IDENTIFICATION OF MUTATIONS IN rpoB GENE (β-SUB UNIT OF DNA DEPENDENT RNA POLYMERASE) IN RIFAMPICIN RESISTANT *MYCOBACTERIUM TUBERCULOSIS* AMONG RETREATMENT TUBERCULOSIS PATIENTS IN NEPAL

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IN MICROBIOLOGY

BY

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JULY 2018

DECLARATION

Thesis entitled "**Identification of Mutations in rpoB Gene (β-Sub Unit of DNA Dependent RNA Polymerase) in Rifampicin Resistant *Mycobacterium tuberculosis* Among Retreatment Tuberculosis Patients in Nepal**" which is being submitted to the Central Department of Microbiology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Anjana Singh, Central Department of Microbiology, Tribhuvan University and cosupervised by Prof. Dr. Prakash Ghimire, Central Department of Microbiology, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

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RECOMMENDATION

This is to recommend that **Mr. Dhruba Kumar Khadka** has carried out research entitled **"Identification of Mutations in rpoB Gene (β-Sub Unit of DNA Dependent RNA Polymerase) in Rifampicin Resistant *Mycobacterium tuberculosis* Among Retreatment Tuberculosis Patients in Nepal"** for the award of Doctor of Philosophy (Ph.D.) in **Microbiology** under our supervision. To our knowledge, this work has not been submitted for any other degree.

He has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

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On the recommendation' of **Prof. Dr. Anjana Singh**, Supervisor and **Prof. Dr. Prakash Ghimire**, Co-supervisor, this Ph. D. thesis submitted by **Mr. Dhruba Kumar Khadka**, entitled "**Identification of Mutations in rpoB Gene (β-Sub Unit of DNA Dependent RNA Polymerase) in Rifampicin Resistant *Mycobacterium tuberculosis* Among Retreatment Tuberculosis Patients in Nepal**" is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, T.U..

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ABSTRACT

Tuberculosis (TB) remains one of the major public health problems in Nepal. The increasing burden or trend of multi drug resistant tuberculosis and extensively drug resistant tuberculosis (MDR-TB and XDR-TB) is a big challenge to National TB Programme. The priority of rapid diagnosis and appropriate treatment management of any kind of tuberculosis throughout the country has become crucial. With this objective in the present study, a total of 159 sputum samples; 42(26.4%) sputum smear negative (ss-ve) and 117(73.6%) sputum smear positive (ss+ve) were collected from the retreatment tuberculosis (TB) patients i.e. females 64(40.3%) and males 95(59.7%) with the median age of 30 years. The samples were processed for mycobacterial culture and analyzed by Gene Xpert MTB/RIF assay that detected *M. tuberculosis* and rifampicin resistance (RR) for all (100%) of them. There was no significant difference in distribution of age wise (p=0.253) and sex wise smear results (p=0.152). The majority of RR or MDR TB cases by Gene Xpert MTB/RIF assay fell within the age group of 15-60 years (p=0.050) but no sex wise differences (p=0.225). Among the total, 115(73.3%) were culture positive, 34(21.4%) culture negative and 10(6.3%) were contaminated by primary isolation culture. There was no significant difference in gender wise culture positivity (p=0.213), whereas, it was high among smear positive cases (p<0.001) and majority fell among males of 15-60 years age group. The sensitivity, specificity, predictive value of positive test and predictive value of negative test of Gene Xpert and smear microscopy results were evaluated with reference to culture and found to be 106(92.2%) at 95% confidence interval (CI) within lower limit 85.1 and upper limit 96.1, 29(85.3%) at 95% CI; 68.2 and 94.5), 106(95.5%) at 95% CI; 89.3 and 98.3, 29(76.3%) at 95% CI; 59.4 and 88.0 respectively. From 115 pure cultures identified as *M. tuberculosis*, first line anti–tuberculosis drug susceptibility test (FLD-DST) was performed following proportion method, of which 109(94.78%) positive samples (3 from ss-ve and 106 from ss+ve) showed both isoniazid (I) and rifampicin (R) resistance (MDR) with or without streptomycin (S) and ethambutol (E) resistance (p=0.001). Out of 115 positive cultures, 2 (one each; 1.74%) showed mono resistance to I and R respectively, 1(0.87%) to S and I and 4(3.47%) were susceptible to all 4 drugs (SIRE:

streptomycin, isoniazid, rifampicin, ethambutol). There was no significant difference of age wise (p=0.532) and sex wise (p=0.775) distribution of MDR-TB cases identified by Gene Xpert, conventional culture and FLD-DST. Second line anti-tuberculosis drug susceptibility test (SLD-DST) on 29(26.6%) of 109 MDR confirmed *M. tuberculosis* isolates was performed by Line Probe Assay (LiPA Genotype MTB/DR*sl* version 1.0), of which 17(58.6%) for *gyrA* and 8(27.6%) for *emb B* and all 29(100%) for *rrs* genes were identified as wild type. Whereas, 12(41.4%) were resistant to *gyr A* gene i.e. 3 cases resistant to fluoroquinolone alone and 9 cases along with ethambutol. Similarly, 10(34.5%) were resistant to ethambutol (*emb B* genes) only and 7(24.1%) were susceptible to all SLDs. Out of 29 MDR confirmed *M. tuberculosis* isolates, 12(41.4%) were found to be pre-extensively drug resistant (pre-XDR-TB) but none was found XDR TB. The maximum number of age/sex wise pre-XDR TB distribution was found in 15-60 years of age group and among females and males equally. There were no differences of age (p=0.531) and sex wise (p=0.428) findings of pre-XDR TB. Among 159 RR/MDR-TB, 128(80.5%) cases were analyzed for time difference between previous cure, current diagnosis of MDR-TB and treatment outcome. The treatment outcomes were categorized into favorable (cured and treatment completed) and unfavorable (died, lost to follow up, not evaluated and treatment failure) groups. The age wise treatment outcomes for a total of 128(100.0%) cases; 1(1.1%), 80(62.5%), 8(9.0%) were among the favorable group and 1(2.6%), 34(26.6%), 4(10.3%) were among unfavorable groups of <15, 15-60 and > 60 years of age respectively. Similarly, 2(1.6%) i.e. 1(0.8%) in 5-11 months and 1(0.8%) in 1-4 years groups were not evaluated, 3(2.3%) i.e. 1(0.8% fell in 1-4 years and 2(1.6% fell in ≥5yrs) groups respectively were treatment failure. There were no significant differences of age (p=0.897), sex (p=0.357), time difference (p=0.474), diagnosis by Gene Xpert alone or along with C/DST (p=0.746) and treatment outcomes. This is the first analyzed report on retreatment TB patients in Nepal.

Key words: *Gene Xpert, Line Probe Assay, Multi and Extensively Drug Resistant Tuberculosis (MDR-XDR TB), Time Difference, Treatment Outcome.*

LIST OF ABBREVIATIONS

AC	Amplification Control
AFB	Acid Fast Bacilli
Amk	Amikacin
Amx/Clv	Amoxycillin/Clavulanate
BSC	Biological Safety Cabinet
CAT-I	Category-I
CAT-II	Category-II
CC	Conjugate Control
CDC	Centers for Disease Control and Prevention
Cm	Capreomycin
CPC	Cetylpyridinium Chloride
Cs	Cycloserine
CSF	Cerebrospinal Fluid
DOTS	Directly Observed Therapy System
DR-TB	Drug Resistant Tuberculosis
DST	Drug Susceptibility Test
E	Ethambutol
ECDC	European Centre for Disease Control
EPTB	Extra Pulmonary Tuberculosis
Eto	Ethionamide
FLD	First Line Anti Tuberculosis Drugs
H or I	Isoniazid
HIV	Human Immunodeficiency Virus
IGRAs	Interferon-Gamma Release Assays
IUALTD	International Union against Tuberculosis and Lung Disease/the Union
Km	Kanamycin
LiPA	Line Probe Assay
L-J	Lowenstein-Jensen
MDR TB	Multidrug Resistant Tuberculosis
Mfx	Moxifloxacin

MGIT	Mycobacterial Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MTB	*M. tuberculosis* Complex
MUT	Mutation
NALC	N-acetyl L-cysteine
NaOH	Sodium Hydroxide
NTC	National Tuberculosis Centre
NTP	National Tuberculosis Programme
Ofx	Ofloxacin
PAS	Para-Aminosalicylic Acid
PCD	Pulmonary Clinically Diagnosed
PCR	Polymerase Chain Reaction
PNB	Para-Nitrobanzoic Acid
Pre-XDR TB	Pre- Extensively Drug Resistant Tuberculosis
PTB	Pulmonary Tuberculosis
QRDR	Quinolone Resistant-Determining Region
RIF	Rifampicin
RR-TB	Rifampicin Resistant Tuberculosis
RT-PCR	Real time PCR
SAARC	South Asian Association for Regional Cooperation
SEA	South East Asia
Se	Sensitivity
SM	Streptomycin
SLD	Second Line Anti Tuberculosis Drug
Sp	Specificity
TB	Tuberculosis
TCH	Thiophene-2-Carboxylic Acid Hydrazide
TST	Tuberculin Skin Test
TUB	Tuberculosis Complex
WHO	World Health Organization
WT	Wild Type
XDR TB	Extensively Drug Resistant Tuberculosis
Z	Pyrazinamide

ZN	Ziehl-Neelsen

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CHAPTER 1

1. INTRODUCTION AND OBJECTIVES

1.1 Introduction

Tuberculosis (TB) is an airborne disease caused by *Mycobacterium tuberculosis* (MTB). It is an infectious disease that transmits from one person having such organisms to another by means of a droplet containing MTB through coughing, sneezing, talking and singing. TB is mainly classified as Pulmonary (PTB) and Extra pulmonary (EP TB). TB is a top infectious disease killer worldwide. With nearly two billion people (one third of the world's population) harboring latent infection, TB is a global threat. World Health Organization (WHO) reported that in 2014, 9.6 million people fell ill with TB and 1.5 million died from the disease. TB death occurs in low and middle-income countries over 95% and it is among the top 5 causes of death for women aged 15 to 44 years. Globally in 2014, an estimated 480 000 (an estimated 3.3% of new TB cases and 20% of previously treated cases) people developed multidrug-resistant TB (MDR-TB) and 190, 000 people died of MDR-TB. An estimated 43 million lives were saved through TB diagnosis and treatment between 2000 and 2014. Extensively drug-resistant TB (XDR-TB) had been reported by 105 countries till 2015. An estimated 9.7% of people with MDR-TB have XDR-TB (WHO, 2015). In the SAARC region, 34% of the global burdens of TB out of which 1.79 million are estimated to be sputum smear positive infectious cases. Among the estimated cases, a total of 81,142 MDR-TB cases were notified in 2013 in the SAARC region, of which 41% were new pulmonary cases and 59% were previously treated cases (STAC, 2014).

TB remains one of the major public health problems in Nepal. During 2014, a total of 37,025 cases of TB were registered out of which, pulmonary TB was 51 %. Highest reported cases were among 15-24 (20%) years age group though most cases reported among the middle aged (40-60 years) group. WHO estimated 4.6 thousand people died from TB in 2014 (WHO, 2015). TB-HIV co-infection rate in Nepal is 2.4% (HIV among TB) and 11.6% (TB among HIV) based on the sentinel survey, 2011/12. Nationwide, the proportion of MDR-TB was 2.2% among new cases and 15.4%

among retreatment cases based on survey carried out in 2011/12 (NTPN, 2015). In 2014, a total of 349 MDR-TB and 25 XDR-TB were enrolled for treatment. One of the concerned aspects of drug resistance in Nepal is the high level of resistance to fluoroquinolones (26.4%), which leads to heavy burden of pre-XDR and XDR-TB among MDR-TB patients (8% of the cases were found to be XDR among MDR-TB during the same survey). This problem of drug resistance might be because of over the counter availability and unregulated use of fluoroquinolones. To combat the excess mortality related to XDR-TB, it is recommended to perform drug susceptibility test (DST) for second line-drugs (SLD) to all MDR-TB cases before starting of treatment. It seems important for Nepal to strengthen its capacity to perform SLD-DST, either by solid/liquid culture & DST or molecular biological technique like line probe assay or LPA (NTPN, 2015).

