tubercular-drugs work effectively singly or in combination doses, they have no 100% guarantee of cure. No single vaccine or drug have been made so far to completely cure the disease.

4. The gene conferring resistance to anti-tubercular-drugs are more than one in case of all anti-tubercular-drugs known with more than one target. But in case of RIF resistance, *rpoB* gene is the only gene involved and >95% resistance can be detected within the hotspot region of this gene.

CHAPTER 3

OBJECTIVES OF THE PRESENT STUDY

1. To estimate the prevalence of rifampicin resistance cases of pulmonary tuberculosis in the population of Sikkim.

2. To determine the validity and reliability of *rpoB* mutation for early detection of rifampicin resistance and treatment outcomes of DOTS.

SCOPE OF THE STUDY

The scope of the study includes:

1. Early detection of rifampicin resistance in sputum smears at the beginning of treatment will benefit TB patients by helping to get appropriate treatment under DOTS prior to confirmation of sensitivity result by culture.

2. Presence of mutated *rpoB* gene would imply >90% chances of presence MDR-TB strains in sputum and this in turn will help in controlling transmission of MDR-TB strains in the community.

3. Amplification of normal *rpoB* gene in positive sputum would indicate no rifampicin resistance and will reassure clinicians to continue DOTS with confidence.

4. It will be relatively economical in the resource constrained settings as culture would be more expensive in terms of laboratory set up, operational costs, time, materials, manpower, associated hazards, and the clinical deterioration faced by patients in waiting for 3 to 5 month to get culture sensitivity report.

5. It will be safer than culture as a technician need only to prepare a sputum smear and heat fix at around 75°C followed by ethanol inactivation. No special arrangement for storage or transport would be necessary.

6. Apart from installing a PCR as initial cost in a referral center, there is no need to incorporate additional logistic measures in the current DOTS program, as the slide could be delivered from the periphery to the PCR center by a DOTS inspector. Running cost per sample for processing would be significantly less than that incurred on culture and susceptibility tests.

7. DRS will be much simpler as detection of MDR isolates will be rapid (about 48 hrs).

8. Specific mutation/deletion of a strain can be mapped and thus provides an additional means of typing the resistant strains. Moreover, the possibility of using positive sputum slides which are stored at room temperature for longer duration of more than one year for DNA extraction and *rpoB* gene amplification would become useful for doing study on old slides stored at DOTS centres. This will contribute to molecular epidemiology.

CHAPTER 4

REVIEW OF LITERATURE

The risk of infection with drug-resistant mycobacteria is an increasing problem, posing a threat to control and eradication of the disease. By the late 1990s, scientific experts across the world stated *M. tuberculosis* bacteria could survive and become drug resistant in patients whose treatment could not be properly monitored or completed. Drug resistance in *M. tuberculosis* of two types:

1. Primary resistance or 'Drug resistance among new cases' develops in a person without having a history of previous TB treatment and becomes infected with tubercular strain already resistant to anti-tubercular-drugs (55, 56).

2. Acquired drug resistant or 'drug resistance among previously treated patients' is resistance among those TB patients who were initially infected with drug-sensitive tubercular strain and was treated for TB for at least one month and in course of time acquired drug resistance due to inappropriate treatment (initial drug-sensitive bacilli gets replaced by few mutant strains growing in the bacterial population) (55, 56).

Factors that the bacteria adopted to resist the action of antimicrobial agents against them include:

1. Their mycobacterial cell-wall, surrounded by highly specialized, hydrophobic cell wall, having the property of decreased permeability to many compounds (55, 57, 58).

2. The feature of active drug efflux systems in bacterial cell.
3. Spontaneous mutations [point mutations, deletions, or insertions] for all first-line drugs (isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin), and for several second-line and newer drugs (ethionamide, fluoroquinolones, macrolides, nitro-imidazopyrans)] (59-62).

Rifampicin, the first line anti-tubercular drug

Background

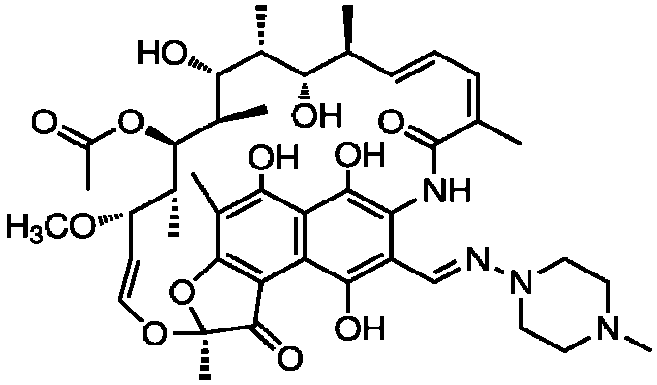


Fig 1: *Chemical structure of rifampicin* ($C_{43}H_{58}N_4O_{12}$)

Rifampicin [3-(4-Methylpiperazinyliminomethyl)] (Figure 1)**,** is a semisynthetic antibiotic synthesized in 1965 and introduced in 1971 (63). The molecular weight of the drug is 822.94. Mode of Action: RIF inhibits the assembly of DNA and protein

into mature virus particles. It inhibits initiation of RNA synthesis by binding to β-subunit of RNA polymerase, which results in cell death (64).

RIF is the key sterilizing drug against tubercle bacilli (65).The drug is given preferably 30 minutes before the patient eats, because after food, the drug absorption gets reduced in body. Oral dose of RIF given to patients contain the concentration as [10 (range 8–12) mg/kg (maximum 600mg) three or two times weekly] and this concentration, produce no-toxic effect to body and produce a serum concentration about 100 times as high as levels that inhibits growth of *M. tuberculosis,* thus RIF made TB treatment a success by shortening treatment duration (66). In wild *M. tuberculosis* strains, proportion of RIF resistant mutants ($1:10^8$) are substantially present in low number, than that of INH-resistant mutants ($1:10^6$). RIF mono-resistance occurs rarely and is preceded by development of isoniazid resistance and this property make RIF the surrogate marker of MDR-TB (36, 67). In case of RIF resistance, treatment becomes prolong and patients outcome turns fatal (55, 68).RIF works against TB bacilli by its both early bactericidal effect on metabolically active *M. tuberculosis* strains and late sterilizing action on semidormant organisms undergoing short bursts of metabolic activity. This late sterilizing effect of RIF on dormant bacilli, along with the additional effectiveness of pyrazinamide, TB treatment was cut from one year to six months' time (55, 69).

In >90% of TB cases, RIF resistance occurs due to mutation in *rpoB* gene. But for other anti-tubercular drugs, several targets genes determine drug resistance (70) like katG, inhA, aphC and kasA for INH resistance (71-73), rpsL and rrs for streptomycin resistance, embB for ethambutol resistance and pncA for pyrazinamide resistance (32, 74).

Rifampicin possess adverse side effects as follows (75).

1. Some TB patients do not tolerate current drug dosage due to toxicity that causes serious hepatotoxicity (RIF accelerates hepatic cytochrome p450 pathway, in overt hepatitis case, all hepatotoxic drugs should be stopped until clinical and biochemical hepatitis resolves and non-hepatotoxic drugs, like streptomycin, ethambutol, and fluoroquinolones (except ciprofloxacin, excreted by liver) are given. After hepatitis resolves, anti-TB-drugs are reintroduced in a phased manner.

2. Orange-red discoloration of body secretions (urine, faeces, tears, and sweat).

3. Cutaneous syndrome (usually starts during the first month) consisting of flushing and /or pruritus, with or without rash, involving particularly the face and scalp, often with redness and watering of eyes.

4. An abdominal syndrome (over the first six months) consisting of pain and nausea, sometimes accompanied by vomiting or, less commonly, diarrhea, a "flu" syndrome (observed only with intermittent regimens, begins in third to fifth month of treatment, is immunologic change which is mild and require no treatment change but on persistence, change to daily drug administration is useful) consisting of attacks of fever, chills, malaise, headache, and bone pains, a respiratory syndrome (shock might develop, with a sudden fall in systolic blood pressure and anuria, requiring hospitalization) characterized by shortness of breath are some of other manifestations produced as drug effect.

5. Purpura and other rare reactions, such as acute haemolytic anaemia, shock, andrenal damage with or without impaired kidney function and elevated transaminase serum levels are also seen (RIF should be stopped and never

given again). The first four syndromes typically begins two to three hours after single morning dose of RIF. Patients may exhibit more than one syndrome simultaneously at one time.

6. RIF reduces serum levels of many drugs, including antifungal agents, corticosteroids, warfarin, and oral hypoglycaemic agents.

7. RIF reduces levels of protease inhibitors and non-nucleoside reverse transcriptase inhibitors used to treat HIV, leading to rapid development of resistance in HIV strains to the protease inhibitors.

Genetic basis of rifampicin resistance in *M. tuberculosis*

Genome of *M. tuberculosis* $H_{37}R_v$ consists of 4.4×10^6 bp and contains approximately 4,000 genes (Fig.2) sequenced in 1998 (76). Over 200 genes are annotated as encoding enzymes for metabolism of fatty acids, comprising 6% of total. Among these 100 genes are predicted to function in β-oxidation of fatty acids. This large number of *M. tuberculosis* enzymes that putatively use fatty acids may be related to ability of the pathogen to grow in tissues of infected host, where fatty acids are the major carbon source.

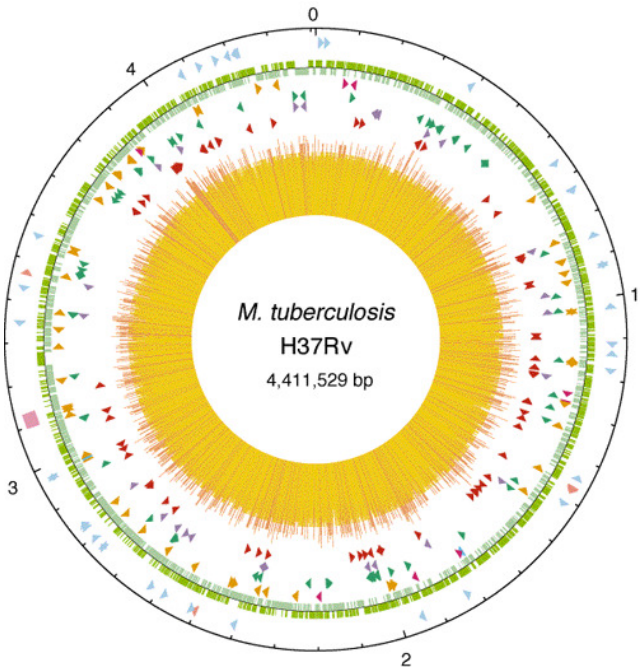


Figure 2: *The M. tuberculosis genome* (77). *Circular map of chromosome of M. tuberculosis* $H_{37}R_v$. *The outer circle shows scale in megabases, with 0 representing the origin of replication. The first ring from exterior denotes positions of stable RNA genes (tRNAs are blue, and others are pink) and the direct-repeat region (pink cube); the second ring shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (center) represents the G+C content, with <65% G+C in yellow and >65% G+C in red. The figure was generated with software from DNASTAR. Reprinted from reference* (11, 76).

Presently with the latest re-annotation of the *M. tuberculosis* genome, it is possible to assign a function to 2058 protein coding sequences (52% of the 3995 proteins predicted). Only 376 putative proteins share no homology with known proteins and thus could be unique to *M. tuberculosis* (78)

Another feature of *M. tuberculosis* genome is presence of unrelated PE (Pro-Glu) and PPE (Pro-Pro-Glu) families of acidic, glycine-rich proteins, found in two conserved N-terminal regions in each of these protein families and are approximately 110 and 180 amino acids long, respectively. The 172 genes (104 of PE class and 68 of PPE), comprise over 4% of genes in *M. tuberculosis* (79) involved in virulence (80). Proteins encoded by 104 PE genes are further subdivided into three classes, containing 29 proteins with PE region alone, 8 proteins in which PE region is followed by unrelated C-terminal sequences, and 67 proteins forming PE-PGRS subfamily. This group of proteins has conserved PE domain followed by C-terminal extensions with multiple repeats of Gly-Gly-Ala or Gly-Gly-Asn that are in PGRS (polymorphic GC-rich repetitive sequences) domains. Size variation has been observed in members of PE-PGRS subfamily family in clinical TB strains, and many of these proteins are localized in cell wall and cell membrane (81). Antigenicity of these proteins have led to hypothesis that at least some of these proteins have involvement in antigenic variation of *M. tuberculosis* during infection (81).

Among transcriptional regulatory proteins, *M. tuberculosis* has 13 siga factors (proteins conferring transcriptional specificity on RNA polymerases), corresponding to 0.3% of total genes and, 22 other regulatory proteins, including 13 two-component response regulators (usually transcriptional regulators that are activated by and serve to transduce environmental signals), corresponding to 0.6% of total gene (82) and it has been postulated for ability of TB bacilli to adapt to radically changing

environmental conditions by gene duplication and divergent evolution and the number of predicted transport proteins for this is 125 genes for transport functions, corresponding to 3% of total.

Mechanism of RIF resistance in *M. tuberculosis* is based on a study from DNA-dependent RNA polymerase of *E. coli* consisting of a complex oligomer containing four different subunits (α,β,β' and σ) assembled in two major forms: 1) a core enzyme ($\alpha_2\beta\beta'$) and 2) a holoenzyme ($\alpha_2\beta\beta'$ plus σ subunit) (66, 83). The core enzyme performs RNA polymerization but require a σ subunit to initiate site-specific transcription at promoter site. The genes encoding subunits α,β,β', and σ were designated as rpoA, *rpoB*, rpoC, and rpoD (66) and in a reconstitution experiments showed that RNA polymerase containing α and β' subunits from a resistant *E. coli* strain and β subunit from a susceptible *E. coli* strain was sensitive to rifampin, while RNA polymerase containing the α and β' subunits from a susceptible strain and β subunit from a resistant strain was resistant to rifampin. In rifampin susceptible *E. coli* strains, RIF binds to β subunit of RNA polymerase and leads to abortive initiation of transcription (66). The majority of mutations causing RIF resistance in *E. coli* was mapped to three distinct loci near the center of the *rpoB* gene (66). Other mechanism found causing RIF resistance are permeability barrier or membrane proteins acting as drug efflux pumps (33, 41, 84).

RIF attacks mycobacterial cell by inhibiting transcription and interfering with RNA elongation by targeting DNA-dependent RNA polymerase (Figure 3).

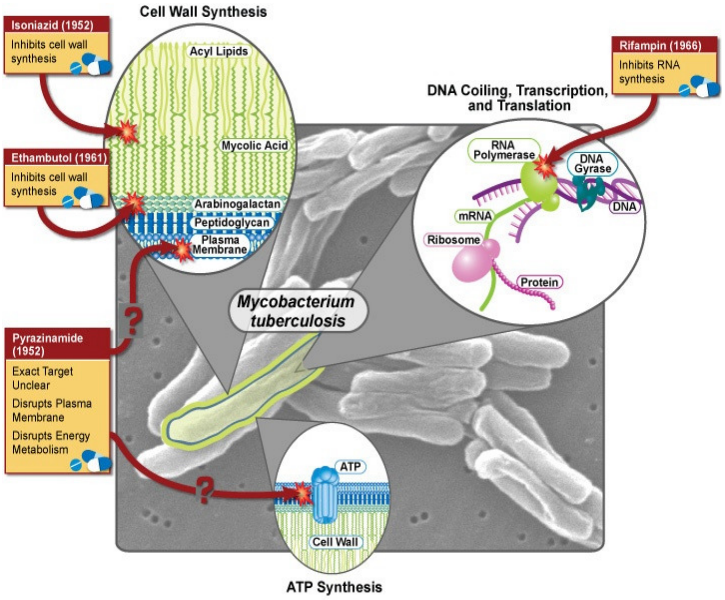


Fig 3: *Rifampicin inhibits RNA synthesis by acting on RNA polymerase and blocking protein transcription. First Line treatment of tuberculosis for drug susceptible strains (Obtained from the National Institute of Allergy and Infectious Diseases* (NIAID) www.niaid.nih.gov.)

>95% to 98% of RIF resistant *M. tuberculosis* isolates have missense mutations or less commonly small deletions or insertions within an RRDR of *rpoB* gene corresponding to codons 507-533 (35, 36, 85).

Majority of *rpoB* mutations (70 to 95%) occur at codon 516, 526 or 531 in region of high MDR prevalence (35, 43, 86-88). But frequency and nature of these mutations vary according to geographical locations (43, 89, 90). Commercial LiPA kit cover these four most frequently occurring mutations (D516V, H526Y, H528D and S531L) within hot spot region of *rpoβ* (41). 2-5% of RIF resistance causing mutation occur outside RRDR (91). >50 mutations have been characterized by automated DNA sequencing within hotspot region (36, 43, 86, 87).

RIF resistance development in *M. tuberculosis* is a "single-step" high-level resistance pattern (63). In wild strains not exposed previously to antibiotic, mutants arise spontaneously at a rate of 10^{-7} to 10^{-8} (one-step mutational event) (92) with high frequency of insertions and deletions in hotspot region. Many strains of *M. tuberculosis* with identical IS6110 restriction fragment length polymorphism pattern have same variant *rpoB* allele and some do not, and this suggest occurrence of evolutionary divergence at clone level (43).

Not all mutations within 81bp region exhibits same level of resistance to RIF. Alteration in codon 526 or 531 results in high level RIF resistance (MIC >32mg/ml) and in codons 511, 516, 518, and 522 exhibits low-level resistance to rifampin, rifamycin derivative (rifapentin), but susceptible to two other rifamycins (rifabutin and rifalazyn) (55, 84, 93). High-level RIF resistance also is correlated with multiple mutations in *rpoB* gene of tuberculosis bacilli. Substitution of limited number of highly conserved aminoacids encoded by *rpoB* gene cause single stephigh-level RIF resistance in *M. tuberculosis*. Most reference laboratories examine only 81bp region, and rare mutations occur in amino-terminal region of *rpoB* gene (outside 81bp region) (55, 94, 95).

Global scenario of drug resistance tuberculosis

World drug susceptibility test surveillance projects to investigate prevalence of anti-tubercular drug resistance was started by WHO and IUATLD (International union against tuberculosis and lung disease) in 1994 after declaration of global emergency in 1993. Reports of three surveillance projects (3 years each) reflected the magnitude of worldwide problems due to tuberculosis.

Between 1994 and 2007, global project collected data from 50% of world's TB cases. Countries having SRLs submitted drug sensitivity testing results to second-line anti-TB drugs. Seeing failure to measure trends in drug resistance from high burden countries, a new resolution was made to scale up diagnostic networks as a part of Global Plan to stop TB, 2006-2015. Success was in collecting baseline data on first-line anti-TB drugs resistance only and second line resistance test could not become possible due to limited SRLs facility. Japan and Republic of Korea showed high proportion of XDR-TB among MDR, however these two countries data are biased towards most ill patients with small population of MDR-TB cases. Former Soviet Union showed 4% to >20% of XDR-TB among MDR-TB (96)(96)(97)(98) (102,130,128 &129).

Survey conducted using *M. tuberculosis* isolates from forty-eight countries network of SRL during 2000-2004 found 19.9% MDR-TB isolates of which 9.9% met criteria for XDR-TB (97).

It is expensive and difficult to treat XDR-TB than MDR-TB, with worse patient's outcome (98, 99) and 4th global report data suggests XDR-TB is becoming widespread, with 45 countries reporting at least one case (96)(96)(97)(98) (102, 130, 129, 128). China ranks number one in terms of total MDR-TB cases worldwide, along with India and Russian Federation, together accounts 62% global estimates of MDR-TB (32, 74)**.** Prevalence of MDR-TB is increasing throughout the world both among new TB cases and previously-treated ones as seen from data collected between 2002-2007 (96)(96)(97)(98) (102, 128 - 130). In 2007, among 22 high-burden countries accounting 80% of global TB burden, countries with highest prevalence was India (2.0 million cases), China (1.3 million), Indonesia (530,000), Nigeria (460,000), and

South Africa (460,000). MDR cases estimated was 500,000 (including 289,000 new cases), of which 131,000 were in India, 112,000 in China, 43,000 in Russia, 16,000 in South Africa, and 15,000 in Bangladesh.

In 2008, 4,40,000 cases of MDR-TB (390,000–510,000) was actual global estimate consisting of eighty-one countries, out of which, ninety-thousands XDR-TB strains were detected from forty-five countries (100)(100)(101)(102) (106, 132 - 134). In 2009, worldwide reports of *M. tuberculosis* strains resistant to anti-tubercular drugs were mostly from Eastern Europe and countries with endemic TB and HIV co-infection showing 13% of all new cases resistant to at least one first line drug and 1.6% resistant to both INH and RIF (101, 102).

First WHO/IUATLD global project on anti-tubercular drug resistance surveillance was carried out in 35 countries during 1994-1997. Overall percentage of resistance to different anti-tubercular-drugs obtained from different surveys done during this period is shown in table 1 (103).

Table 1: WHO-IUATLD, 1994-1997 surveillance: median and range of percentage of anti-tubercular drug resistance.

Median Resistance to Any drug in % (range)		**Median Resistance to Isoniazid in % (range)**		**Median Resistance to Streptomycin in % (range)**		**Median Resistance to RIF in % (range)**		**Median MDR in % (range)**	
Primary	Acquired	Primary	Acquired	Primary	Acquired	Primary	Acquired	Primary	Acquired
9.9 (2.0-40.6)	36.0 (5.3-100.0)	7.3 (1.5-31.7)	-	6.5 (0.3-8.0)	-	1.8 (0-16.8)	-	1.4 (0-14.4)	13. (0-54.4)

Second WHO/IUATLD global project on drug resistance surveillance was conducted among 58 countries during 1996-1999. Overall percentages of resistance to different anti-TB-drugs obtained from different surveys are listed in Table 2 (103).

Table 2: WHO-IUATLD, 1996-1999 surveillance: median and range of percentage of anti-tubercular drug resistance.

Median Resistance to Any drug in % (range)		**Median Resistance to Isoniazid in % (range)**		**Median Resistance to Streptomycin in % (range)**		**Median Resistance to RIF in % (range)**		**Median MDR in % (range)**	
Primary	Acquired	Primary	Acquired	Primary	Acquired	Primary	Acquired	Primary	Acquired
10.7 (1.7-36.9)	23.3 (0.0-93.8)	6.2 (0-28.1)	19.6 (0.0-50.0)	5.2 (0.3-32.0)	12.4 (0.0-53.4)	1.2 (0-15.8)	12.0 (0.0-50.0)	1 (0-14.1)	9.3. (0.0-48.2)

Third WHO/IUATLD global project on drug resistance surveillance was conducted among 77 countries during 1999-2002, representing 20% of global total of new smear positive TB cases. It included 39 settings not included in previous global projects. The overall percentages of resistance to different anti-tubercular drugs obtained from different surveys are listed in Table 3 (103).

Table 3: WHO-IUATLD, 1999-2002 Surveillance: median and range of percentage of anti-tubercular drug resistance.

Median Resistance to Any drug in % (range)		**Median Resistance to Isoniazid in % (range)**		**Median Resistance to Streptomycin in % (range)**		**Median Resistance to RIF in % (range)**		**Median MDR in % (range)**	
Primary	Acquired	Primary	Acquired	Primary	Acquired	Primary	Acquired	Primary	Acquired
10.2 (0-57.1)	18.4 (0.0-82.1)	5.7 (0-42.6)	14.4 (0.0-71.0)	6.3 (0.5-15)	11.4 (0.0-77.1)	1.4 (0-15.6)	8.7 (0.0-61.4)	1.1 (0-14.2)	7.0. (0.0-58.3)

WHO-IUATLD, 1999-2002 global anti-tubercular drugs resistance surveillance data analyzed the distribution of MDR prevalence among new cases from 74 settings. Countries /geographical sites with high prevalence (>5%) of MDR-TB among new cases were identified and termed as 'hot spots'. The cut off value for hot spots was reset to MDR prevalence of more than 6.5% among new cases, where ten countries classified as hot spots among new cases were Ecuador (6.6%), Henan (7.8%), Latvia (9.3%), Lithuania (9.4%), Liaoning (10.4%), Estonia (12.2%), Uzbekistan (13.2%), Tomsk Oblast (13.7%), Israel (14.2%) and Kazakhastan (14.2%). Trend analysis in this third surveillance revealed variation in increase or decrease of percentages of resistance (to any drug or MDR) to different drugs in different countries and are listed in table 4 (103).

Table 4: Trend analysis of prevalence of resistance (to any drug or MDR) observed during 1999-2002, WHO-IUATLD global anti-tubercular drugs resistance surveillance.

	Resistance in new cases		**Resistance in previously treated cases**	
	Increased	**Decreased**	**Increased**	**Decreased**
Any drug	Botswana, New Zealand Poland Tomsk Oblast	Thailand	Botswana Cuba Switzerland	USA
MDR	Tomsk Oblast Poland	Hong Kong SAR Thailand USA	Estonia Lithuania Tomsk Oblast	Slovakia USA

In 2003, 184 countries anti-tubercular drugs resistance surveillance estimated 4, 58,000 MDR-TB among new cases worldwide with 2, 76,000 of cases (60%) reported from high burden countries. China and India constituted 3.2% of all new TB cases (104).

In 2007, there was an estimate of 0.5 million cases of MDR-TB. Countries that ranked first to fifth in terms of total numbers of MDR-TB cases were as follows; India (1,31,000), China (1,12,000), the Russian Federation (43,000), South Africa (16,000) and Bangladesh (15,000). By November 2009, 57 countries reported at least one case of XDR-TB (105)(105)(106)(107)(108)(108)(108)(108)(108)(108)(108)[108](108) (111, 137 - 139).

Data on prevalence of any drug resistance among new cases of TB from 2002 to 2007 were provided by 72 countries and 2 SARs of China (95). The overall drug resistance ranged from 0% (Iceland), 1.4% in Bosnia and Herzegovina, and 1.5% in Sri Lanka, to 49.2% in Georgia, 51.2% in Tashkent (Uzbekistan), and 56.3 in Baku city (Azerbaijan). Thirteen countries reported prevalence of resistance to any drug of 30% or higher in order of highest to lowest as: Baku city (Azerbaijan), Tashkent (Uzbekistan), Georgia, Republic of Moldova, Donetsk Oblast (Ukraine), Heilongjiang Province of China, Armenia, Latvia, Guatemala, Jordan, Viet Nam.

Prevalence of MDR-TB among new cases from 2002 to 2007 (95) ranged from 0% (Andorra, Cuba, Luxembourg, Malta, Slovenia, Aragon, Spain and Uruguay) to 19.4% in Republic of Moldova, and 22.3% in Baku city (Azerbaijan). Fourteen countries reporting prevalence of MDR-TB among new cases, higher than 6% from highest to lowest were as: Baku city (Azerbaijan), Republic of Moldova, Donetsk Oblast, Ukraine, Tomsk Oblast, Russian Federation, Tashkent, Uzbekistan, Estonia, Mary El Oblast, Russian Fed, Latvia, Lithuania, Armenia, Orel Oblast, Russian Fed,

Inner Mongolia Autonomous Region of China, Heilongjiang Province of China, Georgia (Table 5).

Table 5: Multidrug-resistant TB among new cases by WHO region, 2002 to 2007

WHO African region	6 countries (United Republic of Tanzania, Senegal, Rwanda, Madagascar, Ethiopia, Coted'Ivoire). The median sample size was 471 new cases. MDR-TB among new cases ranged from 0.7% (95% CLs, 0.2% - 1.8%) in Madagascar to 3.9% (95% CLs, 2.5-5.8) in Rwanda.
WHO region of the Americas	11 countries reported. The median sample size was 335 for new cases, and ranged from 169 new cases in Cuba to 1809 in Peru. No MDR was found among new cases in Cuba or Uruguay. The highest proportion of MDR-TB was seen in Guatemala (3%; 95% CLs, 1.8-4.6) and Peru (5.3%; 95% CLs, 4.2-6.4)
WHO Eastern Mediterranean region	Five countries reported. The median sample size was 264 for new cases, and ranged from 111 new cases in Jordan to 1049 in Morocco. MDR-TB among new cases ranged from 0.5% (95% CLs, 0.2% - 1.1%) in Morocco to 5.4% (95% CLs, 2.0-11.4) in Jordan.
WHO European region	Thirty eight countries reported data. MDR-TB were highest in the Baltic countries among all TB cases reported. Of the eight countries conducting surveys on subnational data, seven were countries of the former Soviet Union. The prevalence of MDR-TB among new cases (country with highest to lowest prevalence were Baku city, Republic of Moldova; Donetsk Oblast, Ukraine; Tomsk Oblast, Russian Fed; Tashkent, Uzbekistan; Mary El Oblast, Russian Fed; Armenia; Orel Oblast, Russian Fed; Georgia; Romania) ranged from 2.8% (95% CLs, 1.8-4.2) in Romania to 22.3% (95% CLs, 18.5-26.6) in Baku city, Azerbaijan, 28.6%.
WHO South-East Asia Region	Six countries (including four settings in India, ie prevalence from highest to lowest as Hoogli district, West Bengal; Gujarat; Ernakulam district, Kerala; Mayurbhanj district, Orissa) reported data. The median number of new cases tested was 547, and ranged

	from 101 in Mimika district in the Papua province of Indonesia to 1571 in Gujarat, India. MDR among new cases ranged from 0.2% (95% CLs, 0.0-1.0) in Sri Lanka and 0.7% (95% CLs, 0.1-2.5) in Mayurbhanj district, Orissa state, India to 4% (95% CLs, 2.6-5.7) in Myanmar.
WHO Western Pacific Region	Seven countries and two SARs of China reported. Six countries reported data distinguished by treatment history, including four settings in mainland China. For these six countries, the median number of new cases tested was 1004, and ranged from 250 in New Zealand to 3271 in Hong Kong SAR. MDR-TB among new cases (MDR-TB prevalence highest to lowest in Inner Mongolia Autonomous Region of China ; Heliongjiang Province, China; Philippines; Shanghai municipality, China; Viet Nam; Republic of Korea; Beijing municipality China; China Macao, SAR; China, Hong Kong, SAR; Japan; New Zealand; Singapore) ranged from less than 1% in Hong Kong SAR, Japan, New Zealand and Singapore to 7.2% (95% CLs, 5.6-9.4) in Inner Mongolia Autonomous Region of China.

Non-MDR RIF resistance, an important indicator in terms of programmes screening for MDR-TB, on basis of RIF testing alone (95). RIF resistance unaccompanied by INH resistance is rare and value >3%, suggest errors in either RIF or INH testing. Of 93 settings reporting, 80% reported <1% non-MDR-TB RIF resistance; only three settings reported non-MDR-TB RIF resistance >3%.

Table 6: Prevalence of non-MDR rifampicin resistance among all TB cases, 2002 to 2007.

Prevalence of non-MDR rifampicin resistance (%)	**Number and location of settings**
0.0	30 settings
0.1-1.0	47 settings
1.1-3.0	13 settings: - Armenia - Beijing municipality, China - Donetsk Oblast, Ukraine - Ernakulum district, Kerala, India - Ethiopia - Guatemala - Lebanon - Paraguay - Republic of Korea - Republic of Moldova - Romania - Shanghai municipality, China - Tomsk Oblast, Russian Federation
>3.0	3 settings: - Heilongjiang Province, China - Inner Mongolia Autonomous Region, China - Jordan
Non-MDR rifampicin resistance= tuberculosis with resistance to rifampicin but susceptible to isoniazid	

Thirty-five countries and two SARs reported data on XDR-TB, through routine surveillance data (twenty-five countries and two SARs) and drug resistance surveys (ten countries) (95).

Table 7: Representative survey or surveillance data (new and previously treated cases combined) for countries reporting data on XDR-TB, 2002-2007.

Region	Country	Year	MDR	MDR tested	XDR	XDR%
WPR	Japan	2002	60	55	17	30.9
	Hong Kong SAR, China	2005	41	41	6	14.6
	Australia	2002-2005	43	43	1	2.3
	Singapore	2002-2005	14	14	0	0
	Macao SAR, China	2005	9	9	0	0
	New Zealand	2005	4	4	0	0
	Republic of Korea	2004	110	110	2	1.8
EEUR	Estonia	2003-2006	248	245	58	23.7
	Latvia	2003-2006	712	688	53	7.7
	Tomsk Oblast, RF	2003-2005	468	458	30	6.6
	Lithuania	2003-2006	656	173	25	14.5
	Baku, Azerbaijan	2007	431	431	55	12.8
	Armenia	2007	199	199	8	4
	Donetsk, Ukraine	2006	379	20	3	15
	Georgia	2006	105	70	3	4.3
AMR	Argentina	2005	36	36	2	5.6
	USA	2000-2006	925	601	18	3
	Canada	2003-2006	55	55	2	3.6
EUR	Czech Republic	2003-2006	38	25	5	20
	Spain, Barcelona	2002-2005	43	37	3	8.1
	Romania	2003-2006	50	44	2	4.5
	Israel	2003-2006	45	44	2	4.5
	Ireland	2003-2006	8	3	1	33.3
	Slovenia	2003-2007	3	3	1	33.3
	Sweden	2003-2006		18	1	5.6
	Netherlands	2003-2006	34	33	1	3
	France	2003-2006	152	149	1	0.7

	UK	2003-2006	174	62	0	0
	Belgium	2003-2006	31	12	0	0
	Switzerland	2003-2006	25	22	0	0
	Poland	2003-2006	17	6	0	0
	Norway	2003-2006	11	11	0	0
	Croatia	2003-2006	5	1	0	0
	Denmark	2003-2006	5	5	0	0
	Spain, Galicia	2006	2	2	0	0
	Republic of Moldova	2006	203	47	3	6.4
	Spain, Aragon	2005	4	4	1	25
AFR	Rwanda	2005	32	32	0	0
	UR Tanzania	2007	6	6	0	0
WPR= WHO Western Pacific region; AMR= WHO region of the Americas; AFR= WHO African region; EUR= WHO European region; EEUR= Eastern WHO European region						

Table 8: Countries reporting data on XDR-TB, non-nationally representative samples 2002-2007.

Region	Country	Year	Cases	MDR tested	XDR	XDR%
AFR	South Africa	2004-2007	Retrospective review (National health lab data)	17615	996	5.7
AFR	DR Congo, Kinshasa	2006-2007	Category I failures	144	0	0
AFR	Burundi	2006-2007	Category II failures	23	0	0
WPR	Philippines	2005-2006	Confirmed MDR	293	10	3.4
SEAR	Myanmar	2007	Category II failures	43	0	0
SEAR	Bangladesh	2003-2006	Retreatment	300	3	1
WPR= WHO Western Pacific region; AFR= WHO African region; SEAR= South East Asian Region; MDR= Multi drug resistant; XDR= Extensively drug resistant						

Nineteen countries (four countries reported surveillance data, but XDR-TB case found was not during years 2002-2007 for which surveillance data was reported) reported at least one case of XDR-TB since 2001.

Table 9: Countries reporting at least one case of XDR-TB since 2002-2007

Region	Country	Source
AMR	Brazil	1
	Chile	1
	Ecuador	1
	Peru	1
	Canada (one case reported outside of surveillance data reported to Euro TB)	NTP report
EMR	Iran	2
WPR	Vietnam	NTP report
AFR	Mozambique	NTP report
	Botswana	NTP report
SEAR	India	4
	Thailand	NTP report
	Mexico	1
	Nepal	NTP report
EUR	UK (one case reported outside of surveillance data reported to Euro TB)	1
	Poland (one case reported outside of surveillance data reported to Euro TB)	NTP report
	Norway (one case reported outside of surveillance data reported to Euro TB)	NTP report
	Germany	1
	Italy	3
	Portugal	1

WPR= WHO Western Pacific region; AMR= WHO region of the Americas; AFR= WHO African region; EUR= WHO European region; EEUR= Eastern WHO European region; SEAR= South East Asian Region

1= Emergence of *M. tuberculosis* with XDR-TB worldwide, 2000-2004. MMWR 2006; 55:301-305

2= Masjedi MR, Farnia P, Sorooch S, *et al*. XDR-TB: 2 years of surveillance in Iran. Clin Infect Dis 2006; 43(7): 841-7.

3= Migliori GB, Ortmann J, Girardi E, *et al*. XDR-TB, Italy and Germany. Emerg Infect Dis 2007; 13(5):780-2.

4= Thomas A, Ramachandran R, Rehaman F, *et al*. Management of MDR-TB in the field: Tuberculosis Research Centre experience. Indian J Tuberc 2007; 54(3): 117-24.

In total, 45 countries and 1 SAR identified at least one case of XDR-TB since 2000. Of the settings conducting routine surveillance, three countries and one Oblast of Russian Federation reported between 25 and 58 cases of XDR-TB over a four-year period representing 6.6% to 23.7% of MDR-TB burden in Tomsk Oblast (Russian Federation). The United States reported 17 cases over a six-year period, representing 1.9% of MDR-TB cases tested for second anti-TB drugs during 2002-2007. Over a four year period, Barcelona, Spain reported three cases and the Czech Republic reported five cases representing 8.1% and 20% of MDR-TB cases respectively. During the same period, Australia, France, Ireland, Netherlands, Slovenia and Sweden reported one case; and Israel, Romania, and Canada reported two cases. Aragon, Spain reported one case in 2005. Eight countries (Belgium, Croatia, Denmark, Norway, Poland, Switzerland, Singapore and United Kingdom) reported no XDR-TB cases over a four year period. Four settings - China, Macao SAR, Galicia (Spain) and New Zealand reported no cases in one year reporting period. Of countries conducting surveys, proportion of XDR-TB among MDR-TB ranged from 0% in Rwanda and UR Tanzania to 12.8% in Baku city, Azerbaijan, and 15% in Donetsk Oblast, Ukraine.

Since the start of global project in 1994, data was collected from 138 settings in 114 countries and 2 SARs of China, consisted 48% of world's population and 46% of total TB burden (95).

Table 10: Population coverage of drug resistance data reported to WHO, 1994-2007

Region	Country	Total population	Total TB case	Total smear positive TB case	Total retreatment TB case
Global	117 (55%)	3,134,350,210 (49%)	2,518,270 (46%)	1,088,826 (45%)	246,137 (37%)
SEAR	6	318,225,607 (19%)	450,687 (23%)	163,774 (19%)	34,463 (14%)
SEAR= WHO South East Asia Region					

Twenty countries reported data before year 2000. The weighted mean of resistance to individual drugs varied across WHO regions (95).

Table 11: Global weighted mean of resistance to first-line anti-TB drug by treatment history by WHO region, 1994-2007.

Global	New	Previous	Combined
Countries	105	94	114
Settings	127	109	138
Any H	10.3 (8.4-12.1)	27.7 (18.7-36.7)	13.3 (10.9-15.8)
Any R	3.7 (2.8-4.5)	17.5 (11.1-23.9)	6.3 (4.7-7.8)
Any S	10.9 (8-13.7)	20.1 (12.2-28)	12.6 (9.3-16)
Any E	2.5 (1.7-3.2)	10.3 (5-15.6)	3.9 (2.6-5.2)
Any resistant	17 (13.6-20.4)	35 (24.1-45.8)	20 (16.1-23.9)
MDR	2.9 (2.2-3.6)	15.3 (9.6-21)	5.3 (3.9-6.6)
H= Isoniazid; R= RIF; S= Streptomycin; E= Ethambutol; MDR= Muti-drug-resistant 95% confidence levels are given between brackets			

The proportion of resistance to all first line anti-tubercular drug and MDR-TB was highest in Eastern Europe, and lowest in Africa and Western Europe. The global

weighted mean of MDR-TB was 2.9% among new cases, 15.3% among previously treated cases and 5.3% among all TB cases. The highest proportions of resistance was seen to INH and S, followed by RIF and E among patients with history of previous treatment. Without regard to treatment history, with exception of previously treated cases in Eastern Mediterranean region, RIF resistance was higher than INH resistance.

Stable proportion of MDR-TB was reported among new cases in Baltic countries. Data reported to Global project from the Orel and Tomsk oblasts (Russian Federation) indicated statistically significant increase in proportion of MDR-TB among new cases (95). Declining trends in MDR-TB among all TB cases was seen in United States and Hong Kong SAR (significant decrease in any resistance among all cases, and INH resistance and MDR-TB among new cases). Denmark showed significant declines in any drug resistance in both new and combined TB cases. Puerto Rico showed declining trends in any resistance and MDR-TB among combined cases. Singapore showed significant decrease in prevalence of MDR-TB among all TB cases.

World-wide distribution of *rpoB* gene mutation in *M. tuberculosis* isolates

Compilation of data from diverse countries (Taiwan, India, Greece, Japan, Monterrey, Mexico, Poland, Latvia and China) indicated RIF resistance in *M. tuberculosis* was mainly due to distinct mutations located within an 81-bp RIF resistance-determining region of the *rpoB* gene (84, 89, 106-114). However, relative frequencies of *rpoB* mutations described for *M. tuberculosis* isolates varies from different geographic locations and these differences reflects complex and crucial interaction between RIF and targets at molecular level, where the position of affected nucleotide changes are

variable(73, 115-117). Frequencies for *rpoB* mutations reported in isolates from various parts of world were 41% and 36% for codons 531 and 526of the *rpoB* gene (35). Mutation at codons 531 (TCG→TTG), 526 (CAC→ TAC), 516 are the most commonly found mutations in decreasing order of frequency (118), where S531L, H526Y, and H526D are three reliably detected and differentiated using INNO LiPA Rif.TB test (109). However some study describe mutation at codon, 510 (CAG→_AG), 511 as sites with most frequently occurring mutations worldwide(71, 73, 113, 117-119) and point mutations in 531 and 515 are most prevalent worldwide *rpoB* mutations (73, 120-124).