Isoniazid (INH) with rifampicin (RIF) forms the cornerstone of short course chemotherapy for tuberculosis and resistance to either drug hampers the complete cure of patients. *M. tuberculosis* strains resistant to at least these two major frontline drugs (INH and RIF) develop MDR-TB (WHO, 1997). Approximately 95% RIF resistant *M. tuberculosis* strains have mutations in an 81 bp hot spot region (codon 507-533) of *rpoB* gene that encodes RNA polymerase beta subunit (Telenti et al., 1993). This region is therefore an ideal target for molecular tests for RIF resistance (Sam, et al., 2006; Bartfai et al., 2001).

Globally, more than half of all TB cases are not detected due to weak result of health care system or the inadequacy of available technology. If a diagnosis is not proper, patients do not get treated, thus transmission may continue, patients suffer needlessly and may eventually die. As a diagnostic tool of tuberculosis, sputum smear microscopy may vary between 30% and 70% depending on a number of factors relating to how the test is implemented (Long, 2001).

Cepheid (Cepheid, Sunnyvale, CA) has recently introduced the GeneXpert MTB/ RIF assay for research use only (Boehme et al., 2010). The GeneXpert assay is a real-time time polymerase chain reaction (RT-PCR) test that will simultaneously identify *M. tuberculosis* and detect rifampin resistance (RR) directly from clinical specimens (Helb et al., 2010). Rifampicin resistance can serve as a marker for multidrug-

resistant tuberculosis (MDR-TB) and has been reported in 95% of the multidrug-resistant *M. tuberculosis* isolates (Morris et al., 1995). The GeneXpert assay detects an 81-bp "core" region of the *rpoB* gene. The test utilizes five molecular beacons that detect mutations in the core region that are associated with rifampin resistance. Previously, the sensitivity of the GeneXpert system in detecting smear-positive specimens has been reported to be 98 to 100% (Marlowe et al., 2011).

Diagnosis and control of TB is difficult to achieve as it involves composite measures, which may lead to drug related side effects, disruption of daily life and social isolation. So, it is very important that the methods used to diagnose tuberculosis should be extremely precise (Burman and Reves, 2000). Studies have shown that cultures of MTB from clinical specimens are presently close to the "gold standard" for diagnosis of TB (Getahun, et al., 2007; Moore et al., 2006). An erroneous or reprehensible drug sensitivity testing (DST) result may lead to recognition of susceptible cases as resistant or vice versa and may lead to inappropriate or inadequate treatment, which may affect the treatment outcome (Kim, 2005).

So, in high burden resource-poor countries, it is essential to carefully prioritize the tools of diagnosis with clinical relevance for maximal utilization of available healthcare facilities (Sharma, et al., 2013). The use of the rapid test Xpert MTB/RIF has expanded substantially since 2010, when WHO first recommended its use. By 2015, 69% of countries recommended using Xpert MTB/RIF as the initial diagnostic test for people at risk of drug resistant (DR) and/or MDR-TB (WHO, 2015).

1.2 Rationale

National Tuberculosis Programme is a priority one programme in an integrated health system of Nepal. National Tuberculosis Program has introduced multiple approaches for controlling the disease including directly observed treatment short course (DOTS), expansion of culture and drug susceptibility testing centres in different parts of the country, introduction of molecular diagnostics (Gene Xpert) for early detection of the drug resistance and institution of the first/second line of treatment as implicated by the test results. However, national data clearly indicates that the drug resistant cases including MDR/XDR are on rise. To detect DR/MDR/XDR-TB cases early in the infection and for early institution of the appropriate treatment (Cat 1 or Cat 2

treatment failure), identification of *rpoB*, *gyrA/gyrB*, *rrs* and *embB* genes has become increasingly important. So, this study embarked on the molecular detection of the infection and drug resistance in previously treated TB cases visiting TB treatment centres with recurrence of sign and symptoms either due to relapse or re-infection. The findings of the study could be helpful for the national programme in implementing policy changes, also for the treating physician and the patient in need for moving from first line to second line shown by the laboratory results. The study findings may be useful for the programme in planning strategies for early and effective reduction of disease transmission.

1.3 Objectives

1.3.1 General Objective

To identify mutations of *rpoB* gene in rifampicin resistant (RR) MTB isolates from retreatment TB cases in Nepal using Gene Xpert MTB/RIF and compare the results with drug resistance patterns of conventional DST following proportion method.

1.3.2 Specific Objectives

1. To identify *rpoB* gene from rifampicin resistant (RR) MTB isolates using Gene
   Xpert MTB/RIF assay from retreatment TB cases.
2. To compare DST patterns detected by Gene Xpert MTB/RIF assay, proportional DST and MTBDR*sl* LPA.
3. To detect XDR-TB by MTBDR*sl* LPA.
4. To detect possible relapse/re-infection cases based on time difference between previous cure and current diagnosis of MDR-TB cases.

CHAPTER 2

2. LITERATURE REVIEW

2.1 History

Rajayakshma, Yakshma or Kshya (wasting) is the name given for TB mentioned in ancient Sanskrit Vedas. Still the word ‘Sukenaas’ (gradual loss of weight) can be found to indicate TB in various communities in Nepal. Due to the disease had occurred first time in Raja (the King), so the word given Rajayakshma for TB. TB was well recognized by the time of Hippocrates (377-400 B.C.) who gave an excellent clinical description of the disease. The Dutch Physician Fransciscus Sylvius (1614-1672) deduced from autopsies that TB characterizes by the formation of nodules, which he named 'tuberculosis'. During the course of time, the modern concept of TB started from the work of Rene Theoclore Laennec (1781-1816), French clinician who himself was a consumptive and succumbed to the disease. In 1819, he invented stethoscope and accurate description of tuberculosis lesions; he described follicular (milliary) and exudative forms of TB. Later Robert Koch announced to discovery of tubercle bacillus in 24 March 1882 and succeeded in culturing it on inspissated serum (Grange, 2007).

2.2 *Mycobacterium*

Mycobacterium is a genus of *Actinobacteria*, given its own family the *Mycobacteriaceae*. The genus includes pathogens known to cause serious diseases in mammals, including TB and leprosy. The Latin prefix "*Myco*" means both fungus and wax; its use here relates to the "waxy" compound in the cell wall. Organisms belonging to the genus *Mycobacterium* are thin, nonsporing, pleomorphic, straight or slightly curved rods between 0.2-0.6µm long. They are aerobic and non-motile bacteria that are characteristically acid-alcohol fast. The high Guinine+Cytosine (G+C) content of the DNA of *Mycobacterium* species (61 to 70 mol % except for *Mycobacterium leprae* 55%) is within the range of those of the other mycolic acid containing genera including *Nocardia* (64 to 72 mol %), *Rhodococcus* (63 to 73 mol %), *Gordonia* (63 to 69 mol %), *Tsukamurella* (68 to 74 mol %), (Goodfellow, 1998).

TB is a disease mainly caused by *M. tuberculosis* complex (MTBC) that includes *M. tuberculosis, M. africanum, M. bovis, M. Microti*, *M. laprae,* and *M. pinnipedii*. Over 100 species of *Mycobacterium* are currently recognized (O'Neill, et al., 2015). Other *Mycobacteria* that may be associated with human disease are collectively known by different names such as atypical, anonymous, non-tuberculosis, tuberculoid, opportunistic and mycobacteria other than tuberculosis bacilli (MOTT). Many MOTTS are found in the environment but they can also colonize in man, as in the part of previously damaged respiratory tract and cause clinical infection (Rastogi, et al., 2001).

2.3 Cultural Characteristics

Tubercle bacilli are obligate aerobes and will not grow in the absence of oxygen, even a moderate reduction in the oxygen tension result in an appreciable decrease in the metabolism of the bacilli. The bacilli grow slowly, the generation time *in vitro* being 14-15 hours. Colonies appear only in about two weeks and sometimes may be delayed up to 6 to 8 weeks. Optimum temperature is 37°C and growth does not occur below 25°C or above 40°C. Optimum pH is 6.4 to 7.0 and grows only in especially enriched media containing egg, asparagines, potatoes, serum and meat extract (Forbes, 1998). In culture on solid media; *M. tuberculosis* (MTB) produces dry, rough, raised and irregular colonies with a wrinkled surface. While in culture on liquid media; MTB produces growth that appears first at the bottom and then grasps up to the sides and produces a surface pellicle that may extend along sides above the medium (Parijaa, 2012).

2.4 Tuberculosis Disease

TB is a mankind disease from time of immemorial. The evidence of spinal disease has been found in Egyptian mummies of several thousand years BC and references to TB are found in ancient Babylonian and Chinese writings. Recent molecular genetic studies have shown that MTB, the most common cause of TB in humans worldwide, has a progenitor ~3 million years old (Gutierrez et al., 2005). TB is chronic bacterial infection caused by collectively known as MTB complex (MTBC). Human tuberculosis is mostly caused by MTB and less commonly by *M. africanum, M. bovis, M. Microti, M. caprae* and *M. pinnipedii* characterized by the formation of granulomas in infected tissue and by cell mediated hypersensitivity (WHO, 1997).

TB is an infectious disease that is caused by MTBC, which is characterized by the presence of tubercles in lungs or other organs. TB of lungs is called pulmonary TB, which is contagious type of TB. Whereas TB of nervous system, gastro-intestinal tract, genitourinary tract, bones etc. are called extra pulmonary TB, which may be non-contagious (Grange, 2007).

2.5 Aetiological Agent

Over 100 species of mycobacteria are recognized. MTBC is the collective term used to describe as the main causative agent of TB. Saprophytic mycobacteria which are non-tubercle bacilli and isolated from a number of environmental sources are loosely grouped under the term 'atypical' or 'anonymous' mycobacteria. They have also been called 'paratubercle', 'tuberculoid' and MOTT (Collins, 1985). They are notably resistant to commonly used antitubercular drugs (Cole et al., 1998). They can also colonize man and cause clinical infection more commonly in the presence of predisposing factors like weakened immunity, diseased condition, malnutrition, poverty and crowded living. Clinically important MOTT are *M. kansaii, M. genavense, M. marinum, M. simiae, M. scrofulaceum, M. szulgai, M. avium, M. hamophilum, M. intracellulare, M. malmoense, M. ulcerans, M. xenopi, M. abscessus, M. chelonae, M. fortuitum,* and rarely *M. smegmatis* (Horsburgh, 1996).

2.6 Virulence Factors

The virulence of tubercle bacilli appears to be related to their ability to survive within macrophages. Tubercle bacilli inhibit the fusion of phagosome with lysosome, by a mechanism that is poorly understood; the bacilli secrete several compounds (ammonium ions, polyglumtamic acid, cyclic adenosine monophosphate or cAMP and sulpholipids) that affect cell membranes, but their role in vivo is uncertain. It has been suggested that the alternation in membrane function may isolate the bacilli from nutrients as well as from the lysosomal contents and this may be the mechanism of bacterial dormancy and persistence (Grange, 2007).

2.7 Transmission

TB is spread from person to person through the air by droplet nuclei or particles of 1 to 5µm in diameter that contain MTBC. There are four factors that determine the transmission of MTB: (1) the number of organisms being expelled into the air (2) the concentration of organisms in the air determined by the volume of the space and its ventilation (3) the length of time an exposed person breathes the contaminated air and (4) presumably the immune status of the exposed individual (Horsburgh, 1996). For infection transmission, the particles must carry a viable organism. One time coughing can produce 3,000 - 5,000 droplet nuclei. Transmission generally occurs from dark environment where droplet nuclei can stay in the closed air for a long time. Ventilation removes droplet nuclei and direct sunlight quickly kills tubercle bacilli. Two factors determine an individual's risk of exposure: the concentration of droplet nuclei in contaminated air and the length of time he breaths that air (Park, 2005).