Mutation in codon 531 in *M. tuberculosis* isolates are such as: from India – TCG→TGG, TTG, Russia – TCG→TGG, CAG or TGT, China – TCG→ TTG, Japan- TCG→ TTG, Korea- TCG→ TTG, Taiwan- TCG→ TTG and Ser→ Gln(73). Frequency of mutations in codon 526 (CAC to GAC) among Italian isolates (Europe) was 40.1%, in Greece isolates (CAC to GAC) 17.6% and CAC to GAC was reported more prevalent among American isolates 27.9% (73). No changes in codon 510 (73, 117, 120, 121) or very seldom CAG mutation of codon 510 (deletion or CTG or CAC or CAT) was detected in most countries (37, 73, 125). Mutation CAG→CAT in codon 510 was found in India (71), in Russia (Europe) – CAG→CAT, in Belarus CAG→GAG, TAG, in Lithuania (Europe) CAG→GAG and in Poland (Europe) CAG→GAG(73, 120, 121, 123, 126, 127). A rare mutation at codon 513, resulting in a Gln→Glu substitution, was reported once, in an isolate from Taiwan. Similarly, deletion of codon 518 was observed in only two studies from Africa and Belgium(73).

90 RIF resistant *M. tuberculosis* strains from ten countries (Bangladesh, Canada, India, Indonesia, Korea, Malaysia, Myanmar, Nepal, Philippines, Thailand, Yemen) (Figure 5) mostly from Asia [two RIF resistant isolates from Bangladesh (Asp 516

Val mutation in one isolate and no mutation in one isolate), two from Indonesia (His 526 Tyr mutation in one isolate, none in one isolate), twenty-seven from Malaysia (Gln 513 Pro in one isolate, 514 Phe ins.in one isolate, Asp 516 Tyr in one isolate, Asp 516 Val in two isolates, His 526 Tyr in two isolates, Ser 531 Leu in seven isolates), fifteen from Myanmar (Gln 513 Pro for one isolate, Asp 516 Val for two isolates, His 526 Asp for one isolate, His 526 Tyr for one isolate, Ser 531 Leu for nine isolates and none in one isolate), fourteen from Thailand (Gln 513 Pro, Asp 516 Val, His 526 Arg, and His 526 Asp individual mutation in each two RIF resistant isolates, His 526 Tyr for one isolate, Ser 531 Leu for four isolates, none in one RIF resistant isolate) and three from India (His 526 Asp for one isolate, Ser 531 Trp for two isolates), two from Nepal (Leu 511 Pro was seen in one isolate, Asp 516 Val in one isolate)] showed approximately 85% of RIF resistant isolates having mutations in one of three dominant amino acids (531-Leu mutation was in 48 of 90 RIF-resistant isolates (53.3%), mutation at Asp-516-Val was 16.7%, followed by His-526-Tyr mutation with 14.4%) between amino acid position 511 and 533 in hot-spot (75bp) variable region of *rpoB* gene (Figure 5). 11 mutations were found (ten point mutations, and two isolates contained a 3base insertion encoding aphenylalanine residue inserted between Gln-513 and Phe-514). No other missense mutations, deletion mutations, or two mutations in separate codons were found in any of 90 RIF resistant *M. tuberculosis* isolates. No mutations were observed within 305bp region for any of RIF sensitive strains tested similar to results of others (36) mostly from Europe, Africa, United States and Japan(116, 128). Five RIF-resistant isolates (5.6%) contained no mutations within 305 bp region of *rpoB* gene, implying a mutation in another part of *rpoB* gene or existence of at least one additional gene that participates in RIF resistance (41).

Table 12: Frequency of mutations in rifampin-resistant isolates reported by four groups as given by Hirano K *et al.*(1999).

Mutation position	**Frequency of codon substitution (no. [%] of isolates)[a]**			
	Telenti *et al.* (*n* =66) European and African countries	Williams et al. (*n* = 110) United States	Suzuki *et al.* (*n*= 46) Japanese isolates	Kazue *et al* (*n*=90) Mostly Asian countries (Bangladesh, Canada, India, Indonesia, Korea, Malaysia, Myanmar, Nepal, Philippines, Thailand,Yemen)
Leu-511	2 (3.0))			1 (1.1)
Gln-513	2 (3.0)	1 (0.9)		5 (5.6)
514-Phe ins.[b]		1 (0.9)		2 (2.2)
Met-515			1 (2.1)	
Asp-516	6 (9.1)	8 (7.3)	4 (8.5)	13 (14.4)
Asn-518 del.[c]	1(1.5) 1 (0.9)	1 (0.9)		
Leu-521		1 (0.9)		
Ser-522	1 (1.5)	2 (1.9)	1 (2.1)	1 (1.1)
His-526	18 (27.3)	37 (33.6)	13 (27.6)	15 (16.7)
Ser-531	33 (50.0)	46 (41.7)	23 (48.9)	48 (53.3)
Leu-533	1 (1.5)	3 (2.7)		
Ser-509,His-526[d]		1 (0.9)		
His-526,Lys527[d]		1 (0.9)	1 (2.1)	
None	2 (3.0)	8 (7.3)	3 (6.4)	5 (5.6)

***a* Refer to the reports by Telenti *et al.*(1993), Williams *et al.*(1994), and Suzuki *et al.*(1995)**

***b* Phe ins= phenylalanine residue inserted between Gln-513 and Phe-514**

***c* Asn del= Asn deletion. *d*= Double mutation.**

In year 2010, comparison study was done to study RIF resistant mutation profile of *M. tuberculosis* clinical isolates from Guizhou to different regions of China [Guizhou, Sichuan, Beijing, Shanghai, Shandong, Zhejiang, Hong Kong] and nine countries of

Asia, Europe and America(Vietnam, Singapore, India, Iran, Spain, Brazil, Turkey, Russia and USA) by sequencing 688bp fragment of *rpoB* gene covering entire RRDR of *M. tuberculosis* DNA (Figure 6).Thirteen missense mutations, all caused by single base substitutions were identified in 32 RIF-resistant strains at codons 509, 511, 516, 522, 526, 531, 533, 550 and 572. Most frequent mutation observed was at codon 531 (Ser→Leu, 42.1%), followed by mutations at codons 526 (18.4%), 516 (10.5%) and 511 (7.9%), at codons 522, 533 and 550 (5.3%), codons 509 and 572 (2.6%). 15.6% RIF-resistant clinical isolates had two or more missense mutations (two isolates with mutations at codons 522 and 550; one isolate with mutations at codons 511 and 516; one isolate with mutations at codons 511 and 526; and one isolate with three mutations at codons 509, 511 and 516). Two new mutations at codons 550 (GTG→TTG, Val→Leu) and 509 (AGC→AGG, Ser→Arg) were identified in two isolates and deposited in GenBank with accession numbers GQ250580 (Val550Leu) and GQ250581 (Ser509Arg). The findings made clear that *rpoB* gene mutation profile in RIF-resistant *M. tuberculosis* clinical isolates from Guizhou differed from other regions of China and other countries of Asia, Europe and America (32).

Table 13: Comparison of mutation frequency at different codons of the *rpoB* gene in rifampicin resistant *M. tuberculosis* from different geographical regions of the world as reported by Chen *et al*. (2010).

	Mutation frequency[a] (%)									Total mutations	RIF-resistant isolates
	533	**531**	**526**	**522**	**516**	**513**	**511**	**other**	**Multiple[b]**		
Guizho, China **Ling Chen *et al*. (2010)**	5.3	42.1	18.4	5.3	10.5	0	7.9	10.5	15.6	38	32
Sichuan,China **Guo J.H *et al*. (2008)**	6.9	60.4	12.1	1.7	10.3	0	6.9	1.7	5.0	58	60
Beijing,China **Jiao W.W *et al*. (2007)**	0	64.6	13.8	0	3.1	0	0	18.5	0	65	71
Shanghai, China **Fan X.Y *et al*. (2003)**	2.4	51.2	22.0	0	9.8	2.4	7.3	4.9	7.7	41	39
Shandon, China **Ma X *et al*. (2006)**	5.0	55.0	26.7	0	6.7	0	3.3	3.3	1.7	60	58
Zhejiang, China **Sheng J *et al*. (2008)**	1.9	3.7	75.8	0	1.9	11.1	0	5.6	0	54	56
Hong Kong **Chan R.C *et al*. (2007)**	2.8	65.1	20.9	1.4	3.3	2.3	1.4	2.8	3.9	215	207
Vietnam **Caws M *et al*.(2006)**	3.6	40.6	29.7	0	14.4	2.7	2.7	6.3	11.5	111	104
Singapoe **Lee A.S *et al*. (2005)**	0	56.0	24.0	2.0	12.0	2.0	0	4.0	2.0	50	51
India **Mani C *et al*.(2001)**	1.9	52.8	18.9	0	3.8	1.9	5.6	15.1	9.1	53	44
Iran **Doustdar F *et al*. (2008)**	6.5	51.6	19.4	3.2	9.7	3.2	0	6.5	6.7	31	30
Spain **Chaves F *et al*.(2000)**	3.1	37.5	12.5	0	15.6	3.1	9.4	18.8	10.3	32	29
Brazil **Valim A.R *et al*. (2000)**	1.1	52.9	21.8	2.3	8.1	2.3	3.5	8.0	8.9	87	79
Turkey **Cavusoglu C *et al*.(2002)**	4.8	54.8	19.0	4.8	7.1	2.4	0	7.1	2.4	42	41
Russia **Mikhailovich V *et al*.(2001)**	7.7	66.6	16.3	1.6	5.4	0.8	1.6	0	0.8	129	128
USA **Caoili J.C *et al*.(2006)**	0	51.5	15.2	0	18.2	0	3.0	12.1	3.1	33	32

a = mutations at a specific codon as a percentage of the total number of mutations.

b = isolates with two or more mutations as a percentage of the total number of RIF-resistant isolates.

WHO Eastern Mediterranean Region:

Twenty-one RIF resistant *M. tuberculosis* isolates from Yemen showed mutations as Ser 522 Leu (1 isolate), His 526 Tyr (2 isolates), Ser 531 Leu (11 isolates), None (1 isolate), Asp 516 Val (4 isolates), 514 Phe ins.[b](1 isolate), Gln 513 Pro (1 isolate) and no mutation in RIF susceptible isolates (3 isolates) in a study (41).

Western Pacific Region:

In Singapore, direct sequencing was done for entire RRDR (RIF Resistance Determining Region) of fifty-one RIF-resistant and four RIF-sensitive *M. tuberculosis* isolates and genetic alterations was seen with a high frequency of 96.1% (129). Mutations at codon 531 were observed in 28 (55%) isolates, at codon 526 in 12 (24%) isolates, and at codon 516 in 6(12%) isolates. Most commonly mutated residues were at Ser-531 [Ser→Leu substitution at codon 531, present in 25 (49%) isolates], His-526 [His→Tyr substitution at codon 526 in 7 (13.7%) isolates], and Asp-516, as demonstrated by studies worldwide (35, 41, 130). Twelve different missense mutations involving codons 513, 514,516, 522, 526, and 531 were identified in forty-nine RIF-resistant strains. Mutation at codon 513, resulting in a Gln→Glu substitution identified in one isolate is rare and similarly deletion of codon 518 was observed in one isolate. One isolate had double mutations in both codons 514 and 516. No genetic alterations were detected in 2 (3.9%) of 51 RIF-resistant isolates. No mutations were found in four RIF-sensitive isolates used as controls (129).

WHO region of Americas:

Thirteen RIF resistant *M. tuberculosis* isolates from Canada had mutation in His 526 Arg and Ser 531 Leu and no mutation was present in RIF susceptible isolates (41).

WHO European region:

In St. Petersburg, Russia, mutations at codon 531 was found in majority of RIF-resistant strains (131).

Mutations in 193bp of *rpoB* gene was evaluated using automated sequencing to detect RIF resistant and susceptible *M. tuberculosis* strains isolates from Brazil (25 strains) and France (37 strains). Mutation was detected in 100% (16/16) RIF resistant isolates from France and 89% (16/18) from Brazil. No mutation was detected in 28 RIF-susceptible strains. Double point mutation was detected in one strain from France. Among French resistant strains, mutations were found in codons 531 (31.2%), 526, 513 and 533 (18.7% each). In Brazilian strains the most common mutations were in codons 531 (72.2%), 526 (11.1%) and 513 (5.5%). The heterogeneity was found in French strains (127).

WHO South-East-Asian-Region drug resistance TB scenario

WHO South-East-Asia-Region is home to four high-burden TB countries in the world. Important progress has been made throughout region in initiating plans for MDR-TB treatment. Except Thailand, all countries got identified laboratory capacity to scale up diagnosis and treatment to reach the target outlines in global plan to stop TB, 2006-2015 (95).

Since 1994, 6 of 11 (India, Indonesia, Myanmar, Nepal, Sri Lanka and Thailand) countries reported drug resistance data, from areas representing 23% of all TB cases in the region (95). India reported data from three districts and one state, and Indonesia reported data from one district. Of the countries reporting, Mayurbhanj district in Orissa state India (132), Sri Lanka, and Thailand reported <l2% MDR-TB among new

cases. Ernakulum district in Kerala (133), and Gujarat in India as well as Mimika district, of Papua province in Indonesia and Nepal reported between 2-3% MDR-TB among new cases. Myanmar reported 3.9% MDR-TB among new cases. The population-weighted mean of MDR-TB based on all countries that reported in the WHO South-East Asia Region was 2.8% among new cases, 18.8% among previously treated cases and 6.3% among combined cases. There were an estimated 149 615 incident MDR-TB cases in the region in 2006, with 74% of these cases estimated to be in India (102, 128 - 130).

Table 14: SEAR weighted mean of resistance to first-line-anti-tubercular drugs by treatment history by WHO region, 1994-2007.

SEAR	New	Previous	Combined
Countries	6	5	6
Settings	13	6	14
Any H	10.3 (6.9-13.7)	36.8 (26.7-47)	15.7 (10.5-20.9)
Any R	3.4 (2.4-4.4)	19.3 (14.1-24.5)	6.9 (4.8-9)
Any S	8.9 (5.9-11.8)	21.7 (13.3-30.2)	11.7 (7.5-16)
Any E	3 (0.7-5.4)	13.8 (0.3-27.3)	4.7 (2.2-7.2)
Any resistant	15.8 (11.6-20)	42.3 (32.3-52.3)	20.8 (14.2-27.4)
MDR	2.8 (1.9-3.6)	18.8 (13.3-24.3)	6.3 (4.9-8.4)

H= Isoniazid; R= RIF/rifampicin; S= Streptomycin; E= Ethambutol;
MDR= Muti-drug-resistant
95% confidence levels are given between brackets

The result from survey in Gujarat, India, showed MDR-TB among new cases 2.4% and 17.2% among retreatment cases. XDR-TB has been reported in country evidenced by survey in Gujarat and Maharashtra (96, 134). Myanmar nationwide survey showed

3.9% MDR-TB among new cases and 15.5% among re-treatment cases, and report was estimated with 4251 incident MDR-TB cases in 2006. Mimika district of Papua province in Indonesia showed moderate levels of resistance. Sri Lanka showed low proportion of resistance but data was not fully quality assured. Nepal showed MDR-TB among new cases from 1% - 3% in four surveys conducted since 1996. MDR-TB among new cases was estimated 2.9% and 11.7% among retreatment cases. Thailand reported data from three surveys showing stable trends in resistance, with MDR-TB under 2% among new TB cases. Damien Foundation showed low levels of drug resistance from monitoring data on drug resistance in rural population of Bangladesh for past 10 years. Republic of Korea reported re-treatment cases comprising 18% of notified cases, suggesting a considerable burden of MDR-TB and indicating drug resistance might be higher than in other countries.

rpoB gene mutation study from Asia

From Tehran (border of Iran), DNA sequencing determined resistance in 81bp region of *rpoB* gene using RIF resistant *M. tuberculosis* isolates and found predominant nucleotide change in codons 531 (TCG/TTG, TCG/TGG), 515 (ATG/ATA), 510 and 526. Missense mutations with 20 types of amino acid substitutions in codons 531, 526, 515, 510, 566, 476 and 490 were revealed. Mutations in codon 531, 510, 526, and 523 were associated with high-level RIF resistance (>100mg/L). Mutation in codon 516 was associated with low-level RIF resistance ($p<0.005$). Mutations in different codons and single mutation was also seen. RIF resistant strains with no mutations was also seen (73).

Indian scenario of drug resistant TB

India is second-most populous country in the world and is a developing nation. India has more new TB cases annually than any other country. Malnutrition is highly prevalent in India. Tobacco use is highly prevalent and is a potent contributor to TB-related mortality, one of the risk factors to develop TB epidemiology in India (135).In 2009, estimated TB global annual incidence was 9.4 million cases, out of which 2 million occurred in India, contributing to a fifth of global TB burden. 40% of Indian population is infected with TB bacillus. On a national scale, high burden of TB in India is illustrated by accounting 17.6% of deaths from communicable disease and 3.5% of all causes of mortality. WHO estimated TB mortality in India as 280,000 (23/100,000 population) in 2009 (135).

TB Research Centre and National TB Institute have standardized methodology, proper elicitation of previous treatment history, sample selection, uniformity in bacteriological procedures, good quality drug powders used for susceptibility testing and quality assurance studies (136). Since 1999, TRC carried out several operational research studies in model DOTS areas of Tiruvellore district, Tamil Nadu, including measurement of drug resistance among patients. Interim data (1999-2003) on 1,610 patients with no history of previous treatment revealed 11.8% isoniazid resistance and 1.6% MDR respectively (TRC 2004, unpublished). Likewise, a study on drug resistance carried out on HIV/TB patients (2000-02) revealed 13% isoniazid resistance and 4.3% MDR respectively (TRC 2003, unpublished).

NTI, Bangalore conducted drug resistance surveillance in four districts of Mysore (2001), Hoogly (2003), Mayurbhanj (2003) and Bangalore city (1999) where MDR-

TB levels amongst patients with no history of previous treatment observed was 1.2%, 3.0%, 0.7%, and 2.2% respectively (132, 137, 138)

A retrospective analysis of various randomized clinical trials conducted by TRC with various RIF containing regimens in the initial intensive phase, and with and without RIF in the continuation phase, revealed an overall emergence of 2% RIF resistance and 18% INH, either alone or in combination with other anti-TB drugs (TB Research Centre, ICMR, 2001).

Global project on TB drug resistance surveillance by the WHO/IUATLD during year 1999 to 2002 included 3 centers from Tamil Nadu, Karnataka, and Maharashtra over a period of 3 to 10 months for new sputum positive cases and found primary MDR to be 0.5% to 2.8% (139).

Since available data from India cover only some area of the vast country, there is need for a surveillance of drug resistance of the whole country or at least representative areas by a network of investigators using a WHO protocol, with an emphasis on quality control assurance, which will serve as a useful parameter in evaluation of RNTCP programs. The Central TB Division, Government of India has finalized a protocol for carrying out representative drug resistance study at state level and provide valuable assistance to investigators interested in carrying out DST in respective areas. Ongoing studies of ICMR on molecular basis of drug resistance and DNA finger-printing of these isolates will yield valuable information on drug resistance across the country**.**

RNTCP covered the whole nation by end of March 2006 and evaluated >24 million TB suspects by examining >100 million sputum slides. >6.7 million patients are put on treatment, saving more than a million TB deaths and the programme has achieved

target of nearly 70% of new sputum positive case detection and over 85% of global target cure rates (70). While the RNTCP is by and large has become a success story, the problem of drug resistance is emerging over the years. Drug resistance against INH and RIF in India, are many hospital-based, anecdotal, and susceptibility testing wasn't done by quality controlled laboratories, with exception of a few reports from TRC Chennai, NTI Bangalore and few others, failed to give an exact idea of national situation as a whole in India (70). Subsequently several surveys in different parts of country reported total prevalence of primary MDR-TB from 0-5% and acquired MDR-TB from 6-60% (101, 136, 140).

Early in pre-rifampicin era, studies were undertaken by ICMR during 1965-67, resistance to INH ranged from 11-20%, to streptomycin 8-20% and to both drugs 4-11%. The second study showed resistance to INH from 15-69%, to streptomycin 12-63% and to both drug from 5-58%. The relation of drug resistance level was proportionate to duration of previous treatment taken. A subsequent study from Chennai carried out a decade later reported similar results to those from earlier ICMR surveys, indicating the prevalence of initial drug resistance did not change (141). Subsequently after about two decades (1980's), 11 other studies from different parts of the country reported similar resistance to INH and S like those found in earlier studies with notable exception of RIF resistance being observed in 10 of 11 publications and level of MDR-TB in all the centres was observed <5% (70).

Studies from TRC Chennai on prevalence of primary drug resistance through control clinical trials showed resistance to RIF started appearing around 1990 but remained at around 1% with a similar figure for MDR-TB. The global project on TB Drug Resistance Surveillance by WHO/ IUATLD during the period 1999-2002 included

three centres from India -North Arcot (Tamil-Nadu), Raichur (Karnataka) and Wardha (Maharashtra) over a period of 3-10 months for new cases. Any resistance was found between 19.8-27.7%, to isoniazid from 15.2-23.4%, to RIF 0.5-2.8%, to ethambutol 1-4.6%, to streptomycin 7.2-12.4% and multi drug resistance varied from 0.5-2.8% (70, 139), thus, primary MDR in India is around3% (70, 142). On the other hand, acquired MDR-TB rate range from 9.6-100 % (136).

Most of the published studies were carried out before the RNCTP (70). India, has five accredited laboratories performing culture and drug sensitivity testing (70). RNTCP accepts figure for acquired MDR-TB from 13-17% (70, 142). To describe a country's burden of MDR-TB, percent prevalence and absolute number must be considered and with this regard, a relatively low prevalence of multi-drug resistance with high TB burden represent high absolute number of cases of MDR-TB. The RNTCP is undertaking the exercise of strengthening laboratory in expansion phase (70). XDR-TB was reported on 21st May 2007, by American Thoracic Society International Conference, depicted 32% MDR-TB and 8% XDR-TB cases among 1,274 *M. tuberculosis* isolates at Hinduja National Hospital, Mumbai, India (143). 58.2% MDR-TB was found among suspected drug-resistant 177 TB cases from tertiary care hospital, Christian Medical College, Vellore, in South India from 2003 to 2007 (103). 60% cases met the criteria of XDR-TB, out of 75 MDR-TB cases tested for second-line drug susceptibility test (45), in comparison to worldwide data on XDR-TB as 6.6% cases of MDR-TB(144). But the data is not considered to reflect the national situation as hospital reporting the study were tertiary care referral centres and the observations need further scientific scrutiny and more experience from accredited laboratories (70).

Present MDR-TB cases are managed on an individual basis having inherent problems of further default as the patient has to arrange the drugs for treatment, but soon DOTS-Plus under RNTCP will undertake whole nation in years to come (70). Following the training of STDC staff in DRS techniques, and of field staff in patient intake and sample collection mechanisms, state representative DRS surveys were undertaken in Gujarat (56m population) and Maharashtra (107m) in 2005-2006 with results of the surveys indicating prevalence of MDR-TB <3% amongst new cases and 12-17% in re-treatment cases estimating annual incidence of 131,000 cases of MDR TB in the country (70). Results of second line DST on MDR isolates from Gujarat DRS survey showed no XDR amongst new cases and the prevalence amongst re-treatment cases as to be 0.5%. The Medical College National Task Force has acknowledged the immediate need for action by all practitioners to prevent the development of MDR-TB and XDR-TB and issued a consensus statement to adopt the national and international guidelines on management of drug resistant TB and promote the rational use of second line anti TB drugs (70, 142). The accreditation document for the state level intermediate reference laboratories for performing culture and drug sensitivity testing is approved in Laboratory Committee meeting. Similarly accreditation of Medical College laboratories is developed by Central TB Division. The New WHO Stop TB Strategy has one of its six principal components to address the TB/HIV and MDR-TB to realize the global TB-related MDG. Reliable and periodic updates on prevalence of drug resistance are needed by a network of quality control laboratories. TB control is a long term battle and will require extended political support (70).

Prevalence of MDR-TB among new cases of pulmonary TB patients in India collected from earlier studies was summarized by S.K. Sharma *et al* in 2011(145) (Table 15).

Table 15: Prevalence of MDR-TB among new cases of pulmonary tuberculosis patients in India summarized by Sharma *et al*. (2011)

Location	Period of study	No. of isolates	MDR-TB (%)
Bangalore	1980s	436	1.1
Wardha	1982-1989	323	5.3
North Arcot	1985-1989	2779	1.6
Pondicherry	1985-1991	1841	0.8
Kolar	1987-1989	292	3.4
Jaipur	1989-1991	1009	0.9
New Delhi	1990-1991	324	0.6
Pune	1992-1993	473	1.0
Tamil Nadu	1997	384	3.4
North Arcot	1999	282	2.8
Lucknow	2000-2002	318	13.2
Hyderabad	2001-2003	714	0.14
Ernakulum	2004	305	2.0
Gujarat	-	1571	2.4
Mumbai	2004-2007	493	24
New Delhi	2008-2009	177	1.1

India under RNTCP a nation-wide laboratory network, encompassing over 12,500 DMCs, supervised by IRL at state level, and NRL & Central TB division at national level. Efforts have been made to consolidate laboratory network into a well-organized one, with a defined hierarchy for carrying out sputum microscopy with EQA, DRS, mycobacterium culture and DST and DOTS-Plus related activities. TB mortality in

country reduced from over 42/100,000 population in 1990 to 24/100,000 population in 2008 and reduction in prevalence of TB from 568/100,000 population in 1990 to 185/100,000 population by 2008 as per WHO Global TB control 2009 report. Two more surveys underway are in states of Andhra Pradesh and Uttar Pradesh with a plan to undertake a future survey in Orissa. The programme is also in process of establishing a network of accredited culture and drug susceptibility testing IRLs across the country in a phased manner for diagnosis and follow up of MDR-TB patients. 9 IRLs (Gujarat, Maharashtra, Kerala, Andhra Pradesh, Tamil Nadu, Delhi, Rajasthan, Orissa, West-Bengal) are accredited as per RNTCP accreditation protocol in 2008/2009. The IRLs of Haryana, Jharkhand and UP (Lucknow) are in advanced stages of proficiency testing and rest of the IRLs will be starting the accreditation process in coming time. The procurement of culture and DST equipments for another fourteen IRLs (Assam, Bihar, Sikkim, Manipur, Arunachal Pradesh, Uttar Pradesh, Punjab, Himachal Pradesh, Srinagar, Jammu, Pune, Karnataka, Madhya Pradesh & Goa) is done as per World Bank guidelines through UNOPS. DOTS plus for management of MDR-TB has been rolled out in states of Gujarat and Maharashtra in March, 2007 and in Andhra Pradesh, Delhi, West Bengal and Kerala in 2008. By the end of the 4th quarter of 2009 the MDR-TB treatment services scaled up to cover ~200 million population in 105 districts across 10 states. Over 6000 MDR suspects were examined and over 1000 MDR cases were put under treatment. Culture & DST Laboratory accreditation work is entrusted with NRLs. Current four National Reference Laboratories (NRL) are TB Research Centre (TRC), Chennai; National TB Institute (NTI), Bangalore; Lala Ram Swarup Institute of TB and Respiratory diseases (LRS), Delhi and JALMA Institute, Agra. The NRLs work closely with IRLs, monitor and supervise the IRL's activities and undertake periodic training for the IRL staff in EQA, culture & DST activities. To maintain uniformity in testing procedures NRLs

conduct 2-4 week culture and DST trainings to the microbiologists and laboratory technicians of laboratories undergoing accreditation.

A total of twelve TDR-TB patients were detected with resistance to twelve drugs, out of which, one died from Hinduja Hospital [US-based Clinical Infectious Diseases (CID) peer review journal]. However, findings cannot be relied upon as its laboratory is not accredited to detect such cases.

rpoB gene mutation study from India

rpoβ mutations was determined by sequencing various hot-spot loci prevalent in 93 RIF resistant *M. tuberculosis* isolates from patient having had history of taking anti-tubercular drugs from North India (Largest number of samples was from New Delhi, followed by Chandigarh, Ahmedabad, Agra, Shimla, Jaipur) Bangalore and Chennai in a study by workers from various institute (Centre for DNA Fingerprinting and Diagnostics, Hyderabad, National Institute of Immunology, New Delhi TB Centre, and Department of Medicine, A.I.I.M.S., New Delhi, Central Jalma Institute of Leprosy, Agra and Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore India). *rpoB* region was amplified and sequenced for a stretch of 30 amino acids from 432 to 458 for mutations (146). Mutations in *rpoB* loci was similar to other parts of world (35). Codon 531was the most vulnerable site of mutations in RIF resistant isolates. 28 had missense mutation, Ser531Leu and 8 had substitution Ser531Trp. The next most common mutations were amino acid substitutions Asp516Val or Asp516Gly (20 isolates) and His526Tyr, His526Leu, or His526Arg (19 isolates). Two isolates with Gln510His changes was also found. Silent mutations at amino acids Leu511 (present only in isolates with >1 mutation at the *rpoB* locus) and

Leu521 was also found. Mutations in codons 516 and 521 conferred low level RIF resistance (MIC <40µg/ml), whereas mutations in codons 510, 526, 527, 528, and 531 conferred high levels resistance (MICs ≥64µg/ml) (147). Double mutations seen in few cases had additive effect on degree of resistance. Significantly, the frequency of mutations was higher at codon 516 and lower at codon 526 in Indian isolates. Mutation not reported earlier (24% of total mutations in 93 isolates studied) were at codons Ser509Arg, Leu511Val, Asn518Thr, Ser522Gln, Lys527Asn, Arg528Pro, and Arg528His. These novel mutations needs to be considered when designing tools for detection of MDR-TB. Study done in PGI Chandigarh by Sharma et al. in 2000, also showed that the most common mutation among New Delhi isolates was in codon 516, Asp-->Val using fluorescence based PCR- single strand conformation polymorphism (SSCP) (148). A study from TRC, Chennai consisting of 44 RIF resistant *M. tuberculosis* clinical isolates (Andhra Pradesh, 6; Delhi, 1; Goa, 3; Kerala, 4; Karnataka, 5; Sikkim, 5; and Tamil Nadu (TN), 20) and six RIF sensitive *M. tuberculosis* clinical isolates strains (four from Tamil Nadu and two from Andhra Pradesh) when analyzed to determine *rpoB* mutations within 81bp RIF resistance determining region (RRDR) of *rpoB* gene by PCR and sequencing (107). Fifty-three mutations of 18 different kinds, 17 (95%) point mutations (involving 10 codons) and one deletion, was observed in 43 of 44 resistant isolates (Table 16). One RIF resistant isolate with mutation outside amplified region of gene was also present. Three novel mutations and three new alleles within RRDR, along with two novel mutations outside RRDR, were found. DNA sequence analysis of 44 RIF resistant isolates showed, 39 had a single mutation, two had triple [One isolate from Andhra Pradesh containing a triple mutation had two mutations in codon 516 (GAC to AAA), another at codon 531 (TCG to TTG) and also contained a mutation outside RRDR (CCC to CAC at codon 535). Second isolate containing a triple mutation was from Tamil Nadu

and had mutations at codon 508 (ACC to AGC), codon 512 (AGC to AGG), and codon 526 (CAC to GAC)] mutations] and two resistant strains had quadruple mutations [two isolates from Tamil Nadu contained a novel mutation of GCG to GCA at codon 532. In addition, one of the two Tamil Nadu isolates had mutations at codon 518 (AAC to CAC), codon 531 (TCG to TTG), and codon 533 (CTG to CTT), while the other had mutations at codon 511 (CTG to ATG), codon 512 (AGC to AGG), and codon 526 (CAC to GAC)] in the 81-bp region of the *rpoB* gene. In Karnataka, four isolates had the same mutation, TCG to TTG at codon 531, while the fifth exhibited a mutation at codon 526, CAC to GAC. Overall findings suggested, that the codons most frequently involved in mutation were codon 531 with frequency of 53% (TCG (Ser) to TTG (Leu) 49%) and codon 526 (19%) similar to other studies (89, 149). CAC to GAC mutation at codon 526 was found only 6% in contrast to same mutation obtained from Greece (19%) (89), and Italy (30%) (149). The higher occurrence of mutation of TCG to TTG at codon 531 in isolates from all the states except from Andhra Pradesh, where codon 526 was the most involved can be explained by such mutants ability to survive as they have a higher mean relative fitness (150). Five different types of mutations were seen at codon 526. Mutation was absent in six RIF sensitive isolates. Mutations not detected in earlier studies were found at codon 532 from GCG (Ala) to GCA (Ala) in two isolates, at codon 508 from ACC (Thr) to AGC (Ser) and deletion at codon 517 alone of CAG (Gln). New alleles for mutation reported was, AGC (Ser) to AGG (Arg) at codon 512, GAC (Asp) to AAA (Lys) at codon 516, and CTG (Leu) to CTT (Leu) at codon 533. New mutations outside the RRDR were GGG to GAG at codon 534 and CCC to CAC at codon 535. High MICs for isolates was seen with mutations at codon 516 or codon 533 and 532 in contrast to other's study (147).

Table 16: Distribution of mutations by state found in RRDR of the *rpoB* gene in rifampicin resistant *M. tuberculosis* isolates from India (Mani *et al*., 2001).

Codon no.	Mutation	Distribution by state [a]
508	ACC 3 AGC[b]	TN, 1
511	CTG 3 CCG	K, 1; TN, 1
	CTG 3 ATG	TN, 1
512	AGC 3 AGG[c]	TN, 2
513	CAA 3 AAA	G, 1
516	GAC 3 TAC[c]	S, 1
	GAC 3 AAA[d]	AP, 1
517	CAG 3 del[b,e]	AP, 1
518	AAC 3 CAC	TN, 1
526	CAC 3 CTC	S, 1; TN, 2
	CAC 3 TAC	AP, 1; K, 1
	CAC 3 GAC	KA, 1; TN, 2
	CAC 3 CGC	AP, 1
	CAC 3 ACC	AP, 1
531	TCG 3 TGG	S, 1; TN, 1
	TCG 3 TTG	S, 2; G, 2; K, AP, 2; D, 1;
532	GCG 3 GCA[b]	TN, 2
533	CTG 3 CTT[c]	TN, 1

a **KA, Karnataka; S, Sikkim; G, Goa; D, Delhi; K, Kerala.**

b **Novel mutation.**

c **New allele.**

d **Double mutations in the same codon.**

e **del, deletion.**

In Tamil Nadu, a study using PCR and DNA sequencing study was done for 101 *M. tuberculosis* isolates (50 *M. tuberculosis* isolates were new cases and 51 re-treatment patients) consisting of 67 from Hyderabad (37 RIF resistant and 30 showed mutations

either within or outside RRDR region) and 34 (one RIF resistant isolate had no mutation either within or outside RRDR region) from Koraput (114). All RIF susceptible isolates showed *rpoβ* sequence similar to $H_{37}R_V$. Thirty out of 38 (79%) RIF resistant isolates showed mutations at either within (28 isolates) or outside the RRDR region (Table 17). Within the hot spot region, point mutations were observed at codon 531(47%), 526 (17%), 516 (13%) along with other uncommon mutations in 26 isolates and multiple mutations in two isolates. Mutations outside the RRDR region occurred in 5% (two) isolates [one isolate showed multiple mutations at 145, 170, 173,174, 180, 181 and 184, reported for the first time (Gene bank accession number GQ395623)]. 94% (48 isolates out of 51) cured patients did not have any mutations and 77% (20 out of 26) treatment failure patients had *rpoB* gene mutations. Mutations at codon 531, 526 and 516 was at high frequency common with a slight varied occurrence across geographical regions of world (151, 152). Certain uncommon mutations such as 511, 513, 518, 519, 528 and 529, was found like in other places (130)**.** Combined occurrence of mutation at 511 along with mutations at one or the other codons as found outside India was also seen (146). A rare mutation, V176L was seen (153). Multiple silent mutations from a patient with clinically and bacteriologically confirmed treatment failure case, was found between the codons145-184 (outside RRDR region) for first time (GQ395623). No mutations were found in 21% of RIF resistant isolates, which was in concurrence with similar reports of others (107, 152, 154). Thereby the study highlighted the diversity in *rpoB* mutations of *M. tuberculosis* as observed in other studies (41, 155).

Table 17: List of *rpoB* gene mutations detected in *M. tuberculosis* isolates from Andhra Pradesh (Hyderabad and Koraput), India by Lingala *et al.* (2010)

Mutation codon	Amino acid and nucleotide change	No of isolates
	Mutations in RRDR region	
513	CAA (Gln)- GAA (Glu)	1
516	GAC(Asp) -GCA (Ala)	1
516	GAC(Asp) - TAC (Tyr)	2
516	GAC(Asp) - GTC (Val)	1
518	AAC(Asn) - TAC (Tyr)	1
526	CAC(His) - CTC (Leu)	1
526	CAC(His) - AAC (Asn)	2
526	CAC(His) - TAC (Tyr)	2
529	CGA (Arg) - CAA (Gln)	1
531	TCG(Ser) - TTC (Phe)	1
531	TCG(Ser) - TGG (Trp)	1
531	TCG(Ser) - TTG (Leu)	12
511,516,528	CTG(Leu) -CTA(Leu), GAC(Asp) - GTC (Val), insertion	1
513, 519, 531	Deletion, Deletion, TCG (Ser) - TTG(Leu)	1
	Mutations outside RRDR	
145,170, 173, 174, 180, 181 and 184	(T-T); (G-G); (R-R); (V-V); (V-V) ; (R-R); (G-G)	1
176	Val GTC-TTC Phenyl	1
	Total	30

From Sankara Nethralaya hospital in Chennai, Absolute concentration method was used to determine MIC of RIF resistance for 44 *M. tuberculosis* isolates, (21 respiratory, 3 ocular, 3 cerebrospinal fluid and 17 biopsies) and were performed automated DNA sequencing to characterize *rpoB* mutations. Five isolates found as RIF resistant with MIC greater than 128 mg/ml showed mutations. Missense mutation

at codon 531(Ser→Leu) was present in two isolates and the third isolate had a novel mutation containing three insertions at end of codon 519 (CAG AAC AAC repeat) reported first time. The other two isolates with absence of mutation was attributed to presence of mutations in a region outside the specific sequence of the primers used for amplification. High level drug resistance (MIC >50μg/ml) (35) were found associated with missense mutations in codons 513(5%), 526(33%) and 531(53%)(93)**.**

Drug resistant TB scenario in Sikkim, India

Sputum conversion rate and cure rate needs improvement (State level Performance based on quarterly reports for 1st quarter 2009). Estimated no. of new sputum positive cases was 75/100,000 population per year (based on recent ARTI report). The State has been consistently achieving RNTCP twin targets of 70% new sputum positive case detection rate and 85% new sputum positive cure rate. All four districts started service delivery under RNTCP on 1st March 2002.

Increasing number of MDR-TB cases in state is an area of concern with reported 135

during 2008-09 (3rd Common Review Mission Report for Sikkim). District-wise

performance (based on quarterly reports for 1st quarter 2009) cure rate in new sputum positive cases (against >85%) was 85% (Gangto-East Sikkim), 81% (Namchi-South Sikkim), 80% (Gyalshing-West Sikkim), 67% (Mangan -North Sikkim) recorded, below standard cure rate of DOTS. TB was shown main cause of death among all identified diseases in Sikkim between 2003 to 2005 (PME Division, H & F.W. Deptt). Sikkim is an open challenge for drug resistance surveillance, as no previous research work had been done, to provide information about MDR-TB in the state.

***rpoB* gene mutation study report from Sikkim, India**

Five *M. tuberculosis* RIF resistant isolates from Sikkim was analyzed to determine *rpoB* mutations within 81bp RIF resistance determining region (RRDR) of *rpoB* gene by PCR and sequencing (107). Mutation were obtained at codon region, 531(3), 526 (1) and 516 (1). Rest, no other work has been carried out with the *M. tuberculosis* samples from Sikkim.

Available diagnostics tests for rifampicin resistance detection in *M. tuberculosis* isolates

Diagnosis of TB has advanced so much at this 21st century, that the identification test are available upto the species level for MTBC, both consisting of conventional and molecular test. Various TB diagnosis test available today are as:

I. Culture and drug susceptibility testing

Culture is accepted gold standard test even today, in presence of newer molecular methods. It is reliable as it can discriminate between live and dead bacilli and it's the most sensitive test, with capacity to detect as few as 10-100 CFU/ml sputum and thus guide the patient's treatment effectively. Drug susceptibility testing by culture method is determined by macroscopic observation of growth in drug-free and drug-containing media. Culture can be done on two types of state, one is solid culture and second is liquid culture.