Inhalation of even a single viable organism has been found to be the cause of infection, although close contact has been taken as usual cause. Regarding MTB, 15% to 20% of the infected persons have developed disease. Disease usually occurs some years after the initiation of infection when the patient's immune system breaks down for some reason other than presence of tubercle bacilli in lungs. The disease becomes systemic affecting a variety of organs in low percentage (Forbes, 1998). Common forms of EPTB include glands, bones and joints, TB meningitis, abdominal, milliary, laryngeal and genitourinary TB (NTPN, 2012).

2.8 Pathogenesis

WHO has estimated that one-third of the total world population is latently infected with MTB and 5% -10% of the infected individuals will develop active TB disease during their life time. However, the risk of developing active TB is 5%–15% every year and lifetime risk is ~50% in HIV individuals in latent cases (WHO, 2009; Wells et al., 2007). PTB may be primary or post-primary depending on the time of the infection and the type of host immune response. More than 85% of active TB case occurs in high TB incidence countries due to higher rates of transmission. While most, if not all, EPTB result from haematogenous spread from a primary focus, which is not always detected. In order of frequency, the sites most commonly affected

include lymph nodes, pleura, genitourinary tract, bones and joints, meninges, peritoneum and pericardium (Chakraborty, 2013; Parijaa, 2012). EPTB is common in low TB incidence countries and the developed countries, particularly among HIV-infected individuals and immigrants originating from TB endemic countries (Golden & Vikram, 2005).

2.9 Diagnosis

Rapid detection of current infection is extremely important for early therapy or for disease control. The contribution of the microbiology laboratory to the diagnosis and management of TB involves the detection, isolation and identification of the mycobacterial species or MTBC. It also includes the determination of susceptibilities of the organisms to antimycobacterial drugs (ATS/CDC, 2000; Kent and Kubica, 1985). Definitive diagnosis depends on clinical suspicion, the demonstration of acid fast bacilli (AFB) by microscopy, isolation and identification of MTBC by culture methods and more recently, nucleic acid amplification assays (Tiwari, et al., 2003).

2.9.1 Clinical Diagnosis

The course of PTB is highly variable and symptoms can vary from mild, persistent cough and an acute pneumonia-type syndrome to chronic cough, haemoptysis, fever, night sweat and weight loss. However, some patients with active TB may even be asymptomatic (Leiner and Mays, 1996).

2.9.2 Radiological Diagnosis

Despite its several disadvantages, chest radiography remains an important supporting tool in TB surveys and clinical management of active disease (Hopewell, et al., 2014). In a study from Kenya, the sensitivity and specificity of the chest X-Ray (CXR) score TB among smear negative suspects were 80% and 67% respectively. CXR is used as a screening tool in all suspects and sensitivity and specificity of the score any pathology were 92%, 63% respectively (van Cleeff, et al., 2005). So, the introduction of a well defined scoring system, clinical conferences and a system of CXR quality control can contribute to improve diagnostic performance. The radiographic presence of pleural effusion, cavities and hilaradenopathy were also more specific than sensitive for TB.

Among all of these factors, the presence of pleural effusion (98%) and cavities (97%) had the highest specificities for TB (Davis, et al., 2010).

2.9.3 Tuberculin Skin Test (TST)

TST is currently the standard tool to detect latent TB infection, although it is far from a 'gold' standard. TST is based on the detection of delayed-type hypersensitivity to purified protein derivative (PPD), a mixture of antigens shared by several mycobacteria that gives rise to a skin reaction. Two visits are required for the test, one for PPD inoculation (Mantoux technique uses intracutaneous injection by needle and syringe) and another after 48–72 hours for interpretation of the result based on the size of the skin reaction (Andersen, et al., 2000).

2.9.4 Microscopy

Microscopy is used to examine clinical specimens or cultures for the presence of AFB. The three most commonly used stains are Ziehl Neelsen (ZN), auramine–rhodamine, fluorochrome and Kinyoun stains (Attorri, et al., 2000). Microscopy indicates that AFB is present in the sample, but does not always indicate viable organisms or that the organism is MTBC. Approximately 40–50% of patients with PTB are smear positive; sputum must contain at least 5000 bacilli/ml for them to be detectable by microscopy (Dye, et al., 1999). AFB appears red against a blue background on ZN stained smear, while in the fluorochrome procedures, it appears as fluorescent rods, yellow to orange (the color may vary with filter system used) against a pale yellow or orange background (WHO, 1998).

2.9.5 Culture

Culture is the most sensitive of currently available tests (sensitivity rates of up to 98% have been reported) that also permits identification and DST. However, it may require up to 6–8 weeks for the isolation of MTBC from a clinical specimen and in 10–20% of cases the bacillus is not successfully cultured (Andersen, et al., 2000). All clinical specimens suspected of containing mycobacteria should be inoculated (after appropriate digestion and decontamination) onto culture media for four reasons: (1) Culture is much more sensitive than microscopy, being able to detect as few as 10 bacteria/ml of sample (Yeager, et al., 1967) (2) Necessary for species identification; (3) DST (4) Genotyping of cultured organisms. In general, the sensitivity of culture is 80–85% with a specificity of approximately 98% (Ichiyama, et al., 1993; Morgan, et al., 1983). Traditionally mycobacterium is grown on solid media, containing a cocktail of antimicrobial agents that permit only mycobacterium to replicate. The media are egg-based Lowenstein–Jensen (LJ) and Ogawa media or agar-based Middlebrook 7H9, 7H10 and 7H11.

2.9.6. Anti Tuberculosis Drugs and Mycobacterial Genetic Relation

Each anti TB drug has its one target to the mycobacterial enzymes encoded with different genes in a normal condition and this is called “drug susceptible to the organism”. Even a single amino acid change due to any nucleotide mutation may create “resistance to certain drugs”. Basic anti TB drugs (first line and second line) and how do they act over several bacterial organelles are shown and described in Table 1, Table 2 followed by subsequent texts.

Table 1: Suggested target sites for the anti-TB activity of first line drugs (Narain, 2002)

Drugs	Target sites	Encoding genes	References
Isoniazid	Catalase –peroxidase	*katG*	(Zhang, et al., 1992)
	Enoyl-ACP reductase	*inhA*	(Banerjee et al., 1994)
	β- ketoacyl ACP synthase	*kasA*	(Mdluli et al., 1998)
Rifampicin	β- subunit of RNA-polymerase	*rpoB*	(Levin and Hatfull, 1993) (Telenti, et al., 1993)
Streptomycin	12S ribosomal protein / 16S ribosomal protein	*rpsl/rrs*	(Finken, et al., 1993)
Ethambutol	Arabinosyl transferase	*embCAB*	(Belanger et al., 1996)
Pyrazinamide	Pyrazinamidase	*pncA*	(Scorpio and Zhang, 1996)

*ACP: (acyl-carrier-protein)

Table 2: Suggested target sites for the anti-TB activity of second line drugs

Drugs	Target sites	Encoding gene	References
Fluoroquinolone	Topoisomerase II	*gyrA*	
	Topoisomerase IV	*gyrB*	(Musser, 1995)
*Capreomycin	16S ribosomal RNA	*rrs*	(Finken, et al., 1993)
*Kanamycin	16S ribosomal RNA	*rrs*	(Ho, et al., 1997)

* AG/CP (amino glycoside /cyclic peptide): AMK-KM-CM all are amino glycosides

It is now clear that the occurrence of MDR-TB among retreatment (Cat1 and Cat 2 treatment failures) cases alerts the NTP managers for prompt diagnosis of TB/DR/MDR/XDR-TB using reliable and rapid diagnostics tools based on molecular biological techniques. So, the present study has been giving priority to identify MDR-TB and XDR-TB among retreatment cases using the rapid diagnostics tools like RT-

PCR based Gene Xpert MDR/RIF assay in reference with culture and DST on first line anti TB drugs (FLD-DST) and Genotype MTBDR*sl* line probe assay (LPA) for second line anti TB drugs (SLD-DST).

2.9.6.1 Mechanism of Action of Anti TB Drugs

2.9.6.1.1 First-Line Anti-TB Drugs

2.9.6.1.1.a Streptomycin

Streptomycin was originally isolated from the soil microorganism *Streptomyces griseus* and the first antibiotic to be successfully used against TB. Unfortunately, as soon as it was prescribed, resistance to it emerged, a result of being administered as monotherapy (Crofton and Mitchison, 1948). Streptomycin is an aminoglycoside effective against actively growing bacilli and its mode of action is the inhibition of the translation of protein synthesis (Moazed and Noller, 1987). More specifically, streptomycin acts at the level of the 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA coded by the genes *rpsL* and *rrs* respectively (Finken, et al., 1993). As a result, the major mechanisms of resistance to streptomycin (60%–70%) are due to the mutations in *rpsL* and *rrs*. Among the mutations reported in *rpsL*, a substitution in codon 43 from lysine to arginine has been found to be the most common (Gillespie, 2002).

2.9.6.1.1.b Isoniazid

Isoniazid was implemented as an anti-TB drug in 1952, which together with rifampicin, is the basis for the treatment TB. Isoniazid is only active against metabolically active or replicating bacilli. Isoniazid is also known as isonicotinic acid hydrazide, which is a pro-drug that requires activation by the catalase/peroxidase enzyme KatG, encoded by the *katG* gene to exert its effect (Zhang, et al., 1992). Isoniazid interrupts the synthesis of bacterial cell wall formation by inhibiting mycolic acids through the NADH-dependent enoyl-acyl carrier protein reductase (ACP), encoded by *inhA*. Isoniazid resistance has been found to be associated with mutations of *katG*, *inhA*, *ahpC*, *kasA* genes (Ramaswamy et al., 2003; Rawat, et al., 2003). Among these, the most prevalent gene mutation has been identified as S315T in *katG* resulting in an isoniazid product deficient in forming the isoniazid-NAD

adduct needed to exert its antimicrobial activity. The second most common mutation occurs in the promoter region of *inhA* at position −15C/T causing an over expression of InhA or less frequently, a mutation in its active site, which decreases its affinity for the isoniazid-NAD adduct (Larsen, 2002; Rozwarski, et al., 1998; Banerjee, et al., 1994). Mutations in *inhA* not only cause resistance to isoniazid but also to the structurally related ethionamide, which shares the same target. A recent study found that a mutation in the *inhA* regulatory region together with a mutation in the *inhA* coding region produced high-level isoniazid resistance and also cross-resistance to ethionamide (Machado et al., 2013).

2.9.6.1.1.c Rifampicin

Rifampicin is derived from rifamycin that was introduced as an anti-TB agent in 1972. Rifampicin is one of the most effective anti-TB drugs, which together with isoniazid constitutes the basic treatment regimen for TB. Rifampicin is active against growing and slow metabolizing bacilli (Mitchison, 1979). Rifampicin acts in MTB by binding to the β-subunit of the RNA polymerase, inhibiting the elongation of messenger RNA (Blanchard, 1996). The majority of rifampicin-resistant isolates of MTB harbor mutations in the *rpoB* gene that codes for the β-subunit of the RNA polymerase. As a result of this, conformational changes occur that decrease the affinity for the drug and results in the development of resistance (Telenti, et al., 1993). In about 96% of MTB isolates resistant to rifampicin, there are mutations in the so-called hot-spot region of 81-bp spanning codons 507–533 of the *rpoB* gene. This region is also known as the rifampicin resistance-determining region or RRDR (Ramaswamy and Musser, 1998).