1. Solid culture: The growth time required for solid culture takes incubation time of 2-8 weeks. Medias available for solid culture are egg-based (Lowenstein-Jensen, Petragnani, American Trudeau Society, and Ogawa) and agar-based (Middlebrook 7H10 and 7H11).Solid culture susceptibility tests can be done by three methods:

a) Absolute concentration method - this method uses a standardized inoculum grown on drug-free media and media containing graded concentrations of the drug (s) to be tested. Several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth; i.e., MIC. This method is greatly affected by inoculum size and the viability of the organisms.

b) Resistance ratio method - it compares the resistance of unknown strains of tubercle bacilli with that of a standard laboratory strain. Parallel sets of media, containing two fold dilutions of the drug, are inoculated with a standard inoculum prepared from both the unknown and standard strain of tubercle

bacilli. Resistance is expressed as the ratio of the MIC of the test strain divided by the MIC for the standard strain in the same set.

c) Proportion method - This method is the standard reference method chosen worldwide presently (156). The resistance is calculated by dividing the total number of colonies growing on drug-containing medium by the total number of colonies growing on drug-free medium, indicates the proportion of drug resistant bacilli present in the bacterial population. Resistance is defined when growth appears greater than 1% of an inoculum of bacterial cell in presence of critical concentration of the drug (the drug concentrations used are described in detail by the National Committee for Clinical Laboratory Standards, 2003). Proportion method is only method considered valid for achieving critical drug concentrations (The critical concentration of a drug represents the lowest concentration of the drug that inhibit 95% of wild-strains of *M. tuberculosis* without inhibiting strains that have been isolated from patients not responding to the treatment) and also the drug resistance proportions has very well had been shown to correlate to clinical condition of patients. It enables precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of inoculum are planted on to both control and drug–containing media. At least one dilution should yield isolated countable (50-100) colonies. When these numbers are corrected by multiplying by the dilution of inoculum used, the total number of viable colonies observed on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be determined. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total

population tested. The proportion method is currently the method of choice due to the simple version of bacterial suspension and interpretation of results.

2. Liquid culture: Liquid media available for culture are Middlebrook 7H9 and Dubos medium. Liquid culture has shortened culture growth time as compared to solid culture with a mean of 10-12 days. eg Semi-automated (BACTEC 460TB system) or automated (BACTEC MGIT 960, ESP Culture System II, and MB/BacT ALERT 3D system) liquid culture systems (157, 158). Liquid based culture method uses the minimum inhibitory concentration as the cut off point for isolates from patients who were never exposed to anti-tubercular drugs to calculate the drug resistance (45). Liquid culture faces the drawback of getting high false resistant results (159-161) due to contamination (162).

II. Phage based assay:

a) Lysis with mycobacteriophage: is a phage based techniquefor rapid detection of RIF resistance in *M. tuberculosis* isolates(Gali,2003).The turnaround time for PhaB assay is 2 to 3 days(163) by detecting live mycobacteria in clinical samples (FAST Plaque TBTM method use mycobacteriophage to detect presence of *M. tuberculosis* directly from sputum specimen) where phages infects and replicates inside mycobacterial cells as indicators. The system has the advantage of speed and high sensitivity of 100%, 97.7% specificity, and 95.2% predictive value for RIF resistance (164).

b) Luciferase reporter gene assay: In this assay, sputum is placed into medium and is then transfected with a luciferase-containing mycobacterial phage. If viable *M. tuberculosis* is present in the sample, it will take up the phage and the luciferase gene will function, producing visible light when luciferin is added to assay. Drug susceptibility testing is tested by inoculating clinical sample into antibiotic-containing medium (165). The current version of assay, the "Bronx Box," uses polaroid film for the readout and is a self-contained unit that may be capable of detecting viable *M. tuberculosis* and drug inhibited strains in as few as 2 days (166).

III. Reverse line blot assay (RLBA):

It is a sensitive, specific test developed and evaluated in India for detection of genotypic resistance to RIF. The assay is based on reverse hybridization principle and it simultaneously detects 13 different mutations affecting 6 independent codons, including the most prevalent mutations at positions 531 and 526. The test application with a panel of 292 MDR-TB isolates and susceptible strains collected from five different cities in India showed 98% concordance with sequencing results. Thus the assay seemed promising as rapid, simple, economical, and highly sensitive method as an alternative to sequencing for genotypic evaluation of RIF resistance in *M. tuberculosis* (167).

IV. Molecular beacons:

is a rapid and sensitive method for making diagnosis and identifying mutations associated with antibiotic resistance (168, 169). It uses molecular

beacons that are molecules that emit light when a chemical reaction occurs involving binding of primers with specific target DNA (170).The assay result indicates whether a patient is infected with *M. tuberculosis*, concentration of bacilli present in sample and RIF resistance mutations in *M. tuberculosis* DNA extracted directly from sputum in New York using molecular beacons (fluorogenic nucleic acid hybridization probes) in a single-tube assay in less than 3 hours duration enabling immediate decision to whether to prescribe a more rigorous course of antibiotic treatment (171). Disadvantage of the assay is its high cost due to need of spectrofluorometric instruments and need of sophisticated technology. The assay utilizes five differently colored molecular beacons (probes interrogating entire 81bp core), each of which binds specifically to a different target segment within the core region. Each molecular beacon doesn't bind to its target if the target sequence differed from the RIF-susceptible sequence by as little as a single nucleotide substitution. Molecular beacons fluoresce when they are bound to their targets. Absence of any one of five colors in assay means, that the bacilli in the sample is RIF resistant.

V. Ligase chain reaction:

facilitates detection of a mismatch of even one nucleotide in the sequence of resistance genes.

VI. Restriction fragment length polymorphism (RFLP):

RFLP patterns have been used to categorize and compare drug sensitive and drug resistant isolates of *M. tuberculosis.* As the DNA finger prints of *M. tuberculosis* have been observed not to change during the development of drug resistance, RFLP analysis has also been used as a molecular epidemiological tool to track the spread of drug resistant strains. Molecular markers in use for epidemiologic and evolutionary studies of drug resistance are Interspersed Repeat Unit Variable Number Tandem Repeats (IRU-VNTR) and fluorescent amplified fragment length polymorphism (FAFLP).

VII. Validation and Demonstration of Newer Technologies for Diagnosis of TB and MDR-TB by RNTCP in collaboration with Foundation for Innovate and New Diagnostics (FIND), India

In this eve of growing era of drug resistant *M. tuberculosis* as MDR-TB and XDR-TB, there is need for development of rapid newer technologies that will enhance accuracy of TB diagnosis a well as anti-tubercular drugs sensitivity pattern of the tubercular bacilli in very short time as possible. Realizing these needs, RNTCP initiated projects to validate and demonstrate newer technologies in collaboration with Foundation for Innovate and New Diagnostics (FIND), India. These newer technologies under study are - Line probe assay (LPA), Automated liquid culture systems for Culture & DST (Drug sensitivity testing), and LED Fluorescence microscopy are under validation in various IRLs (Intermediate Reference Labs) and NRLs (National Reference Labs). IRLs of Gujarat and Andhra Pradesh, and JALMA Institute are validating Line probe assay for detection of isoniazid and RIF resistance. Liquid culture systems have got validated in Gujarat and LRS Institute, Delhi. LED

Fluorescence Microscopy is under validation at New Delhi TB centre, JALMA Institute (Agra) and CMC Vellore. It is anticipated that validation and demonstration studies would provide enhanced reach to programme for diagnosis and follow-up of the MDR-TB.

a) Reverse Line Probe Assays (HAIN's MTB-DR kit): The assay is the advanced modification of INNO LiPA Rif.TB assay. It too works on the same principle of reverse based hybridization. The assay can detect RIF and INH resistance both in a single strip. The assay is now being popularized under a demonstration project, called post-STAG approval project, which is being conducted at six programme sites in India. Under this project LPA (Line Probe assay) mechanism has been successfully piloted in the RNTCP settings. The results so far of this pilot study are submitted to the National Lab committee and the mechanism has now been approved for use throughout the country lab for scale-up plan. Under this project the validation of LPA with solid culture as reference has so far been completed. The results of the in-country validation was submitted to National lab committee and LPA got approved for carrying drug resistant TB diagnosis at the treatment initiation time under the project. Gujarat is front runner in this regard. Kailasben 45 year female, is the first TB patient, to get diagnosed as MDR-TB patient by this project using Line Probe Assay in the molecular lab set up at IRL Ahmedabad on 18th August 2009. After pre-treatment evaluation at B.J. Medical College, DOTS-Plus Site, Honorable health minister of Gujarat Mr. Jay Narayan Vyas gave the first dose of Directly Observed treatment. Thus India's first MDR-TB patient diagnosed by LPA is now put on cat IV treatment at Ahmedabad.

b) Liquid culture lab preparedness study: It is a post STAG approval project. This project was conducted at four programme sites in India, where validation of

Liquid culture (MGIT-Mycobacterium Growth Indicator Tube) with Solid culture as reference was done and is now completed. The results of the validation was presented to National Lab committee and got approved for the drug resistant TB diagnosis and treatment initiation under this demonstration project.

c) iLed and Gene-Xpert demonstration projects: is a part of the FIND multi-country demonstration project being conducted to collect data for submission to WHO-STAG. Of these iLED project has been completed and the data has been presented to STAG. Gene-Xpert project is currently in the stage of blinded lab validation and Gene-Xpert results are not being used currently for patient management.

INNO LIPA RIF.TB assay studies done across the globe

LiPA was used in following studies using DNA extracts from culture growth.

INNO LiPA Rif.TB was evaluated using culture isolated DNA for the first time with 107 *M. tuberculosis* isolates with known *rpoB* sequences, 52 non- *M. tuberculosis* complex strains, and 61 and 203 clinical isolates found to be sensitive and resistant, respectively, by in vitro testing. Their study found that the *M. tuberculosis* complex probe was 100% specific, when compared to sequencing. All strains sensitive by in vitro susceptibility testing were correctly identified by LiPA except for 4 (2%)

resistant strains by in vitro susceptibility testing due mutation lying outside the RRDR or 81bp region (Figure 3) of the *rpoB* gene(109). The strains used were collected from different countries of the world (Algeria, Azerbaijan, Bangladesh, Belgium, Benin, Burkina Faso, Canada, Egypt, Guinea, Pakistan, Peru, Romania, Rwanda, Tunisia.)

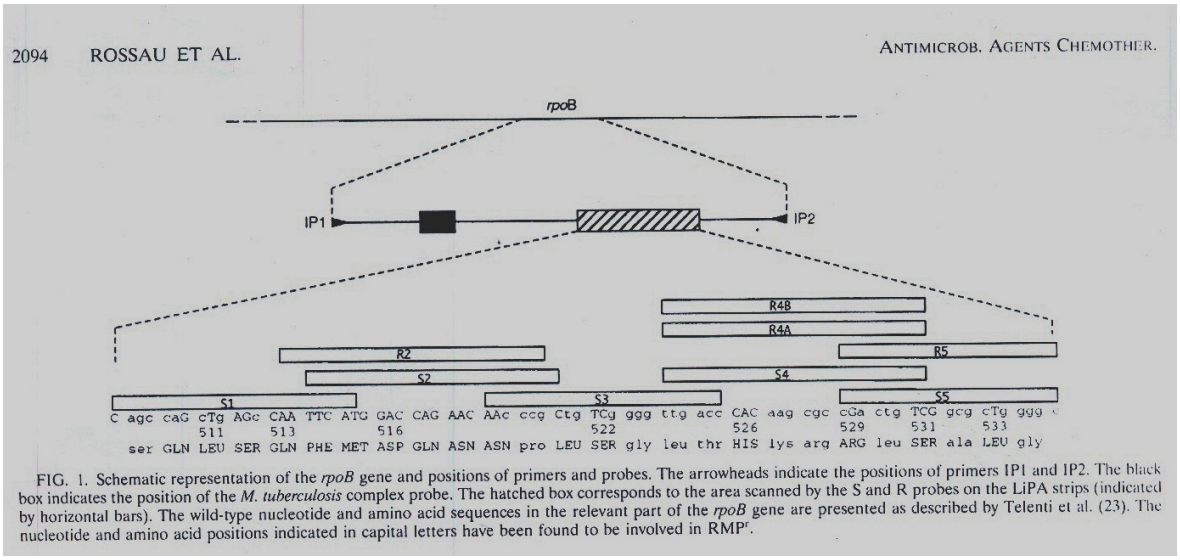


FIG. 1. Schematic representation of the *rpoB* gene and positions of primers and probes. The arrowheads indicate the positions of primers IP1 and IP2. The black box indicates the position of the *M. tuberculosis* complex probe. The hatched box corresponds to the area scanned by the S and R probes on the LiPA strips (indicated by horizontal bars). The wild-type nucleotide and amino acid sequences in the relevant part of the *rpoB* gene are presented as described by Telenti et al. (23). The nucleotide and amino acid positions indicated in capital letters have been found to be involved in RMP^r.

Fig 4. *The 81bp region of rpoB gene corresponding to codon region 511 to 533 covered by LiPA primers supplied in the amplification kit for MTBC.*

The INNO LiPA Rif.TB assay was applied directly to Lithuanian and Danish clinical specimens stored at -20^{o}C to detect RIF resistance and obtained results in 78.3% of clinical specimens which was explained that samples stored at -20^{0}C deep freezer might damage the DNA. LiPA's result was concordant with those obtained by BACTEC 460.Comparison of results obtained by LiPA and DNA sequencing on 36 MDR-TB isolates found exact type of mutation in 85% of isolates. In Denmark the most frequent mutation was Ser531Leu mutation, followed by His526Tyr. In Lithuania the Ser531Leu mutation was the most frequent, followed by the Asp516Val mutation (172).

INNO LiPA Rif.TB assay was also used in a study using Turkish *M. tuberculosis* complex strains, to compare with sequencing and Genotype MTB-DR assay in 2006 to detect mutations within the 81-bp hotspot region of *rpoB* gene (173).

In Tunisia, Standard proportion agar method, INNO-LiPA Rif.TB, and DNA sequencing was applied on 544 clinical MTBC strains isolates from a university hospital collected between 2004 and 2006 had frequently occurring *rpoB* mutations at codon 531 and lower at codon 526 and no mutations in codon 516 (174).The INNO-LiPA showed different patterns of mutations in codon 526 or 531. In all cases, there was a single nucleotide mutation in codon 531, with substitutions of serine to tryptophan (T*C*G to T*G*G), serine to leucine (T*C*G to T*T*G) and serine to alanine (TCG to *G*CG). Three *M. tuberculosis* strains showed triple point mutations in two different codons (codon 526, His *CA*C to Cys *TG*C, and codon 531, Ser *T*CG to Ala *G*CG), which was not reported previously(174).

Two DNA line probe assays, GenoType MTB-DR and INNO LiPA Rif.TB were compared for their abilities to detect resistance to INH and RIF using 80 MTBC isolates. The test results were compared to those obtained by conventional drug susceptibility testing (DST), DNA sequencing and/or PCR-restriction fragment length polymorphism (RFLP) analysis of regions of interest of MTBC genome. For RIF resistance, GT-MTBDR and INNO-LiPA test results were concordant with DST for 74 of 80 (92.5%) and 76 of 80 (95%) strains, respectively. The GT-MTBDR test results correlated with sequencing results for 77 of 80 (96.2%) while INNO-LiPA results for 79 of 80 (98.7%) isolates(131).

From Greece, INNO LiPA Rif.TB assay and DNA sequencing was used to characterize mutations in *rpoB* gene of 13 RIF-resistant and 6 susceptible *M. tuberculosis* isolates (175). No mutation was found in six RIF susceptible strains.

Specific mutations were found in 8 isolates, in addition, mutations in 4 RIF-resistant isolates in which was present specific base changes within the target region, wasn't determined by INNO LiPA Rif.TB suggesting mutations in external regions and insertions to be possibly detected only by automated DNA sequence analysis while one of the RIF resistant strain was identified as RIF-susceptible which was confirmed by DNA sequencing. The majority (8 of 13) of resistant isolates involved base changes at codon 531 of the gene. Concordance of line probe with phenotypic RIF susceptibility test was 94%. The changes in codons Ser531 and His526 accounted for majority of RIF resistance (131)

INNO LiPA Rif.TB assay studies done in India

Only few studies had been done from India, and the assay was done using DNA isolated from culture growths.

- In a comparison study of INNO LiPA Rif.TB assay with proportionate susceptibility testing method, 36 (thirty RIF resistant strains and 6 RIF sensitive strains) strains of *M. tuberculosis*, assay was found 100% specific. The most frequently observed mutation in *rpoB* gene was His-526-Tyr. Correlation between LiPA and proportionate susceptibility testing was 100%. Thirty RIF resistant strains by proportion method were tested using LiPA assay and detected two types of mutational pattern. In 28 of 30 samples (93.3%), one sample showed mutation at R4A (His-526-Tyr). In other 2 (6.66%), the precise mutation could not be localized as both did not hybridize with S3 probe and none gave a positive result with mutant (R) probes, which could be due to mutant probes not incorporated in LiPA strips for Indian

isolates. Sensitive strains by proportion method were correctly identified by LiPA (176).

- 55 *M. tuberculosis* isolates deposited from various regions of India (Ranchi, Manipal, New Delhi, Chandigarh, Agra, Bangalore, Ahmedabad, Cochin and Jammu & Kashmir) in Mycobacterial Repository Centre at CJILOMD, Agra were tested for RIF at various concentrations of drug: 10, 40, 64, 128 μg/ml on Lowenstein-Jensen medium. Their 260bp *rpoB* gene fragment were amplified and hybridized using INNO LiPA Rif.TB kit and mutations detected were correlated with the degree of RIF resistance. All sensitive isolates (identified by MIC) were RIF sensitive (100%) by LiPA. Two of five isolates, resistant at 10μg/ml and 40μg/ml had either D516V, H526Y mutations or unknown mutations. Thirty (85.71%) isolates resistant at clinically relevant levels (64, 128μg/ml) exhibited single, double, triple or more 'R' type mutations [R2(D516V), R4a(H526Y), R4b(H526D), R5(S531L)] as well as unknown mutations present at 'S' probes region. Remaining isolates did not show any mutation by this method. LiPA identified with definitiveness 60% (21/35) isolates as RIF resistant as mutations observed in others were also present in isolates with low levels of resistance, suggesting in 60% microbiologically resistant isolates, mutations are reliably correlated. Unknown mutations (lack of hybridization to some probes) are common with low or significant levels of RIF resistance. Some mutations present in isolates with low degree of resistance are still microbiologically sensitive to RIF, suggests need to improve LiPA by exclusion of some of current probes and inclusion of more probes. No mutations were found in ten sensitive isolates. Deletion of wild

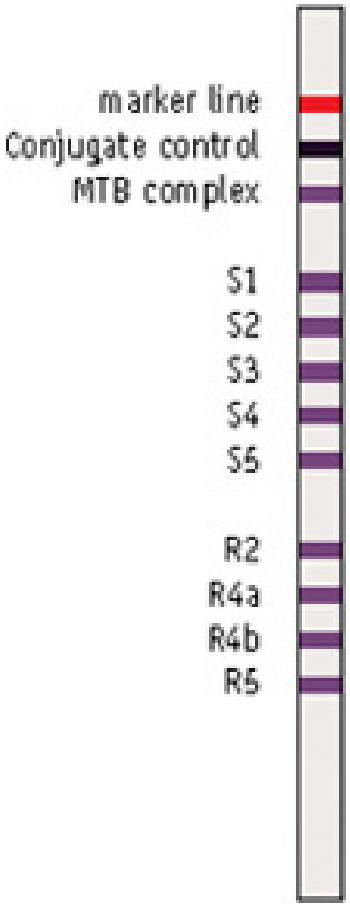
type S probes (S1,4,5) show unknown mutations in these regions. The combination of R2 and R4a mutation observed in isolates resistant at 10 and 40 µg/ml RIF (categorized as sensitive by microbiological criteria) as well as isolates resistant at 64 and 128 µg/ml levels showed that, these mutations cannot be used as definitive criteria of resistant for *M. tuberculosis* (177).

- To validate LiPA, comparison study between LiPA, DNA sequencing and proportionate method of drug susceptibility testing was done for 19 RIF resistant and 11 RIF susceptible isolates. DNA extracted from culture growth suspended in 400µl of 1X TE buffer was amplified for 256bp RRDR region with specific biotinylated primers. Amplified product of 81bp region was used for DNA sequencing. 18 RIF resistant isolates yielded 6 types of hybridization patterns with each isolate exhibiting no reaction with one of wild type S probe. One RIF resistant isolate yielded sensitive pattern by kit. GAC to GTC in codon 516 (D516V) accounted for resistance in one isolate. S5 probe mutation was found in four RIF resistant strains, and on sequencing exhibited nucleotide substitution at codon 531 (mutation S531W). Heteroresistant mutation was seen in three isolates with positive band for three R-probes and sequence mutation as D516V, H526Y, S531L. Double mutation was present in four isolates with absence of band at S3 and S5 probe region. Mutation CAG to GAC in codon 526 (H526D) accounted for resistance in two isolates. All susceptible strains yielded hybridization patterns with all five wild type S probes and DNA sequence yielded sequence same as wild type *M. tuberculosis*. Hybridization pattern obtained from R probes correlated well with sequence results. Specific mutation identified by LiPA were confirmed

by DNA sequence. In one RIF resistant strain by proportion method, both sequencing and LiPA showed no mutation. Nucleotide substitution at codon 526 (mutation H526D) in two isolates correlated with S4 mutation. LiPA and DNA sequencing identified 18 isolates as RIF resistant with specific mutation, while one RIF resistant strain was identified as RIF susceptible by both LiPA and DNA sequencing, with a concordance of 94.73% with phenotypic drug susceptibility result. 42.1% (8 of 19) majority of resistant isolates involved base changes at codon 531 of *rpoB* gene (mutation TCG to TTG in codon 531 (S531L) accounted for RIF in four isolates) (178).

Importance of INNO LiPA Rif.TB assay as an important diagnostic tool (Innogenetics, Belgium)

INNO LiPA Rif.TB is a commercial line probe assay designed to rapidly detect RIF resistance from fresh culture isolates mainly. LiPA testing is simple and rapid-tool to detect RIF resistant of *M. tuberculosis* in less than 48 hrs in clinical samples. This system is based on the reverse hybridization of the *rpoB* amplicons to specific probes immobilized on membrane-based strips. The assay can identify RIF resistant bacteria from mix bacterial population too. It allows for detection of MTBC too.



INNO-LiPA RifTB simultaneously detects the *Mycobacterium tuberculosis* complex and the presence of mutations in the rpoB gene associated with resistance to rifampicin (RMP), which is considered a marker for multidrug resistant strains.1

The strip contains 5 probes for detection of sensitive genotypes (S1-S5) and 4 probes for detection of resistant genotypes (R2, R4a, R4b, R5). RMP resistance is indicated by the absence of one or more "sensitive" probes, possibly accompanied by the appearance of one or more "mutant" probes.

Fig 5 (a)

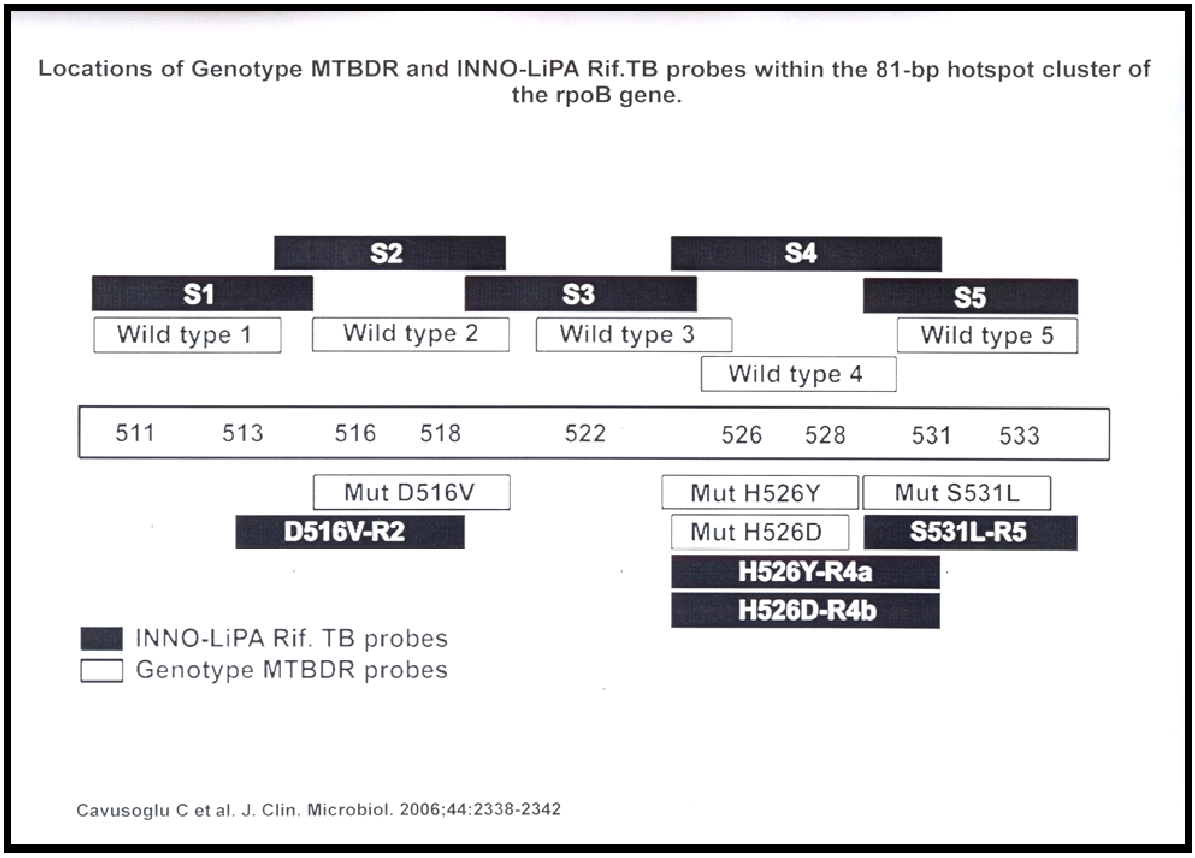


Fig 5 (b)

Fig 5 (a) and 5 (b): *Sensitive probes (S1, S2, S3, S4 and S5) and resistant probes (R2, R4a, R4b and R5) in INNO LiPA Rif.TB assay.*

Features & benefits of INNO LiPA Rif.TB assay

- Detects MTBC and sensitivity/resistance to RIF simultaneously on one strip.
- Detects mixed populations, allowing early detection of emerging resistant strains.
- Targets the *rpoB* gene where mutations associated with RIF resistance are located.

- Quick visual interpretation.
- Fast resistance profile obtained before standard susceptibility testing.
- Automation is possible using Auto-LiPA.

Molecular typing (Spoligotyping and MIRU-VNTR typing)

Advances in molecular technology have helped us in understanding genetic structure of *Mycobacterium tuberculosis* complex (MTBC) providing insights regarding the population dynamics and spread of MTBC locally as well as globally. The information obtained by molecular typing of MTBC isolates is important for understanding TB epidemics and for preventing the spread of TB (179) (180) . Current studies indicate that the outcome of TB infection may be related to strain diversity of MTBC (181, 182). For example, "Beijing strain" of MTBC has been reported to be more virulent in animal models (181) and is often reported to be responsible for causing outbreaks (183, 184). Moreover, knowledge on genetic diversity of MTBC is very useful for assessing impact of TB control programme (185). Although numerous studies on the genetic diversity of MTBC have been conducted in India (185-213) yet no such types of studies are available from north-eastern (NE) region of India.

To better understand the genetic diversity of multidrug resistant (MDR) and non-MDR MTBC circulating in NE region of India, characterization of 300 MTBC isolates from two states of NE region namely, Assam and Sikkim has been done using spoligotyping and 15-loci mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) typing. Spoligotyping is a PCR-based reverse-hybridization blotting technique based on polymorphisms in presence or absence of

"spacers" in the Direct Repeat locus of MTBC (214). Typing of MTBC using spoligotyping led to the creation of database "SpolDB4" in 2006 which gave first overview into the global diversity and phylogeography of MTBC spoligotypes (214). MIRU-VNTR typing has been shown to have high discriminatory power and can be used to further resolve strain lineages based on spoligotypes (215).

Treatment for tuberculosis

Antibiotic era for TB began when streptomycin was discovered in the year 1940 by Schatz and Waksman (12). This discovery was followed by the introduction of other anti-tubercular drugs such as: INH, RIF and pyrazinamide to treat TB patients (11). TB is currently treated by means of combination therapy consisting of three to four drugs having different properties given under DOTS therapy for four category groups with regime of two periods as 1) Intensive phase for two to three months and 2) Continuation phases for four to five months. The first line anti-tubercular drugs given under DOTS regimen are RIF, INH, Z, E and S. Second line anti-tubercular drugs are aminoglycosides, polypeptides, fluoroquinolones (Ofloxacin, Capreomycin, Kanamycin, Moxifloxacin, Leofloxacin), thioamides (Ethionamide), cycloserine, and PAS (para-amino-salicyclic acid). Second line drugs with bactericidal activity are aminoglycosides, capreomycin, and thioamides. Drugs with low bactericidal activity are fluoroquinolones. Drugs with bacteriostatic effect are cycloserine and PAS. BCG (Bacillus calmette Gueirin) vaccine was developed in France in 1908. A live attenuated vaccine strain made by sequential passage of a virulent *M. bovis* strain later on in 1920s from Paris, by Calmette and Guerin, called the BCG and was introduced in 1922. For more than 80 years, BCG vaccine has been in use (216). But the BCG is

not effective against the growing challenge of multidrug-resistant *M. tuberculosis* strains everywhere in the world, and the question is wether the scientist can become successful in inventing a more promising vaccine based on antigen of the organism can be made (216). In the present trial era, DNA vaccine (Mtb8.4/hIL-12 chimeric gene vaccine) seems to give higher efficacy by reducing colony forming unit (CFU) counts in organs of experimental mice (217).

TB control in India

To resolve the problem of TB in India, NTP was implemented in 1962. To further improve & strengthen TB control activities, the Government of India launched RNTCP in 1997 and covered almost whole country with excellent results by the end of 2005 with DOTS with meaning that patient swallows short course anti-TB drugs in presence of health worker or other trained individual.

Prevention of tuberculosis

BCG (Bacillus Calmette Gueirin) vaccination: is given as a specific prevention for tuberculosis. Some countries routinely vaccinate children with BCG, made by company, Merck & Co Inc. The vaccine doesn't give full 100% protection against TB but provide only partial immunity. The effectiveness of BCG in preventing TB is better in children and limited in adult. 80% of world's children, are vaccinated with BCG. The vaccine showed upto 80% protection in clinical trials performed in Europe, but more recent trials in India and Africa showed little value. In the USSR, all newborns are vaccinated, and all persons are revaccinated up to the age of 30.

Anti-tubercular-drug: INH in combination with para-amino-salicyclic acid, is administered daily, generally for a two to three month period twice a year, to children, adolescents, and adults living in close contact with TB patients, discharging mycobacteria in sputum. These drugs are also administered to other high-risk individuals, including those exhibiting a positive reaction to tuberculin test, a pronounced reaction to the mantoux test, or non-active tuberculous changes in the lungs.

Other factors: TB can also be prevented by combining efforts of state-support and community programs, like constructing apartment or buildings with public facilities, improved sanitation at work places, environmental protection, and raising economic and cultural level of the population. Resistance to TB can also be increased by including measures such as physical culture, hardening, hiking, sports, and proper hygienic conditions for children in nurseries, children's homes, and schools. To prevent infection within a family, family members with the disease should be instructed to maintain separate rooms for the TB patient with good ventilation and sunlight facing. TB can also be prevented by raising educational level and teaching patients to observe the rules of personal hygiene, hospitalizing infected persons, and removing infected persons from work in children's institutions and enterprises involving direct contact with food. Veterinary measures include disinfection of milk and other food products, and the isolation of slaughter diseased cattle. Above all timely detection of TB is most important preventive measure to avoid development of drug resistance.

CHAPTER 5

MATERIALS AND METHODS

Settings of the study

Sikkim, is a small hilly state in North Eastern part of India, with elevation from 600 feet to over 28,509 feet above sea level. Total area consist of 7096 sq.km with an altitude of 5840 feet nested in Himalayas. The boundary on three sides are Tibet (North-East), Bhutan (East), Nepal (West) and Darjeeling district of West Bengal (south). Total population is 607688 (according to 2011 Census). Population with the most populated district being Gangtok-East district (281 293) followed by Namchi-South district (146 742), Geyzing-West district (136 299) and least populated district being Mangan-North district (433 54).The main three ethnical group of Sikkim are Lepcha (19%), Bhutia (16%) and Nepali (56%). State implements health care and family welfare sector through a network of 147 PHSC, 24 PHC, 4 CHC and 300 bedded State Referral Hospital (STNM) at State Capital, Gangtok and 400 bedded Central Referral Hospital cum Medical College set up in collaboration with Manipal Pai Foundation Group under Private management (Health care, human services and family welfare). All four districts started service delivery under RNTCP on 1st March 2002. RNTCP was implemented in a phased manner since 2004, and 100% of state got covered in the same year. Infrastructure consists of total districts- 4, TUs- 5, DMCs- 20**.** There are now 24 primary health centers (PHC) and 147 sub-centers functioning throughout the State. These centers have medical and para-medical staff capable of rendering the basic Primary Health Care. Construction of 100 bedded Community Health Centres at Singtam, Namchi, Gyalsing and Mangan have been

completed and functioning. TB has been the major health hazard in the State. One 60 bedded district TB Centre (DTC) is functioning at Namchi with 10 bedded mini DTC in the other 3 Districts. TB Control Programme has been given top priority and the District TB Control Societies have implemented DOTS regimens, funded by the World Bank in all the four Districts of Sikkim.

Source, storage and identification of isolates

A total of 320 sputum positive Category I patients were enrolled in the study between 6th October 2007 and 28th December 2009 from four district DOTS centres in Sikkim. The samples were brought to Microbiology Department, Central Referral Hospital (SMIMS), Tadong, Gangtok. Samples were collected in a screw lid-universal containers, well labeled with patients identification number. Sputum samples were retested for presence of AFB by Ziehl Neelson's staining method. The sputum samples were graded according to the RNTCP criteria and two extra slides smears were made from each sample. The slides were given lab number and heat fixed followed by inactivation using ethanol. The smears were kept inside a slide box and stored at room temperature. The remaining sputum was mixed 1:2 ratio with 1% CPC solution and transported within ten days to RMRC (ICMR), Dibrugarh, Assam, following biohazard precautions for sample transportation. Samples were cultured on LJ medium and incubated for upto 8 weeks at 37^{0}C. Identification tests included were colony morphology, growth period of more than two weeks& biochemical tests (Nitrate test, Catalase test, Para-nitro-benzoic-acid test and Niacin test).

The study design

Case definition

All the patients of pulmonary tuberculosis from four districts of Sikkim who came to the DOTS centers and had at least two of their sputum samples positive for AFB at first report and also sputum positive after 2 months intensive phase of Category I regimen or later up to 5 months of DOTS during the study period, were considered as "Cases".

Medical history

Symptoms of pulmonary TB like productive, prolonged cough of more than two weeks, chest pain, and hemoptysis were obtained. Systemic symptoms included low grade remittent fever, chills, night sweats, appetite loss, weight loss, easy fatigability, and production of sputum. Other parts of medical history included prior TB exposure, infection or disease; past TB treatment; demographic risk factors for TB; and medical conditions that increase risk for TB disease such as HIV infection.

Microscopy

Mucopurulent portion of sputum was spread on two new and clean glass slides of an area 1x2 cm was air-dried, heat fixed and done Ziehl Neelson staining followed by oil immersion examination (Appendix-I). Gradings were given according to Revised National TB Control Programme guidelines. For Ziehl Neelson staining, positive control slide was prepared from culture growth of $H_{37}R_V$ (obtained from TB Research Centre, Chennai, India) and a negative control slide was prepared from culture

negative sample for *M. tuberculosis* organism after 8 weeks of incubation. For AFB positive sample, one additional sputum slide was prepared and inactivated using 5% phenol in ethanol for 5 minutes (218).

Digestion and decontamination procedures (CPC & NaCl [Sodium chloride])

Delay of >48-72 hrs was predicted, as the samples collected from remote areas of Sikkim was collected and transported to TB lab at RMRC, ICMR, Assam, India. Therefore sputum samples were collected in a container with 1% cetyl pyridinium chloride and 2% sodium chloride solution, a quaternary ammonium compound, used to decontaminate the specimen while sodium chloride effects liquefaction. This method not only decreases the number of cultures lost by contamination as a result of prolonged transit time but also decreases significantly the laboratory time required for processing the specimens.

Preparation of 1% CPC-sodium chloride solution

Preparation of the solution was done as per RNTCP protocol (219) and the stock solution was stored in dark coloured bottles at room temperature.

Procedure for culture of CPC containing sputum specimen

1. To the specimen with CPC we added 15-20 ml of sterile distilled water (to reduce viscosity).
2. Tightened cap of the container and mixed well by inversion.
3. Centrifuged at 3000xg for 15 minutes.
4. Supernatant carefully poured off.

5. Added approximately 20ml of sterile distilled water to resuspend the sediment and fill up with sterile distilled water to the brim.
6. Centrifuged again at 3000 x g for 15 minutes.
7. Supernatant decanted and deposit inoculated on two slopes of LJ medium with one 5mm loop.

Incubation of cultures

It was done at 35-37^{o}C until growth was observed or discarded as negative after eight weeks. Slopes that were grossly contaminated were discarded.

Culture examination and identification

Cultures were examined 48-72 hrs after inoculation to detect gross contaminants. Thereafter cultures were examined weekly, up to 8 weeks on a specified day of week. Contaminated cultures found during examination were discarded. Colonies of *M. tuberculosis* are rough, crumbly, waxy, non-pigented (buff coloured) and slow-growers appearing two to three weeks after inoculation. A very small amount of growth was removed from culture using a loop and gently rubbed into one drop of sterile saline on a slide. The ease with which organisms emulsified in liquid was noted (tubercle bacilli doesn't form smooth suspensions, unlike some other mycobacteria). The smear was allowed to dry, fixed by heat and stained by Ziehl-Neelsen staining. When no AFB was seen in smear it was reported as contamination.

Table 18: Reading of culture on LJ medium (Standard method).

Colony count	**Culture grading**
No growth	Negative
1-100 colonies	Positive (actual number of colonies)
>100 discrete colonies	Positive (2+)
Confluent growth	Positive (3+)
Contaminated	Contaminated
< 20 colonies of only NTM colonies in one or both slopes	No growth
> 20 colonies of only NTM colonies in both slopes	Negative for *M. tuberculosis*
NTM= Non tubercular mycobacteria	

Preparation of Lowenstein-Jensen medium for culture of MTBC

LJ medium was prepared as per routine given in RNTCP culture and drug susceptibility manual (219).

Sterility check of the LJ medium:

After inspissation, the whole media batch was incubated at 35^{o}C-37^{o}C for 24 hrs as a check of sterility. After 24 hrs 5% of slopes were picked up randomly and continued incubation. In both the cases, contamination rate was not allowed to be >10%.

Storage of LJ medium:

LJ medium were dated and stored with the batch number in refrigerator and kept for upto 4 weeks with the caps tightly closed to prevent drying of medium.

Preparation of LJ medium with sodium pyruvate:

For cultivation of *M. bovis*, LJ was enriched with 0.5% sodium pyruvate as per RNTCP protocol (219).

Identification tests for *Mycobacterium tuberculosis*:

Following tests, used in combination enabled precise identification of *M. tuberculosis* strains (Appendix-II).

1. **Colony characteristics and growth rate on Lowenstein Jensen media**

Non pigmented ruff tuff buff colored colony growth appearing after two weeks onwards upto eight weeks at 37^0C incubation.

2. **Nitrate test**

Substrate for the test was prepared as per RNTCP manual for culture and DST (220) and sterilized by autoclaving. 2ml substrate was dispensed in test-tube where two loop full of four week old culture from LJ medium was emulsified into test tube containing substrate. The tube was incubated upright for two hours in a 37^0C water bath. The tube was removed from water bath and added one drop of reagent 1 (50ml of Conc. HCl to 50ml of distilled water), two drops of reagent 2 (0.2g of sulfanilamide in 100 ml of distilled water), two drops of reagent 3 (0.1g of N-naphthylethylenediamine

dihydrochloride in 100ml of distilled water). Examined immediately for a pink to red color formation in test tube which is positive for nitrate reduction test (Appendix-III). If no color develops, the test is either negative or the reduction had proceeded beyond nitrite. In such condition, a small amount of powdered zinc was added to negative test. If nitrate was still present, it would be catalytically reduced by the zinc and a red color would develop, indicating a true negative. If no color develops when zinc dust was added, the original reaction was positive, but the nitrate was reduced beyond nitrite. In this case test was repeated to confirm the observation. Positive control included as culture growth from *M. tuberculosis* and negative control included reagents without organisms.

3. Susceptibility to p-nitrobenzoic acid (PNB)

This test was included along with drug susceptibility tests. Preparation and storage of PNB media was done as given in RNTCP manual for culture and drug susceptibility testing (219). The neat bacterial suspension prepared for drug sensitivity testing was inoculated on one slope of LJ medium and one slope of PNB at a concentration of 500µg/ml and incubated at 37^0C for each set. Reading was done on 28^{th} day of incubation (Appendix-IV). *M. tuberculosis* does not grow on PNB medium and all other mycobacteria grows.

4. Niacin test

M. tuberculosis and some isolates of *M. simiae* and *M. chelonae* produce niacin during growth. These strains do not metabolize niacin further and therefore accumulates niacin which is excreted into the agar or slant. *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its

definitive diagnosis. Niacin negative *M. tuberculosis* strains are very rare, while very few other Mycobacterial species yield positive niacin tests. Cultures grown on egg medium containing asparagine yield the most consistent results in the niacin test and LJ medium is therefore used. Culture growth must be at least three to four weeks old and must have sufficient growth of at least 100 colonies. Controls: reagents were checked by testing extract from an uninoculated tube of medium (negative control) and extract from a culture of *M. tuberculosis* $H_{37}R_v$ (positive control).

Niacin Reagents: was supplied from Himedia [K047 Niacin detection kit (modified) with syringe]

Niacin kit contents:

- Part A: Reagent (1ml) (1.5% o-tolidine)
- Part B: Reagent (1ml) (10% cyanogens bromide)
- R055: Reagent P (4ml) (niacin extract from a culture of *M. tuberculosis* $H_{37}R_V$)
- Sterile syringe (1ml capacity)

Test sample preparation for niacin test:

- Only more than three weeks old cultures grown on LJ medium slant showing heavy growth was chosen for the test.
- Note: False negative result was avoided by not using cultures with few colonies and no mixed culture with mycobacteria other than *M. tuberculosis* was used.
- 2ml sterile distilled water was added to the slant.
- The slant was stabbed with a needle.

- The slant was kept horizontally for 20 minutes at room temperature.
- The slant was retained upright position for 5 minutes.
- 1ml of the solution was used as a test sample.

Niacin test procedure:

1ml of part A was transferred to 1ml part B. The mixture solution was used as a reagent for further test. 1ml of the test sample was added to the above mixture solution, using a syringe. Development of yellow colour within five minutes was taken as positive reaction (Appendix-V). No development of yellow colour within five minutes was regarded as the negative reaction. Reagent solution should remain colorless.

Positive control included in niacin test:

1ml of part A was added to 1ml of part B. The mixture was used as reagent solution for further test. 1ml of R055 reagent P was transferred to the reagent solution mixture (part A + part B) using syringe. Development of yellow colour within five minutes was considered positive test.

Negative control in niacin test:

1ml of part A was added to 1ml of part B. The mixture was used as reagent solution for further test. 1ml of sterile water was added into reagent solution mixture (part A + part B) using syringe. If solution remained colorless after five minutes, test was considered negative for niacin formation.

Precautions taken while performing niacin test:

The reagents being photosensitive was not exposed to light. After the test, the tubes were neutralized by adding 10% Sodium-hydroxide to each tube before discarding because in acid solution, cyanogen bromide hydrolyses to hydrocyanic acid, which is extremely toxic and disinfectant solution made alkaline by addition of sodium hydroxide neutralizes it.

5. 68^{o}C heat stable catalase test

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen. Oxygen bubbles into the reaction mixture indicate catalase activity. All mycobacteria possess catalase enzymes, except for certain isoniazid resistant mutants of *M. tuberculosis* and *M. bovis*. Mycobacteria possess several kinds of catalase that vary in heat stability. Quantitative differences in catalase activity are demonstrated by 68^{o}C test at pH7, which indicates loss of catalase activity due to heat. Drug susceptible strains of *M. tuberculosis* lose catalase activity when heated to 68^{o}C for 20 minutes. Cultures on LJ were used for the test.

Controls used in catalase test:

Extract from an un-inoculated tube of medium (negative control). Extract from atypical mycobacteria culture medium (Positive control).

Reagents for heat stable catalase test were as given below:

Reagent preparation was done as per RNTCP protocol (219).

- 0.067M Na_2HPO_4 phosphate buffer solution, pH 7.0 (solution 1)
- 0.067 M KH_2PO_4 buffer solution (Solution 2)

  61.1 ml of solution 1 was added to 38.9 ml of solution 2. pH was checked.
- 10% Tween-80
- Complete catalase reagent (Tween-peroxide mixture):

  Immediately before use, equal parts of 10% Tween-80 and 30% hydrogen peroxide was mixed. 0.5ml reagent was used for each strain to be tested.

Procedure for heat stable catalase test:

With a sterile pipette, 0.5ml of 0.067 M buffer was aseptically added to 16 x 125 mm screw capped test tubes. A loopfull of culture was suspended in buffer solution, using sterile loop. 0.5ml of freshly prepared Tween-Peroxide mixture was added to each tube. Tube containing emulsified culture was placed in a previously heated water bath at 68^0C for 20 minutes (Time & temperature are critical). Tubes were removed from heat and cooled to room temperature. Formation of bubbles appearing on surface of liquid indicated positive test (Appendix-VI). Precaution was taken not to shake the tube because Tween-80 forms bubbles when shaken, resulting in false positive test. Negative tube was held for 20 minutes before discarding.

Preparation of drug containing media for susceptibility testing by proportionate method

Concentration of drugs used were as follows:

- Isoniazid 0.2µg/ml
- Ethambutol 2µg/ml
- Dihydrostreptomycin sulfate 4µg/ml
- RIF 40µg/ml

Preparation of drug stock solution was done along with drug free LJ media and PNB media, as a set for each wild strain being tested. One set consisted of four LJ slopes, one for 10^{-1} (neat), next three for other three dilutions 10^{-2}, 10^{-3} and $10^{-4.}$ Four drug containing slopes for individual drugs H (Isoniazid), R (RIF), Z (Pyrazinamide), E (Ethambutol) & S (Streptomycin) (one each for 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} suspensions) and one PNB slope, total 14 LJ slopes. Four acid control slopes for Z control was prepared using LJ media made pH 4.8 by adding 2N HCl. Each LJ slope required approximately 5-6 ml of LJ fluid. One positive culture showing more than 1+ growth in LJ slope was selected for DST.

Drug-containing LJ slopes are made by adding appropriate amounts of drugs aseptically to LJ fluid before inspissation. Drugs (Appendix-VII) solutions were prepared based on the potency of drug in sterile distilled water for S,H,Z,E and for RIF, absolute methanol was instead of sterile distilled water. The solutions of INH and E were sterilized by filtering through 0.22µm membrane filter. These drugs solutions were prepared fresh on day of drug media preparation. Drugs solution

prepared were added to the LJ fluid, dispensed in 5 ml amounts and inspissated once at 85^{o}C for 50 minutes. The medium prepared were stored in the cold for 3-4 weeks.

Preparation of Macfarland nephelometer barium sulfate standards (Paik 1980)

1. 1% aqueous barium chloride and 1% aqueous sulfuric acid solutions were prepared. [100 mg of barium chloride (anhydrous) in 10ml of SDW and 0.1 ml of sulfuric acid (AR) in 10ml of SDW].
2. 0.05ml of 1% barium chloride was added to 9.95ml of 1% sulphuric acid to obtain Mac Farland standard, which matches with 1 mg/ml of *M. tuberculosis*.
3. The tube was sealed and labeled as 0.05 Mac-Farland tube with date of preparation (Appendix-VIII).

Table 19: First line anti-tubercular drugs used for proportionate method of sensitivity testing.

First line anti-tubercular drugs used for proportionate method of sensitivity testing (Sigma Aldrich)	**Quantity of drugs dissolved in appropriate solvent**
RIF, R-3501, 5g	100 mg drug was dissolved in 25ml methanol
Isonicotinic acid hydrazide, I-3377, 5g	2 mg drug was dissolved in 100ml of sterile DW
Dihydro streptomycin sesquisulphate, D-7253, 5g	10 mg drug was dissolved in 25ml of sterile DW
Ethambutol dihydrochloride, E-4630, 25g pack	5 mg drug was dissolved in 25ml of sterile DW
Pyrazinamide	250 mg drug was dissolved in 25ml of sterile DW
DW= distilled water	

Drug susceptibility tests by proportion method

This method of sensitivity testing enables precise estimation of proportion of mutants resistant to a given drug. Several 10-fold dilutions of inoculum are planted on to both control and drug containing media. One dilution should yield isolated countable (50-100) colonies. When these numbers are corrected by multiplying by dilution of inoculum used, the total number of viable colonies observed on control medium, and number of mutant colonies resistant to drug concentrations tested is determined. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of total population tested. The proportion method is currently the method of choice and the simple version of bacterial suspension and interpretation of results are given below. The economic variant of proportion method used for DRS is given subsequently.

The standard strain *M. tuberculosis*, $H_{37}R_v$ (Appendix-IX) was used as control strain with each new batch of sensitivity testing.

1. Arrange the bottles contained with six glass beads were arranged according to bottles for dilution and labeled with the isolates number along the corresponding dilution.

2. 1 ml of sterile distilled water (SDW) was added in bottle containing beads (S1), added 1.8ml of SDW in to bottles for dilution (S2, S3 and S4).

3. 2/3 loopfull colony of 3mm internal diameter 24 SWG wireloop, was mixed into 0.2 ml of sterile distilled water in a 7 ml bijou bottle containing six 3mm glass beads, marked as Neat/S1. This was vortexed for 30 s to produce a uniform bacterial suspension. To this was added 3.8 ml of sterile distilled and mixed well. This suspension was kept on bench to let coarse particles settle down for 15 minutes.
4. While waiting for 15 minutes, sample number was written on to the LJ slopes, including the drug containing slope.
5. The supernatant from S1 dilution was transferred to a fresh bottle and its bacterial suspension turbidity was compared with Mcfarland tube no. 0.05 and if required, adjusted the turbidity by adding SDW drop by drop.
6. 0.2ml of S1 dilution was transferred into S2 bottle containing 1.8ml of SDW and mixed well.
7. 0.2ml of S2 dilution was transferred into S3 bottle containing 1.8ml of SDW and again mixed well.
8. Same way, again 0.2ml of S3 dilution was transferred into S4 bottle containing 1.8ml of SDW and again mixed.
9. The caps of LJ slopes was loosened, just one thread, and with the help of 3mm internal diameter 27 SWG wire loop, delivering 0.01 ml, one standard loopfull was inoculated uniformly on to drug-free as well as drug-containing LJ slopes, starting from S4, S3, S2 and S1 (Neat) (table 20).

Table 20: Inoculation of bacterial suspension on control drug-free LJ medium and drug containing LJ medium by proportionate method of susceptibility testing.

Suspension	Control drug-free	S 4µg/ml	H 0.2µg/ml	R 40µg/ml	Z 100µg/ml	AC	E 2µg/ml	PNB 500µg/ml
S1 (neat)	XX	X	X	X	X	X	X	X
S2 (10^{-1})	XX	X	X	X	X	X	X	
S3 (10^{-2})	XX	X	X	X	X	X	X	
S4 (10^{-3})	XX	X	X	X	X	X	X	

S= Streptomycin, H= Isoniazid, R= RIF, E= Ethambutol, AC= Acid control for Pyrazinamide, PNB= para-nitro-benzoic-acid, S1= neat,

S2,S3,S4= serial bacterial suspension dilution

Incubation and reading of sensitivity test media:

Incubation of media were done at 37^{o}C. The results were red for the first time on 28th day of incubation. Colonies were counted on slopes seeded with inoculum producing exact readable counts (up to 100 colonies on slope). The average number of colonies obtained on drug-containing slopes indicated number of resistant bacilli contained in

inoculum. Dividing the number of colonies in drug containing slopes by number of colonies in drug free slopes was taken as the proportion of resistant bacilli existing in the strain. Below critical proportion, the strain was classified as sensitive and above critical proportion value, it was classified as resistant (Appendix-X). The proportions were reported as percentages. If, the result of the reading made on 28th day was "resistant", no further reading of the test for that drug was required and the strain was classified as resistant. If the strain was resistant for all four drugs on 28th day, then sensitivity reading was taken on the same day. If result at 28th day was "sensitive", a second reading was made on 42nd day for that sensitive strain (Appendix-XI). The final definitive results for all four drugs were reported on 42nd day.

Recording of colony count was done as given below

+ + +	confluent growth
+ +	>100 colonies
1-99 colonies	actual number of colonies

When the number of colonies on a given dilution was <15, number of colonies with the next larger inoculum was counted, or estimated if >100. (No attempt was made to estimate number of colonies if the growth was + + +).

Interpretation of sensitivity test by proportionate method: was based on 42nd day reading. For each strain, the number of organisms resistant to each drug concentration was expressed as a percentage of number of organisms growing on drug-free slope.

The selection of slopes for estimating the growth on drug-free and drug containing media was done as given below in following order of preference.

Drug-free slope colonies:

- 5–19 colonies
- >70 colonies

Drug-containing slopes:

- 5-100 colonies in the same row or the row nearest to the control slope
- 1-4 colonies in the same row or the row nearest to the control slope
- No colonies in the farthest row
- >100 colonies, if there are no other acceptable counts

Criteria of resistance in proportionate method

Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs- RIF, INH, E and S is classified as resistant to that drug. For calculating the proportion of resistant bacilli, the highest count obtained on the drug-free and on the drug-containing medium should be taken (regardless of whether both counts are obtained on the 28^{th} day, both on the 42^{nd} day, or one on the 28^{th} day and the other on the 42^{nd} day).

Precautions taken while performing drug sensitivity test by proportionate method were

- While picking the colonies with a loop, it was taken care not to touch the media.
- The loop was sufficiently cooled down before picking the colonies.
- Loopfull of colonies was taken in one sweep, by touching all colonies on the LJ slope.
- While scrapping the colonies, touching the water of condensation was avoided.
- Inoculation was done with uniform suspension all over the slopes.

Flow chart for proportion method of sensitivity testing

1ml sterile distilled water with six 3mm glass beads

+

1 loopfull (3 mm internal diameter) of culture

↓

vortexed for 20–30 s

↓

Added 4ml of sterile distilled water to the above

↓

Adjusted turbidity with McFarland 0.05 with sterile distilled water

↓

S1 (Neat)

(Contain 4ml sterile distilled water with bacterial suspension adjusted to 0.05 Mc Farland standard)

↓

S2 -10^{-1} dilution (contain 0.2ml suspension from neat and 1.8ml sterile distilled water)

↓

S3 -10^{-2} dilution (contain 0.2ml suspension from S2 dilution and 1.8ml sterile distilled water)

↓

S4 -10^{-3} dilution (contain 0.2ml suspension from S3 dilution and 1.8ml sterile distilled water)

Points remembered while taking the sensitivity reading by proportionate method

1. Reading of the sensitivity test was proceeded, if there was no growth in the PNB, if growth was noticed in PNB, the isolate was ignored for reading and subjected the culture for identification tests with niacin and rate of growth.
2. The test was repeated,
    - If there was <100 colonies growth in neat suspension.
    - If growth in S4 control slope (plain LJ) was confluent and counting was not possible to be done.
    - If no countable colonies were obtained in any of the control slopes.
    - If all the slopes were grossly contaminated.
3. During counting fused colonies should were counted as a single colony.
4. Peaks of the fused colonies were not counted as individual colonies.
5. The confluent bottom growth was counted as a single colony.
6. If growth along with other contamination was noticed, the colony counts was recorded with star mark (*). In such cases sensitive results could not be accepted and the test was repeated.

Verification of proportionate method results was done using the following tests:

1. Proficiency testing conducted by National Institute for Research in Tuberculosis (NIRT) [TRC, Chennai].

Proficiency testing, also called as External Quality Assessment by WHO standards refers to a system of retrospectively and objectively comparing results from different laboratories by means of programs organized by external agency, such as a reference laboratory in agreement with a reference standard. Material for testing was prepared by National Institute for Research in Tuberculosis (NIRT) [TRC, Chennai] and was distributed to RMRC, ICMR, Dibrugarh TB lab (lower level lab).As the participant lab in accreditation programme, we took part in culture and sensitivity test conducted by TRC and presented results to reference laboratory to assess proficiency. This test was a part of quality assurance programme. Also included for this test was my 15 research samples from this study (10 MDR-TB samples and 5 non MDR-TB samples). The result of this research samples was verified by the TRC, Chennai lab and now RMRC (ICMR), Dibrugarh lab has been accredited for culture & DST of MTB.

2. Bactec 460 drug susceptibility testing done for verification of proportionate method sensitivity result of the study samples for RIF and INH drugs at Auroprobe lab, Delhi, India

24 samples (23 MDR-TB samples and 1 Non MDR-TB samples were sent for doing BACTEC 460 drug susceptibility testing for first line anti-tubercular drugs, out of

which 7 samples could not grow for carrying out the BACTEC 460 test, rest 17 samples (all MDR by proportionate method) were found as MDR-TB by the BACTEC 460 drug susceptibility testing (Table 21).

Table 21: Comparison of sensitivity results between proportionate method and Bactec 460 method for rifampicin and isoniazid.

S. NO	Sample ID of RMRC (ICMR), lab	Proportionate sensitivity result [from RMRC (ICMR), lab]	Sample ID of Auroprobe lab, Delhi	Bactec 460 sensitivity results [Auroprobe lab, Delhi]
1	1390	MDR	11180	MDR
2	1251	MDR	11181	NG
3	1015	MDR	11182	MDR
4	1488	MDR	11183	MDR
5	1539	MDR	11184	MDR
6	1717	MDR	11185	NG
7	1112	MDR	11186	NG
8	1009	MDR	11187	MDR
9	1591	MDR	11188	MDR
10	1047	MDR	11189	MDR
11	1587	MDR	11190	MDR
12	1127	MDR	11191	MDR

13	1753	MDR	11192	NG
14	1469	Non-MDR	11193	NG
15	1624	MDR	11194	MDR
16	1205	MDR	11195	MDR
17	1527	MDR	11196	MDR
18	1644	MDR	11197	NG
19	1465	MDR	11199	MDR
20	1340	MDR	11200	MDR
21	1789	MDR	11201	NG
22	1066	MDR	11202	MDR
23	1585	MDR	11203	MDR
24	1598	MDR	11204	MDR
MDR= multi-drug-resistant, NG=No growth				

3. HAIN's MTB-DR plus assay done for sensitivity verification for study samples in RMRC (ICMR), Dibrugarh, Assam.

12 samples of this research study (4 non MDR-TB samples, 8 MDR-TB samples) were tested by HAIN's MTB-DR assay plus assay and the results matched with our study samples.

Genotypic methods:

A) DNA extraction from positive sputum slides and its use in INNO LiPA Rif.TB assay

Materials required:

- Autoclave.
- Centrifuge machine (Siga 2-16k).
- Vortex (vx100, labnet).
- pH meter 3310 Jenway.
- Balance (cp225d, sartorius).
- Butter paper/ weighing plate.
- UPS (Uninterrupted power supply) supply.
- Speed-vac (Eppendorf concentrator 5301).
- 4^0C refrigerator.
- Dry heating bath for 37°C and 55°C [(Accu block^{tm} digital dry bath, Labnet international, Inc) and (Genei dry bath)].
- Deep-freezer -80^0C (New brunswick scientific, ultra low temperature freezer, U570 premium).
- Ice maker machine and ice bucket.
- Hot air oven (Thermo scientific, Heraeus oven).
- 37^0C incubator (311ds Labnet, shaker control system).
- -40^0C defrigerator (Heto).
- Pipette (Axypet autoclavable).
- Pipette tips (aerosol barrier recommended).
- Tissue paper.
- Parafilm (Himedia).
- PCR tubes.

- apparatus (Axygen submerged gel electrophoresis).
- Gene Amp^{R} PCR system 9700, AB applied biosystems.
- Mini spin plus (Eppendorf).
- Tarsons micro-centrifuge tube 1.5 ml and 2ml for DNA preparation.
- Ethanol (95%–100%), Catalog nos. E7023, E7148, or 459836.

Chemicals required for DNA extraction directly from heat fixed sputum smear:

- NA2120 1 kit lot 42k9291 Siga, Genelute bacterial genomic DNA kit, mini sufficient for 350 preps for molecular biology, stored at room temperature.
- Amresco lysozyme, egg white, code: 0663-10g, lot: 2609b156, ultrapure grade, stored at 20^{0}C.
- Amresco, Proteinase K, biotechnology grade, code: 0706-100mg, lot # 0989b226, stored at 20^{0}C.

Table 22: Materials provided in Genelute™ bacterial genomic DNA kit.

Reagents provided	**Cat.No.**	**NA2120 350preps**
Gram-positive lysis solution	L7539	90 ml
Lysis solution C	B8803	90 ml
Wash solution 1	W0263	225 ml

Wash solution concentrate	B6553	90 ml
Elution solution (10 mm TRIS-HCl, 0.5 mM EDTA, pH 9)	B6803	180 ml
Column preparation solution	C2112	225 ml
Proteinase K	P2308	2 × 100 mg
Gen-elute nucleic acid binding columns in tube	C9471	5 x 70 each
Collection tubes, 2 ml capacity	T5449 or T7813	15 x 70 each

Materials required for DNA concentration by ethanol precipitation method:

- Ethanol, absolute for analysis (Merck, Index No. 603-002-00-5).
- Sodium acetate 3M.
- TRIS pH8.0
- 0.5M EDTA (Ethylenediamine tetra acetic acid disodium di-sodium salt dehydrate 99%, SIGMA-ALDRICH, E 5134-500G, Batch 056K0078).
- Speed Vac (Eppendorf concentrator 5301).
- Pipette (Axypet autoclavable).

Materials required for INNO LiPA Rif.TB assay:

- INNO LiPA Rif.TB amplification kit.
- INNO LiPA Rif.TB hybridization kit.

- Water bath with shaking platform (80 rpm; with inclined lid; temperature adjustable to minimum 62°C ± 0.5°C).
- Aspiration apparatus.
- Adjustable pipettes to deliver 1-20µl, 20-200µl, and 200-1000µl.
- Dispensing multipipette (Eppendorf).
- Timer, 2 hours (± 1 min).
- Vortex mixer or equivalent.
- Calibrated thermometer.
- Distilled or deionized water.
- Disposable gloves.
- Disposable sterile pipette tips (preferably cotton-plugged).
- Tweezers for strip handling.
- Graduated cylinders (10, 25, 50, and 100 ml).
- Rocking platform shaker (the shaking angle should not exceed 13° to avoid spilling of liquid and there commended speed is 50 rpm).

I. Strain selection: 29 MTBC isolates producing positive growth on LJ medium was selected randomly for use in INNO LiPA Rif.TB assay.

II. DNA extraction from sputum positive slides:

Preparation instructions:

1. Two heating block was preheated to respective 55°C and 37°C for use with gram-positive bacteria only.

2. The reagents were thoroughly mixed and examined if precipitation any. If any reagent had precipitate, reagent was warmed at 55–65°C until precipitate dissolved and cooled to room temperature before use.

3. Washed solution concentrate was diluted with 360 ml (350 prep package) of 95–100% ethanol. After each use, diluted wash solution was tightly capped to prevent evaporation of ethanol.

4. Proteinase K was reconstituted by dissolving 100mg of proteinase K in 5ml of sterile distilled water to obtain a 20 mg/ml stock solution. For long-term storage, the solution was aliquoted and stored at –20°C until needed.

   Note: Proteinase K solution was added directly to each sample every time.

5. Lysozyme solution was prepared by dissolving 2.115 x 10^6 unit/ml stock solution of lysozyme (L7651) (approximately 45mg/ml) was prepared usinggram-positive lysis solution (L7539) as the diluents (To make 1ml oflysozyme solution, 2.115 x 10^6 units of lysozyme (45mg) in 1ml of gram-positive lysis solution). The mixture was pipetted up and down instead of vortexing to prevent foaming). For each DNA preparation, 200 µl of lysozyme solution was required.The lysozyme solution was prepared on the day of use.

Procedure for DNA extraction directly from sputum positive slides:

All work was carried inside a biosafety level-II cabinet (Appendix-XII).

1. Harvest of cells and digestion of cell wall: Sputum slide was gently scrapped after wetting the smear with 200µl lysozyme solution for 1-2 minutes, with a

sterile blade. The scrapped material was transferred into a microcentrifuge (Eppendorf) and incubated at 37°C for 30 minutes.

2. Lysis of cells: 20µl of proteinase K (20mg/ml) was added to the sample, followed by 200µl of lysis solution C. Vortexing was done thoroughly about 15 s and incubated at 55°C for 10 minutes.

3. Column preparation: 500µl of column preparation solution was added to each pre-assembled Gen-Elute Miniprep binding column (with a red o-ring), seated in a 2ml collection tube. Centrifugation was done at 12,000 x g for 1 min. Flow-through was discarded.

4. Binding of DNA to column: 200µl of ethanol (95-100%) was added to lysate and mixed thoroughly by vortexing for 5-10 s to get a homogeneous mixture.

5. Loading of lysate: The entire contents of the tube was transferred into binding column by using wide bore pipette tip to reduce shearing of DNA, while transferring the contents into the column. Centrifuged at 6500 x g for 1 min. Collection tube containing the elute was discarded and the column was placed in a new 2ml collection tube.

6. First wash: 500µl of wash solution 1 (W0263) was added to the column and centrifuged for 1 min at 6500 x g for 1 minute. The collection tube containing the elute was discarded and placed the column in a new 2ml collection tube.

7. Second wash**:** 500µl of wash solution was added to the column and centrifuged for 3 minutes at maximum speed (12,000-16000 x g) to dry the column. It was made sure that the column was free of ethanol before eluting the DNA. The column was centrifuged for an additional 1 min at the maximum speed if residual ethanol was seen (for this emptying and reusing the collection tube was done if needed this additional centrifugation step). The

collection tube containing the elute was discarded and placed the column in a new 2ml collection tube.

8. Elution of DNA: 50µl of elution solution (B6803) was added directly onto center of the column and incubated for 5 minutes at room temperature to increase elution efficiency. Then centrifuged at 6500 x g to elute DNA.

III. DNA concentration by ethanol precipitation method

2.5-3 volumes of an ethanol/acetate solution was added to DNA sample in a microcentrifuge tube, placed in an ice-water bath for 10 minutes. Then precipitation was done by incubating DNA at -20^{0}C overnight. To recover precipitated DNA, the tube was centrifuged, the supernatant discarded, and DNA pellet was rinsed with a more dilute ethanol solution. After a second centrifugation, supernatant was again discarded, and DNA pellet was dried for ten s in a speed-vac.

1. 50µl aqueous DNA sample was taken in a 1.5ml microcentrifuge tube and added 142µl of 95% ethanol and 8µl of 3M sodium acetate. Microcentrifuge tube was inverted to mix, and incubated in an ice-water bath for at least 10 minutes. The sample was further incubated overnight at -20^{0}C at this stage.

2. The sample was then centrifuged at 12,000 rpm for 15 minutes at 4^{0}C. Supernatant obtained was carefully decanted and microcentrifuge tube was drained inverted on a paper towel.

3. 100µl of 80% ethanol (corresponding to about two volume of original sample) was added to microcentrifuge tube and incubated at room temperature for 5-10

minutes followed by centrifugation again for 5 minutes. The supernatant was decanted and drained the tube, as above.

4. DNA pellet was dried in a savant speed-vac by keeping for about 5-10 minutes.

5. 25µl of 10mM TRIS-HCl, pH 7.6-8.0, 0.1 mM EDTA (termed 10:0.1 TE buffer) was added to dried DNA.

6. Purified DNA was stored at -80^{0}C until further processing.

IV. INNO LiPA Rif.TB assay for detection of *rpoB* mutation directly from positive sputum slides.

Steps involved in the assay were:

a) Amplification of rifampicin resistance region of *rpoB* gene.

b) Hybridization of biotinylated amplicon to the strip, followed by stringent wash.

c) Addition of conjugate and substrate, resulting in color development.

d) Visual interpretation of signal pattern.

Note: The kit was stored at 2-8^{0}C, isolated from any source of contaminating DNA, especially amplified DNA products. All reagents and plastic tubes containing test strips were brought to room temperature (20-25°C) approximately 30 minutes before use and was returned to 4^{0}C immediately after use.

Table 23: Reagents supplied in INNO LiPA Rif.TB kit.

Strips	1 x 20	58306	Contains twenty INNO LiPA Rif.TB strips marked with a red marker line.
Denaturation solution	1 x 1 ml	56718	Alkaline solution containing EDTA. Vial is closed immediately after use. Prolonged exposure of solution to air was avoided to prevent rapid deterioration of denaturing strength.
Hybridization solution	1 x 80 ml	57219	SSC buffer containing 0.1% SDS, pre-warmed to a temperature of at least 37°C and not exceed 62°C.
Stringent wash solution	1 x 200 ml	57220	SSC buffer containing 0.1% SDS, pre-warmed to a temperature of at least 37°C and not exceed 62°C.
Rinse solution 5x	1 x 80 ml	56721	Phosphate buffer containing NaCl, triton® and 0.05% MIT/0.5% CAA as preservative, to be diluted 1:5 in distilled water before use. Quantity of solution needed was 4 ml diluted rinse solution for each test trough + 5 ml in excess for manual testing.
Conjugate diluents	1 x 80 ml	56951	Phosphate buffer containing NaCl, triton, protein stabilizers and 0.01% MIT/ 0.1% CAA as preservative.
Conjugate 100x	1 x 0.8 ml	56952	Streptavidin labeled with alkaline phosphatase in tris buffer containing protein stabilizers and 0.01% MIT/0.098% CAA as preservative, to be diluted 1:100 in conjugate diluent. Quantity needed for test was 1 ml diluted conjugate for each test trough + 1 ml in excess for manual testing.
Substrate buffer	1 x 180 ml	56953	Tris buffer containing NaCl, $MgCl_2$, and 0.01% MIT/0.1% CAA as preservative.
Substrate BCIP/NBT 100x	1 x 0.8 ml	56954	BCIP and NBT in DMF, to be diluted 1:100 in substrate buffer. Quantity needed for test was 1 ml diluted substrate for each test trough + 1 ml in excess for manual testing.
Incubation tray	2	-	Contains 12 troughs each.
Reading card	1	-	For identification of positive lines.

a) Amplification of 81 bp region in *rpoB* gene of MTBC

Table 24: Reagents supplied in LiPA amplification kit.

Component	Quantity	Ref.	Description
Amplification buffer	1x (0.30ml)	56982	Containing all, dNTPs and 0.05% NaN_3 as preservative.
Primer mix	1x (0.30ml)	58307	Containing biotinylated primers, and 0.01% NaN_3 as preservative.
$MgCl_2$ solution	1x (0.30ml)	55701	Containing 0.01% NaN_3 as preservative.

Materials required but not provided in the kit for amplification:

- DNA extracted from sputum positive slide as mentioned in above procedure.
- Adjustable pipettes to deliver 1-20µl, 20-200µl, and 200-1000µl.
- Disposable sterile cotton-plugged pipette tips.
- PCR / Microtube racks (Appendix-XIII).
- PCR tubes / Sterile microtubes.
- Microtube centrifuge ([Mini spin plus (Eppendorf)].
- DNA thermal cycler and equipment (Gene Amp® PCR system 9700, AB Applied Biosystems).
- Ampli Taq gold DNA polymerase, 1000 units, 5U/ µl (Roche, Applied Biosystems, lot no. F11901).
- Vortex.
- Milli-Q water (Millipore).

- Hand glove (Kimberly-Clark, Purple nitrile-XTRA*, powder-free exam gloves).

Reagents description, preparation for use and recommended storage conditions:

- All reagents were stored at -20^{o}C upon arrival.
- The reagents were stored isolated from any source of contaminating DNA, especially amplified DNA products. Reagents for amplification processes were handled in a room free from amplified DNA products. Autoclaved tubes and cotton-plugged pipette tips were used. Microbial contamination of reagents was avoided.
- All reagents were brought to room temperature (20-25^{o}C) approximately 30 minutes before use and was returned to the freezer immediately after use.

Amplification protocol:

1) Determined the number of "N", where N= (Number of samples to be amplified) + (one Negative control) + 1. Using a cotton-plugged pipette tip, a master mix in an autoclaved 1.5ml tube was prepared.

   (N x 15.8µl) autoclaved distilled water

   + (N x 10µl) Amplification buffer

   + (N x 10µl) Rif.TB primer mix

   + (N x 10µl) $MgCl_2$ solution

   + (N x 0.2µl) Taq polymerase (N x 1 U) (5 U/µl)

The total volume of this amplification mix becomes (N x 46 µl) is vortexed briefly and aliquoted 46 µl of master mix into (N-1) autoclaved amplification tubes.

The primers (IP1 and IP2), each used at a concentration of 20 pmol per reaction mixture, had the following respective sequences: 5□ -GGTCGGCA TGTCGCGGATGG-3□ and 5□-GCACGTCGCGGACCTCCAGC-3□. They were

biotinylated at their 5□ ends (109, 221).

2) 4µl of the DNA is pipette into each amplification mix. 4µl of Milli-Q water was added to the negative control tube. These mixtures were not vortexed and it was made sure that the amplification mixture remained at the bottom of the tube.
3) Samples were placed into the preheated and calibrated thermal cycler. The amplification program designed for the INNO LiPA Rif.TB amplification was as given below.

INNO LiPA Rif.TB amplification profile:

Step		**Temp**	**Time**	
1	Denature	95^{o}C	5minutes	
2	Denature	95^{o}C	1minutes	repeat cycle
3	Anneal primers	55^{o}C	1minutes	step 2 to 4,
4	Extend primers	72^{o}C	1minutes	30 times
5	Elongate	72^{o}C	10minutes	

4) After the amplification process, the amplified DNA was immediately used with the INNO LiPA Rif.TB kit for hybridization.

Procedure applied for increasing amplification efficiency:

To increase the amplification efficiency by removing PCR inhibitors, the DNA providing negative amplification products were re-purified for second time by ethanol precipitation and subjected to amplification.

Amplification results visualization:

The presence of the amplification product was checked on a 2% agarose gel. 4µl of the amplified product was loaded per slot. The amplicon appeared as a single band with a length of approximately 260bp.

Amplification results validation:

One positive and negative control was included each time a test was performed avoiding contamination. A positive band was observed with the positive control and a no band was seen in the negative control used.

b) Gel-electrophoresis:

Materials required for horizontal gel-electrophoresis (Appendix-XIV).

- Premixed TAE buffer 10X for using as buffer in electrophoresis (REF 11 666 690 001, LOT 12498100, Roche).
- SeakemLe agarose for gel electrophoresis (Cambrex, catalogue no 50004, lot no AG 4845).
- Balance / Weighing machine (CP225D, Sartorius).
- Butter paper.
- Ethidium bromide.
- Levelling table (Catalog 170-4046, Bio rad).
- Balance for leveling table for gel preparation.
- Power pac (Axygen, AXY-PS1) (Appendix-XV).
- Gel casting apparatus (Axygen submerged gel electrophoresis).
- Levelling table (catalog 170-4046, Bio Rad) (Appendix-XVI).
- Balance for leveling table for gel.
- UV transilluminator 2000 (Bio-Rad).
- Gel-Documentation machine (Appendix-XVII).
- Hand glove, kimberly-clark, purple nitrile-xtra, powder free gloves (UPC code 036000506037, kc500 latex free).
- 6X Loading dye (G190A 21314501 Blue / Orange 6X Loading dye).
- 100bp DNA ladder (Takara) (code 341 0A Lot A401-1).
- 6X Loading buffer (Takara) (Lot A145)
- Milli-Q water (Millipore).
- Pipette (Axypet autoclavable).
- UV transilluminator 2000 (Bio-rad)

Procedure for submerged gel-electrophoresis:

1. Gel casting apparatus was adjusted on a levelling table using balance. Combs for slots were kept ready.

2. 2g of SeakemLe agarose was taken weighed on a butter paper placed on a weighing balance. The agarose was transferred into a beaker. 100ml of premixed TAE 10X buffer was added to the agarose in beaker. The agar was dissolved in the above buffer by melting in an oven for few minutes and cooled to around 55^{o}C. Then added 7µl of ethidium bromide and mixed well, avoiding formation of bubbles.
3. Slowly the gel solution was poured into the gel casting tray apparatus and immediately the comb was set into the gel and the gel was left to solidify.
4. Upon solidification the gel was transferred to an electrophoresis chamber, and the gel was placed in such a way, that the slots / wells of gel were towards the negative electrode side. Then 10X TAE buffer was poured into the electrophoresis chamber, till the gel was completely submerged in the buffer.
5. 5µl of amplified DNA was mixed with 1µl 6X loading dye and loaded into the gel slots along with a negative control, positive control and a 100bp ladder / marker (1µl 6X loading buffer + 1µl 100bp ladder + 4µl Milli-Q water).
6. The electrophoresis unit was run for 15-20 minutes at 90-100 volts power pac.
7. After electrophoresis, the gel was checked in UV illuminator for presence of amplified target and documentation was done in a gel-doc machine (Appendix-XVIII).

c) Hybridization step in INNO LiPA Rif.TB assay

Samples for hybridization:

- Mycobacterial *rpoB* gene amplified product (10µl).
- Blank amplified control sample (Negative control) (10µl).

Hybridization procedure:

1. Shaking water bath was heated to 62°C ± 0.5°C. Temperature was checked using a calibrated thermometer, and adjusted the temperature if necessary. The hybridization solution and stringent wash solution was pre-warmed to at least 37°C not exceeding 62°C. The solutions were mixed before use.
2. Required number of INNO LiPA Rif.TB strips from tube (1 strip per sample) was removed with a tweezer and an identification number above the red marker line on the strip was written by a pencil. A strip for negative control sample (no DNA added) was included too.
3. Required number of test troughs (1 trough per strip) was taken and placed in tray.
4. 10µl of denaturation solution was pipetted into upper corner of each trough. Vial of denaturation solution was immediately closed after use.
5. 10µl amplified biotinylated product, or 10µl negative control sample was added to denaturation solution and carefully mixed by pipetting up and down (cotton-plugged sterile pipette tips was used). Denaturation was allowed to proceed for 5 minutes at room temperature (20-25°C).
6. Pre-warmed ready-for-use hybridization solution was shacken and added gently 1ml to denatured amplified product into each trough and mixed by

gentle shaking. Care was taken not to contaminate neighbouring troughs during pipetting.

7. Strip was immediately placed into trough with marked side (red marker line) of membrane facing upwards and completely submerged in solution.
8. The tray was placed into 62°C ± 0.5°C shaking water bath (approximately 80 rpm), closed the lid, and incubated for 30 minutes.

d) **Stringent washing procedure**

1. After hybridization, the tray was removed from water bath.
2. The tray was held at a low angle and the liquid was aspirated from the trough with a pipette, attached to a vacuum aspirator. 1ml pre-warmed stringent wash solution was added into each trough and rinsed by rocking the tray for 60s at room temperature. The solution was aspirated from each trough.
3. Washing step was repeated once.
4. The solution was aspirated and each strip was incubated in 1ml pre-warmed stringent wash solution in shaking water bath at 62°C ± 0.5°C for 10 ± 2 minutes with closed lid of water bath. Before incubation, temperature of water bath was checked using a calibrated thermometer, and temperature adjusted if necessary.

Note: rinse solution and conjugate solution was prepared during stringent washing incubation.

e) Color development in LiPA assay

All subsequent incubations were carried out at 20-25°C on a shaker. Precaution was taken not to make temperature fall below 20°C, to avoid obtaining weaker results. Also temperature was not exceeded above 25°C, to prevent high background and/or false positive signals. During the incubations, the liquid and test strip was kept moving back and forth in trough for homogeneous staining.

1. Each strip was washed twice for 60-90s (seconds) using 1 ml of diluted rinse solution. The liquid was aspirated.
2. 1 ml of diluted conjugate solution was added to each trough and incubated for 30 ± 3 minutes while shaking. Liquid was aspirated.

   Note: Substrate was diluted about 10 minutes prior to end of conjugate incubation.
3. Each strip was washed twice for 60-90s using 1ml of diluted rinse solution and again washed once more using 1 ml substrate buffer. Liquid was aspirated.
4. 1 ml of prepared substrate solution was added to each trough and incubated for 30 ± 3 minutes while shaking. Liquid was aspirated.
5. The color development was stopped by washing strips in 1 ml distilled water while shaking for at least 3 minutes.
6. The strips were removed from troughs by tweezer and placed on absorbent paper for drying completely before reading the results. Developed dry strips was stored and stored in dark at room temperature (20-25°C).

f) Results reading

A line was considered positive when a clear purple band appeared at end of test procedure for each probe on the strip. The conjugate control line (Conjugate control)

provided an internal control for color development reaction. MTB line was specific probe for *M. tuberculosis* complex.

g) Validation of the assay

One negative control was included each time a test was performed. Additional positive and negative control was included until a high degree of confidence was reached in ability to correctly perform the test procedure.

For each negative control used, no apparent signal was expected for any of lines on strip, except for the conjugate control line.

h) Interpretation of the results

- The uppermost red line is the marker line.
- The first positive line is the conjugate control line lined up with conjugate control line on plastic reading card. This line controls for the addition of reactive conjugate and substrate solution during detection procedure. The line should always be positive and should have approximately the same intensity on each strip in the same test run.
- The second positive line ("*M. tuberculosis* complex" on reading card) contains a probe specific for the MTBC, and controls for addition of amplified material for hybridization. This line is positive if DNA amplicons from MTBC strains are present. Strains from other microbial taxa react negatively on this probe.
- When all the S-probes (S1, S2, S3, S4 and S5) give a positive signal while all the R-probes are negative, the *M. tuberculosis* strain is sensitive to RIF (Figure 2A).