2.9.6.1.1. d Ethambutol

Ethambutol was first introduced in the treatment of TB in 1966 and is part of the current first-line regimen to treat the disease. Ethambutol is bacteriostatic against multiplying bacilli interfering with the biosynthesis of arabinogalactan in the cell wall (Takayama and Kilburn, 1989). In MTB, the genes *embCAB*, organized as an operon, code for arabinosyl transferase, which is involved in the synthesis of arabinogalactan, producing the accumulation of the intermediate D-arabinofuranosyl-P-decaprenol (Mikusova, et al., 1995). The ethambutol resistance occurs due to the mutations in the

embB gene at position *embB*306, which is the most prevalent site (Sreevatsan et al., 1997).

2.9.6.1.1.e Pyrazinamide

Mitchison, (1985) in his study described that pyrazinamide was introduced into tuberculosis treatment in the early 1950s and now become a part of the standard first-line. Pyrazinamide is slightly different in chemical structure to nicotinamide and it allowed reducing the length of treatment to six months. It's characteristic is to inhibit semi-dormant bacilli residing in acidic environments usually found in the TB lesions. Being a pro-drug, pyrazinamide needs to be converted to its active form, pyrazinoic acid, which is completed by the enzyme pyrazinamidase/nicotinamidase coded by the *pncA* gene (Scorpio et al., 1997; Scorpio and Zhang, 1996; Konno, et al., 1967). The proposed mechanism of action of pyrazinoic acid is to disrupt the bacterial membrane energetic inhibiting membrane transport. Pyrazinamide under acid pH would enter the bacterial cell by passive diffusion and after conversion to pyrazinoic acid it is excreted by a weak efflux pump (Zhang and Mitchison, 2003). In replicating MTB bacilli, the fatty acid synthase type I is important, which is inhibited by pyrazinoic acid and its n-propyl ester (Zimhony, et al., 2007; Zimhony, et al., 2000). The most common finding in pyrazinamide resistant strains is due to the mutations in the *pncA* gene that are scattered throughout the gene but most occur in a 561-bp region in the open reading frame or in an 82-bp region of its putative promoter (Jureen, et al., 2008; Scorpio, et al., 1997).

2.9.6.2 Second-Line Anti-TB Drugs

2.9.6.2.a Fluoroquinolones

Fluoroquinolones are currently in use as second-line drugs in the treatment of MDR-TB. Both ciprofloxacin and ofloxacin are synthetic derivatives of the parent compound nalidixic acid, discovered as a by-product of the antimalarial chloroquine (Goss, et al., 1965). Moxifloxacin and gatifloxacin are new generation quinolones that are being evaluated in clinical trials. These drugs are proposed as first-line anti TB treatment regimen with the purpose of shortening the length of treatment (Palomino and Martin, 2013; Rustomjee et al., 2008). Fluoroquinolones inhibit two critical enzymes for bacterial viability; topoisomerase II (DNA gyrase) and topoisomerase

IV. These enzymes are encoded by *gyrA*, *gyrB*, *parC* and *parE* genes respectively. In MTB, only type II topoisomerase (DNA gyrase) is present thus, is the only target of fluoroquinolone activity (Aubry, et al., 2004). Type II topoisomerase is formed by α and β subunits and coded by *gyrA* and *gyrB* genes respectively, which catalyzes the supercoiling of DNA (Takiff et al., 1994). The fluoroquinolone resistance develops by chromosomal mutations in the quinolone resistance-determining region (QRDR) of *gyrA* or *gyrB* in MTB. The most frequent mutations found are at position 90 and 94 of *gyrA* but mutations at position 74, 88 and 91 have also been reported (Sun et al., 2008; Cheng et al., 2004). A recent systematic review of fluoroquinolone-resistance-associated gyrase mutations in MTB has been published (Maruri et al., 2012).

2.9.6.2.b Capreomycin (CM)

Food and Drug Administration (FDA) provided a label of polypeptide antibiotic to capreomycin. CM clearly interacts with the ribosome and inhibits protein synthesis though the mode of action is not fully understood. The up-regulation of several ribosomal proteins (RpsR, RplI, RplY and RplJ), Rv2907c (16S rRNA processing protein) and Rv1988 (methyltransferase) have been demonstrated by a gene-chip experiment in MTB. It has been explained that the expression data support the interaction of CM with ribosomal components although a number of genes unrelated to protein synthesis are also affected. As CM has such potent activity against the persistent forms of TB the drug may have a target or secondary target outside the ribosome; the gene chip data may lead to discovery of new targets for CM (Fu and Shinnick, 2007). The resistance mechanism of CM is associated with ribosomal changes in the 16S rRNA; there is possible cross-resistance with streptomycin, but this is not always complete (Di Perri and Bonora, 2004).

2.9.6.2.c Kanamycin, Amikacin, Viomycin

Kanamycin and amikacin are aminoglycosides and viomycin is cyclic peptides that have the same mechanism of action inhibiting protein synthesis. These are used as second-line drugs in the management of MDR-TB. Kanamycin and amikacin inhibit protein synthesis by alteration at the level of 16S rRNA. Mutations of the *rrs* gene at position 1400, 1401 and 1483, conferring high-level of kanamycin and amikacin resistance are most common. It has been reported that the mutations at position −10

and −35 of the *eis* promoter region led to an over expression of the protein and low-level resistance to kanamycin but not to amikacin (Alangaden et al., 1998; Suzuki et al., 1998).

2.9.6 Nucleic Acid Amplification Tests (NAATs)

NAATs are molecular systems, which are able to detect small amounts of DNA or RNA target sequences from the micro-organism. If the target organism is not present in the sample, no amplification will occur. NAATs are most promising development for rapid diagnosis of TB and rapid DST. For MTBC, a number of nucleic acid amplification techniques are available as commercial. There are varieties of amplification methods in used, amplification of nucleic acid such as PCR or amplification of a nucleic acid probe such as a ligase chain reaction (Louie, et al., 2000).

2.9.6.1 Loop Mediated Isothermal Amplification (LAMP)

LAMP is a novel method of amplifying nucleic acid. LAMP relies on auto cycling strand displacement DNA synthesis performed by large fragment of *Bacillus stearothermophilus* (*Bst*) DNA polymerase. LAMP amplicons are cauliflower-like structures with multiple stem-loops DNA structures with several inverted repeats of the target. The LAMP reaction with a 60 minutes incubation and visual inspection has a sensitivity equivalent to that of the amplicor test. The sensitivity of LAMP in smear and culture-positive sputum specimens was 97.7% (173/177 specimens, 95% confidence interval (CI) 95.5 - 99.9) and in smear-negative, culture-positive specimens 48.8% (21/43 specimens, CI 33.9 - 63.7) (Boehme, et al., 2010).

2.9.6.2 Nucleic Acid Hybridization Tests (NAHTs)

The ability of NAHTs is to specifically align and to associate complementary nucleic acid strands to form stable double-stranded complexes. The Accu PROBE SYSTEM uses a single-stranded DNA probe with a chemiluminescence label that is complementary to the ribosomal RNA of the target organism's ribosomal RNA to form a stable DNA:RNA hybrid. The differentiation of non-hybridized and hybridized probe is allowed by the selection reagent. The labeled DNA:RNA hybrids are measured in a Gen-Probe luminometer (Kohne, 1984).

2.9.6.3 Transcription Mediated Amplification (TMA)

TMA, an isothermal target-based amplification system development by Gen-Probe incorporation has been combined with a homogenous detection method to detect MTB in clinical specimen (Jonas et al., 1993). In this method rRNA is amplified via TMA in which the target sequences are copied into transcription complex by using reverse transcriptase and then RNA polymerase is used to make numerous RNA transcripts of the target sequence from the transcription complex. The process then repeats automatically. Detection of the amplified sequence is achieved by using an acridinium ester-labeled DNA probe specific for MTB (Bloom, 1994).

2.9.6.4 Polymerase Chain Reaction (PCR)

PCR is one of the most significant advances in DNA based technology, which is rapid, sensitive, inexpensive and simple means of producing relatively larger number of copies of DNA molecules from minute quantities of source DNA from viable or non-viable cell material (Sambrook et al., 1989). PCR has proved to be a most useful tool for the rapid diagnosis of infectious disease. In PCR, oligonucleotide primers are used to direct the amplification of target nucleic acid sequences through repeated rounds of denaturation, primer annealing and primer extension (Mullis and Faloona, 1987). In general, the amplification process can be completed in 2 to 6 hours of obtaining a processed specimens and the detection assay can be completed in an additional 2-24 hours (Bloom, 1994). The sensitivity of a carefully performed quality controlled PCR would be expected to be 90–100% on smear positive and 60-70% on smear negative; culture positive sputum samples (Drobniewski, et al., 2003).

2.9.6.5 Multiplex Polymerase Chain Reaction (MPCR)

MPCR is a modified PCR that uses multiple pairs of specific primers in a single PCR mixture to different DNA sequences. PCR system alone can provide the information by targeting multiple genes at the same time, but other system would require more amounts of reagents and take longer performance time. The standard PCR usually uses one pair of primers to amplify a specific sequence. Whereas, MPCR allow the amplification of more than one target sequence in a single reaction by using a more than one set of primers (Rodriguez and Hernandez, 2013). This saves considerable time and effort and decreases the number of reactions that need to be performed to detect the desired targets in the single sample. Whereas, the presence of multiple set

of PCR primers in a single reaction can cause some problems, such as the increased formation of primer dimers and the amplification discrimination of longer DNA fragments. For MPCR, primers should be chosen with similar annealing temperatures. The lengths of amplicons should be similar as large differences in the lengths of the target DNA may favor the amplification of shorter targets over the longer ones, which results in differential yields of amplified products (Sambrook, 1989).

Most of the PCR based studies have used a single target (e.g. *IS6110* segment or *MPB64* gene) for the amplification of MTB DNA. However, single target can result in false negative results and the reliable result can be obtained by targeting more than one target gene for amplification (Sharma, et al., 2012; Kusum et al., 2011). The insertion sequence, *IS6110* may be absent in 10-40% of MTB isolates especially in geographically specific endemic areas (Chauhan et al., 2007; Daley, et al., 2007). As an alternative approach, MPCR can be used to diagnose TB in which several target genes for MTBC are amplified simultaneously to increase the sensitivity and specificity of the test. MPCR also has several strengths, such as cost effectiveness and reduced possibility of PCR contamination. In the application of two nested PCR targeting *MPB64* and *IS6110* regions for detection of MTB genome in culture proven clinical specimen both sensitivity and specificity were 100% respectively (Therese et al., 2013).

2.9.6.6 Gene Xpert MTB/RIF Assay

The Gene Xpert (Xpert assay Cepheid Sunnyvale, USA) MTB/RIF technique is a fully automated, cartridge based and real time PCR (RT-PCR) assay, which is designed to detect the presence of MTBC and rifampicin (RIF) resistance within 2 hours. The Gene Xpert can be utilized as a rapid diagnostic test for tuberculosis in patients with presumptive PTB as well as rapidly exclude MTBC from non tuberculous mycobacteria (NTM) and to determine RIF resistant strain (WHO, 2011). The Gene Xpert utilizes molecular beacon technology to detect DNA sequences amplified in a hemi-nested RT-PCR assay (Tyagi and Kramer, 1996). There are five different nucleic acid hybridization probes used in the same multiplex reaction. All the five probes are complementary to a different target sequence within the *rpoB* gene of rifampicin susceptible MTBC. The probes are labeled with differently colored fluorophore. Collectively, these overlapping probes span the entire 81bp core region

of the *rpoB* gene (Piatek et al., 2000; Piatek et al., 1998). The molecular beacons are oligonucleotide sequences that contain a probe sequence inserted between two arm sequences. The two 'arm' sequences are designed to be complementary to each other. Under assay conditions, they hybridize to form a stem-and-loop secondary structure; the probe is located within the loop structure. A fluorophore is covalently linked to the end of one arm and a non-fluorescent quencher to the other. With the probe in its free and non-hybridized state, the close proximity of the quencher and fluorophore molecules suppresses fluorescence. However, when the probe sequence binds to its complementary DNA target, the molecular beacon undergoes conformational change. This causes separation of the two arms and the fluorophore and quencher molecules, resulting in the onset of bright fluorescence (Tyagi and Kramer, 1996).