- When at least one negative signal with the S-probes is obtained, the *M. tuberculosis* strain is resistant to RIF (Figure 2B, C-G). If the resistance to RIF is caused by one of the four most frequently observed mutations (D516V, H526Y, H526D, S531L), a positive reaction should also be obtained with one of the four R-probes (R2, R4A, R4B, R5, respectively). A positive reaction of R-probe should be accompanied with a negative reaction on corresponding S-probe (S2, S4, S4, OR S5, respectively) (Figure 2 C-F). There are however some exceptions (Table 25: INNO LiPA Rif.TB regular probe patterns), when the pattern obtained may deviate from the regular patterns when, the sample contains more than one strain or contamination. In conclusion, a sample is considered as harboring a resistant strain if any INNO-LiPA pattern is observed that deviates from wild type pattern.

Table 25: INNO LiPA Rif.TB regular probe patterns.

Probes										**Interpretation**	**Pattern**
Rif.tb	S1	S2	S3	S4	S5	R2	R4A	R4B	R5		
-	-	- (1)	-	-	-	-	-	-	-	No *M. tuberculosis*	-
+	+	+	+	+	+	-	-	-	-	Wild type (2)	WT
+	-	+	+	+	+	-	-	-	-	Mutation in probe region 1	ΔS1
+	+	-	+	+	+	-	-	-	-	Mutation in probe region 2	ΔS2
+	+	-	+	+	+	+	-	-	-	D516V	R2

+	+	+	-	+	+	-	-	-	-	Mutation in probe region 3	ΔS3
+	+	+	+	-	+	-	-	-	-	Mutation in probe region 4	ΔS4
+	+	+	+	-	+	-	+	-	-	H526Y	R4A
+	+	+	+	-	+	-	-	+	-	H526D	R4B
+	+	+	+	+	-(3)	-	-*	-*	-	Mutation in probe region 5	ΔS5
+	+	+	+	+	-	-	-*	-*	+	S531L	R5

* These probes may react weakly positive when S5 is negative.

(1) May occasionally be positive with non- *M. tuberculosis* strains.

(2) Theoretically, there is a possibility that a sample contains a mixture of a wild type strain and a mutant strain (not being recognized by one of the R-probes), in this case all S-probes becomes positive and all the R-probes becomes negative. If there is no noticeable difference in color intensity of different S-lines, the mixture is interpreted as sensitive.

(3) When dealing with a mutation at codon 533, it is possible that the S5 probe does not disappear completely.

Table 26: Example chart followed for interpretation of results obtained in hybridization strips using INNO LiPA Rif.TB assay.

Strips	Probes										Pattern	Result
	TB	S1	S2	S3	S4	S5	R2	R4A	R4B	R5		
A	+	+	+	+	+	+	-	-	-	-	Wild type	Sensitive
B	+	-	+	+	+	+	-	-	-	-	ΔS1	Resistant
C	+	+	-	+	+	+	+	-	-	-	R2	Resistant
D	+	+	+	+	-	+	-	+	-	-	R4A	Resistant
E	+	+	+	+	-	+	-	-	+	-	R4B	Resistant
F	+	+	+	+	+	-	-	-	-	+	R5	Resistant
G	+	+	+	+	+	-	-	-	-	-	ΔS5	Resistant
H	-	-	-	-	-	-	-	-	-	-	-	No *M. tuberculosis*

Limitations of the LiPA procedure

False positive reactions of probes R4A, R4B, and/or S5 may occur when a mutation is present in the S5 probe. For instance, when dealing with mutation S531L, weak false positive reactions of probes R4A and R4B will be visible in addition to a regular R5-pattern (meaning MTB, S1, S2, S3, S4, and R5 are positive). When dealing with mutation L533P, a weak false positive reaction of the S5 will occur, together with false positive reactions of probe R4A and R4B. During evolution towards resistance, a mixture of a wild type strain and a mutant strain (not being recognized by one of R-probes) may be interpreted as sensitive.

Waste disposal

Biological materials were disposed off after autoclaving for at least 15 minutes at 121°C, followed by incineration. Liquid waste (neutralize liquid waste that contains acid before adding sodium hypochlorite) was mixed with sodium hypochlorite so that the final concentration becomes ± 1% sodium hypochlorite, then allowed to stand overnight before disposal.

Statistical analysis:

We analyzed the data using Epi-Info version 3.5.4 (epiinfo@cdc.gov) and SPSS 17 (Chicago: SPSP Inc.).

The descriptive statistics was done by place, person and time. Frequency distribution of various parameters was done. The difference between categorical variables (proportion) was done using Chi square test. Difference in continuous variables were estimated using independent t-test. F-test was done to compare analysis of variance.

The analytical statistics was done to deal with analysis of time to events. We applied Kaplan-Mayer test for this analysis. We also calculated sensitivity, specificity, positive predictive value and negative predictive value for validity estimation.

Ethical clearance:

The ethical clearance was taken from the Institutional ethics committee, SMIMS.

B) DNA isolation from fresh cultures and its use in spoligotyping and MIRU-

VNTR typing (Additional research work done)

Materials and Methods

Ethics statement

Surveys carried in the study regions were approved by the Ethical Committee of Regional Medical Research Centre. Written informed consent was obtained from all the participants or their guardian in case of minors who provide sputum samples. Patients found positive for AFB were referred to the nearest DOTS centre for treatment.

Bacterial culture, identification and DNA extraction

A total of 300 MTBC strains collected during 2009 – 2012 from two states of NE region viz., Assam and Sikkim were used in this study. Sputum samples were decontaminated according to modified Petroff's method and all the samples were subjected to culture on solid LJ media at 37^{o}C for 6 to 8 weeks. MTBC were identified using biochemical methods and identification was confirmed using MicroSeq™ 16S rDNA bacterial identification system (life technologies | Applied Biosystems).

Drug sensitivity testing (DST)

Hain's Genotype® MTBR plus (Hain life sciences) which is a line probe assay was used to detect resistance to refampicin and isoniazid for 234 MTBC strains according to the instructions of manufacturers. For Hain's test we used confirmed multiple drug

resistant (MDR) strains of MTBC (based on DST carried by proportion method using solid LJ media) and completely sensitivity strains (H37Rv) in each batch of the test for quality control.

DNA isolation

DNA was extracted from fresh cultures by the cetyl-trimethyl ammonium bromide (CTAB) method (222)

Spoligotyping

Spoligotyping for detection of presence or absence of 43 spacers was done on all isolates as described by Kamerbeek, Schouls (223) using commercially available kit (Isogen Biosciences®, BV, Maarsen the Netherlands now Ocimum Biosolutions). Briefly, the direct repeat (DR) region was amplified with primer pair Dra, 5' - GGTTTTGGGTCTGACGAC - 3' (biotinylated 5' end) and DRb, 5' – CCGAGAGGGGACGGAAAC – 3'. The DNA amplification was carried out in GeneAmp® PCR system 9700 of Applied Biosystems. The amplified PCR products were hybridized with nitrocellulose membrane having covalently linked 43 spacer oligonucleuotides following the manufacturer's instructions. The hybridized fragments were detected using enhanced chemiluminescence system (GE Healthcare, UK Ltd., Buckinghamshire, UK). The spoligotypes were initially reported as 43 digits binary representation of 43 spacers, one was scored for positive hybridisation and zero for no hybridisation. Finally the spoligotypes were converted into fifteen digit octal code (224) to facilitate comparison of our results with international database SpolDB

4.0 (214). Spoligotyping data was also analysed by cluster analysis using Jaccard's coefficient in SPSS® version 17 to get an insight to the genetic structure of MTBC isolates from NE region.

MIRU-VNTR typing

MIRU-VNTR typing was performed by amplifying fifteen hypervariable MIRU loci of all 300 isolates of MTBC from NE region. These 15 MIRU Loci used for typing in this study are: ETR-A, ETR-B. ETR-C, MIRU2, MIRU4, MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU26, MIRU27, MIRU31, MIRU39, and MIRU40. The details of primer pairs and PCR reaction conditions are given by Dymova, Liashenko (225). However, the PCR products were run in Lab Chip (Caliper life sciences Inc., USA). The copy number of tandem repeats was calculated as a function of size of PCR product. The results were entered in SPSS for hierarchical cluster analysis using nearest neighbour joining algorithm (SPSS® version 17).

Discriminatory power of MIRU_VNTR typing system was calculated using the Hunter-Gaston discriminatory index (HGDI) (226)

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} X_j (X_j - 1)$$

where N = the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the j^{th} type.

Principal Components analysis

Spoligotyping data from MTBC isolates from Assam and Sikkim along with similar data from Kanpur, Mumbai and other parts of mainland India, Pakistan, Vietnam, Thailand, China, Korea, Japan, Sri Lanka, Uganda, South Brazil , Brazil and Columbia were subjected to principal component analysis to elucidate major patterns of variation in genetic diversity of MTBC isolates. Data on spoligotypes present in various other regions used in the above analysis was obtained from various sources like (Purwar, Chaudhari (194), Sharma, Chauhan (203), Kulkarni, Sola (210), Rajapaksa, Victor (227), Hasan, Tanveer (228), Cerezo, Jimenez (229)) and also from the international database SpolDB 4.0.

For evaluation of the pattern of MTBC strains in NE region as compared to other geographical regions. A correlation coefficient matrix of spoligotype abundance data was generated for extraction of principal components. Varimax rotated first two principal components were used to make a scatter plot. Geographical areas with similar spoligo patterns tended to be together in the reduced multivariate data space without loss of information due dimensionality reduction.

CHAPTER 6

RESULTS AND DISCUSSIONS

To estimate the prevalence of rifampicin resistance cases of pulmonary tuberculosis in the population of Sikkim.

Results

Table 27, below shows that the overall cure rate for DOTS treatment outcome from year 2004 to 2009 was 50.4%. Highest cure rate was obtained in 2004 and lowest in 2009 with 28.8% indicates, decline in cure rate was observed year by year. Failure rate comprise of 15.6% in 2004 with 9.9% in 2009. Highest death rate was 4.1% in 2008 and lowest in 2009 with 2.7%. Males in Sikkim (56.9%) are more vulnerable to TB than females (43.1%), suggesting their daily work outdoor, increasing their chance of exposure to the infectious TB cases.

Table 27: Demographic profile of tuberculosis patients attending DOTS centers in Sikkim, India during period 2004-2009.

Variables		Total	Year of DOTS treatment						Chi square (p value)
			2004	2005	2006	2007	2008	2009	
Sex	Female	1878 (43.1%)	153 (33.0)	289 (37.7%)	521 (45.7%)	384 (45.2%)	202 (43.8%)	329 (48.4%)	40.9 (<0.01)
	Male	2482 (56.9%)	311 (67.0)	477 (62.3%)	619 (54.3%)	465 (54.8%)	259 (56.2%)	351 (51.6%)	
	Total	**4360**	**464**	**766**	**1140**	**849**	**461**	**680**	

Type of TB patients	New	2751 (71.0%)	325 (71.0)	545 (71.4%)	499 (61.5%)	515 (73.2%)	354 (77.1%)	513 (75.4%)	196.7 (<0.01)
	Failure	198 (5.1%)	33 (7.2%)	25 (3.3%)	70 (8.6%)	35 (5.0%)	12 (2.6%)	23 (3.4%)	
	Relapse	567 (14.6%)	54 (11.8)	157 (20.6%)	166 (20.4%)	92 (13.1%)	38 (8.3%)	60 (8.8%)	
	Default	118 (3.0%)	24 (5.2%)	24 (3.1%)	27 (3.3%)	22 (3.1%)	5 (1.1%)	16 (2.4%)	
	Others	242 (6.2%)	22 (4.8%)	12 (1.6%)	50 (6.2%)	40 (5.7%)	50 (10.9%)	68 (10.0%)	
	Total	**3876**	**458**	**763**	**812**	**704**	**459**	**680**	
DOTS treatment outcome	Cured	1692 (50.4%)	346 (76.2)	491 (66.7%)	541 (50.3%)	186 (28.6%)	128 (28.8%)	NA	632.6 (<0.01)
	Treatment completed	1003 (29.9%)	2 (4%)	92 (12.5%)	321 (29.9%)	338 (51.9%)	250 (56.3%)	NA	
	Default	134 (4.0%)	22 (4.8%)	29 (3.9%)	48 (4.5%)	25 (3.8%)	10 (2.3%)	NA	
	Failure	421 (12.5%)	71 (15.6%)	102 (13.9%)	129 (12.0%)	75 (11.5%)	44 (9.9%)	NA	
	Died	110 (3.3%)	13 (2.9%)	22 (3.0%)	36 (3.3%)	27 (4.1%)	12 (2.7%)	NA	
	Total	**3360**	**454**	**736**	**1075**	**651**	**444**	**NA**	
Treatment category	Cat I	1268 (44.9%)	0(0%)	97 (34.3%)	282 (40.9%)	314 (49.1%)	235 (51.0%)	340 (50.0%)	236.6 (<0.01)
	Cat II	807 (28.6%)	66 (94.3)	128 (45.2%)	215 (31.2%)	128 (20.0%)	106 (23.0%)	164 (24.1%)	
	Cat III	629 (22.3%)	4 (5.7%)	46 (16.3%)	158 (22.9%)	164 (25.6%)	102 (22.1%)	155 (22.8%)	
	ND	119 (4.2%)	0 (0%)	12 (4.2%)	34 (4.9%)	34 (5.3%)	18 (3.9%)	21 (3.1%)	
	Total	**2823**	**70**	**283**	**689**	**640**	**461**	**680**	
Disease class	P	1663 (64.7%)	116 (63.0)	402 (66.3%)	395 (61.7%)	307 (66.6%)	NA	443 (65.1%)	
	EP	908 (35.3%)	68 (37.0)	204 (33.7%)	245 (38.3%)	154 (33.4%)	NA	237 (34.9%)	
	Total	**2571**	**184**	**606**	**640**	**461**	**NA**	**680**	

ND= Non DOTS patients, P= Pulmonary TB patients, EP= Extra Pulmonary TB patients

Cured= Initially sputum smear-positive patient who has completed treatment and had negative

sputum smears, on two occasions, one of which was at the end of treatment

Treatment completed= Sputum smear-positive patient who has completed treatment, with negative smears at the end of the intensive phase but none at the end of treatment. Or: Sputum smear-negative TB patient who has received a full course of treatment and has not become smear-positive during or at the end of treatment. Or: Extra-pulmonary TB patient who has received a full course of treatment and has not become smear positive during or at the end of treatment.

Died= Patient who died during the course of treatment regardless of cause

Failure= Any TB patient who is smear positive at 5 months or more after starting treatment. Failure also includes a patient who was treated with Category III regimen but who becomes smear positive during treatment.

Defaulted= A patient who has not taken anti-TB drugs for 2 months or more consecutively after starting treatment.

Transferred out= A patient who has been transferred to another TB Unit/District and his/her treatment result (outcome) is not known.

Table 28: Age distribution of tuberculosis patients (all categories) attending DOTS centers in Sikkim, India, during period 2004-2009.

Year of treatment	Total number of TB patients	Mean Age	Std. Deviation of Age	Std. Error of Mean Age	F (p value)
2004	464	33.13	16.838	.782	Combined=10.97 (<0.01)
2005	766	28.47	14.713	.532	Linearity =10.04 (0.02)
2006	1141	28.29	14.296	.423	Deviation from linearity=11.2
2007	849	27.18	13.363	.459	(<0.01)
2008	461	30.00	14.866	.692	
2009	680	28.46	15.911	.610	
Total	4361	28.83	14.891	.225	

Table above shows 27.18 to 33.13 years age group people in Sikkim are mainly affected with tuberculosis. These age group are the ones who are active earning

citizens of the state, exposed to infectious cases outdoor and vulnerable to develop disease due to hard work and also they are not controlled by anyone for their discipline in the act of smoking and alcoholism.

Figure 6: Age distribution of tuberculosis patients attending DOTS centers in Sikkim, India during 2004-2009.

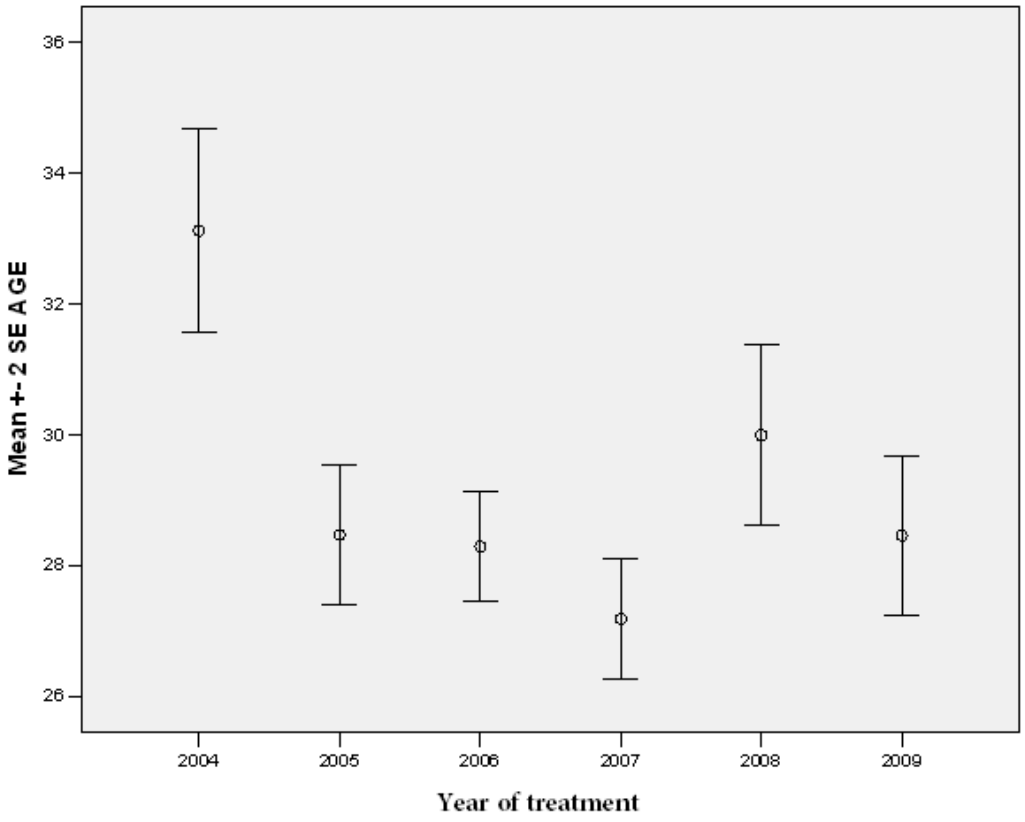


Table 29: Demographic profile of smear positive category-I pulmonary tuberculosis cases in Sikkim (India), between 6th October 2007 and28th December 2009.

Factors		Number of individuals (n=320)	Percentage
Age duration	<25 years	177	55.3
	26-50 years	115	35.9
	>50 years	28	8.8
Sex	Male	185	57.8
	Female	135	42.2
Ethnicity	Bhutia	62	19.4
	Lepcha	24	7.5
	Sherpa	25	7.8
	Nepali	197	61.6
	Others	12	3.8
Treatment period of Category-I DOTS regimen	Pre treatment	253	79.1
	End of intensive phase	39	12.2
	End of extensive phase	11	3.4
	End of continuation phase	11	3.4
	End of extensive continuation phase	1	0.3
	End of treatment	5	1.6

TB was found more prevalent among young age group in Sikkim. More than half (55.3%) of the category-I TB patients belonged to age group <25 years of age. Only 28 (8.8%) patients were >50 years of age. Among the 320 Category I pulmonary TB patients of Sikkim 57.8% were males. 61.6% of patients belonged to Nepali ethnicity. Out of total 320 cases, 253 (79.1%) were pre treatment patients. Other samples were collected from patients who were at different stages of treatment.

Table 30: Sputum conversion shown at different stages of treatment period between 6th October 2007 and 28th December 2009 in Sikkimese Category-I tuberculosis patients.

AFB grading	Stage of Category-I treatment period		
	End of IP (2/3 months)	**End of CP (4/5/6 months)**	**End of treatment (6/7/8 months)**
	Frequency (%)	**Frequency (%)**	**Frequency (%)**
1+	71(22.2)	14(5)	4(1.7)
2+	40(12.5)	25(9)	17(7.1)
3+	32(10)	24(8.6)	11(4.6)
scanty	10(3.1)	6(2.2)	0 (0.0)
Sputum negative cases	167(52.2)	210(75.3)	207(86.6)
Total	320	279*	239*

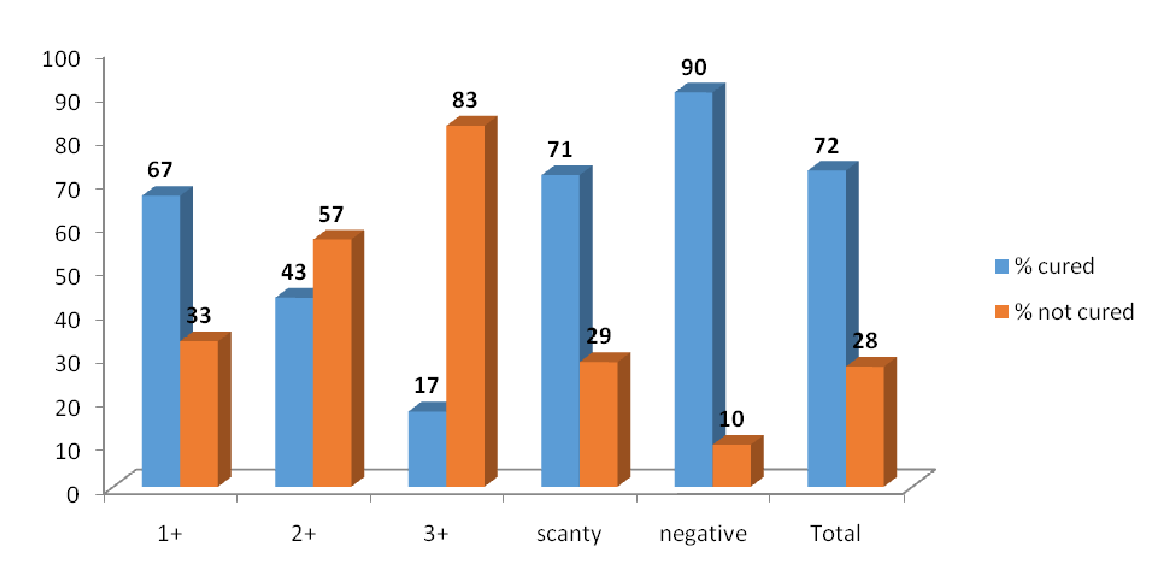
AFB= AFB , 3+= >10 AFB per oil immersion field, 2+= 1- 10 AFB per oil immersion field, 1+= 10-99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields, Negative = No AFB in 100 oil immersion fields, IP= Intensive phase, CP= continuation phase.

***denotes decreased number of cases due to patient's outcome as default, death and transferred out.**

Total sputum positive cases collected before starting category-I treatment was 320. Sputum positivity decreased to 47.8% at end of intensive phase (2/3 months), 24.7% at end of continuation phase (4/5 months) and 13.4% at end of treatment. Sputum screening at end of continuation phase of treatment showed good indication towards patient's clinical outcome under DOTS therapy, similar to findings of other's study (134) (2007), suggesting treatment response could be correctly predicted based on six-

months smear result and those who remain positive could be considered for a change of treatment. AFB grading also decreased as the treatment duration increased.

Figure 7: Sputum conversion at end of intensive phase (two/three months) and end of treatment period for sputum positive DOTS category-I TB patients in Sikkim.

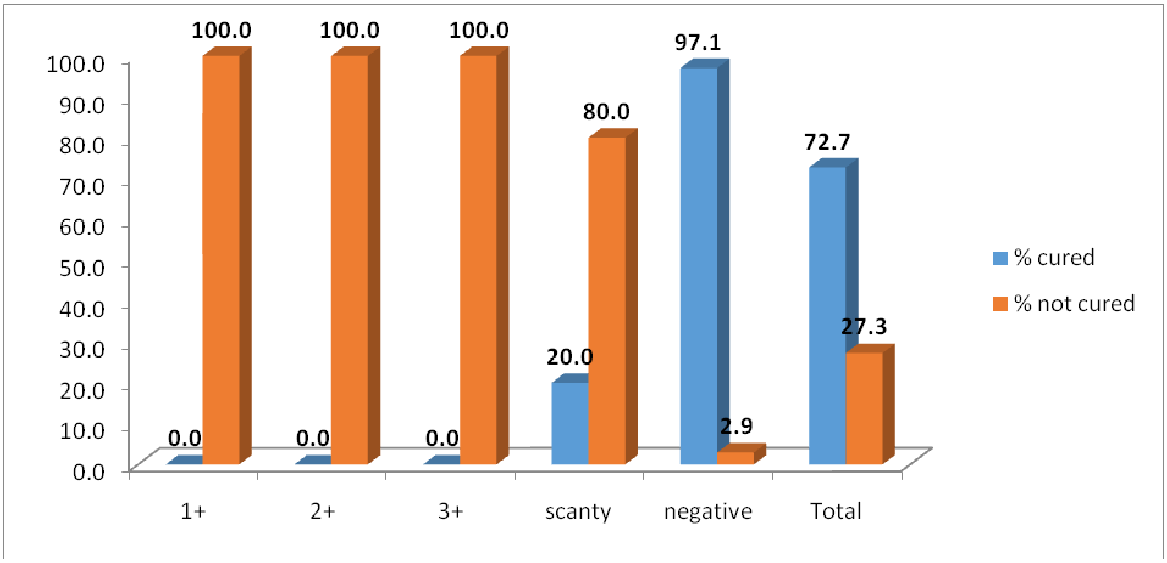


3+= >10 AFB per oil immersion field, 2+= 1-10 AFB per oil immersion field, 1+ = 10-99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields, Negative = No AFB in 100 oil immersion fields, Cured= Initially sputum smear-positive patient who has completed treatment and had negative sputum smears, on two occasions, one of which was at the end of treatment, Not cured= included patients whose treatment outcome turned out to be as defaulters, failures, treatment completed, death and transferred out patients.

The data above show cure rate of TB patients by the end of intensive phase (two or three months) of DOTS treatment. The highest cure rate was observed in TB cases

with sputum negativity at intensive phase (two months) of treatment and the proportions of cured and uncured individuals were significantly different (p value for one-tailed $z<0.001$) in this group. Significant difference in the proportions of cured and not cured patients were observed among the patients who had sputum AFB grading 2+ (p value for one-tailed $z<0.001$) and 3+ (p value for one-tailed $z<0.001$) The lowest cure rate was observed among those patients who were having sputum AFB grade of 3+ even at intensive phase of treatment.

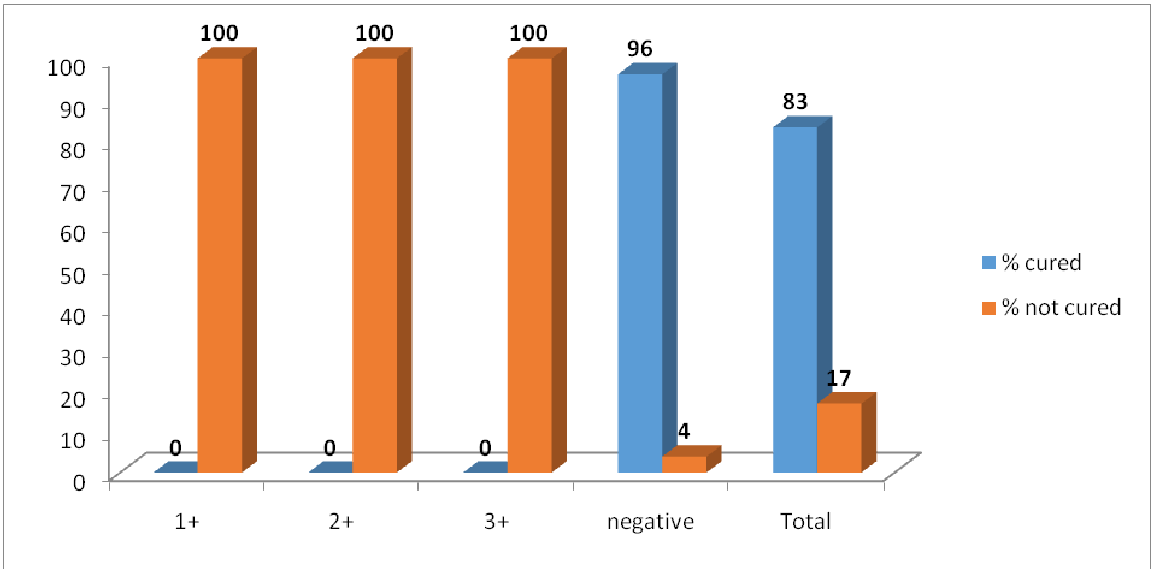
Figure 8: Sputum conversion at end of DOTS continuation phase (four to six months) and treatment outcome of sputum positive category-I TB patients in Sikkim, India.



3+= >10 AFB per oil immersion field, 2+= 1-10 AFB per oil immersion field, 1+= 10-99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields, Negative= No AFB in 100 oil immersion fields, cured= Initially sputum smear-positive patient who has completed treatment and had negative sputum smears, on two occasions, one of which was at the end of treatment, Not cured=includes those patients belonging to default, failures, treatment completed, death and transferred out patients as DOTS treatment outcome.

The data above show cure rate of TB patients at end of continuation phase (four/ five/six months) of treatment. Cure rate was highest with sputum negative patients. Lowest cure rate was observed among those TB patients having sputum 2+ and 3+ AFB grading. However, proportions of cured and not cured cases were significantly different in different five AFB grades patients (p value for z test <0.05 in all categories).

Figure 9: Sputum conversion at end of DOTS regimen completement and final treatment outcome of sputum positive category-I TB patients.



3+= >10 AFB per oil immersion field, 2+= 1-10 AFB per oil immersion field, 1+= 10-99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields, Negative = No AFB in 100 oil immersion fields, Cured= Initially sputum smear-positive patient who has completed treatment and had negative sputum smears, on two occasions, one of which was at the end of treatment, Not cured= includes those patients belonging to default, failures, treatment completed, death and transferred out patients as DOTS treatment outcome.

The data above show cure rate of TB patients at end of treatment under DOTS regimen. Highest cure rate was observed in sputum negative TB patients at the end of treatment. Lowest cure rate was observed in patients with sputum AFB grade of 2+, followed by sputum AFB grade 3+ patients. There was no scanty positive. Cured and not cured proportion in patients with different sputum grades were significantly different (p value for z test <0.05).

Table 31: Association of MTBC culture growth on LJ-medium and AFB grading of sputums collected between 6th October 2007 and 28th December 2009 from sputum positive category-I tuberculosis patients under DOTS centres in Sikkim, India.

AFB sputum grade (n=318*)	Growth of MTBC on Loweinstein Jensen medium		Unadjusted OR	95% C.I. for EXP(B)
	No growth (n=131)	Growth (n=187)		
1+ (n=105)	56 (42.7)	49 (26.2)	1	
2+ (n=84)	33 (25.2)	51 (27.3)	1.77	0.99-3.16
3+ (n=129)	42 (32.1)	87 (46.5)	2.37	1.39-4.03

***Since there was only two scanty positives (1-9 AFB per 100 oil immersion fields) and showed no culture growth they were deleted from the analysis.**

3+= >10 AFB per oil immersion field, 2+= 1-10 AFB per oil immersion field, 1+= 10-99 AFB per 100 oil immersion fields.

A significant association was found between culture growth and sputum gradings. Univariate odds ratios showed that sputum samples with 3+ AFB grade had two times higher possibility to grow in culture (Univariate OR 2.37, 95% CI:1.39-4.02) as compared to 1+ AFB grade sputums, due to high bacilli load in 3+ sputum, as compared to 2+, 1+ and scanty grading sputums.

Table 32: Association between growth duration of MTBC on LJ medium and AFB grading using sputum positive samples from category-I TB patients under DOTS in Sikkim, India.

AFB smear grade (n=318)	**No growth (n=131)**	**2-3 weeks (n=141)**	**> 3 weeks (n=46)**	**Chi-square (p value)**
1+ (n=105)	56 (42.7)	40 (28.4)	9 (19.6)	13.6 (0.009)
2+ (n=84)	33 (25.2)	34 (24.1)	17 (37.0)	
3+ (n=129)	42 (32.1)	67 (47.5)	20 (43.5)	
AFB = AFB , 3+= >10 AFB per oil immersion field, 2+= 1-10 AFB per oil immersion field, 1+= 10-99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields, Negative = No AFB in 100 oil immersion fields.				

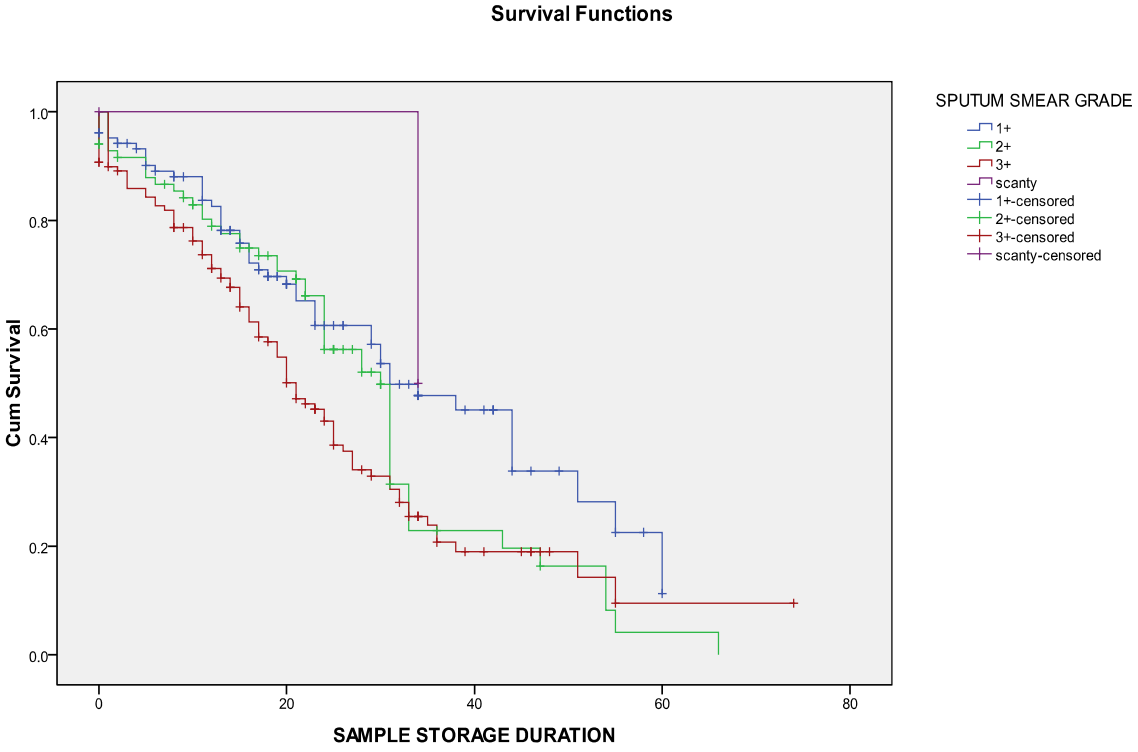
Statistical analysis show there was significant association between AFB gradings and growth duration on LJ slants using sputum positive samples.

Table 33: Mean and median storage time for colony growth of sputum with different grades.

AFB smear grade	Mean		Median	
	Estimate	**95% Confidence interval**	**Estimate**	**95% Confidence interval**
1+	34.3	29.6-39.1	31	23.4-38.6
2+	28.5	24.0-32.9	30	26.5-33.5
3+	26	21.5-30.5	21	17.2-24.8
Scanty	34	34.0-34.0	34	
Overall	29.7	26.8-32.5	27	23.3-30.7
AFB = AFB, 3+= >10 AFB per oil immersion field, 2+= 1-10 AFB per oil immersion field, 1+= 10-99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields, Negative = No AFB in 100 oil immersion fields.				

Above table showed significant difference in growth of *M. tuberculosis* and smear gradings of sputums stored in CPC for different time duration (Chi-square: 10.52, p value=0.0)

Figure 10: Survival plot showing relation between sputum storage duration in 1% CPC and culture positivity of MTBC in LJ medium using sputum positive samples having different smear gradings.



3+= >10 AFB per oil immersion field, 2+= 1-10 AFB per oil immersion field, 1+= 10 -99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields

The above plot show, sputums with 3+ grading had higher rate of culture positivity even after 60 days of storage in 1% CPC solution as compared to 2+ and 1+ grading sputum. It indicated, bacilli load in sputum is related to culture positivity of MTBC on LJ-medium.

Table 34: Measure of agreement regarding rifampicin susceptibility between present study and other laboratories (TRC, Chennai; Ranbaxy, Mumbai; Delhi) conducting first line anti-tubercular drug testing by proportionate method using sputum positive samples from category-I TB patients.

		Rifampicin sensitivity by proportionate method in present study		
		Sensitive	**Resistant**	**Total**
Rifampicin sensitivity by proportionate method in other labs	**Sensitive**	2	0	2
	Resistant	0	34	34
Total		2	34	36

In case of RIF sensitivity test done in present study, the test results were compared to those done in other laboratories. There was 100% concordance between the two laboratory findings by proportionate method (Kappa=1) showing excellent reproducibility

Table 35: Measure of agreement of isoniazid sensitivity in present study with sensitivity result of other laboratories (TRC, Chennai; Ranbaxy, Mumbai; Delhi) conducting first line anti-tubercular drug testing using positive sputums from Category-I tuberculosis patients.

		Isoniazid sensitivity by proportionate method in present study		Total
		Sensitive	resistant	
Isoniazid sensitivity by proportionate method in other labs	**sensitive**	2	0	2
	resistant	1	33	34
Total		3	33	36

INH sensitivity test done in present study was compared to those done in other laboratories. There was 79% concordance between two laboratory findings by proportionate method (Kappa= 0.79) showing excellent reproducibility.

Table 36: Measure of agreement of drug sensitivity test for pyrazinamide in present study with the sensitivity result of other laboratories (TRC,Chennai; Ranbaxy,Mumbai; Delhi) conducting the first line anti-tubercular drug testing using positive sputums from category-I TB patients.

		Pyrazinamide sensitivity by proportionate method in present study		Total
		Sensitive	Resistant	
Pyrazinamide sensitivity by proportionate method in other labs	**Sensitive**	10	0	10
	Resistant	1	6	7
Total		11	6	17

In case of pyrazinamide sensitivity test done in present study, test results were compared to those done in other laboratories. There was 88% concordance between the two laboratory findings by proportionate method (Kappa= 0.88) showing excellent reproducibility of present findings.

Table 37: Concordance study for quality of ethambutol sensitivity test in present study with sensitivity result of other laboratories (TRC,Chennai; Ranbaxy,Mumbai; Delhi) conducting first line anti-tubercular drug testing using positive sputum from category-I tuberculosis patients.

		Ethambutol sensitivity by proportionate method in present study		**Total**
		Sensitive	**Resistant**	
Ethambutol sensitivity by proportionate method in other labs	**Sensitive**	5	0	5
	Resistant	2	28	30
Total		7	28	35

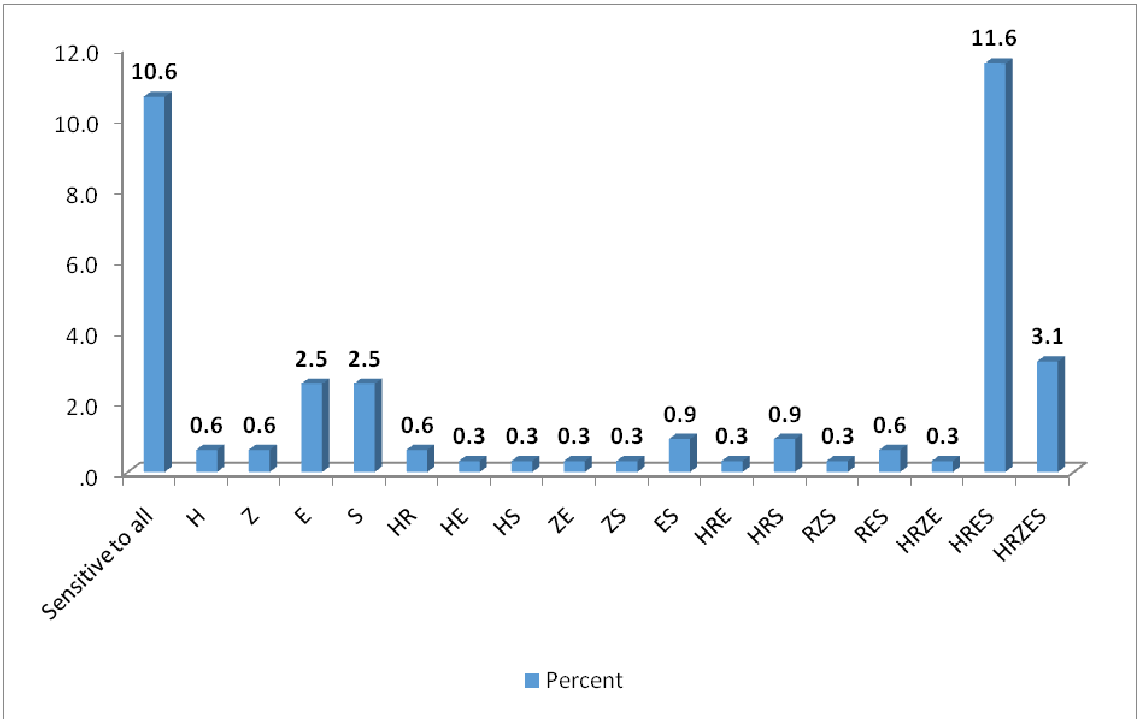
In case of ethambutol sensitivity test done in present study, the test results were compared to those done in other laboratories. There was 80% concordance between two laboratory findings by proportionate method (Kappa= 0.80) showing excellent reproducibility of the present findings.

Table 38: Concordance study for quality of streptomycin sensitivity test outcome in present study with the sensitivity result of other laboratories (TRC, Chennai; Ranbaxy, Mumbai; Delhi) conducting the first line anti-tubercular drug testing using sputum from TB patients.

		Streptomycin sensitivity by proportionate method in present study		
		Sensitive	**Resistant**	**Total**
Streptomycin sensitivity by proportionate method in other labs	**Sensitive**	4	0	4
	Resistant	1	30	31
Total		5	30	35

In case of streptomycin sensitivity test done in the present study, the test results were compared to those done in other laboratories. There was 87% concordance between two laboratory findings by proportionate method (Kappa =0.87) showing excellent reproducibility of the present findings.

Figure 11: Pattern of mono and poly-drug resistance (%) among smear positive category-I TB patients tested by proportionate method of sensitivity test collected during 6th October 2007 to 28th December 2009 from DOTS centres in Sikkim, India.

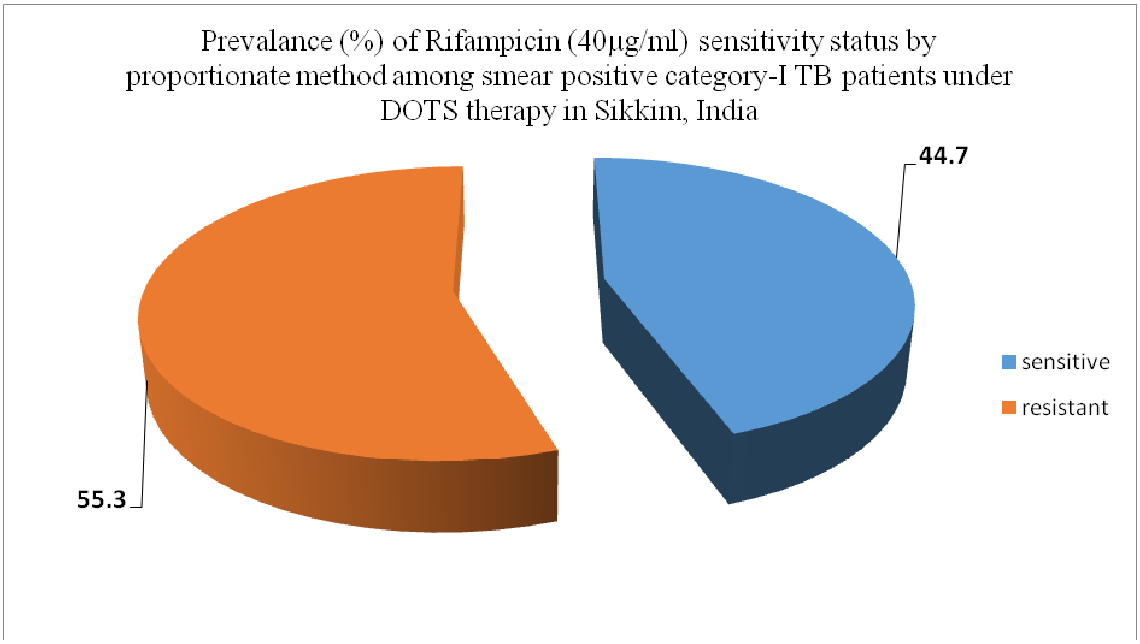


H= isoniazid, R= RIF, Z= pyrazinamide, E= ethambutol, S= streptomycin,

Sensitive to all= sensitive to HRZES

Among the smear positive category-I TB patients, of whom drug sensitivity test by proportionate method was done, the sensitivity pattern showed individual resistance mostly to ethambutol (8/84) and streptomycin (8/84). Highest drug resistance was observed to the combination of drugs INH, RIF, E and S (37/84) followed by combination of all five drugs viz., INH, RIF, Z, E and S. No case was found resistant to only RIF.

Figure 12: Prevalence of rifampicin resistant cases among 103 smear positive category-I tuberculosis patients tested by proportionate method of sensitivity testing under DOTS in Sikkim, India.



Among 320 smear positive Category-I tuberculosis patient's sputum collected during 6th October 2007 to 28th December 2009, 189 samples yielded positive culture for MTBC, out of which 103 culture positive samples were tested by proportionate method of sensitivity, and 57 (55.3%) were found as to be RIF resistant.

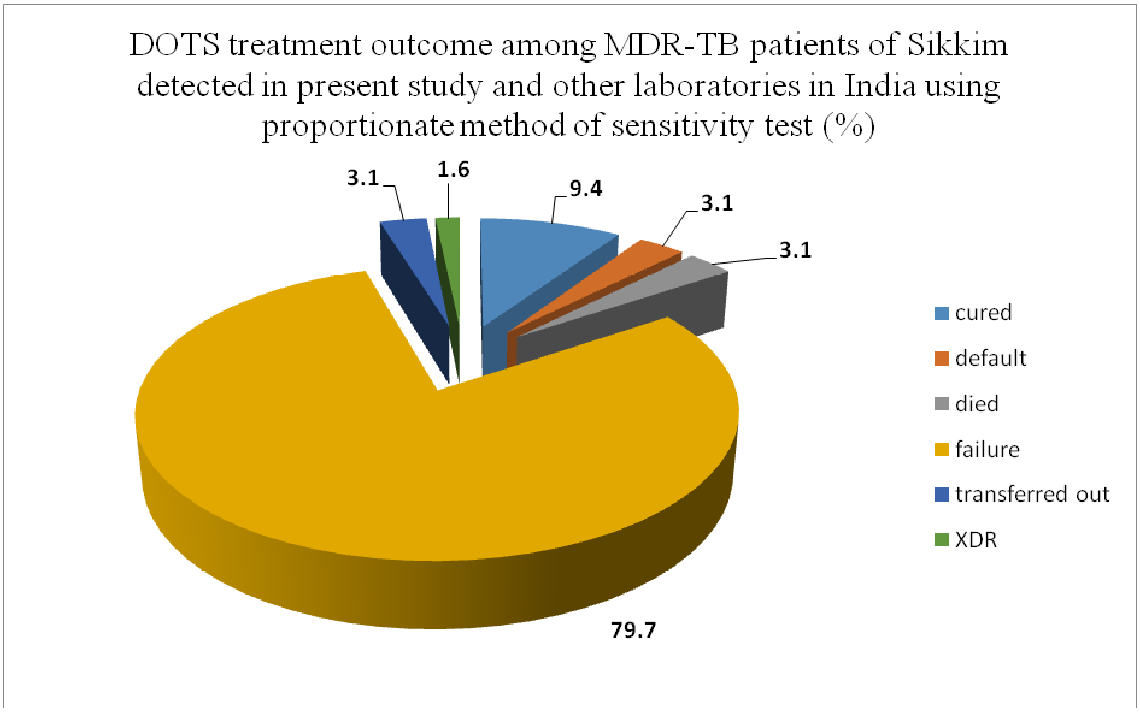
Table 39: Sputum conversion among MDR-TB patients (smear positive category-I TB patients) at end of intensive phase (two/three months), end of continuation phase (four/five/six months) and at end of DOTS treatment (six/seven/eight months) in Sikkim, India.

AFB grading of sputum smear	End of intensive phase (n=54)	End of continuation phase (n=48)	End of treatment(n=36)
	Frequency (%)	Frequency (%)	Frequency (%)
1+	13 (24.1)	6(12.5)	2(5.56)
2+	10 (18.5)	13(27.08)	13(36.11)
3+	20 (37.0)	15(31.25)	8(22.22)
Scanty	1 (1.9)	3(6.25)	0(0.0)
negative	10 (18.5)	11(22.92)	13(36.11)

AFB = AFB, 3+= >10 AFB per oil immersion field, 2+= 1 -10 AFB per oil immersion field, 1+= 10-99 AFB per 100 oil immersion fields, Scanty= 1-9 AFB per 100 oil immersion fields, Negative = No AFB in 100 oil immersion fields.

The sputum conversion (sputum positivity to sputum negativity) of MDR-TB patients after intensive phase was 18.5% (10), after continuation phase was 22.92% (11) and after completion of category-I regimen under DOTS was 36.11% (13). In comparison to non-MDR patients, this group of patients had poor sputum conversion which might be because the resistant tubercular bacilli was not sensitive to the first line anti-tubercular drugs.

Figure 13: Treatment outcome of MDR-TB patients (MDR found in present study as well as other labs= 64) obtained out of total 320 smear positive category-I TB patients between 6th October 2007 and 28th December 2009, under DOTS in Sikkim, India.



Out of 320 smear positive category-I TB patients, 64 patients were proved as MDR-TB patients (including those confirmed by present study [54] and other laboratories-TRC, Chennai; Ranbaxy, Bombay; Ganga-Ram hospital, Delhi) with 79.7% outcome as failures as DOTS outcome. Only 9.4% MDR-TB patients were cured by end of treatment and 1.6% (1) was reported to be XDR-TB.

Table 40: DOTS treatment outcome of fifty-seven RIF resistant (54 MDR-TB) smear positive category-I TB patients by proportionate method of sensitivity testing, collected during 6th October 2007 to 28th December 2009 from DOTS centres in Sikkim, India.

Anti-tubercular drug combination	No. of TB Patients	Cured	Default	Died	Failure	Transferred out	XDR-TB
HR	2	0	0	0	1(50.0%)	1 (50.0%)	0
SHR/HER/SHRE	41	4(9.8%)	2(4.9%)	2(4.9%)	31 (75.6%)	1 (2.4%)	1 (2.4%)
HR+other drugs	11	2(18.2%)	0	0	9(81.8%)	0	0
RZS	1	0	0	0	1 (100%	0	0
RES	2	1 (50.0%)	0	0	1(50.0%)	0	0
Total	**57**	**7(12.3%)**	**2(3.5%)**	**2(3.5%)**	**43(75.4%)**	**2 (3.5%)**	**1(1.7%)**
***Rifampicin mono drug resistant case was not found in present study.**							

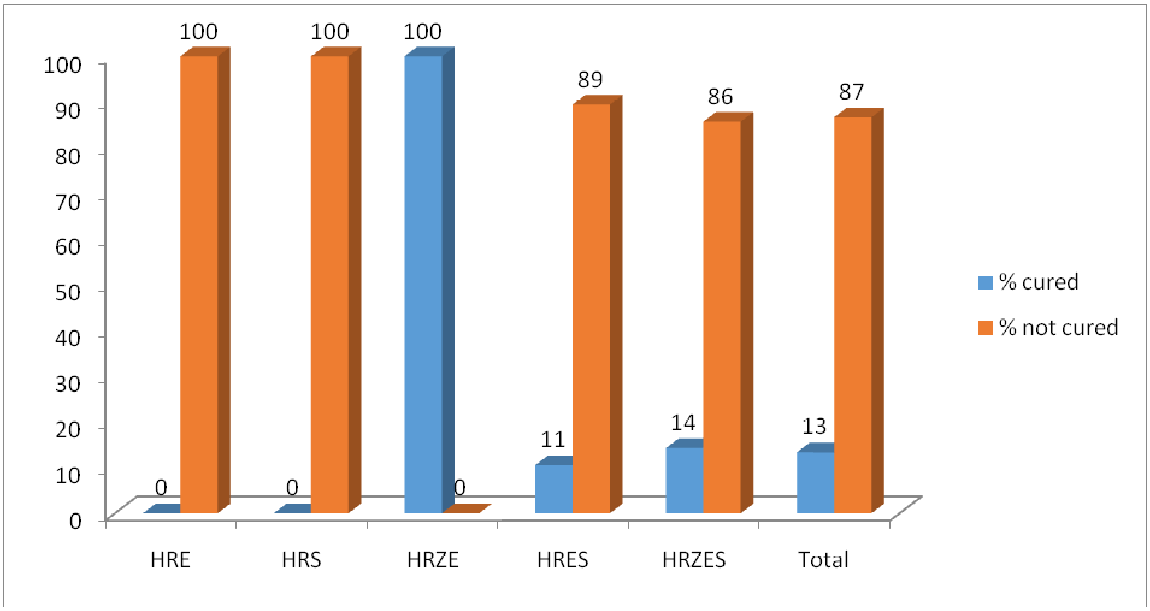
The table above shows that resistance to rifampicin was seen mostly occurring in combination with other drugs and RIF mono-resistant do not usually occur. 54 out of 57 *M. tuberculosis* isolates had RIF resistance along with isoniazid resistance. Most outward outcome of the RIF resistance was failure with 43 out of 57 (75.4%), and multi-drug resistant TB among these failures were 54 cases (94.7%).

Table 41: DOTS treatment outcome of forty-six smear positive category-I TB patients, confirmed as harboring rifampicin sensitive *M. tuberculosis* isolates by proportionate method of sensitivity test, collected during 6th October 2007 to 28th December 2009 from DOTS centres in Sikkim, India.

Anti-tubercular drug combinations	No. of TB patients	Cured	Default	Died	Failure	Transferred out
Sensitive to all (H,R,Z,E,S)	19	15 (79.0%)	2 (10.5%)	1 (5.3%)	1 (5.3%)	0 (0.0%)
H	2	2 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Z	2	1 (50.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (50.0%)
E	8	6 (75.0%)	1 (12.5%)	1 (12.5%)	0 (0.0%)	0 (0.0%)
S	8	6 (75.0%)	0 (0.0%)	1 (12.5%)	0 (0.0%)	1 (12.5%)
HE	1	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)
HS	1	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ZE	1	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ZS	1	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ES	3	2 (66.7%)	0 (0.0%)	1 (33.3%)	0 (0.0%)	0 (0.0%)
Total	**46**	**35 (76.1%)**	**3 (6.5%)**	**4 (8.7%)**	**2 (4.3%)**	**2 (4.3%)**
H= Isoniazid, R= RIF, S= Streptomycin, Z= Pyrazinamide, E= Ethambutol						

Table above shows that 76.1% of smear positive category-I TB patients harbouring rifampicin sensitive isolates are at more advantage of getting cured by end of DOTS treatment.

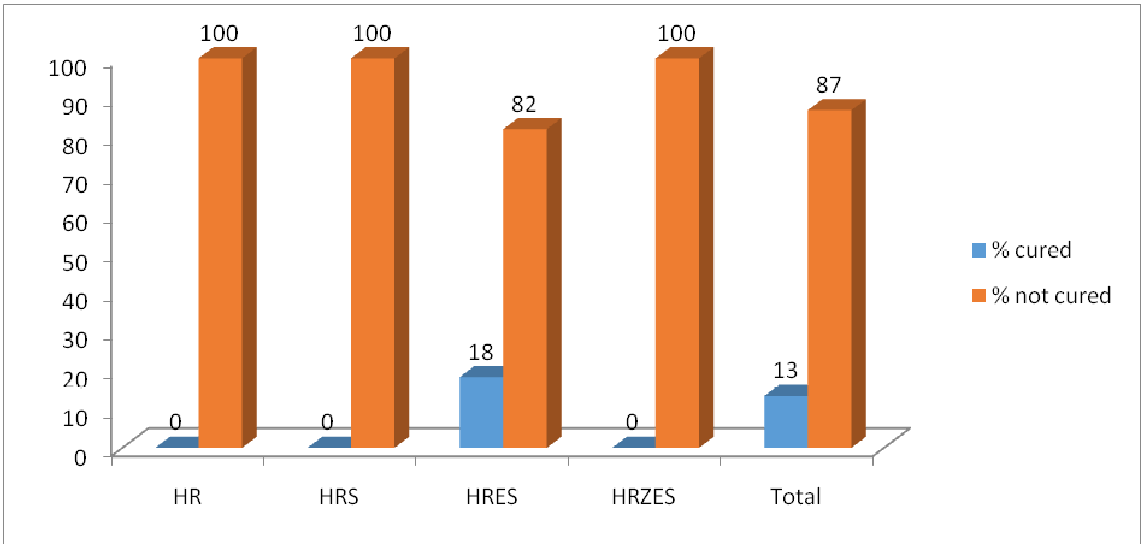
Figure 14: DOTS treatment outcome among 34 MDR-TB patients with different drug resistance pattern by proportionate method in age group < 25 years smear positive category-I TB patients in present study, samples of which were collected between 6th October 2007 and 28th December 2009 under DOTS in Sikkim, India.



H= isoniazid, R= RIF, Z= pyrazinamide, E= ethambutol, S= streptomycin, Cured= Initially sputum smear-positive patient who has completed treatment and had negative sputum smears, on two occasions, one of which was at the end of treatment, Not cured= Includes those patients belonging to default, failures, treatment completed, death and transferred out patients as DOTS treatment outcome.

Among 34 MDR-TB patients in the age group <25 years, highest resistance was observed to combination of drugs HRES, followed by combination of drugs HRZES. There were no significant differences in proportions of cured and not cured patients seen with any of the drugs combination.

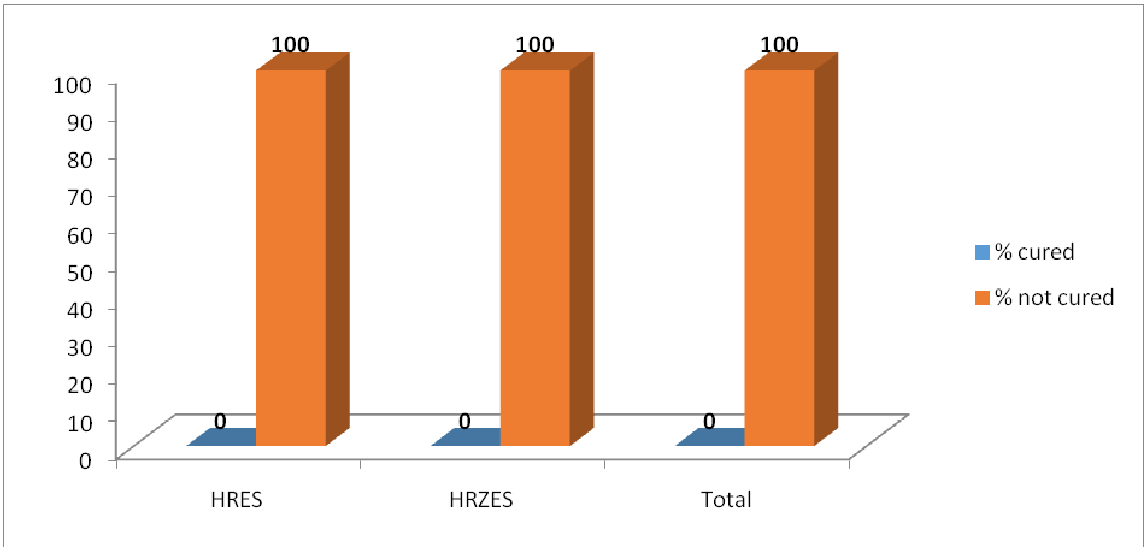
Figure 15: DOTS treatment outcome among 17 MDR-TB patients with different drug resistance pattern by proportionate method in age group <26-50 years smear positive category-I TB patients in present study, samples of which were collected between 6th October 2007 and 28th December 2009 under DOTS in Sikkim, India.



H= isoniazid, R= RIF, Z= pyrazinamide, E= ethambutol, S= streptomycin, Cured= Initially sputum smear-positive patient who has completed treatment and had negative sputum smears, on two occasions, one of which was at the end of treatment, Not cured= includes those patients belonging to default, failures, treatment completed, death and transferred out patients as DOTS treatment outcome.

Among 17 MDR-TB cases in the age group 26-50 years, highest resistance was observed to the drugs combination HRES, followed by drugs combination HRZES. The proportions of cured and not cured patients were not significantly different for any of the drugs combinations.

Figure 16: DOTS treatment outcome among 3 MDR-TB patients with different drug resistance pattern by proportionate method in age group >50 years smear positive category-I TB patients in present study, samples of which were collected between 6th October 2007 and 28th December 2009 under DOTS in Sikkim, India.



H= isoniazid, R= RIF, Z= pyrazinamide, E= ethambutol, S= streptomycin, Cured= Initially sputum smear-positive patient who has completed treatment and had negative sputum smears, on two occasions, one of which was at the end of treatment, Not cured= includes those patients belonging to default, failures, treatment completed, death and transferred out patients as DOTS treatment outcome.

There were only 3 MDR-TB cases in the age group >50 years and their outcome were not cured after treatment completion. Among this 3 MDR-TB cases, 2 were resistant to drugs combination HRES and 1 was resistant to HRZES.

Discussion

India had the second highest total number of estimated MDR-TB cases (99,000) in 2008, after China (100,000 cases) (230). According to World Bank definition, India is currently ranked 119 out of 169 countries on human development, with 41.8% of population living below the international poverty line. Socio-economic determinants such as poverty, overcrowding, food insecurity and malnutrition pose risk factors facilitating transmission of infection and disease, also responsible for inequities in accessing TB care (231). RNTCP was initiated in India in 1997 was implemented in a phased expansion achieving nation-wide programme coverage in March 2006 (232).

In India, free diagnostic and treatment services provided under RNTCP are designed to benefit the poor and vulnerable groups of the society. However, recent evidence from community based Knowledge, Attitudes and Practices (KAP) survey of 2011 conducted by IUATLD, The Union (SEARO) in Delhi suggested that people 'most in need' of free services were not accessing or utilising these services, and that a significant proportion of TB patients, illiterate and from low income rural households were being diagnosed and treated outside the DOTS/RNTCP system, and incurring expenditure (232).

Drug resistance survey conducted in the states of Gujarat and Maharashtra in 2007, and Andhra Pradesh in 2009, showed rate of MDR-TB to be 2-3% in new case and 12-17% among retreatment cases (233). But this does not reflect the data of every states in India. Under RNTCP programme, a new nation /wide drug resistance survey has been planned for 2014-2015. Under this survey, from Sikkim, Singtam TU (TB unit) in east district has been selected for drug resistance surveillance, expected to start from 14th September 2014 for a period of six to twelve months.

The data available for MDR-TB in Sikkim is, at present there are 401 MDR- TB patients registered in central registry under state plan before DOTS Plus began on 14th March 2012 and 14 under DOTS Plus plan (234). Those patients taking drugs under the State plan were mostly tested outside the state in non-accredited private labs, the report data of which could not be accepted for publication. It is mentioned that under the state plan, second line drugs supply were irregular and purchased at expensive rate from sub-standard private pharmaceutical companies. This mostly led to the transmission of MDR-TB among people and development of further XDR-TB in the tuberculosis strains.

RNTCP structure in Sikkim consists of State TB Cell headed by Director Cum State TB officer, 4 district TB Centres (DTCs) with DTOs as Programme Officer to oversee the TB Control activities of the districts, 5 Tuberculosis Unit (TU) – a nodal unit in TB control programme where registrations of patients are done, 31 Microscopic Centre (MC) out of which 20 are designated microscopic centres.

IRL lab (Intermediate Reference lab) became functional from 1st March 2013 and is under the process of getting accredition. Majority of MDR-TB cases were undiagnosed in India as indicated by RNTCP records of 2007-2010 (230). Our present study sensitivity pattern for first line drugs showed >50% new cases to have MDR-TB, supported by records of high failure rate in RNTCP register from east Sikkim to be 11.58% in 2004, 13% in 2005 and 13.6% in 2006 among new sputum positive TB patients. More than 50% RIF resistance rate has also been suggested by Gene-Xpert results at IRL (Intermediate Reference Lab), Gangtok, tested among both new cases and retreatment cases (un-published). The Gene-Xpert technique was recently

installed on 30th July 2013. Our study was initiated even before this facility came up during 2007. Still our study method, has a lot to contribute in public interest, as it not only found out the high MDR-TB prevailing among the TB circulating strains, but also is rapid (within 48 hrs), RIF resistance can be done at affordable approximate cost of Rs 2,971 per test (Gen Elute Bacterial Genomic DNA kit extraction per test was Rs 171, INNO LiPA Rif.TB kit cost for 20 tests was Rs 56,000), serves a useful test for epidemiological study due to biohazard risk free inactivated sputum positive smears from rural areas. As well as the test can be run for patients who cannot expectorate >5ml (219) or >1ml of sputum samples (235).

RNTCP DOTS was initiated in Sikkim on 1st March 2002 (234). The programme has been showing detection of at least 70% of the estimated new smear positive cases from the community and cure rate of 85% of such cases (234), the knowledge about existing MDR-TB percentage among both new and retreatment TB cases is nil, until awareness to certain extent was made by our present study, after finding >50% new cases to be MDR-TB (unpublished). Present study was done before 10 bedded, DOTS-Plus site got established in east Sikkim for the first time on 9th March 2012 (236). Before DOTS Plus started, most of the MDR-TB tests results collected were from non-accredited private laboratories outside Sikkim, sensitivity result of these labs was expensive and not reliable. Therefore the data of such tests could not be accepted for publication.

Inappropriate availability of second line drugs along with unaffordable cost of the medicines led towards increasing MDR and XDR-TB in our community (TB clinician's suggestion with their past experiences) before the DOTS Plus programme got initiated. Still today, to combat the deadly MDR and XDR-TB in our country,

accredited TB labs are few in number (230) (237), all located outside Sikkim. No research study of any kind in field of TB was conducted earlier in Sikkim, which would have been useful to the public interest.

Validity and reliability of *rpoB* mutation for early detection of Rifampicin resistance and treatment outcomes of DOTS.

In total, 29 MTBC strains were tested and successfully amplified, with a clear visible band of approximately 260bp on 2% agarose gel (Figure 17 a, b, c).

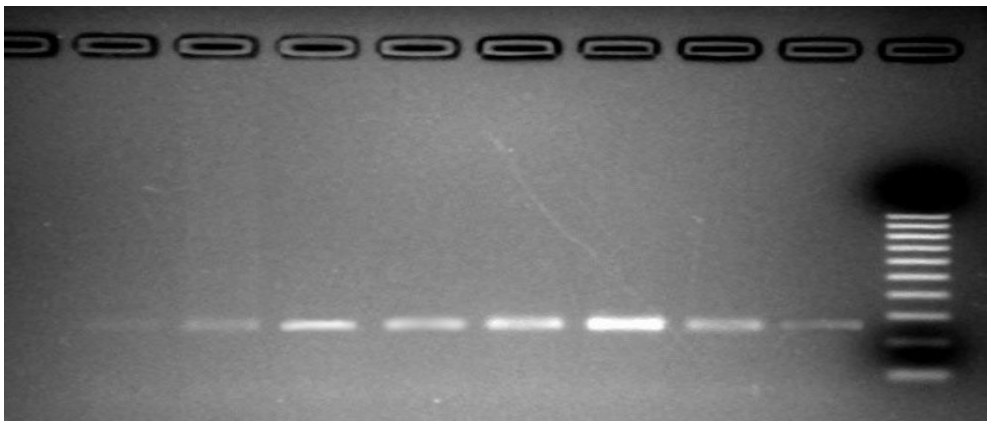


Figure 17 (a): *INNO LiPA Rif.TB amplification of 260bp bands of the rpoB gene characteristic for MTBC in 7 isolates. From left to right, Lane 1=NC, lane 2 to 8 = patients samples, lane 9=PC, Lane 10= 100bp DNA ladder (Takara) (code 341 0A Lot A401-1), NC= negative control for MTBC (added milli-Q water in place of MTB Complex NA), PC= positive control for MTBC ($H_{37}R_V$).*

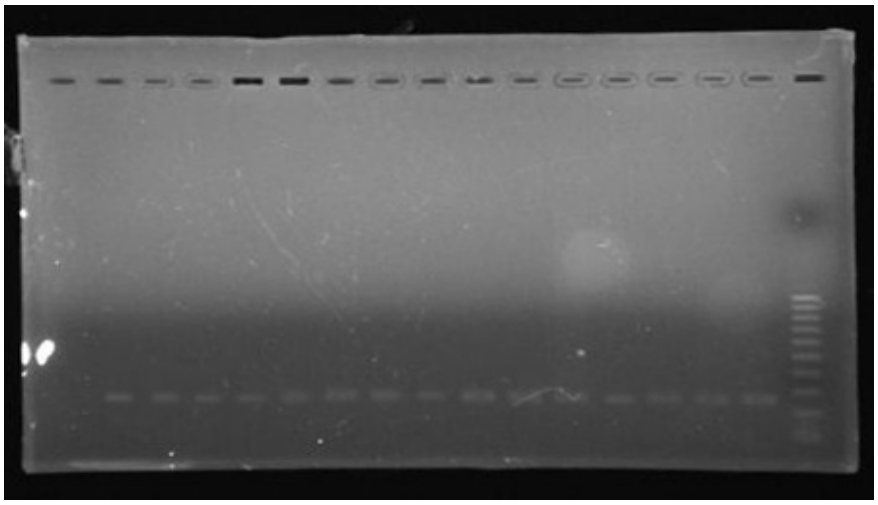


Figure 17 (b): *INNO LiPA Rif.TB amplification of 260 bp bands of rpoB gene characteristic for MTBC in 14 isolates. From left to right, Lane 1=NC, lane 2=PC, lane 3 to 16 = patients samples. Lane 17= 100bp DNA ladder (Takara) (code 341 0A Lot A401-1), NC= negative control for MTBC (added milli-Q water in place of MTB Complex DNA), PC= positive control for MTBC ($H_{37}R_V$).*

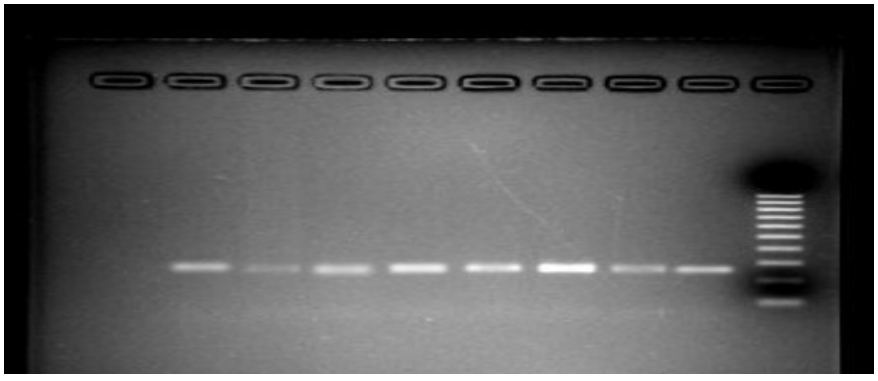


Figure 17 (c): *INNO LiPA Rif.TB amplification of 260bp bands of the rpoB gene characteristic for MTBC in 7 isolates. From Left to right, Lane 1= NC, lane 2= PC, lane 3 to 9 = patients samples. Lane 10=100bp DNA ladder (Takara) (code 341 0A Lot A401-1), NC= negative control for MTBC (added milli-Q water in place of MTBC DNA), PC= positive control for MTBC ($H_{37}R_V$)*

Table 42: DNA extracted from AFB positive sputum smear slides using Gen elute bacterial genomic kit and amplification by INNO LiPA Rif .TB assay.

AFB grading of slides given as per RNTCP manual standard	Total slides used for DNA extraction	Total slides ethanol purified (once) DNA giving positive amplified product for 81 bp *rpoB* gene	Total slides re-ethanol purified DNA giving positive amplified product for 81 bp *rpoB* gene
1+	9	6 (66.7 %)	6 (66.7 %)
2+	11	9 (81.8 %)	9 (81.8 %)
3+	24	20 (83.3 %)	24 (100 %)
PC	1	1 (100%)	1 (100 %)
NC	1	0 (0%)	0 (0 %)
Total patient's slides used for amplification	44	35 (79.5 %)	39 (88.6 %)

Table above showed that 3+ sputum smears had 100% DNA amplification for 81bp region of *rpoB* gene using INNO LiPA Rif.TB assay, followed by amplification of 81.8% in 2+ slides and 66.7% in 1+ slide smear, using DNA extracted directly from inactivated sputum smears by heat and 5% phenol in ethanol for five minutes by using Gen Elute bacterial genomic DNA kit (Sigma).

Table 43: Comparison for rifampicin susceptibility using LiPA assay, proportionate method and its correlation with patient's treatment outcome at end of DOTS Category-I treatment, during 6th October 2007 to 28th December 2009 from DOTS centres in Sikkim, India.

Sample No.	*rpoB* mutation pattern on LiPA probes										mutation pattern	LiPA assay	Proportionate method	Treatment outcome
	MTBC	S1	S2	S3	S4	S5	R2	R4a	R4b	R5				
1582	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1414	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1019	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1738	+	+	+	+	+	-	-	-	-	+	R5	R	R	Failure
1216	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1424	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1371	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1013	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1753	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1213	+	+	+	+	+	-	-	-	-	+	R5	R	R	Failure
1593	+	+	+	+	+	-	-	-	-	+	R5	R	R	Failure
1388	+	+	+	+	+	-	-	-	-	+	R5	R	R	Failure
1015	+	+	+	+	+	-	-	-	-	+	R5	R	R	Default
1177	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1002	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1195	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1221	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1646	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1047	+	+	+	+	+	+	-	-	-	-	WT	S	R	MDR
1457	+	+	+	-	+	-	-	-	-	-	ΔS3,ΔS5	R	R	Cured
1587	+	+	+	+	+	-	-	-	-	+	R5	R	R	Cured
1585	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1539	+	+	+	+	+	-	-	-	-	+	R5	R	R	Cured
1153	+	+	+	+	+	-	-	-	-	+	R5	R	R	MDR
1624	+	+	+	+	+	+	-	-	-	-	WT	S	S	Died
1165	+	+	+	+	+	+	-	-	-	-	WT	S	S	Cured
1155	+	+	+	+	+	+	-	-	-	-	WT	S	S	Cured

Two out of three patients declared cured by DOTS Category-I treatment and having RIF resistant *M. tuberculosis* found by both LiPA assay and proportionate method, relapsed back tuberculosis, with failure outcome after taking DOTS Category-II course, indicated RIF resistant bacilli was latent in patient's body, relapsed in times of poor immunity in patients (25). Alternatively for a sputum to be positive, minimum 5000 bacilli per ml should be present, or else sputum becomes negative upon microscopy examination (47). This highlights need for including culture (87) and LiPA in routine TB diagnosis along with microscopy for better patient's treatment outcome (238) (Table 4). Third Category-I cured patient having RIF resistant *M. tuberculosis*, is still living normal life, may be due to good immunity in him or due to different pathobiology and phylogeography of *M. tuberculosis* strain (239) harbouring in him and such a patient and *M. tuberculosis* strain forms a good study for future.

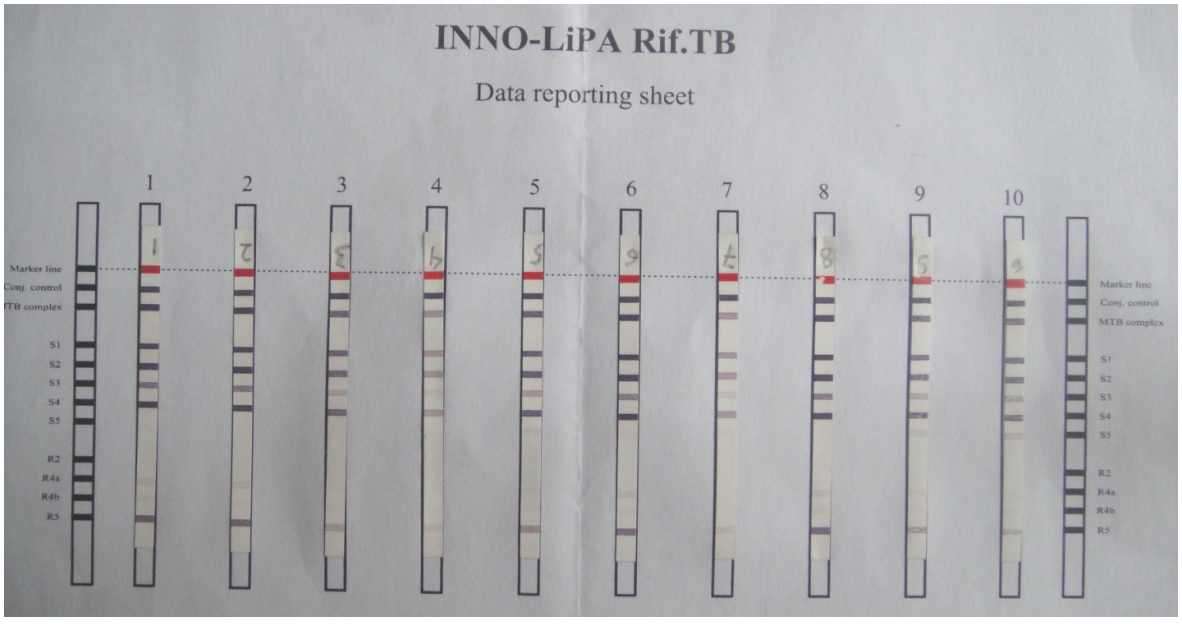


Fig 18 (a) *LiPA hybridization strip from left to right showing result for samples* (1=1213, 2=1587, 3=1414, 4=1153, 5=1177, 6=1002, 7=1221, 8=1019, 9=1738, 10=1593).

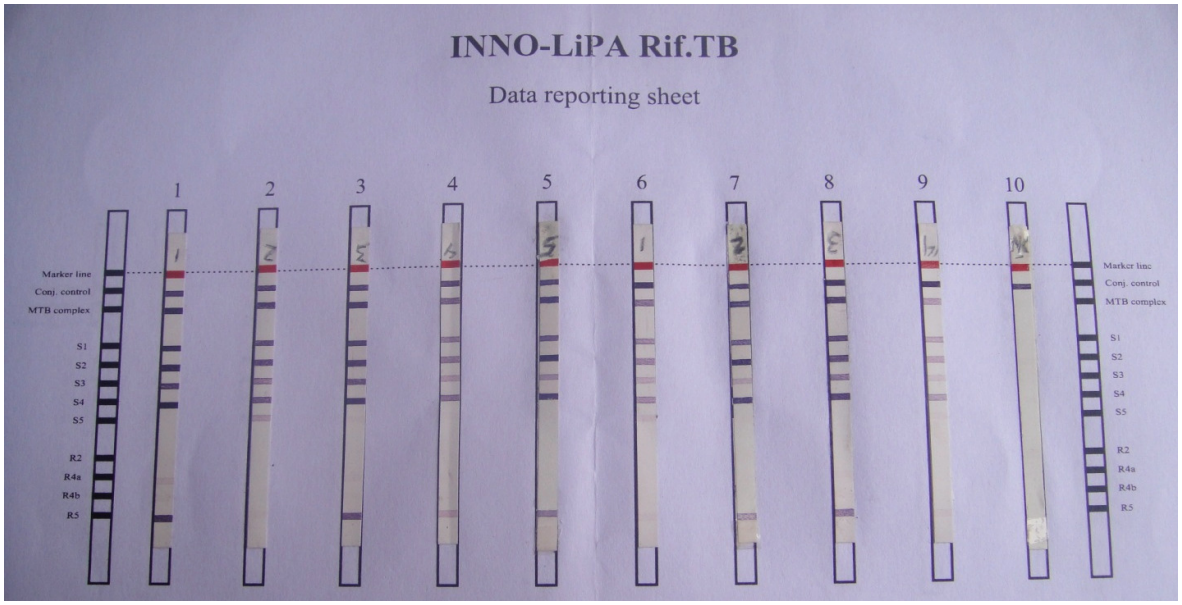


Fig 18 (b): *LiPA hybridization strip from left to right showing result for samples* (1=1195, 2=1388, 3=1646, 4=1585, 5=1015, 6=1371, 7=1013, 8=1216, 9=1539, 10=NC).