In Gene Xpert, MTBC is detected by the five overlapping molecular probes (probes A–E) that collectively are complementary to the entire 81bp *rpoB* core region (Blakemore et al., 2010; Helb, et al., 2010). MTB is identified when at least two of the five probes give positive signals with a cycle threshold (CT) of ≤38 cycles and that differ by no more than a pre-specified number of cycles. The *Bacillus globigii* (*B. globigii*) as internal control will become positive, when the single specific probe produces a CT of ≤38 cycles. The standard user interface indicates the presence or absence of MTBC and rifampicin resistance. The concentration of bacilli as defined by the CT range (high, <16; medium, 16–22; low, 22–28; very low, >28) gives semi-quantitative estimation. Assays showing negative results for MTB and *B. globigii* are reported as invalid assays. The basis for detection of rifampicin resistance is the difference between the first (early CT) and the last (late CT) MTB specific beacon (ΔCT). The system was originally configured such that resistance was reported when ΔCT was >3.5 cycles and sensitive if ≤3.5 cycles. The assay process terminates after 38 cycles. If the first probe CT is >34.5 cycles and the last probe has a CT of >38 cycles, the assay could be indeterminate for rifampicin resistance (Blakemore, et al., 2010; Helb, et al., 2010).

In the first report of the Gene Xpert, the limits of detection of the assay were assessed. By adding MTB genomic DNA directly into the cartridge, the limit of detection (95% sensitivity) was found to be 4.5 (95% CI: 3.3-9.7) genome copies. Clinical sputum samples from non-TB patients were also spiked with known numbers of MTB H37Rv

bacilli (10–300 CFU per ml of sputum) and multiple replicates tested. The limit of detection was found to be 131 (95% CI: 106–176) CFU/ml of sputum (Helb, et al., 2010). In assessment, the assay was tested with a panel of 89 different organisms, including bacteria, fungi and viruses, using strains commonly found in the respiratory tract. Each test was done with 106 genomes of the isolate and all tested negative i.e. 100% specificity (Blakemore, et al., 2010). The sensitivity of the Xpert assay for MTBC detection in Cat 1 was 89.8% (95% confidence interval (CI), 78.5-95.8%), but 66.7% (95% CI, 12.5-98.2%) in Cat 2. The positive predictive values (PPV) ranged from 33.3% (95% CI, 6.0-75.9%) in Cat 2 to 91.3% and 91.7% in categories 1 and 3, respectively. The negative predictive values (NPV) were over 90% in all categories (Huh et al., 2014). Similarly, in the study of Armand, et al. (2011), the sensitivities of the Gene Xpert and the *IS6110* TaqMan assay were 79% and 84% respectively and specificity was 100% for both methods, with respiratory specimens.

2.9.6.7 Line Probe Assay (LPA)

Genotype MTBDRplus assay based on LPA is a rapid molecular technology that detects the mutations conferring rifampicin and isoniazid resistance or MDR-TB. This assay technology was endorsed by the World Health Organization (WHO) in 2008. A new LPA based Genotype MTBDR*sl* assay has been introduced by HAIN Life Science GmbH in 2009, for the rapid determination of genetic mutations associated with resistance to fluoroquinolone, aminoglycosides (kanamycin, amikacin), cyclic peptides (CM), ethambutol and streptomycin. Both the assay formats are similar to each other and allows for testing and reporting results within 24 hours (WHO, 2013; WHO 2009; WHO, 2008). The proportion method was used to determine the phenotypic drug susceptibility or resistance patterns. The mutations inside QRDR of *gyrA* and *gyrB* genes were identified by DNA sequence analyses. Among 206 fluoroquinolone resistance (FQ^r) MTBC strains, 44% (90/206) were MDR isolates and 39% (81/206) were XDR isolates. Mutations in *gyrA* were shown to be the major mechanism of FQ^r in MTBC isolates. The mutations in *gyrA* QRDR can be a good molecular surrogate marker for detecting FQ^r-MTBC in China (Zhu et al., 2012). Among FQ^r-MTBC isolates, mutation at positions 90, 91 and 94 in *gyrA* gene and at positions 495, 516 and 533 in *gyrB* gene have been frequently reported (Pitaksajjakul et al., 2005).

2.10 Treatment and Control

TB therapy generally consists of 6 to 9 months course of isoniazid, rifampicin, streptomycin, thioacetazone, pyrazinamide and ethambutol. Besides these main drugs, other includes cycloserine, ethionamide and capreomycin. There are three main properties of anti-TB drugs: bactericidal activity, sterilizing activity and the ability to prevent resistance (Maher, 1997). Latent TB is treated with a single antibiotic and active TB is treated with combined drugs. Combined drugs reduce the risk of the bacteria developing drug resistance. Drug resistant TB is a public health issue in many developing countries, as treatment is longer and it requires more expensive drugs. MDR-TB is defined as MTB resistance to the most effective two FLDs; rifampicin and isoniazid. MDR-TB developing resistance to fluoroquinolones and at least one of the injectable (KM/CM) aminoglycosides also will result XDR-TB (O'Brien, 1994).

2.11 Tuberculosis Treatment Regimens in Nepal

NTP provides the following fully supervised treatment regimens as described below:

2.11.1 Cat 1 Treatment Regimen

This regimen is used to treat the diagnosed cases of new smear-positive PTB, smear-negative PTB and EPTB. It is given in two phases; an initial intensive phase of two months with HRZE (2 HRZE) and a continuation phase of four months with HR (4 HR). If the 2nd month follow-up sputum exam is still positive, patients will be treated additional one month with the drugs of initial phase.

2.11.2 Cat 2 Treatment Regimen

This regimen is used to treat the relapse, treatment failure and returned after default TB cases (usually sputum smear-positive cases) with two months of HRZE along with injectable (2SHRZE) plus one month of HRZE (1HRZE) and five months of HRE (5HRE).

2.11.3 MDR-TB Treatment Regimen

All newly diagnosed MDR-TB patients receive a standardized regimen of FLDs and SLDs. This includes the use of at least four drugs with an injectable agent (aminoglycoside or capreomycin) for an 8 month intensive phase with a combination

of pyrazinamide (Z), kanamycin (KM), levofloxacin (Lfx) and ethionamide (Eto) and cycloserine (Cs), a 12 month continuation phase with a combination of Z, Lfx, Eto and Cs i.e. 8(Km-Z-Lfx-Eto-Cs)/12(Lfx-Eto-Cs-Z). The treatment duration is minimum of 20 months and extended to 24 months when culture result is still positive after six months.

2.11.4 The Recommended Standard XDR-TB Treatment Regimen

The standard XDR-TB treatment regimen should be given for a minimum of 24 months i.e.12 months of intensive phase with injection capreomycin (Cm), moxifloxacin (Mfx), clofazimine (Cfz), paraaminosalicylic acid (PAS), Cs, amoxycillin/clavulanate (Amx/Clv) and Z and 12 months of continuation phase i.e. Mfx, Cfz, PAS, Cs, Amx/Clv and Z or 12Cm-Mfx-PAS-Cs-Amx/Clv-Z/12Mfx-PAS-Cs-Amx/Clv and extended to 30 months when culture result is still positive after month six (NTPN, 2012).

2.12 Classification of Treatment Outcomes

MDR-TB treatment outcomes were defined as per NTP Nepal guidelines, which have been based on the WHO definitions as cured, treatment completed, died, lost to follow-up, not evaluated and treatment failure.

2.12.1 Cured: A patient has completed treatment and has three or more consecutive negative cultures is taken as cured.

2.12.2 Treatment Completion: It is defined as completed treatment, without evidence of fulfilling the criteria to be cured or treatment failures.

2.12.3 Died: Died referred to death of patients for any reason during the course of treatment.

2.12.4 Lost to Follow-Up: This is referred to interruption of treatment for 60 days or more.

2.12.5 Not Evaluated: A patient for whom no treatment outcome was assigned either due to being transferred out to other facility or still on treatment was classified as ‘not evaluated’.

2.12.6 Treatment Failure: Treatment failure is applied for TB patients under treatment remained continuously smears and culture positives for more than five or more months of the intensive phase (NTPN, 2012).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Material

List of bacteriological media, reagents, chemicals, equipment, glass wares and miscellaneous materials used in this study are given in Appendix B.

3.2 Method

The study is an analytical study to identify rifampicin resistance (RR) and MDR-TB among retreatment TB cases using Gene Xpert MTB/RIF assay, conventional culture based proportional FLD-DST, Genotype MTBDR*sl* for SLD-DST and their comparison.

3.2.1 Study Site

This study was carried out from April 2013 to April 2018, in National Tuberculosis Reference Laboratory/SAARC Tuberculosis Reference Laboratory, at National Tuberculosis Centre, Thimi, Bhaktapur, Nepal.

3.2.2 Sample Size

A total of 159 patients were involved in this study, which was more than the minimum required sample size of 138 using the WHO recommended formula (Appendix A).

Formula: $n = Z^2 (p) (1-p/d^2)$ where,

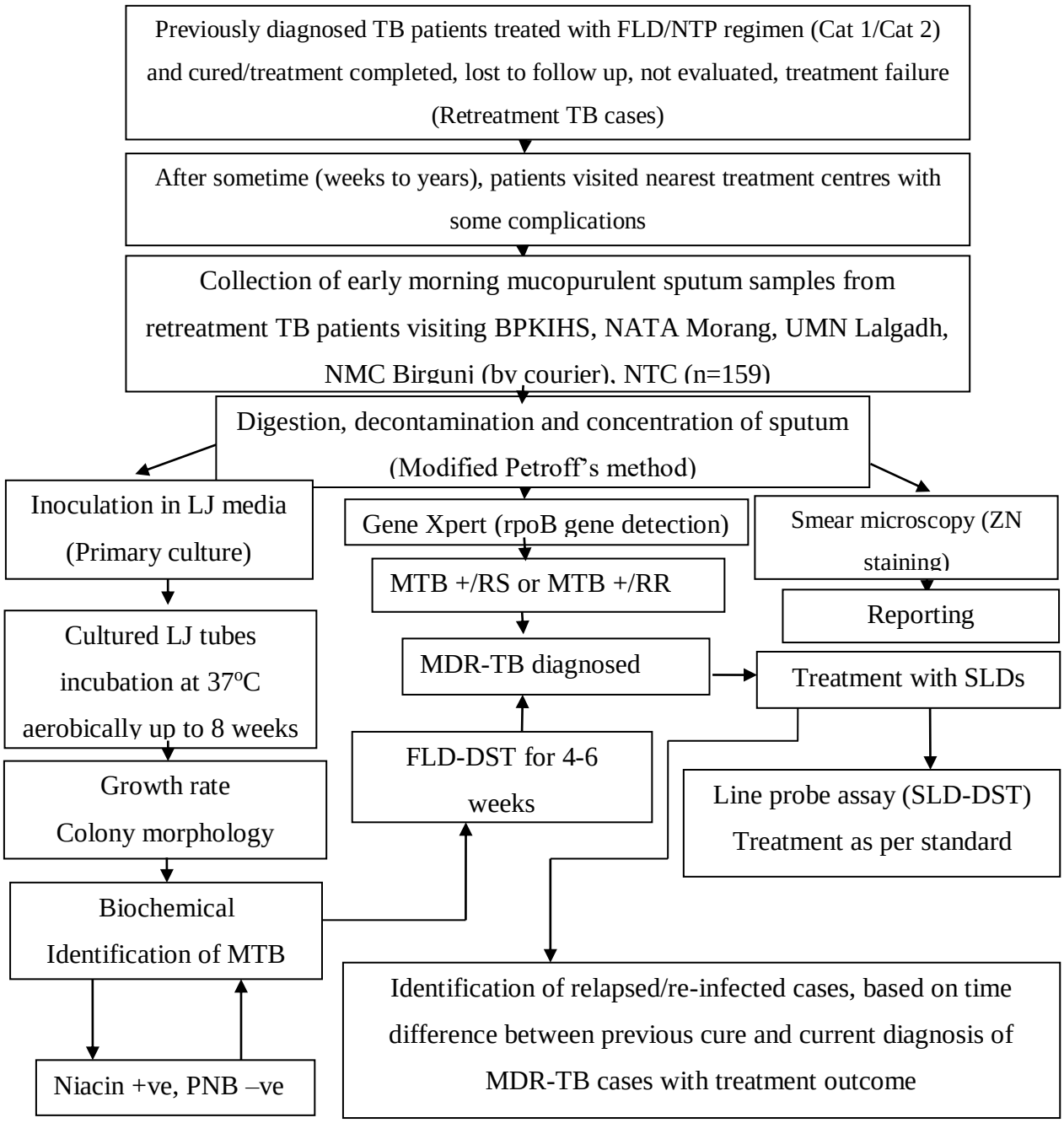
n = sample size

Z = 1.96 (CI 95%)