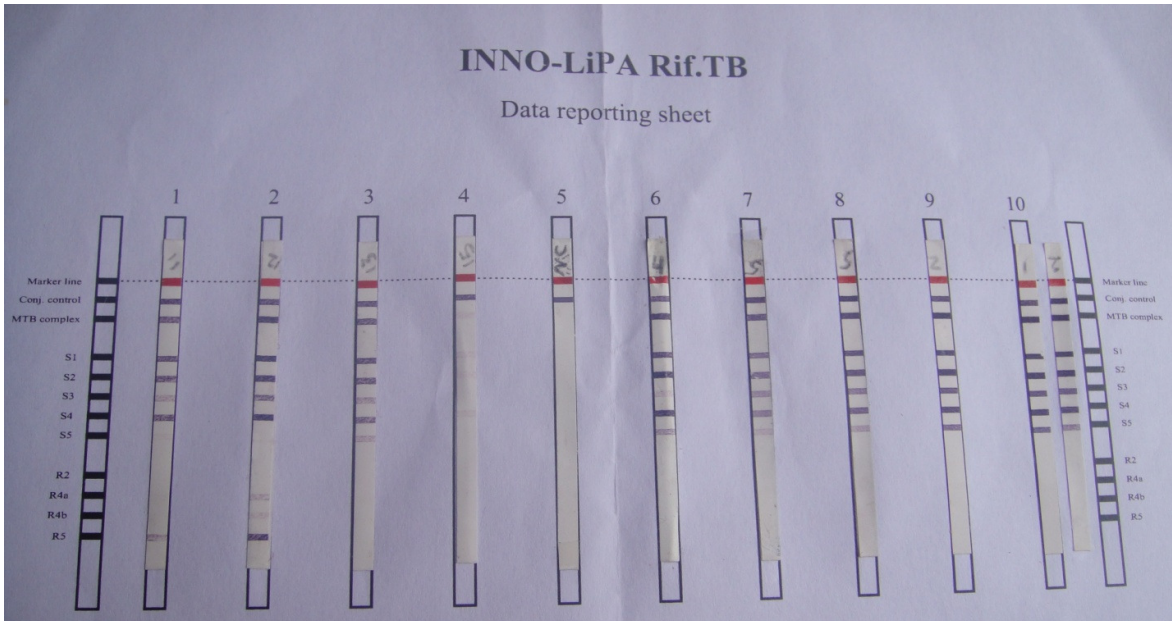


Fig 18 (c): *LiPA hybridization strip from left to right showing result for samples* (1=1582, 2=1394 (PC), 3=1047, 4=1457, 5=NC, 6=1094, 7=1624, 8=1165, 9=1155, 10=$H_{37}R_V$ (NC), 11=1241).

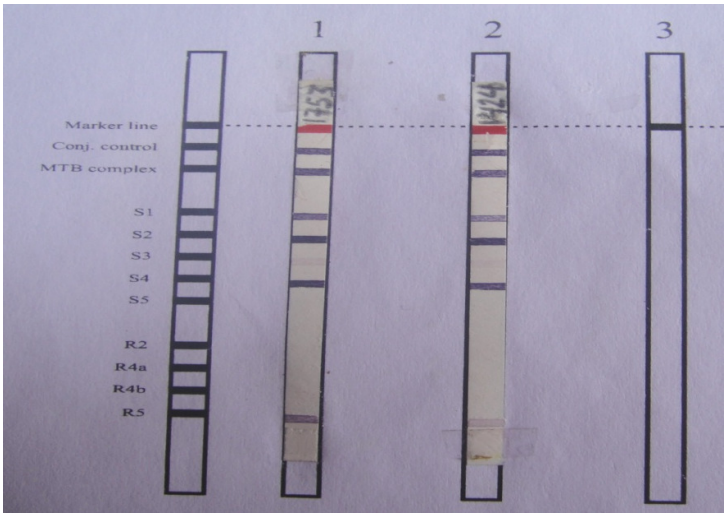


Fig 18 (d): *LiPA hybridization strip from left to right showing result for samples* (1=1753, 2=1424).

Table 44: Comparison of results obtained by INNO LiPA Rif.TB assay and proportionate method of drug susceptibility test for rifampicin sensitivity in smear positive category-I TB cases collected during 6th October 2007 to 28th December 2009 from DOTS centres in Sikkim, India.

INNO LiPA Rif.TB assay result		**Total (n=29)**	**RIF sensitivity by proportionate method**	
			Sensitive (n=5)	**Resistant (n=24)**
N=29	**R5 mutation**	22 (75.9)	0 (0.0%)	22 (91.7%)
	ΔS3, ΔS5 mutation	1 (3.4)	0 (0.0%)	1 (4.2%)
	wild type	6 (20.7)	5 (100%)	1 (4.2%)

The above table showed the relationship between mutation pattern in *rpoB* gene of *M. tuberculosis* and the sensitivity result by proportionate method. Among 24 RIF resistant *M. tuberculosis* isolates tested for *rpoB* mutation by INNO LiPA Rif.TB assay, R5 mutation was present in 22 cases (91.7%). There was significant difference in proportions of RIF sensitive and resistant cases possessing R5 mutation (p value for one tailed $z<0.001$). ΔS3 and ΔS5 mutation was present only in 1(4.2%) *M. tuberculosis* isolate. Remaining 1(4.2%) RIF resistant *M. tuberculosis* isolate identified by proportionate method was missed for mutation detection by LiPA, which might be due to presence of mutation occurring outside the RRDR region of *rpoB* gene. LiPA identified wild type strain (no mutation) in 5 (100%) RIF sensitive *M. tuberculosis* isolates tested by proportionate method.

Table 45: Validity and reliability of INNO LiPA Rif.TB assay results in comparison to rifampicin susceptibility by proportionate method of sensitivity test.

Screening of *rpoB* mutation in MTBC isolates		**RIF resistance by proportionate method of sensitivity test**		
		Resistant	**Sensitive**	**Total**
INNO LiPA Rif.TB assay	***rpoB* mutation present**	23 (95.8%)	0	23
	***rpoB* mutation absent**	1(4.2%)	5 (100.0%)	6
Total		**24**	**5**	**29**

Table 46: Validity and reliability of INNO LiPA Rif.TB assay to identify mutations in MTBC as a screening instrument for identifying rifampicin resistance in smear positive category-I tuberculosis patient's sputum smears collected during 6th October 2007 to 28th December 2009 from DOTS centres in Sikkim, India.

	Estimated Value	95% confidence interval	
		Lower	Upper
Sensitivity	96%	77	99
Specificity	100%	46	100
Positive Predictive value	100%	82	100
Negative predictive Value	83%	36	99
Accuracy	97%		

29 isolates have been used for screening *rpoB* mutation in MTBC and compared with the outcomes of the RIF sensitivity test by proportionate method. The validity and reliability tests were done and it is found that INNO LiPA Rif.TB assay is 96% (95% CI: 77-99) sensitive and 100% (95% CI: 46-100) specific for early detection of RIF resistance in MTBC isolates obtained from TB patients attending DOTS centres in Sikkim. The 97% accuracy of the technique validates the LiPA as a powerful tool for early detection of RIF resistance using clinical samples.

Table 47: Association between *rpoB* mutation and treatment outcome of smear positive category-I tuerculosis cases collected during 6th October 2007 to 28th December 2009 from DOTS centres in Sikkim, India.

Screening for *rpoB* mutation in *Mycobacterium tuberculosis* isolates		**DOTS treatment outcome**		**P value for Fisher's exact test**
		Not Cured	**Cured**	
INNO-LiPA technique to identify mutation in *Mycobacterium tuberculosis*	**Mutation Present (n=22)**	19 (86.4%)	3 (13.6%)	0.009
	Mutation Absent (n=5)	1 (20.0%)	4 (80.0%)	
Total (27)		20	7	

*One patient, with *rpoB* mutation absent and one patient with *rpoB* mutation present was not included under treatment outcome, as the patients died before completion of treatment and hence could not be placed as either cured or not cured.

Not cured= includes those patients with default, treatment completed, transferred out, failures and death outcome at the end of DOTS therapy.

The table shows significant association between *rpoB* mutation and treatment outcome (p=0.009 for Fisher's exact test). Among the 22 individuals having mutation, 86.4% have not been cured by DOTS treatment. It shows that *rpoB* mutation is a responsible factor for MDR-TB.

Table 48: Relationship between clinical outcome of smear positive category-I TB patients attending district DOTS centres in Sikkim (India), during period 6th October 2007 to 28th December 2009 with those rifampicin resistant TB patients (proportion method), whose *rpoB* mutation detection was done using INNO LiPA Rif.TB assay.

DOTS treatment Outcome	Total	*rpoB* mutation by LiPA assay		P value for z-test (one tailed)
		No mutation (n=1)	Mutation (n=23)	
Cured	3 (12.5%)	0 (0.0%)	3 (13.0%)	0.03
Died	1 (4.2%)	0 (0.0%)	1 (4.3%)	0.16
Failure	4 (16.7%)	0 (0.0%)	4 (17.4%)	0.01
MDR	14 (58.3%)	1 (100.0%)	13 (56.5%)	<0.0001
XDR	1 (4.2%)	0 (0.0%)	1 (4.3%)	0.15

The table above shows the relationship between clinical outcomes with the rpo*B* mutation. Z test for proportions shows that significant difference between MDR-TB cases in which mutation was present with those in which there was no mutation present. Significant difference in proportions of mutants with the non-mutants were also observed among cured and failure cases.

Discussion

Among the rapid tests available at market, most of them use fresh culture growth for extracting DNA (240). This delays the test rapidity as the TB bacterial growth takes 10-14 days in liquid medium and 4-8 weeks in traditional solid medium (241). Culture, considered the gold standard method in TB testing, faces the disadvantage

due to time taking more than 3-4 months to produce sensitivity results (219). In molecular biology field, sequencing is considered to be the gold standard, but due to high equipment cost, complexity of the method, requirement of skilled labour, the test becomes too complex for routine widespread implementation in developing countries (235). Line probe assay (MTB-DR Plus assay) was endorsed by WHO to use in public health sectors on June 2008 (240). This test detecting resistance susceptibility to RIF and INH for Sikkim is being done presently under RNTCP in LRS Institute, Delhi which started doing this test from Oct 2011 unwards (242). Though the assay is modification of INNO LiPA Rif.TB technique, with just the addition of hybridization probe for INH to existing RIF hybridization probe, the time required for giving result is two to three weeks, for obtaining DNA from culture growth. In this regard, our study can be implemented in peripheral health centres as the method is rapid (<48hrs), simple, less costly, easy to perform, require only PCR machine and do not require skillfull expertise (mostly manual).

Proportionate method used for drug susceptibility testing in our study was done as a supportive test to validate LiPA's results. Under RNTCP, proportionate method using first line drugs is standardized (220) and is done at accredited labs but for anti-TB second line testing as of Jan 2012, diagnosis of XDR-TB can be confirmed only at three quality assured labs in India. These are National Reference Laboratories (NRL) of TRC/NIRT Chennai, NTI Bangalore and LRS Institute, New Delhi (243). Because of this reason, in our study, we did not do the second line ant-TB testing. First line anti-TB tests were performed to confirm INNO LiPA Rif.TB assay results.

Various workers tried to extract DNA from slides for different purpose like PCR RFLP, spoligotyping, sequencing etc. (130, 218, 244, 245). Whereas we used slide for

doing INNO LiPA Rif.TB test recommended to use fresh culture harvested DNA samples by other studies detecting, characterizing and studying concordance with the classical susceptibility test to be 100% (176) and in another study found sensitivity profile was concordant but failed to match 100% to detect rifampicin resistance (177). In both the study done, they used culture growth, which partially defeat the rapidity of detection. In present study, we tried modifying INNO LiPA Rif.TB assay in a way, by skipping DNA extraction from culture growth and, replacing it with DNA directly extracted from positive smears. Some workers had tried using Ziehl Neelsen stained slides for the purpose of detecting RIF resistance with success rate of 84%. Though the slides were inactivated, the drawback was that the DNA was extracted using chelex, which makes DNA easily degradable if not used immediately after extraction (130). Same way another study detecting rifampin resistance in 91.1% of the samples was done by Naga Suresh et al. in 2007, extracting DNA from ZN-stained sputum smears using 5% chelex followed by twice purification by phenol–chloroform and ethanol precipitation (218). But in our case, DNA samples did not deteriorate when placed at -80^{o}C for long.

In 2010, a study by Wallis et al. stated INNO LiPA kit for molecular detection of MTBC and RIF resistance was stated to give lower sensitivity while performing on clinical samples and high performance when used on fresh culture harvested DNA samples (246). In our study, we used fourty-four smears for DNA extraction [Table 1]. The DNA was kept at -40^{0}C once extracted. Using INNO LiPA Rif.TB amplification kit, the amplification yield was 79.5% (thirty-five samples). Further second round ethanol purification to the negative DNA samples (Seven DNA samples: four 3+ slides extracted DNA samples, two 2+ slides extracted DNA, and one 1+ slide extracted DNA sample) stored at -80^{0}C, four DNA templates (3+),

yielded the desired amplicons with line probe assay, increasing the amplification to 88.6% (thirty-nine samples with 66.7% for 1+, 81.8% for 2+ and 100% for 3+ slide smears). This was due to PCR inhibitors present in the clinical samples, which got removed in second purification by ethanol. The study result suggests, the amplification success rate depends upon the load of bacilli present at the time of smear preparation. Controls used in our study showed result as expected in the manual as: $H_{37}R_V$ DNA extracted using same method was amplified and no product amplified from negative control containing milli-Q water in place of DNA.

Among the thirty-nine 81bp rpo*B* amplied products, we tested 29 samples for hybridization. All the 29 amplied samples showed MTBC isolates in the hybridization strip. With regard to determination of rifampicin susceptibility by LiPA, 22 (91.7%) samples had single *rpoB* mutation (R5), one sample (4.2%) with double *rpoB* mutations (ΔS3,ΔS5) and one more sample (4.2%) with wild type pattern. All twenty-three *rpoB* mutated samples were rifampicin resistant by proportionate method and all non-mutated samples were rifampicin sensitive like other's study (107) except for one wild sample, proved rifampicin resistant by proportionate method. Absence of *rpoB* mutation in one rifampicin resistant sample might be due to presence of mutation lying outside the 81bp region of *rpoB* gene (109, 177) as was found in study from other regions of India (177) or maybe due to hetero-resistance (mixture of susceptible and resistant subpopulations) or changes occurring in genes whose products participate in antibiotic permeation or metabolism or due to some other mechanism like drug efflux system (87) or novel mutations (107).

In our study, the correlation of LiPA with proportionate method was 96% (95% CI: 77-99) sensitive and 100% specific (95% CI: 46-100) as was found in some other studies where LiPA showed good correlation of *rpoB* mutations with levels of RIF

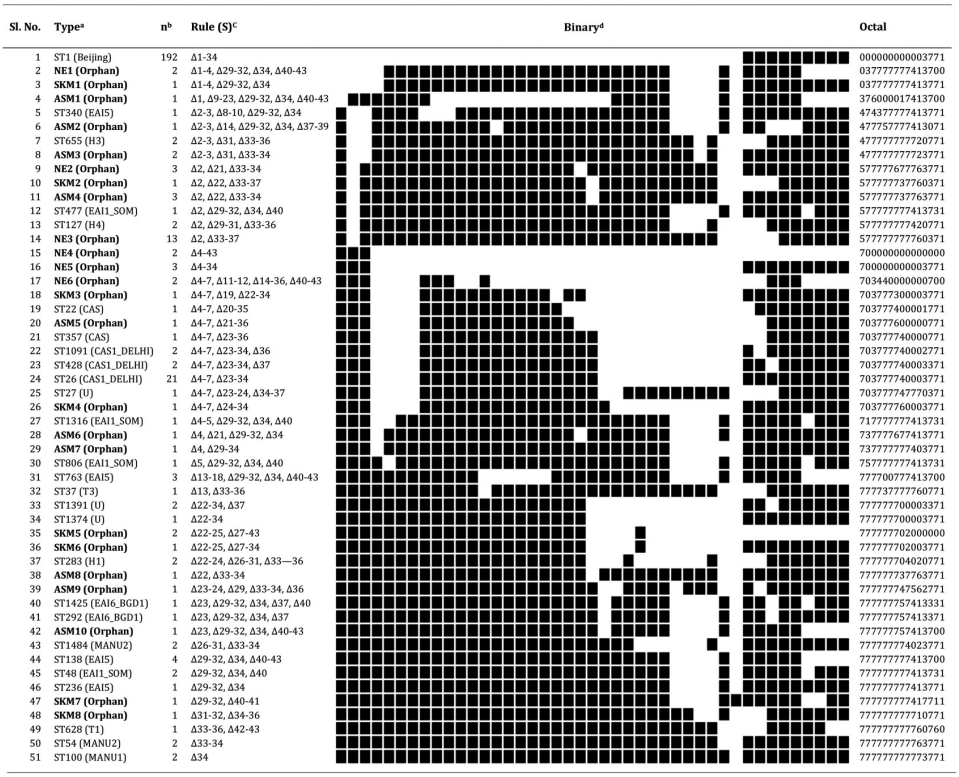
resistance in *M. tuberculosis* complex strains (177). The accuracy of LiPA to detect RIF susceptibility using positive sputum samples was 97% like few other studies (176, 177) making LiPA a convenient tool for tuberculosis control (174), and this verifies that LiPA can become more better if slight modification by including or excluding some hybridization probes are done as suggested by few researchers (107, 177) and it will be best to use along with conventional method for 100% confirmation of RIF resistant cases (87).

LiPA detected *rpoB* mutations in 95.8% (23 out of 24) of RIF resistant cases in sputum samples as compared to other's findings with100% specificity to classical susceptibility method (176) and definitiveness 60% samples as having RIF resistant *Mycobacterium tuberculosis* isolates using culture isolates (177). All RIF resistant cases were resistant to INH drug as was indicated in others's studies (36, 67). Worldwide, most frequently reported mutations are Ser- 531-Leu followed by His-526-Tyr and His-526- Asp, all reliably detected and differentiated by LiPA (109).In a study from Delhi (India), LiPA detected 42.1% of *rpoB* mutation at codon 531and found 94.73% concordance of LiPA with phenotypic method using culture isolated DNA template for LiPA assay(178). Majority of *rpoB* mutations in this study was at R5 probe region (S531L) unlike the majority *rpoB* mutation (His-526-Tyr) reported from Northern India (109). Occurrence of almost same mutation in majority of RIF resistant samples indicates same mutational events occurring in unrelated tubercular strains rather than spreading of related *M. tuberculosis* in community (109). No mix infection with more than one *M. tuberculosis* strains as seen by other's study using LiPA was found in this study (109).

A first insight on the molecular diversity of *Mycobacterium tuberculosis* complex based on spoligotyping and MIRU-VNTR typing profiles in the north-eastern region of India.

Spoligotyping

The 300 MTBC isolates from NE region were found to represent 51 different spoligotypes shown in figure 19.



Sl. No.	Type[a]	n[b]	Rule (S)[c]	Octal
1	ST1 (Beijing)	192	Δ1-34	000000000003771
2	**NE1 (Orphan)**	2	Δ1-4, Δ29-32, Δ34, Δ40-43	037777777413700
3	**SKM1 (Orphan)**	1	Δ1-4, Δ29-32, Δ34	037777777413771
4	**ASM1 (Orphan)**	1	Δ1, Δ9-23, Δ29-32, Δ34, Δ40-43	376000017413700
5	ST340 (EAI5)	1	Δ2-3, Δ8-10, Δ29-32, Δ34	474377777413771
6	**ASM2 (Orphan)**	1	Δ2-3, Δ14, Δ29-32, Δ34, Δ37-39	477757777413071
7	ST655 (H3)	2	Δ2-3, Δ31, Δ33-36	477777777720771
8	**ASM3 (Orphan)**	2	Δ2-3, Δ31, Δ33-34	477777777723771
9	**NE2 (Orphan)**	3	Δ2, Δ21, Δ33-34	577777677763771
10	**SKM2 (Orphan)**	1	Δ2, Δ22, Δ33-37	577777737760371
11	**ASM4 (Orphan)**	3	Δ2, Δ22, Δ33-34	577777737763771
12	ST477 (EAI1_SOM)	1	Δ2, Δ29-32, Δ34, Δ40	577777777413731
13	ST127 (H4)	2	Δ2, Δ29-31, Δ33-36	577777777420771
14	**NE3 (Orphan)**	13	Δ2, Δ33-37	577777777760371
15	**NE4 (Orphan)**	2	Δ4-43	700000000000000
16	**NE5 (Orphan)**	3	Δ4-34	700000000003771
17	**NE6 (Orphan)**	2	Δ4-7, Δ11-12, Δ14-36, Δ40-43	703440000000700
18	**SKM3 (Orphan)**	1	Δ4-7, Δ19, Δ22-34	703777300003771
19	ST22 (CAS)	1	Δ4-7, Δ20-35	703777400001771
20	**ASM5 (Orphan)**	1	Δ4-7, Δ21-36	703777600000771
21	ST357 (CAS)	1	Δ4-7, Δ23-36	703777740000771
22	ST1091 (CAS1_DELHI)	2	Δ4-7, Δ23-34, Δ36	703777740002771
23	ST428 (CAS1_DELHI)	2	Δ4-7, Δ23-34, Δ37	703777740003371
24	ST26 (CAS1_DELHI)	21	Δ4-7, Δ23-34	703777740003771
25	ST27 (U)	1	Δ4-7, Δ23-24, Δ34-37	703777747770371
26	**SKM4 (Orphan)**	1	Δ4-7, Δ24-34	703777760003771
27	ST1316 (EAI1_SOM)	1	Δ4-5, Δ29-32, Δ34, Δ40	717777777413731
28	**ASM6 (Orphan)**	1	Δ4, Δ21, Δ29-32, Δ34	737777677413771
29	**ASM7 (Orphan)**	1	Δ4, Δ29-34	737777777403771
30	ST806 (EAI1_SOM)	1	Δ5, Δ29-32, Δ34, Δ40	757777777413731
31	ST763 (EAI5)	3	Δ13-18, Δ29-32, Δ34, Δ40-43	777700777413700
32	ST37 (T3)	1	Δ13, Δ33-36	777737777760771
33	ST1391 (U)	2	Δ22-34, Δ37	777777700003371
34	ST1374 (U)	1	Δ22-34	777777700003771
35	**SKM5 (Orphan)**	2	Δ22-25, Δ27-43	777777702000000
36	**SKM6 (Orphan)**	1	Δ22-25, Δ27-34	777777702003771
37	ST283 (H1)	2	Δ22-24, Δ26-31, Δ33—36	777777704020771
38	**ASM8 (Orphan)**	1	Δ22, Δ33-34	777777737763771
39	**ASM9 (Orphan)**	1	Δ23-24, Δ29, Δ33-34, Δ36	777777747562771
40	ST1425 (EAI6_BGD1)	1	Δ23, Δ29-32, Δ34, Δ37, Δ40	777777757413331
41	ST292 (EAI6_BGD1)	1	Δ23, Δ29-32, Δ34, Δ37	777777757413371
42	**ASM10 (Orphan)**	1	Δ23, Δ29-32, Δ34, Δ40-43	777777757413700
43	ST1484 (MANU2)	2	Δ26-31, Δ33-34	777777774023771
44	ST138 (EAI5)	4	Δ29-32, Δ34, Δ40-43	777777777413700
45	ST48 (EAI1_SOM)	2	Δ29-32, Δ34, Δ40	777777777413731
46	ST236 (EAI5)	1	Δ29-32, Δ34	777777777413771
47	**SKM7 (Orphan)**	1	Δ29-32, Δ40-41	777777777417711
48	**SKM8 (Orphan)**	1	Δ31-32, Δ34-36	777777777710771
49	ST628 (T1)	1	Δ33-36, Δ42-43	777777777760760
50	ST54 (MANU2)	2	Δ33-34	777777777763771
51	ST100 (MANU1)	2	Δ34	777777777773771

Figure 19: *List of spoligotypes of MTBC present in NE Region (Assam & Sikkim).* *[=] *Unassigned types in the SpolDB4 database are designated as orphan, a = Standard international type strains are designated as in the SpolDB4 database and orphan types have been designated as: Six orphan spoligotypes found both in Assam and Sikkim are named as NE1 to NE6. Ten orphan spoligotypes from Assam are designated as ASM1 to ASM10 and eight orphan spoligotypes from Sikkim are designated as SKM1 to SKM8. b = Frequency of MTBC isolates, c = Rules as identified by absence of spacers, d = Dark boxes represented positive hybridization and empty boxes represents no hybridization i.e. absence of spacers.*

Out of 51 spoligotypes detected 28 (54.9%) were unique and represented by only 1 isolate whereas 23(45.1%) spoligotypes formed clusters. The number of clustering isolates was 272 and the clustering rate was 90.6% (Table 49).

Table 49: Discriminatory power of 43 spacer spoligotyping analysis of 300 *Mycobacterium tuberculosis* complex (MTBC) isolates from north-eastern region of India.

Study area	Total No of type patterns	No of unique types	No of clusters	No of clustered isolated (%)	Maximum no of isolates in a cluster	HGDI
ASSAM	43(N=164)	31	12	133(81.09)	92	0.6779
SIKKIM	20(N=136)	14	6	122(89.70)	100	0.4542
Combined NE	51(N=300)	28	23	272(90.66)	192	0.5840

Out of 300 MTBC isolates, 255 (85%) belong to the known spoligotypes (Figure 20).

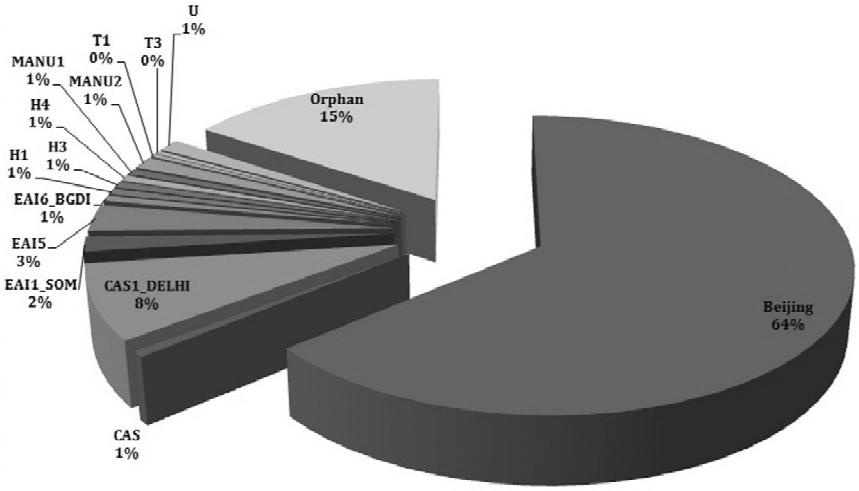


Figure 20: *Distribution of spoligotypes of MTBC isolates in NE region (Assam & Sikkim).*

The remaining 45 MTBC isolates belonged to 24 orphan spoligotypes i.e. those spoligotypes not present in international database SpolDB 4.0 (214). The Beijing clade (ST1) was found to be the most dominant spoligotype representing about 64% of MTBC isolates and CAS1_Delhi was represented 8% of the total isolates (Figure 20). The orphan spoligotypes represented 24 different spoligotypes among them orphan type NE3 (spoligo pattern Δ2, Δ33-37) was the most dominant and contributed 28.88% (n=45) of the total orphan strains (Figure 21).

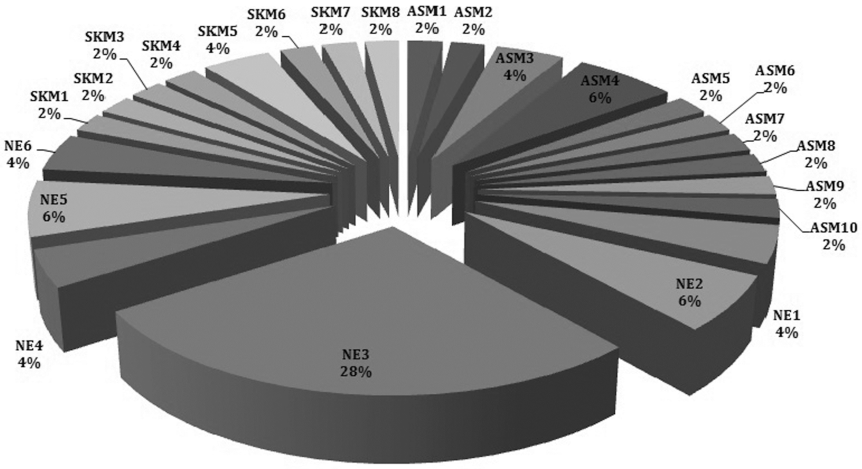


Figure 21: *Orphan spoligotypes of MTBC isolates in NE region (Assam & Sikkim).*

Prevalence of Multiple Drug Resistance according to spoligotypes

The presence of Multiple Drug Resistance (MDR) in MTBC isolates was significantly more (p=0.034) in Beijing strains (26.9%, n=156) as compared to Non-Beijing strains (17.9%, n=78).

Principal components analysis (PCA) of the spoligotypes data

The result of PCA is given in figure 22. Clustering of the geographical areas on the basis of similarity in the pattern of the genetic diversity based on spoligotypes clearly shows that the MTBC isolates from Assam and Sikkim have similar genetic profile with neighbouring southeast Asian countries like China, Thailand, Japan, Korea *etc.* and do not resemble MTBC genotypes circulating in the rest of India.

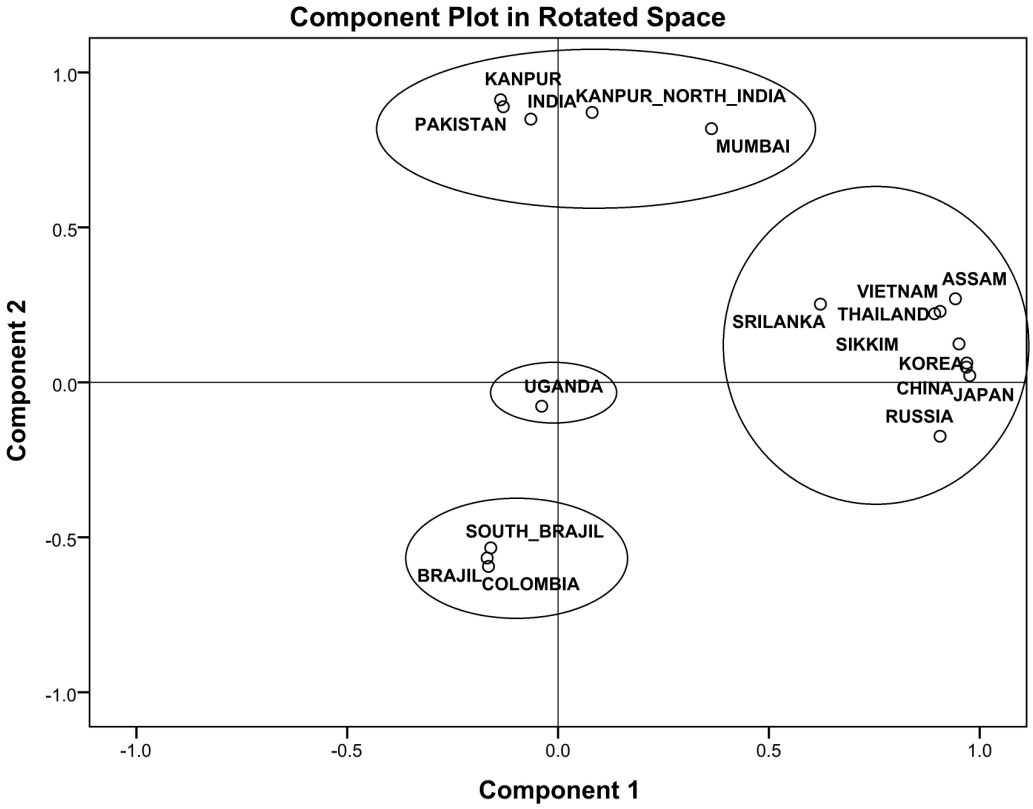


Figure 22: *A scatter plot based on principal component analysis showing relatedness of different geographical regions based on similarity in pattern of genetic diversity in MTBC isolates based on spoligotyping.*

MIRU-VNTR typing

Table 50 summarizes the allelic profiles and diversity of the 15-loci MIRU-VNTR in MTBC isolates from N-E region. MIRU locus 26 and MIRU locus 10 were found to be most discriminatory loci based on allelic diversity generated by Hunter-Gaston Discriminatory Index (HGDI). However, it was found that both these loci showed lower discriminatory power in Beijing strains of MTBC as compared to non-Beijing strains of MTBC (Table 50).

Table 50: The allelic profiles and diversity of each of the 15 VNTR loci in *M. tuberculosis* isolates from NE Region (2009-2012).

			Copy number of tandem repeat unit(s)									Allelic diversity (Total)	BEIJING	NON BEIJING
Sl. No	Alias	Locus	3	4	5	6	7	8	9	10	11			
1	**MIRU02**	**154**	0	3	47	250	0	0	0	0	0	0.281850	0.265052	0.313430
2	**ETR-C**	**577**	0	0	0	0	2	6	53	239	0	0.334783	0.162249	0.539287
3	**MIRU04**	**580**	250	37	11	2	0	0	0	0	0	0.289922	0.153578	0.486501
4	**MIRU40**	**802**	0	10	283	5	2	0	0	0	0	0.109052	0.050993	0.207165
5	**MIRU10**	**960**	0	0	59	190	3	48	0	0	0	0.536299	0.343586	0.645725
6	**MIRU16**	**1644**	1	1	284	14	0	0	0	0	0	0.101962	0.070626	0.156802
7	**MIRU20**	**2059**	2	296	2	0	0	0	0	0	0	0.026488	0.020724	0.036691
8	**ETR-A**	**2165**	0	0	16	281	1	0	2	0	0	0.120156	0.050993	0.233818
9	**ETR-B**	**2461**	7	281	11	1	0	0	0	0	0	0.121159	0.020724	0.280028
10	**MIRU23**	**2531**	0	0	0	288	10	1	1	0	0	0.077525	0.050993	0.124264
11	**MIRU24**	**2687**	0	0	276	24	0	0	0	0	0	0.147692	0.050993	0.292662
12	**MIRU26**	**2996**	0	0	53	2	16	183	46	0	0	0.572196	0.319154	0.665455
13	**MIRU27**	**3007**	9	288	1	0	1	1	0	0	0	0.077726	0.070626	0.090689
14	**MIRU31**	**3192**	0	0	0	0	4	3	1	200	92	0.462765	0.496019	0.254413
15	**MIRU39**	**4348**	0	12	286	0	0	0	0	2	0	0.089810	0.030923	0.187781

A dendrogram based on 15 polymorphic MIRU-VNTR loci generated by neighbour joining hierarchical cluster analysis of 300 MTBC isolates representing Beijing and non-Beijing families from NE are given in figure 23 and figure 24.

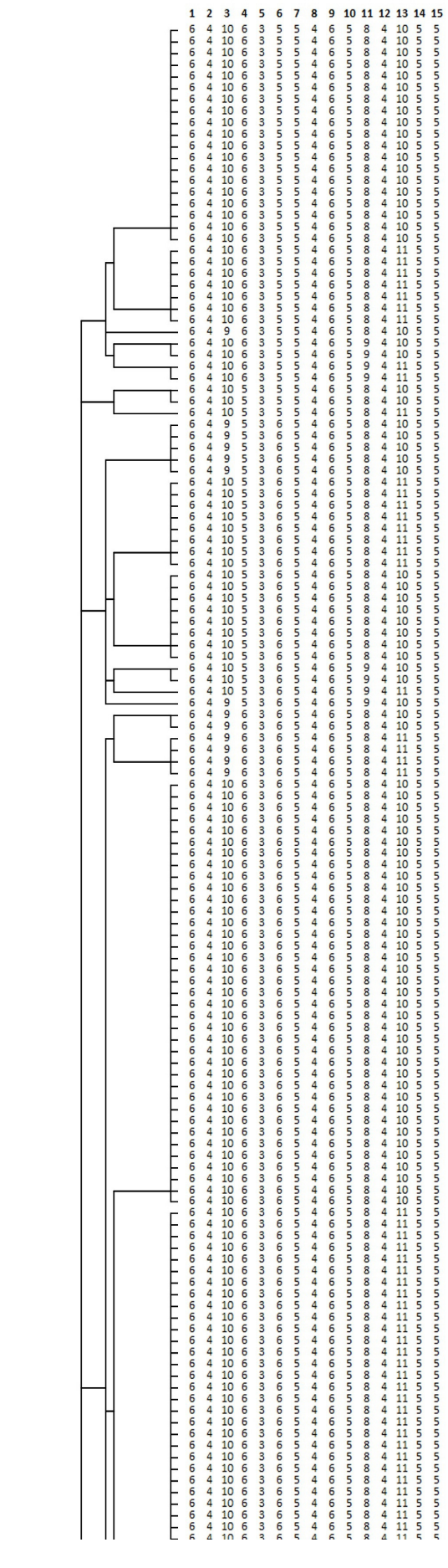

MIRU-VNTR NJ
MIRU-VNTR alleles
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 23: *A neighbour joining dendrogram based on MIRU-VNTR typing of 192 MTBC isolates belonging to Beijing family from Assam and Sikkim. The MIRU-VNTR types 1-15 are as follows: 1=ETR-A; 2=ETR-B; 3=ETR-C; 4=MIRU02; 5=MIRU04; 6=MIRU10; 7=MIRU16; 8=MIRU20; 9=MIRU23; 10=MIRU24; 11=MIRU26; 12=MIRU27; 13=MIRU31; 14=MIRU39; 15=MIRU40.*

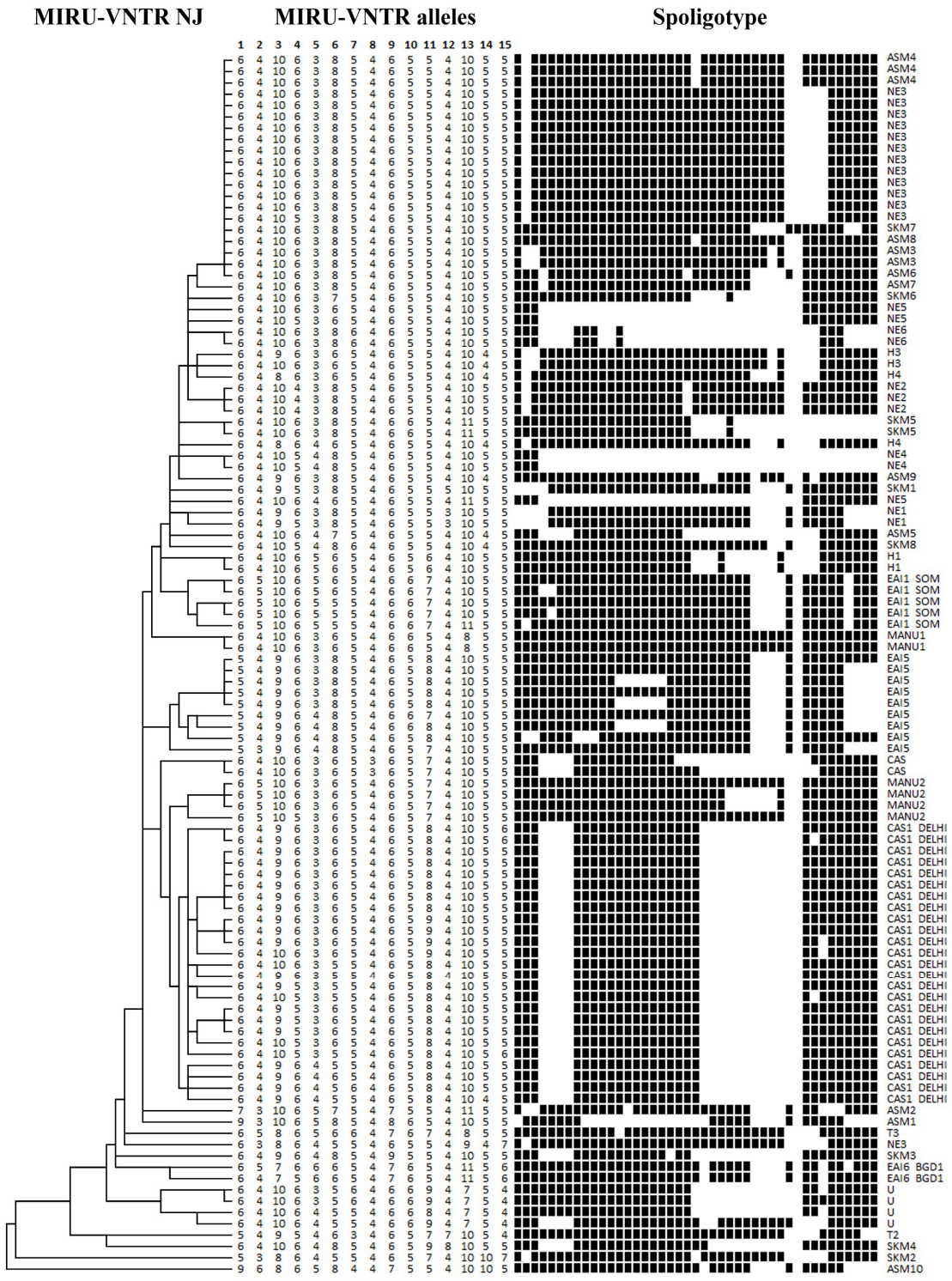


Figure 24: *A neighbour joining dendrogram based on MIRU-VNTR typing of 108 MTBC isolates belonging to Non-Beijing families from Assam and Sikkim. The MIRU-VNTR types 1-15 are as follows: 1=ETR-A; 2=ETR-B; 3=ETR-C; 4=MIRU02; 5=MIRU04; 6=MIRU10; 7=MIRU16; 8=MIRU20; 9=MIRU23; 10=MIRU24; 11=MIRU26; 12=MIRU27; 13=MIRU31; 14=MIRU39; 15=MIRU40.*

The 300 MTBC isolates of NE region were found to represent 99 MIRU-VNTR types out of which 65 types were unique, i.e. each type is represented by only one MTBC isolate and 34 genetic types formed clusters and the clustering rate was (78.33%; Table 51).