p = anticipated prevalence

d = absolute precision

STUDY METHODOLOGY FLOW CHART



3.2.3 Study Population

Retreatment PTB cases (relapse, treatment after failure, and treatment after loss to follow-up) previously treated with Cat 1 and Cat 2 treatment regimen as per National TB Programme (NTP) guidelines based on WHO recommendations were enrolled in this study.

3.2.4 Inclusion/Exclusion Criteria

Retreatment TB cases (Cat 1 and Cat 2 failure) visiting DR-TB Treatment Centres for further diagnosis and diagnosed as sputum smear positive or negative before being registered for and started MDR-TB treatment were included in this study. But the cases already registered and recently undergoing second line anti tuberculosis treatment, blood stained sputum, sputum with food particles, with saliva in greater amount, leaking, dried or if not freshly collected and patients suspected of extra-pulmonary tuberculosis were excluded from this study. The samples showing contamination during culture were not further included in the study.

3.2.5 Sample Collection

Early morning sputum samples (stuffy and mucopurulent, 3-5ml each) were collected from patients in leak proof, wide mouthed, transparent and sterile 50 ml disposable plastic centrifuge tube (Falcon BD, USA); then were appropriately labeled and stored at refrigerated temperature (2-8°C) until dispatched or processed. Samples were submitted directly to NRL (patients attending OPD at NTC) and transported via private courier from other DR-TB treatment centres (BPKIHS, Dharan, Sunsari; NATA, Biratnagar, Morang and NMC, Birgunj, Parsa; Mission Hospital, Lalgarh, Dhanusha).

3.2.6 Sample Processing

Sputum samples were processed in a Biological Safety Cabinet class II (BSC-II AIRTECH, Japan) directly by adding twice the volume of 4.0% NaOH (modified Petroff's method). It was vortexed, mixed and left for 15 minutes at room temperature with occasional shaking (Appendix G). Then phosphate buffer was added up to the level of 45 ml graduation mark. It was vortexed, mixed and centrifuged at 3000x g for 15 minutes in a refrigerated centrifuge at 4°C (KUBOTA, Japan).

3.2.7 Culture on LJ Medium

The supernatants were discarded and sediments/pellets were used for culture; the condensed water in the medium was removed by putting the media tube upside down on spirit cotton and from each of the processed samples, 0.2ml of sediment was inoculated on LJ media in duplicate. The caps of the inoculated medium were loosened and laid the tubes on the slanting bed (putting the slant-face upside). Then all the LJ tubes were incubated at 37°C for 4-8 weeks in an incubator (MEMMERT, Germany). The tubes were examined on 7th day for rapid growers and were checked for growth at 2, 3, 4, 5, 6, 7, 8 weeks. The positive culture results and any contamination in the culture tubes were recorded (Appendix E).

3.2.8 Gene Xpert MTB/RIF Assay

From the remaining of the sediments, Gene Xpert MTB/RIF tests were performed following the manufacturer's guidelines (Gene Xpert IV: CEPHIED, France). Sputum pellets/sediments (processed for primary isolation culture) were treated with sample reagent (SR) i.e. a mixture of NaOH and iso-propanol. The SR was added to the sputum sediments in 3:1 ratio and left for 15 minutes at room temperature. Two ml of the treated samples were transferred to the Gene Xpert cartridges (CEPHIED, France), which were then loaded into the programmed Gene Xpert modules, Gene Xpert device was kept running till the results were observed. The whole process of Gene Xpert was completed within about 2 hours (Appendix K).

3.2.9 Smear Microscopy

A smear of the processed sediment was prepared (size of 2 X 3 cm), air dried at room temperature (RT), heat fixed, stained by Ziehl-Neelsen method, air dried and read under binocular light microscope (Olympus, Japan). AFB was stained pink, straight or slightly curved rod in the blue background (Photograph 2). The results were reported according to NTP Nepal grading scale that is adopted according to WHO/IUATLD grading scale (Appendix E).

3.2.10 Biochemical Identification Tests

Identification tests were performed by biochemical methods (PNB, niacin tests) from the positive growth. All the positive cultures have shown PNB negative and niacin positive.

3.2.10.1 Growth on PNB Identification Test

A loopful of neat bacterial suspension (adjusted to McFarland standard No. 1) was inoculated into one slope of LJ medium and to another slope of p-nitro benzoic acid (PNB) at a concentration of 500 μg/ml and each set was incubated at 37°C. Reading was taken on 28th day.

Results and Interpretation

MTB did not grow on PNB medium. All other mycobacteria were resistant to PNB. MTB $H_{37}Rv$ (PNB susceptible) and *M. kansasii* (PNB resistant) were used as negative control and positive control respectively.

3.2.10.2 Niacin Production Test

BBL Taxo TB Niacin test strips (Becton and Dickinson, USA), absorbent paper strips were used according to the manufacturer's instruction. Using a sterile transfer pipette, approximately 0.6 ml of the positive culture growth extract was transferred to the bottom of 20 mm × 125 mm screw cap test tube. Negative control was also prepared. The strips were dropped with arrow downward into the tubes of positive and negative controls, test cultures and then stopperred immediately. The colors of the extracts were then compared after 15 minutes (Photograph 1). Niacin test strips were then neutralized with 10% NaOH, autoclaved and discarded.

Results and Interpretation:

Niacin accumulation was indicated by vivid appearance of a yellow color in the extract.

Controls

MTB $H_{37}Rv$ and *M. kansasii* were used as positive control as negative control respectively.

3.2.11 Drug Susceptibility Test on First Line Drugs (1% Proportion Method)

First line drug-DST was performed on all the culture positive samples in LJ tubes containing drugs (SIRE) i.e. streptomycin (S: 4.0 µg/ml), isoniazid (I: 0.2 µg/ ml), rifampicin (R: 40.0 µg/ml) and ethambutol (E: 2.0 µg/ml) in duplicate following 1% proportion method. Two LJ slopes without drugs were used as controls (Photograph 3). (All drug powders used were manufactured by SIGMA-Aldrich, USA). Quality control was routinely performed during DST using the reference strain MTB H37Rv (ATCC-27294), which was susceptible to all the 4 FLDs. All the inoculated tubes were then incubated at 37°C. Then resistance pattern of the 4 FLDs/SIRE was checked at 4th week and 6th week (Appendix H).

3.2.12 Drug Susceptibility Test on Second Line Drugs (Line Probe Assay)

Out of 109, a total of 29(26.6%, F12/M17) confirmed MDR-TB isolates were randomly selected and used for SLD-DST to identify how many of them might have developed XDR TB. Second line drug-DST was performed on fluoroquinolones, injectable aminoglycosides and ethambutol by LPA techniques (MTBDR*sl* 96 version 1.0). The whole process of the LPA was followed as per the manufacturer's guideline (HAIN Life Science, GmbH Germany).

3.2.12.1 DNA Extraction (inside a BSC-II)

DNA isolation/extraction from sample materials was done inside a BSC (Micro Flow BSC-IIA, Bioquell, UK). From LJ tube, 1-2 colonies of organisms were harvested and re-suspended in 300 µl of molecular grade water in a cryovial (1.5-2ml). It was kept in a vortex mixer (SONAR, India), vortexed and mixed for homogenize bacterial suspension/pellet. The suspension was heat inactivated at 97°C for 20 minutes and incubated in an ultrasonic water bath (LABTECH, India) for 15 minutes. Then it was centrifuged (KINTARO) for 5 minutes at 13000 X g. The supernatant containing DNA was transferred to another cryotube and stored at 4°C to -20°C until it was processed further. At the end, decontamination of all the instruments, pipettes, racks and bench spaces was done with freshly prepared 0.5% sodium hypochlorite (NaOCl).

3.2.12.2 Primer Nucleotides Mix (PNM) Preparation

PCR master mix was prepared inside a PCR cabinet (LPA Safety Hood, LAB COMPANION). With micropipettes 35µl of PNM, 5µl of 10x buffer (15mM MgCl2), 2 µl MgCl2 (25mM), 3 µl H_2O and 0.2 µl Taq polymerase (Hot star Thermis aqaticus) were added into a cryotube and mixed well carefully. Master Mix was prepared for the determined number of samples, mixed well and aliquoted (45µl) in 1.5ml PCR tube. At the end, all the instruments, pipettes, racks and bench spaces were decontaminated with freshly prepared 0.5% NaOCl.

3.2.12.3 Amplification by PCR

To the aliquoted 45 µl of PNM, 5 µl of sample DNA was added inside a BSC-II and gently vortexed to mix well. The PNM/DNA mixture was placed into the thermal cycler (Thermal Cycler, USA) using Genotype Hot 30 specific progamme i.e. 30 cycles (10 cycles of initial denaturation and elongation then 20 cycles of denaturation, primer annealing and chain elongation). The mixture was run for 1 cycle of denaturation at 95°C for 15 minutes. The amplicon was further processed for 10 cycles of initial denaturation at 95°C for 30 seconds followed by chain elongation at 58°C for 2 minutes. Similarly, additional process for 20 cycles of denaturation at 95°C for 25 seconds, primer annealing at 53°C for 40 seconds and elongation at 70°C for 40 seconds has taken place. The final extension (1 cycle) was performed at 70°C for 8 minutes. After the completion of amplification, the PCR tubes were carefully removed from the thermal cycler and placed into the PCR rack.

3.2.12.4 Hybridization and Detection

Before starting this step, the hybridization buffer (HYB, green) and stringent wash solution (STR, red) were prewarmed at 45°C (Shaking water bath, HAIN, Germany) to dissolve the undissolved precipitates, whereas, rinse solution (RIN) and distilled water (DW) were prewarmed at room temperature (RT). Conjugate concentrate (Con-C) and substrate concentrate (Sub-C) were freshly diluted at 1:100 in the respective dilution buffer and protected from light. Twincubator (Hybridization oven/Twincubator, HAIN, Germany) set with P1 programme was used for Hybridization and Detection.

3.2.12.4.1 Hybridization

Twenty micro liter (20 µl) of denaturing buffer (DEN, purple) was added with 20 µl amplicon and mixed well. The mixture was incubated for 5 minutes at RT on the shaking platform. One milliliter (l ml) of pre-warmed HYB was added using micropipette with filter tips and mixed by tilting the tray back and forth carefully until purple DEN and green HYB mixed homogenously. The tray was then placed on the Twincubator. DNA strip was placed in each well of Twincubator tray with a forceps (number facing upward without forceps touching to the solution in the tray well). DNA strips were covered by the liquid in the wells. As the temperature reached to 45˚C, tray was covered and incubated for 30 minutes at 45˚C (hybridization step).

3.2.12.4.2 Detection

After the completion of hybridization step, HYB was completely aspirated with Pasteur pipette and 1ml of STR was added. It was incubated at 45˚C for 15 seconds. STR was completely removed and 1ml of RIN was added. Incubated for 1 minute at RT. RIN was completely removed and 1ml of diluted conjugate (10 µl Con-C/conjugate C and 990 µl Con-D/conjugate D) was added. Incubated for 30 minutes at RT. Conjugate was then completely removed and 1ml RIN was added. Incubated for 1minute at RT. RIN was completely removed and 1ml RIN was added. Incubated for 1 minute at RT. RIN was completely removed and rinsed with DW for 1 minute. One milliliter of diluted substrate was added (i.e.10 µl Sub-C and 990 µl of substrate buffer or Sub-D) and incubated for 2-10 minutes at RT. The substrate was completely removed and the reaction was stopped by rinsing twice with H_2O for 1 minute (Photograph 4). Finally, DNA strips were removed from tray and dried between two layers of absorbent paper. Detection process was completed using a Twincubator (HAIN Life Science, GmbH Germany). Individual strip after colour development was adhered to the corresponding column of the (HAIN Life Science, GmbH Germany) provided format and resistance pattern was identified. The original strips showing positive bands were kept for NRL record after being scanned for the purpose of present study (Photograph 5).

The molecular grade water was used as conjugate control (CC) and the LPA strip was used as amplification control (AC). The strips for the two negative controls must be positive only at CC and AC bands that were clearly formed in the strips used in this

study, so the test process was valid. The "PCR master mix negative control" verifies that no contaminating nucleic acids/amplicons have been introduced into the master mix.

3.3 Statistical Data Analysis

The study data were analyzed using SPSS version 16.0 software. The chi square test was used to compare the distribution of sputum smear results, culture results, MDR-TB identified by Gene Xpert MTB/RIF assay and conventional culture and FLD-DST results. The Fisher exact test was applied comparing culture and Gene Xpert results at $p<0.05$. Same software was used for the data analysis of XDR/pre XDR-TB cases, time difference between previously cured TB but currently diagnosed and treated MDR-TB with patient's treatment outcome (summarized by Kaplan-Meier survival curve). The *P*-value <0.05 was considered statistically significant for all categories. Mean, Median and standard deviation for the sex wise distribution of cases enrolled in this study were calculated. Sensitivity, specificity, positive predictive value, negative predictive value for smear and Gene Xpert results in reference to culture results were also calculated using MedCalc software and 95% confidence intervals were estimated.

3.4 Ethical Approval

This study was conducted following ethical approval from Ethical Review Board, Nepal Health Research Council (NHRC), (Registration No. 308/2017).

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Results

A total of 159 freshly collected early morning sputum samples from retreatment TB cases were used in this study. All the sputum samples were cultured in L-J media (gold standard for tuberculosis diagnosis), Gene Xpert MTB/RIF assay, stained by Z-N technique and examined under microscope. Out of 159 samples, 115(72.3%) were culture positive, 34(21.4%) culture negative and 10(6.3%) were contaminated. Samples with no growth and contaminated were excluded for further study. FLD-DST was performed on 115(100%) isolates retrieved from the culture, of which 109(94.78%) isolates were identified as MDR-TB. All the 159 sputum samples were tested by Gene Xpert MTB/RIF assay for the identification of *rpoB* gene mutation or RR/MDR-TB; all were Xpert positive. Among the 29(100%) MDR-TB isolates tested for SLD-DST using LPA, 12(41.4%) were found to be pre-XDR-TB, whereas 42(26.4%) and 117(73.6%) were sputum smear negative and sputum smear positive respectively.

Out of 159 retreatment TB cases enrolled in this study, 3(1.9%) were females under 15years, 58(36.5%) females and 84(52.8%) males in 15-60 years, and 3(1.9%) females and 11(6.9%) males in above 60 years group. Among 159, male (M) and female (F) ratio was 1.48:1). The total cases were divided into three age (years) categories, which were <15(1.9%), 15-60(89.3%) and >60(8.8%) respectively. The range of age group of the patients was 11-72 years (Table 3). Three cases (1.9%) were sputum smear positive in <15, 107(67.3%) sputum smear positive and 17(10.7%) sputum smear negative in 15-60 years of age categories. Similarly, 7(4.4%) sputum smear positive and 7(4.4%) sputum smear negative were in >60 years of age categories respectively. Likewise, 42(26.4%; 13F/29M) and 117(73.6%; 51F/66M) were sputum smear negative and sputum smear positive respectively. There was no statistically significant difference in distribution of smear results between age (p=0.253) and sex (p=0.152). Detection of MTB with RR was found in all 159

samples (40.3% F, 59.7% M) by Gene Xpert MTB/RIF technique. Majority of cases fell within the age group of 15-60 years. There were remarkable numbers (14 cases) above 60 years age groups.

4.1.1 Demographic Status of Retreatment TB Cases Enrolled in This Study

Table 3: Demographic status of retreatment TB cases enrolled in this study

Age (years)	Sex, n (%)		Total, n (%)
	Female	Male	
<15	3(1.9)	0(0.0)	3(1.9)
15-60	58(36.5)	84(52.8)	142(89.3)
>60	3(1.9)	11(6.9)	14(8.8)
Total	64(40.3)	95(59.7)	159(100)
Age range (Min-Max)	11-72		
Mean age (years) ± SD	35.86 ±15.95		
Median age (years)	30		

Among 159 cases, 115(72.3%) were culture positive i.e. 106(66.7%) were smear positive/culture positive and 9(5.7%) were smear negative/culture positive. Whereas, 34(21.4%) were culture negative i.e. 2(1.3%) were smear positive/culture negative and 29(18.2%) were smear negative/culture negative. Ten (6.3%; 4 smear negative and 6 smear positive) cases were contaminated. There was an association between smear and culture positive results (p<0.001).

The sensitivity (Se), specificity (Sp), predictive value of positive test (PPV) and predictive value of negative test (NPV) of smear microscopy and Gene Xpert results were evaluated with reference to culture using standard formula. The Se, Sp, PPV and NPV were found to be 106(92.2%) at 95% confidence interval (CI) within lower limit 85.1 and upper limit 96.1, 29(85.3%) at 95% CI; 68.2 and 94.5), 106(95.5%) at 95% CI; 89.3 and 98.3, 29(76.3%) at 95% CI; 59.4 and 88.0 respectively (Table 4). The contaminated cases were excluded from the calculation.

Formula: Sensitivity (Se) = a/(a+c)* 100, Specificity (Se) = d/(b+d)* 100

Positive predictive value (PPV) = a/(a+b)* 100

Negative predictive value (NPV) = d/(c+d)* 100

Where, a = true positive, a+c = total positive (positive test): a, c = disease

d = true negative, b+d = total negative (negative test): b, d = no disease

PPV = positives among total positive predicted

NPV = negatives among total negative predicted

4.1.2 Comparison of Smear and Culture Results

Table 4: Comparison of smear and culture results

	C+ n(%)	**C- n(%)**	**Marginal Row Total**
S+ n(%)	106(66.7%)	5(3.1)	111(69.8)
S- n(%)	9(5.7)	29(18.2)	38(23.9)
Marginal Column Total	115(72.3)	34(21.4)	149(93.7) Grand Total

S+ = smear +ve S - = smear –ve C+ = culture +ve C- = culture -ve

Calculation of Se, Sp, PPV and NPV applying standard formula:

Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
106/115*100 = 92.2 % (85.1 - 96.1)	29/34*100 = 85.3 % (68.2 - 94.5)	106/111*100 = 95.5 % (89.3 - 98.3)	29/38*100 = 76.3 % (59.4 - 88.0)

Out of 159(100%) sputum samples, 42(28.9%) and 117(71.1%) were smear negative and smear positive, 34(21.4%) and 115(72.3%) were culture negative and culture positive respectively. Gene Xpert has identified *rpoB* gene mutations (RR/MDR-TB) in all the sputum samples. Among the randomly selected 29(100%) MDR-TB cases diagnosed by both culture and Gene Xpert, line probe assay (MTBDR*sl*) identified 12(41.4) pre-XDR TB (Table 5).

Gene Xpert was the best diagnostic, which successfully detected *rpoB* gene mutations diagnosing 159(100%) RR/MDR-TB cases in comparison to sputum microcopy 117(71.1%) and culture 115(72.3%).

4.1.3 Comparative Summary between Sputum Smear Microscopy, Culture, Gene Xpert and Line Probe Assay

Table 5: Comparative summary between sputum smear microscopy, culture, Gene Xpert and line probe assay

Diagnostics	Results			Remarks
	Negative n(%)	Positive n(%)	Total n(%)	
Sputum microscopy	42(28.9)	117(71.1)	159(100.0)	
Culture	34(21.4)	115(72.3)	149(93.7)	Contaminated 10(6.3%)
Gene Xpert	0(0.0)	159(100.0)	159(100.0)	
Line probe assay	17(58.6)	12(41.4)	29(100.0)	All 29 cases were diagnosed MDR-TB by C/FLD-DST and Xpert

Out of total 117(100%) positives, the positive grading of sputum smear results was high for 111(94.9%) cases (1+, 2+, 3+) and 6(5.1%) were low/scanty positives (2-6 AFB) as shown in figure 1.

4.1.4 Distributions of Smear Positive Slides as per Grades of AFB Detected

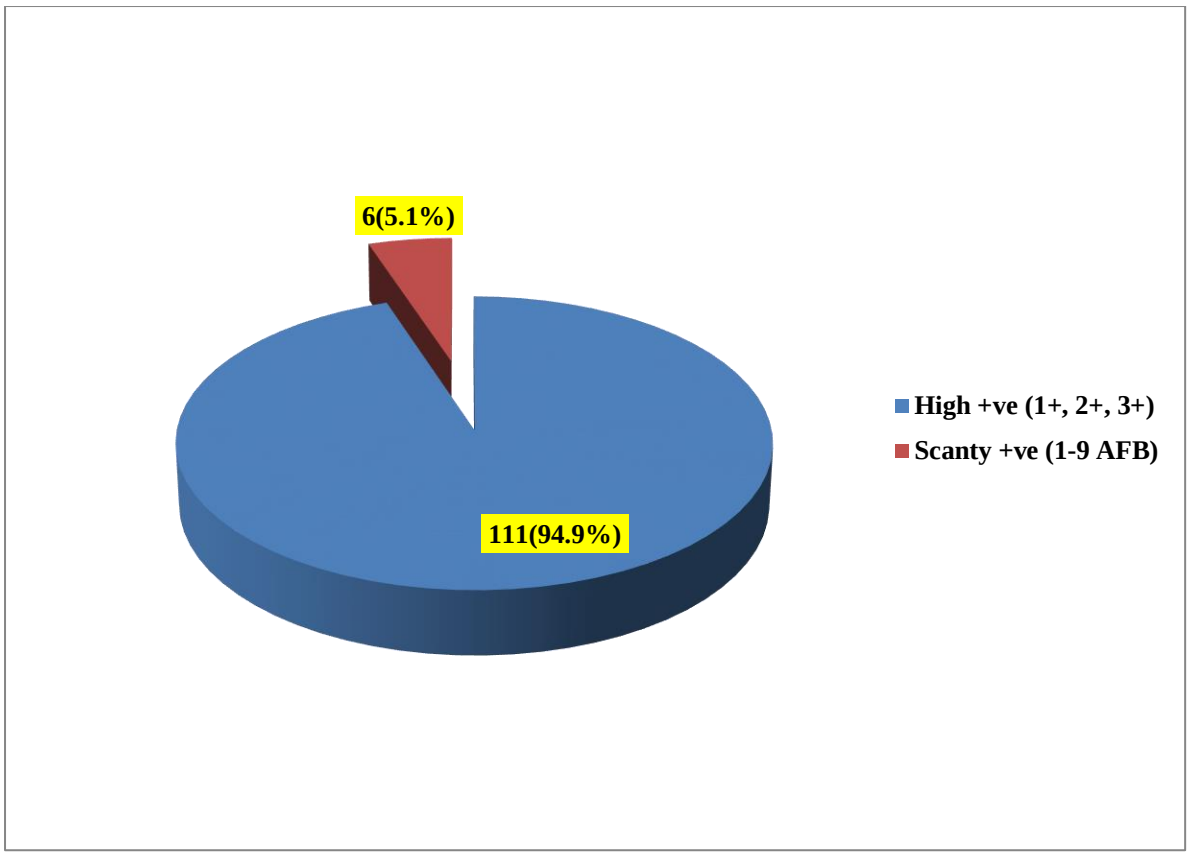


Figure 1 Grading distributions of smear positive results

Among 159 cases, 115(72.3%) were culture positive i.e. 106(66.7%) were culture positive/Xpert positive and 9(5.7%) were culture negative/ Xpert positive (Table 6). These data were analyzed by Fisher exact test and the statistic value was 1. The result was not significant at p <0 .05.