Table 51: Discriminatory power of 15-loci MIRU- VNTR analysis of 300 *Mycobacterium tuberculosis* complex (MTBC) isolates from north-eastern region of India.

Comparison according to Geography	Total No of type patterns	No of unique types	No of clusters	No of clustered isolated (%)	Maximum no of isolates in a cluster	HGDI	Beijing	Non Beijing
ASSAM	64(N=164)	40	24	124(75.60)	21	0.9562	0.8996	0.95266
SIKKIM	45(N=136)	27	18	107(78.67)	27	0.9310	0.8824	0.968254
Combined NE	99(N=300)	65	34	235(78.33)	39	0.9497	0.90499	0.95344
Comparison according to spoligotypes								
BEIJING	50(N=192)	29	21	163(84.89)	39	0.90499	-	-
NON-BEIJING	46(N=108)	22	24	80(78.43)	21	0.95344	-	-

The maximum number of isolates in a cluster was 39 which was formed by Beijing isolates with a clustering rate of 84.9% where as in non-Beijing isolates the maximum

cluster size was 21 and the clustering rate was 78.4%. This difference in clustering rate was statistically significant ($p \leq 0.05$). The 164 MTBC isolates from Assam were represented by 64 MIRU-VNTR types and 136 MTBC isolates from Sikkim were represented by 45 MIRU-VNTR types. The Hunter-Gaston Discriminatory index (HGDI) of 15-loci MIRU-VNTR typing analysis was 0.9497. However, the HGDI was lower in the Beijing strains as compared to non-Beijing strains.

Discussion

This report presents the first insight into the molecular diversity of *Mycobacterium tuberculosis* complex from the north eastern states of India. The Beijing family of MTBC strain was dominant in both the states of Assam and Sikkim representing 64% of the total strains. The dominance of Beijing genotype in NE region is a matter of great concern as this genotype has been associated with high frequency of the drug resistance (247, 248); and treatment failure (249). Moreover, the Beijing genotype is known to cause epidemic outbreaks in several countries because of their high adaptability and also this strain is considered to be less sensitive to BCG vaccination (250, 251). In this study we also found that the frequency of MDR was more in Beijing genotype as compared to non-Beijing genotypes. The principal component analysis has shown that the population genetic structure of MTBC isolates from NE region are quite distinct and are more similar to the MTBC population structure as reported from southeast Asian countries rather than what is found in other states of India. In India Beijing/Beijing-like strains of MTBC are less prevalent and their frequency ranges from 3-7% (207) on the other hand CAS and EAI are more dominant strains of MTBC in rest of India (201-203, 208, 210).

Since spoligotyping is known to have less discriminatory power so we also carried out 15-loci MIRU-VNTR typing for all the 300 isolates in this study. The result of MIRU-VNTR analysis has shown that at least 99 different genotypes of MTBC isolates exist in NE region. The present study has revealed that the 15-loci MIRU-VNTR analysis has a good discriminatory power for non-Beijing MTBC strains; however, for MTBC strains' belonging to the Beijing clade the discriminatory power of 15-loci MIRU-VNTR is quite less. This study has also revealed that 84.9% of MTBC strains show clustering and the maximum size of cluster was 39 MTBC strains belonging to Beijing strain. For non-Beijing strain the largest cluster contained 21 MTBC isolates. The high rate of clustering in MTBC types in NE region indicates that more attention needs to be given in this region for preventing the spread of MTBC in the community and that target will be achieved by strengthening the current TB control programs and making them more effective. On the other hand, in recent studies carried out in the other states of India it has been shown that clustering of MTBC strains is much less extent(203, 252). The present study also indicates that more loci of MIRU-VNTR need to be studied to evaluate genetic diversity of MTBC isolates in this region where Beijing strains are dominant. In conclusion, this study suggest that continuation of the monitoring of clustering in MTBC genotypes needs to be undertaken locally so as to get an insight into the efficiency of TB control programme in Sikkim and Assam of NE region. At present the high clustering rate is an indicator of high scale of active transmission of MTBC going on in NE region.

CHAPTER 7

CONCLUSION

1. 103 culture positive samples were tested by proportionate method of sensitivity, and 57 (55.3%) were found as to be RIF resistant.

2. Finding of 57 (55.3%) cases out of 103 tested for RIF resistance in the study samples tested by proportionate method of sensitivity testing, makes us realize that multi-drug-resistant *M. tuberculosis* are emerging in Sikkim. This warns us the danger of getting MDR infection in mostly public places (parks, markets, restaurants, cinema hall) and working places with too many people in small space (prison, offices, passengers vehicles) etc. Hence the rapid& early detection method such as INNO LiPA Rif.TB tool seems very convenient and great advantage over the sputum microscopy used in DOTS centres.

3. Once the RIF susceptibility status is known for a TB patients, the patient can be put on correct treatment (MDR-TB drugs). This will save the patient from clinically deteriorating his present condition as well as will help in stoping his RIF resistant strains from transmission to other contacts. In total it will save the tome of DOTS workers and also cut down the Government's expenditure on TB drugs, unnecessarily wasted for wrong treatment.

4. Even with the successful upcoming of other rapid techniques in RIF resistance newly introduced in Sikkim, the Gene-Xpert uses fresh sputum collected over

48 hours to the Intermediate Reference Lab from patients living in remote areas, our method of RIF resistance stands more superiorly favourable as the method can be used on fresh as well as sputum stored for more than 48 hours (even ≥4 years) duration. Our study method of RIF resistance do not hold biohazard danger as the smears can be made at the peripheral centres and transported after inactivation using heat fixation and ethanol inactivation of the TB bacilli. Whereas in the techniques (Gene-Xpert) applying presently at IRL lab sputum samples are received without inactivation of the live TB bacilli.

5. LiPA tool competes well with the conventional drug susceptibility method (Proportionate method) and seems to be a promising tool to control MDR-TB in Sikkim.

6. Finding of the majority mutation at R5 region of the LiPA hybridization probe indicated high level RIF resistance causing mutation at codon region 531 as was found in many studies across the globe.

7. Also the mutation missed by the LiPA hybridization probe in one sample, meant there could be the occurrence of *rpoB* mutation outside the hotspot region of the *rpoB* gene in *M. tuberculosis* isolates. This demands slight improvement in the LiPA tool which can be done by inclusion or exclusion of some specific probes in the hybridization strip.

CHAPTER 8

LIMITATIONS OF THE RESEARCH

1. 4% of the RIF resistant isolates have *rpoB* mutations outside the 81bp region of the *rpoB* gene, so these isolates cannot be identified whether they RIF resistant or susceptible based on LiPA tool unless confirmed by sequencing.

2. The study only tests for RIF resistance and not INH resistance, and as >90% RIF resistance is the marker for MDR-TB, rest of the percentage would still mean not MDR-TB.

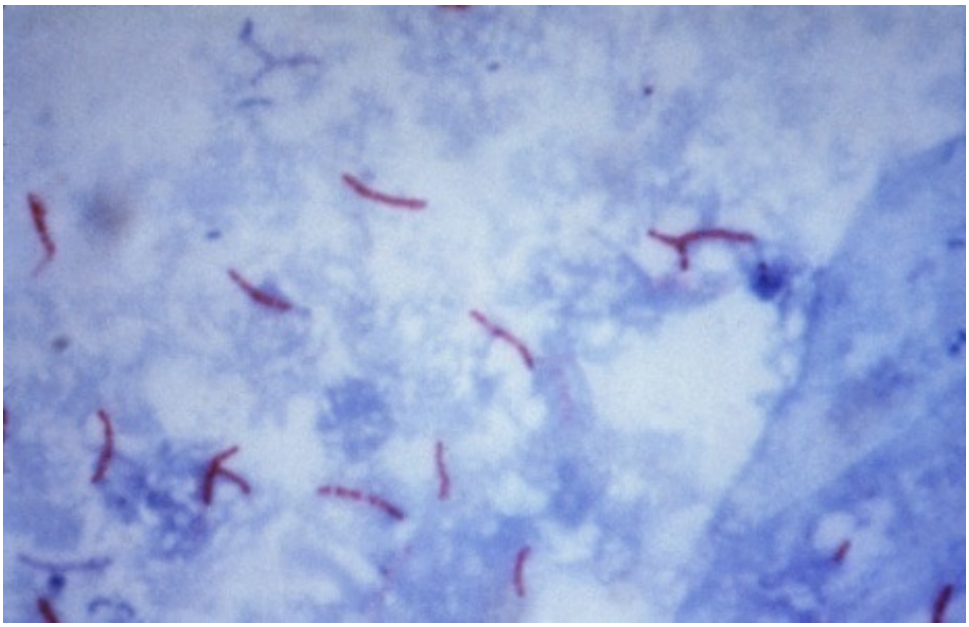
3. The study has been tested using sputum smear positive samples and not on smear negative samples, so its limited to only sputum positive samples.

4. Due to the limited funds for study, molecular methods could be applied using only two kits approximating Rs. 1.2 lakhs. The study was not designed for studying prevalence of MDR-TB in the community.

APPENDICES

APPENDIX I

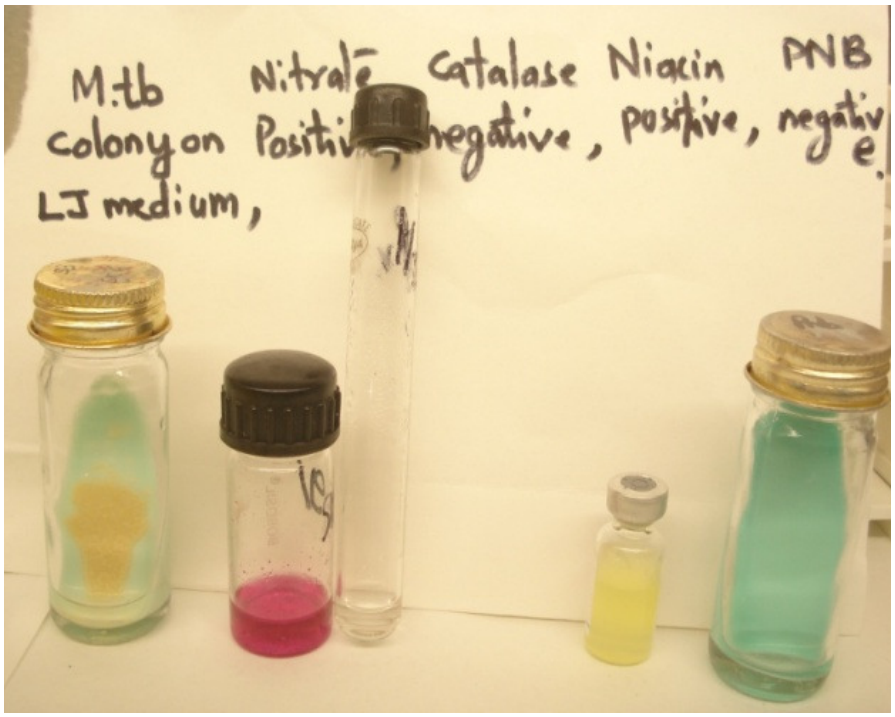
Ziehl Neelson stained sputum smear showing AFB (acid fast bacilli)



APPENDIX II

Biochemical tests for identification of *M. tuberculosis* isolates

(From left to right: positive culture growth of *M. tuberculosis* on LJ medium, positive nitrate test, negative 68^{o}C heat stable catalase test, niacin positive and no culture growth on PNB containing LJ medium).



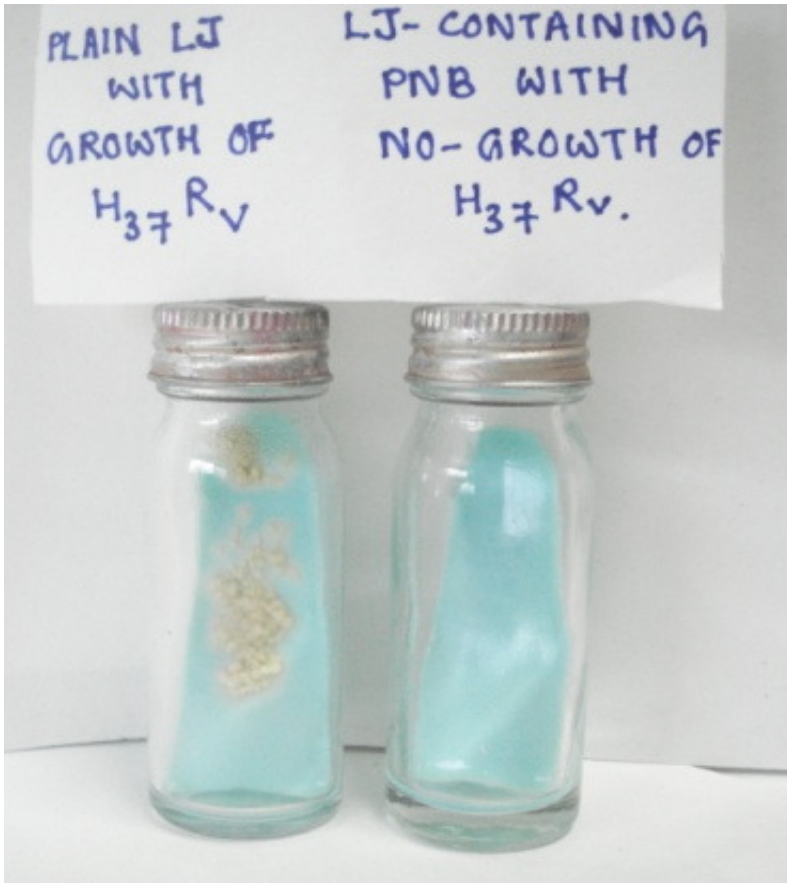
APPENDIX III

Nitrate test

(From left to right: Nitrate positive control ($H_{37}R_V$ growth added), nitrate positive test sample, nitrate negative control (no sample added).

APPENDIX IV

Para-nitro-benzoic-acid test (PNB)



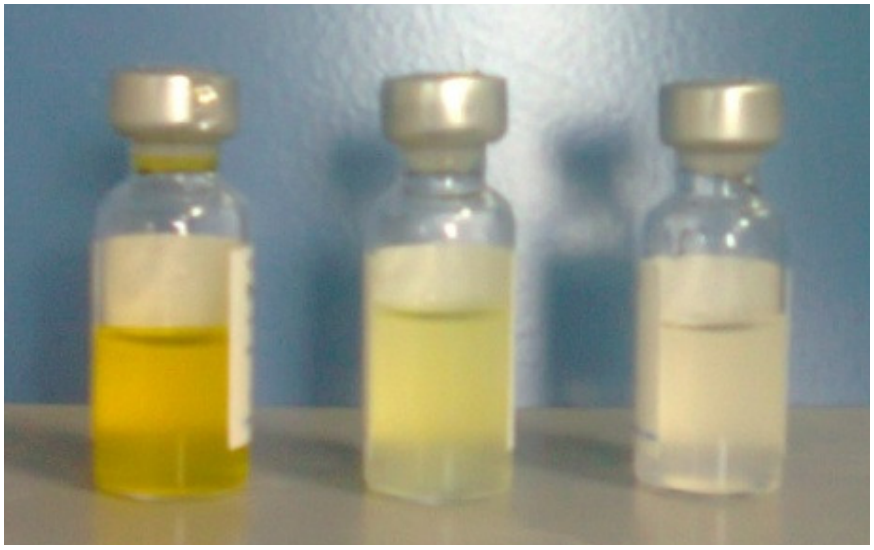
APPENDIX V

Niacin test [done using K047 Niacin detection kit (modified) with syringe from Himedia]

a) **Niacin positive control** (niacin extract from culture of $H_{37}R_V$)
and negative control (no culture extract added) **reagents provided in the kit.**

b) **Niacin positive control, positive niacin test sample and negative test control.**

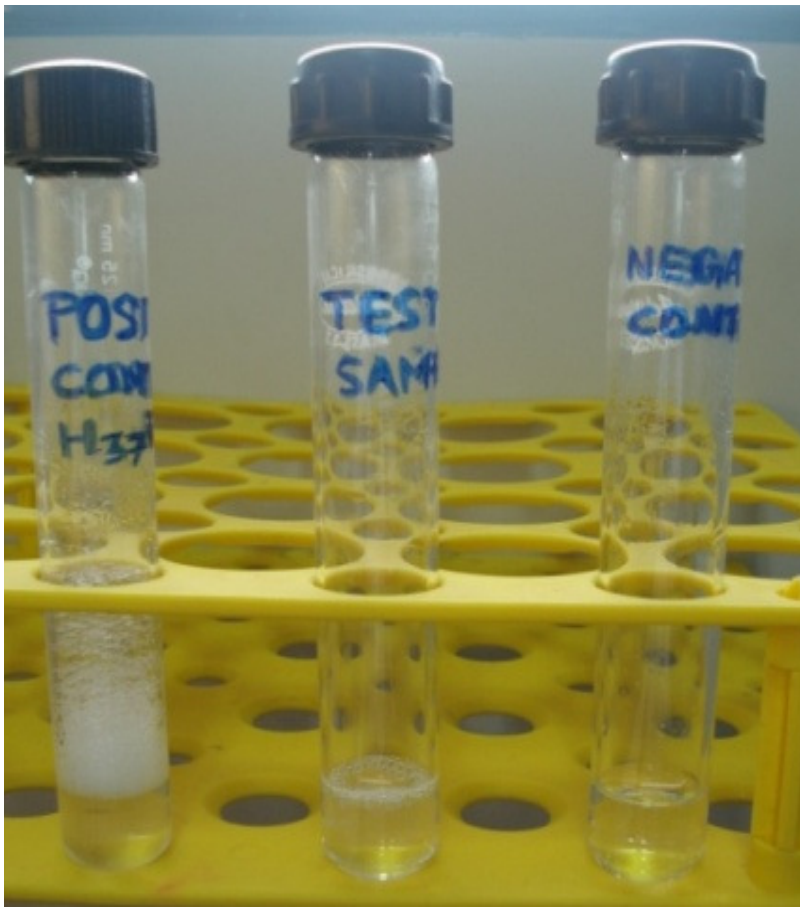
a) 

b) 

APPENDIX VI

68^{o}C heat stable Catalase test

(From left to right: Positive control (Atypical Mycobacteria), positive test sample and negative control ($H_{37}R_V$)



APPENDIX VII

First line anti-tubercular drugs Rifampicin, Isoniazid, Streptomycin and Ethambutol (Sigma-Aldrich)

APPENDIX VIII

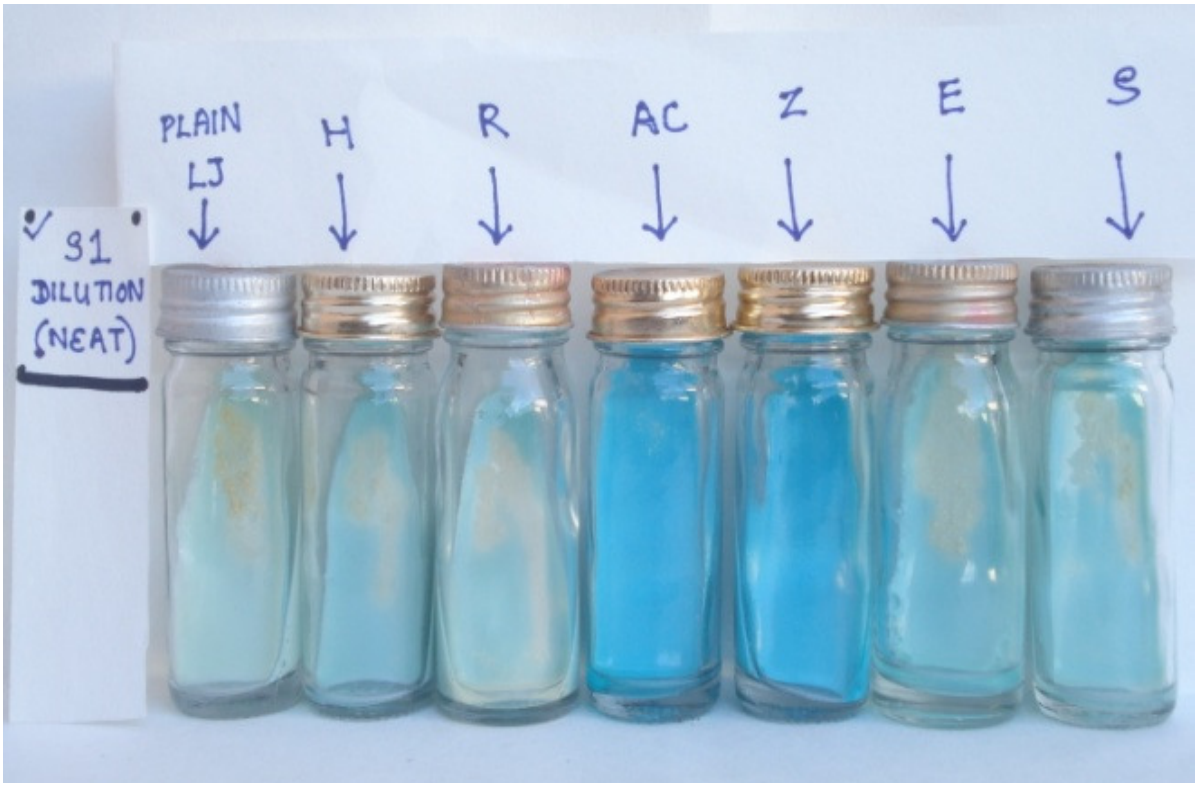
Comparison of turbidity between 0.05 MacFarland tube and neat suspension during proportionate method of sensitivity testing.

APPENDIX IX

Sensitivity pattern of $H_{37}R_V$ strain (Wild strain from TRC, Chennai) using S2 dilution inoculum on plain LJ medium and first line drug containing medium (H=isoniazid, R=rifampicin, AC=acid control for pyrazinamide, Z=pyrazinamide, E=ethambutol, S=streptomycin) by proportionate method.

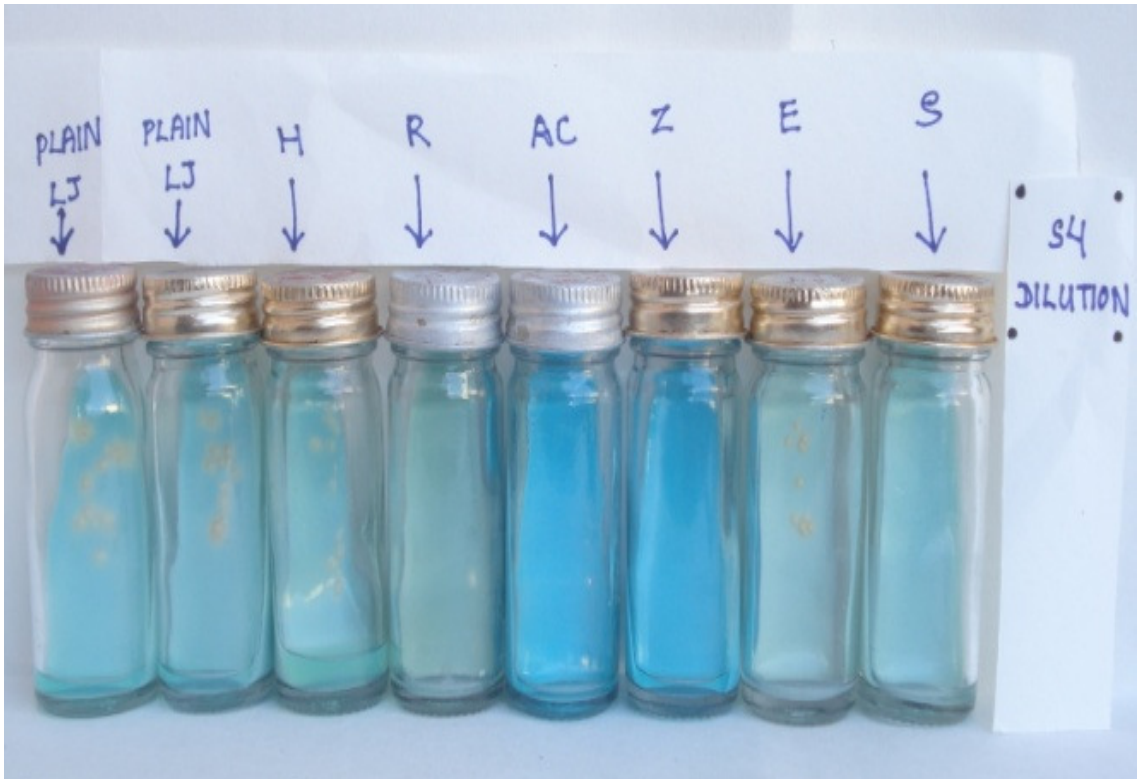
APPENDIX X

Sensitivity pattern of rifampicin resistant *M. tuberculosis* strain on plain LJ medium and first line drug containing medium (H=isoniazid, R=rifampicin, AC=acid control for pyrazinamide, Z=pyrazinamide, E=ethambutol, S=streptomycin) using neat inoculum by proportionate sensitivity test.



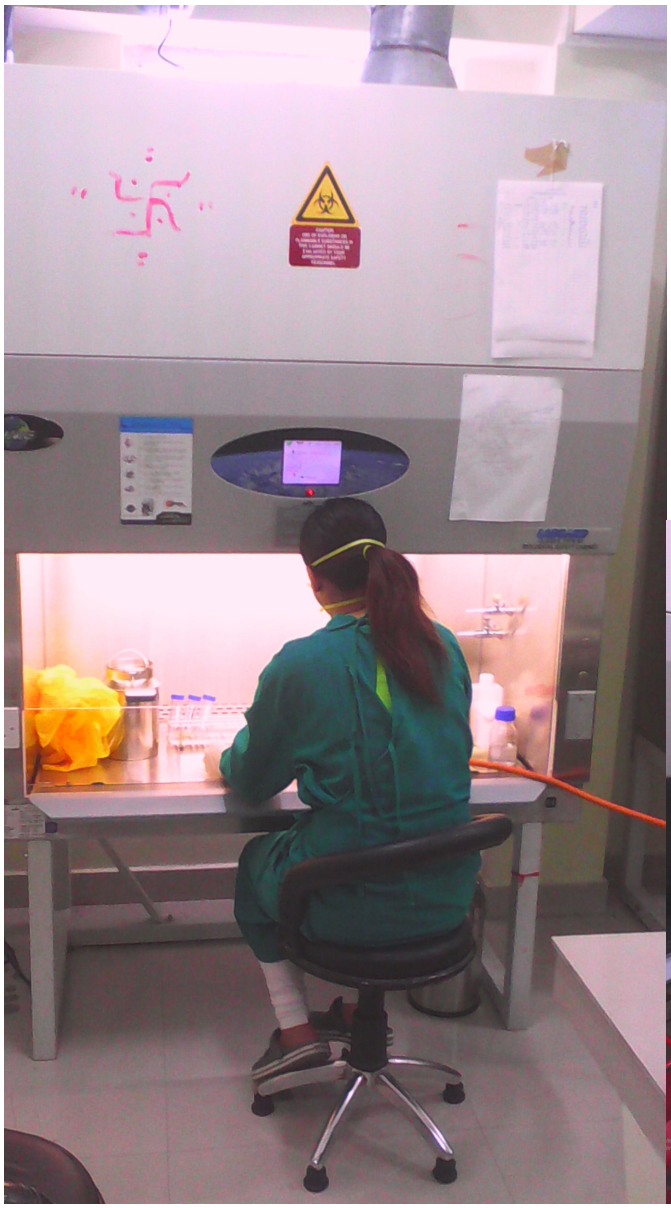
APPENDIX XI

Sensitivity pattern of rifampicin sensitive *M. tuberculosis* strain on plain LJ medium and first line drug containing medium (H=isoniazid, R=rifampicin, AC=acid control for pyrazinamide, Z=pyrazinamide, E=ethambutol, S=streptomycin) using S4 dilution inoculum by proportionate sensitivity test.



APPENDIX XII

Biosafety cabinet class-II

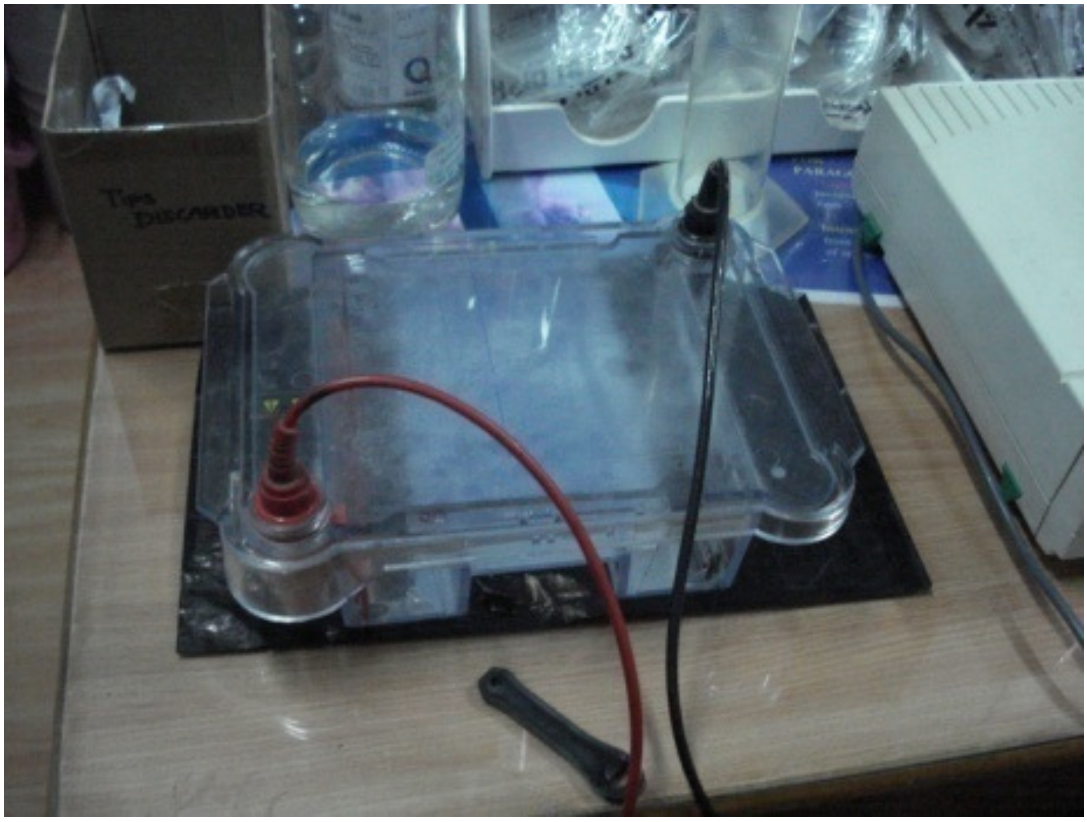


APPENDIX XIII

Gene Amp® PCR system 9700, AB Applied Biosystems.

APPENDIX XIV

Electrophoresis chamber (Bio Rad) filled with premixed TAE buffer 10X (REF 11 666 690 001, LOT 12498100, Roche) and gel made from SeakemLe agarose (Cambrex, catalogue no 50004, lot no AG 4845).

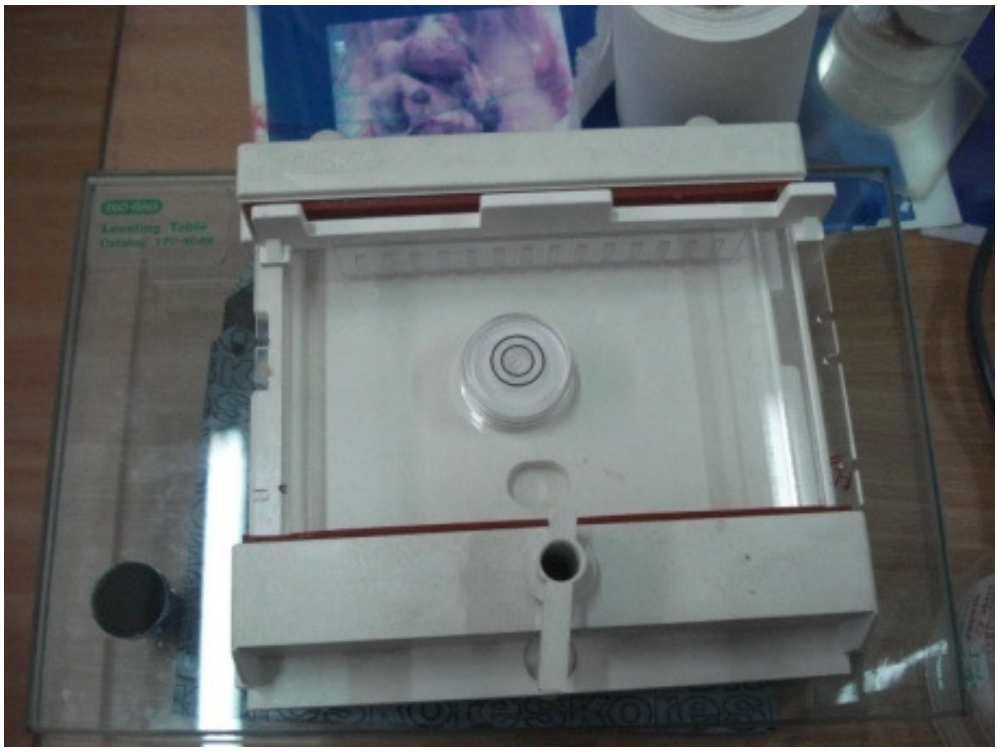


APPENDIX XV

Power pac (Axygen, AXY-PS1), unit for gel electrophoresis

APPENDIX XVI

Levelling table (catalog 170-4046, Bio Rad), balance with Gel casting apparatus (Axygen submerged gel electrophoresis)

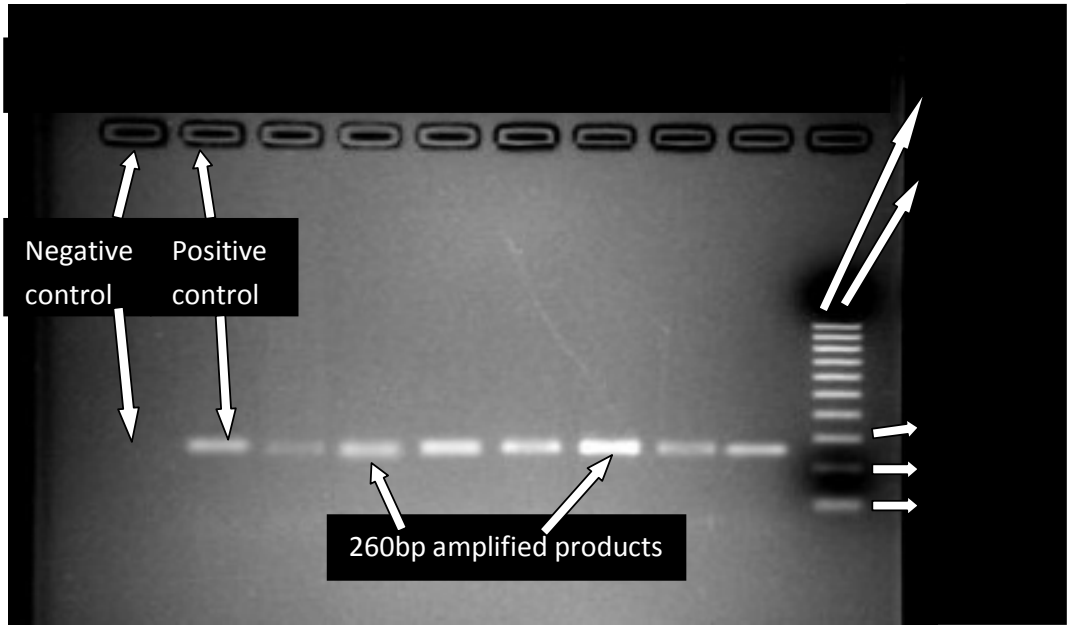


APPENDIX XVII

Gel Documentation (Bio Rad)

APPENDIX XVIII

Gel Documentation of 260bp amplified products causing mutation in 81bp region of rpo*B* gene, causing rifampicin resistance in *M. tuberculosis* isolates.



INNO LiPA Rif.TB amplification of 260bp bands of the rpoB gene characteristic for MTBC in 7 isolates. From Left to right, Lane 1= NC, lane 2= PC, lane 3 to 9 = patients samples. Lane 10=100bp DNA ladder (Takara) (code 341 0A Lot A401-1), NC= negative control for MTBC (added milli-Q water in place of MTBC DNA), PC= positive control for MTBC ($H_{37}R_V$).

PERSONAL DETAILS AND HOUSEHOLD SCHEDULE OF CAT-I TB PATIENTS

Sl.No. ☐☐☐Laboratory No. ☐☐☐☐Examined by: Dr.☐☐☐☐☐☐☐☐☐☐☐

DOTS Center: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Date: ☐☐-☐☐ - 200☐ TB.No. ☐☐☐☐☐☐☐☐☐ DOTS Category: _____

A1. Name of Respondent: _____ **A2.** Age: ___ yrs.

A3. Sex: M [1], F [2] ☐**A4.** BMI _____, Height._____ (cms), Wt._____ (Kgs)

A5. Community: Lepcha [1], Bhutia [2], Nepali [3], Others [4] _____ ☐

A6. Religion: Hindu [1], Christian [2], Buddhist [3], Muslim [4] ☐

A7. Caste: ST [1], SC [2], OBC [3], Minority [4], General [5] ☐

A8. Detailed Address: House No. ☐☐☐☐ Street: ☐☐☐☐☐☐☐☐☐☐☐☐

PIN: ☐☐☐☐☐☐ Village: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Block: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

District of Sikkim: East ☐ West ☐ North ☐ South ☐

Ph.No: ☐☐☐☐☐☐☐☐☐☐

A9. Cough: Persist [1], Absent [2], Reappeared [3] ☐

A10. Classification of case

New sputum positive ☐

New sputum positive after < 1 month treatment with Cat- I regime ☐

Positive after 2 months of Cat I treatment ☐

Positive after 3 months of Cat I treatment ☐

Positive after 5 months of Cat I treatment ☐

Positive after 6 months of Cat I retreatment ☐

A11. Nutritional Status: Undernourished [1], Over-nourished [2], Normal [3] ☐

A12. Type of family: Nuclear [1], Joint [2], Extended [3] ☐

A13. Position in family: Bread Earner [1], Major Decision Maker [2], Dependent [3] ☐

A14. Marital Status: Single [1], Married [2], Widowed [3], Separated [4], Divorced [5] ☐

A15. Living Status: Living Alone [1], With Spouse [2], With only Children/Relatives ☐

A16. Education: Illiterate [1], Literate [2] ☐

A17. Occupation:_____Professional [4],Skilled [3], Unskilled[2], Unemployed [1] ☐

A18. Financial Status: Totally dependent [1], Partially dependent [2], Independent [3] ☐

A19. Any History of Pulmonary TBin the family? Y [1], N [2] ☐

A20. Any History of death in family due to pulmonary tuberculosis? Y [1], N [2] ☐

A21. Did you ever take any anti-tubercular drugs before? Y [1], N [2] ☐

A22. If yes, then who prescribed you the anti-tubercular drug before? _____

A23. Habits: ***(A) SMOKING:***

Regular smoker [1], Ex-smoker [2], Occasional smoker[3], Non-smoker [4] ☐

Habits: ***(B) ALCOHOL CONSUMPTION:***

Regular alcoholic [1], Ex-alcoholic [2], Occasional [3], Non-alcoholic [4] ☐

A24. Did you/Are you suffering from any of the following conditions during D.O.T.S. treatment? Diabetes [1], High BP [2], Low BP [3], Heart Problems [4], Joint Pains [5], Backache [6], Asthma [7], Cough for more than 3 months in the last two years [8], Constipation [9], fits with jerky movements [10], fits without jerky movements [11], Difficulty in initiation of micturition/ wake up too often at night for voiding

[12], Difficulty in holding back the urine for most of the time [13], Paresis/ Paralysis [14], Haemorrhoids [15], Gastritis [16], HIV/AIDS [17]

□,□,□,□,□

MICROSCOPIC ASSESSMENT

Microscopic sputum follow up examination	Date of examination	Grading of AFB smear					Slide.No
Pre-treatment		**3+**	**2+**	**1+**	**Scanty**	**-ve**	□□□□
End of intensive phase		**3+**	**2+**	**1+**	**Scanty**	**-ve**	□□□□
End of extensive intensive phase		**3+**	**2+**	**1+**	**Scanty**	**-ve**	□□□□
End of continuation phase		**3+**	**2+**	**1+**	**Scanty**	**-ve**	□□□□
End of extensive continuation phase		**3+**	**2+**	**1+**	**Scanty**	**-ve**	□□□□
End of treatment		**3+**	**2+**	**1+**	**Scanty**	**-ve**	□□□□

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