4.1.5 Comparison of Culture and Gene Xpert Results

Table 6: Comparison of culture and Gene Xpert results

	Gx+ n(%)	**Gx- n(%)**	**Total**
C+ n(%)	115(72.3%)	0(0.0%)	115(72.3%)
C- n(%)	34(21.4%)	0(0.0%)	34(21.4%)
Total	149(93.7%)	0(0.0%)	149(93.7) (Grand Total)

C - = culture –ve C+ = culture +ve Gx + = MTB detected/rifampicin resistant

Gx - = MTB not detected

Out of 115(48F/61M, 109(94.78%; 9 sputum smear negative: all M and 100 sputum smear positive; 43F/57M)) culture positive samples on FLD-DST showed both isoniazid and rifampicin resistance, whereas, 1 case each (1.74%; 1F/1M) showed mono resistance to isoniazid and rifampicin respectively. Similarly, 4(3.7%; 3F/1M) were susceptible to all 4 FLDs. There was significant difference of culture positive results and MDR-TB detection (p=0.001).

Among the total of 109 MDR-TB cases, 14(12.2%; F3/M11) were resistant to 2 drugs (isoniazid and rifampicin/IR) and higher in the age group of 15-60 years. Similarly, 42(36.5%; F21/M21) cases were resistant to 3 drugs (6.1%; F3/M4 to isoniazid, rifampicin and ethambutol/IRE and 30.5%; F18/M17 to streptomycin, isoniazid and rifampicin/SIR, higher in 15-60 years group respectively. Whereas, 53(46.1%; F24/M29) were found to be resistant to all 4 FLDs (SIRE) that was higher in 15-60 years group. Three (2.6%; F1/M3) cases showed resistance to SIRE among above 60 years age group. Three cases (2.6%; 2 resistant to SIR and 1 to SIRE) were all females below 15 years of age. The age wise distribution of MDR-TB by FLD-DST was 3(2.6%; F3), 101(87.8%; F43/M58) and 5(4.3%; F2/M3) for <15, 15-60 and >60 years group respectively. There was no significant difference of age wise MDR-TB

cases identified by Gene Xpert and conventional C/DST (p=0.532). FLD-DST was run along with the reference strain MTB H37Rv that was pan susceptible. There was no significant difference of sex wise distribution of MDR-TB cases identified by Gene Xpert and conventional C/DST (p=0.775) as shown in table 7. The distribution of FLD-DST patterns of MDR-TB was higher among males of 15-60 years groups.

4.1.6 Age/Sex Wise Distribution of FLD-DST Patterns

Table 7: Age (years)/sex wise distribution of FLD-DST patterns by conventional proportion method on positive cultures detected as rifampicin resistant by Gene Xpert MTB/RIF n(%)

	<15 years		**15-60 years**		**>60 years**		**Total n(%)**		**Grand total (%)**
	F	**M**	**F**	**M**	**F**	**M**	**F**	**M**	
Total Tested	3(2.6)	0(0.0)	46(25.2)	61(26.1)	2(1.7)	3(2.6)	51(44.3)	64(55.7)	115(100.0)
Fully Susc.	0(0.0)	0(0.0)	3(1.7)	1(0.87)	0(0.0)	0(0.0)	3(2.6)	1(0.87)	4(3.5)
Mono Resis.	0(0.0)	0(0.0)	1(0.87)	0(0.0)	0(0.0)	1(0.87)	1(0.87)	1(0.87)	2(1.74)
S	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
I	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.87)	0(0.0)	1(0.87)	1(0.87)
R	0(0.0)	0(0.0)	1(0.87)	0(0.0)	0(0.0)	0(0.0)	1(0.87)	0(0.0)	1(0.87)
E	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Total I^r+ R^r (MDR-TB)	3(2.6)	0(0.0)	43(37.4)	58(50.4)	2(1.74)	3(2.6)	48(41.8)	61(53.0)	109(94.8)
IR	0(0.0)	0(0.0)	2(1.74)	11(4.3)	1(0.87)	0(0.0)	3(2.6)	11(9.6)	14(12.2)
IRE	0(0.0)	0(0.0)	3(2.6)	3(2.6)	0(0.0)	1(0.9)	3(2.6)	4(3.5)	7(6.1)
SIR	2(1.74)	0(0.0)	16(13.9)	17(14.8)	0(0.0)	0(0.0)	18(15.7)	17(14.8)	35(30.5)
SIRE	1(0.87)	0(0.0)	22(19.1)	27(23.5)	1(0.87)	2(1.74)	24(20.9)	29(25.2)	53(46.1)

S: streptomycin I: isoniazid R: rifampicin E: ethambutol I^r: isoniazid resistance

R^r: rifampicin resistant Susc: susceptible Resis: resistant F: female M: male

Among 109 confirmed RR/MDR-TB (by both Gene Xpert and FLD-DST), 29(26.6%, F12/M17; 1M sputum smear negative, 28 sputum smear positive; F12/M16) randomly selected cultures were performed for SLD-DST by LPA to identify how many of the randomly selected isolates could be XDR-TB. Negative controls were clearly developed at CC and AC bands in the strips. MDR-TB isolates showed TUB bands formation in the strips, which is because of the amplicons were generated from all the members of MTBC. Out of 29 confirmed MDR-TB isolates, 17(58.6%; F5/M12) for *gyrA*, all were (100%) found to be wild type for *rrs* and 13(44.8%; F4/M9) for *emb B* genes, whereas 12(41.4%; F6/M6) were mutants for *gyrA*, 16(55.2%, F8/M8) for *emb B* and none were mutants for *rrs* genes.

The bands thus formed on the corresponding nitrocellulose strips were; 17 for *gyrA* WT1, WT2, WT3 probes located in regions from codons 85 to 97 (binding sites for fluoroquinonlones/ofloxacin or levofloxacin), all 29 for *rrs* WT1, *rrs* WT2 probes located in regions for nucleotides 1401, 1402 and 1484 (binding sites for injectable aminoglycoside/capreomycin). Similarly, 13 for *embB* WT1 (binding site for ethambutol) gene probes located in regions from codons 306 were found to be susceptible sites for the corresponding drugs. Whereas, the DNA matched with the mutant probes were; 12 for *gyrA* gene (*gyrA* MUT1, MUT2, MUT3A to 3D) conferring the most frequent mutation codons A90V, S91P, D94A, D94N/Y, D94G, and D94H, 16 for *emb B* (*embB* MUT1A, MUT1B) probes conferring mutations M306V and M306I (base exchange at codon 306; ATG→ATA). None were for AG/CP or *rrs* genes (*rrs* MUT1, MUT2) probes conferring mutations for codons A1401G, C1402T and G1484T. Similarly, *gyrA* gene mutations (fluoroquinolone resistance) alone was 3(10.3%; F2/M1) and together with *embB* gene was found in 9(31.0%; F5/M4) cases mostly of 15-60 years of age, whereas 7(24.1%; F3/M4) were found to be susceptible to all SLDs tested as shown in table 8, photographs 4 and 5 as well. The detection of mutations for *gyrA* gene alone of along with *embB* gene was distinctly higher among 15-60 years groups and similar for both genders.

4.1.7 Age/Sex Wise Distribution of SLD-DST Patterns Identified by Line Probe Assay

Table 8: Age (years)/sex wise distribution of SLD-DST patterns

Description	<15 years		15-60 years		>60 years		Total		Grand total
	F	M	F	M	F	M	F	M	
TUB	0 (0.0)	0 (0.0)	12 (41.4)	14 (48.3)	0 (0.0)	3 (10.3)	12 (41.4)	17 (58.6)	29 (100.0)
gyrA WT	0 (0.0)	0 (0.0)	5 (17.2)	9 (31.0)	0 (0.0)	3 (10.3)	5 (17.2)	12 (41.4)	17 (58.6)
gyrA MUT	0 (0.0)	0 (0.0)	6 (20.7)	5 (17.2)	0 (0.0)	1 (3.44)	6 (20.7)	6 (20.7)	12 (41.4)
rrs WT	0 (0.0)	0 (0.0)	12 (41.4)	14 (48.3)	0 (0.0)	3 (10.3)	12 (41.4)	17 (58.6)	29 (100.0)
rrs MUT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
*emb B*WT	0 (0.0)	0 (0.0)	4 (13.8)	7 (24.1)	0 (0.0)	2 (6.9)	4 (13.8)	9 (31.0)	13 (44.8)
*emb B*MUT	0 (0.0)	0 (0.0)	8 (27.6)	7 (24.1)	0 (0.0)	1 (3.44)	8 (27.6)	8 (27.6)	16 (55.2)

TUB: tuberculosis
gyrA WT: gyrase A gene wild type
gyrA MUT: gyraseA gene mutation
*emb B*WT: ethambutol B gene wild type
rrs WT: capreomycin/kanamycin gene wild type

Out of 29(100%), the distribution of resistance patterns of SLD-DST for one drug were; 3(10.3%) for ofloxacin and 10(34.5%) for ethambutol respectively. Similarly,

the resistance pattern of two drugs was 9(31.0%) for ofloxacin and ethambutol. A total of 7(24.1%) isolates were susceptible to all SLDs (Table 9).

4.1.8 Resistance Patterns of SLD-DST Identified by Line Probe Assay

Table 9: Resistance patterns of SLD-DST identified by line probe assay

	DST patterns of SLDs by Line Probe Assay, n(%)			
Resistance to 1 drug	OF	AG/CP	EMB	**Total**
	3(10.3)	0(0.0	10(34.5)	13(44.8)
Resistance to 2 drugs	OF+AG/CP	OF+EMB	EMB+AG/CP	
	0(0.0)	9(31.0)	0(0.0)	9(31.0)
Susceptible to all drugs	OF+AG/CP+EMB			7(24.1)

OF: ofloxacin, AG: aminoglycosides, CP: cyclic peptide
EMB: ethambutol

Among 29(100%), 12(41.4%; F6/M6) MDR-TB isolates were found to be pre-extensively drug resistant *(pre-XDR-TB) by LPA, but no XDR-TB. The age/sex wise distribution of the SLD-DST pattern showed maximum number of pre-XDR-TB among 15-60 years groups and equal for both females and males i.e. 6(50.0%; F6), 6(50.0%; M5 in 15-60 and 1(8.3%; M) above 60 years. The mutations detected for *emb B* genes were also similar for both females and males (F8/M8). There were no significant differences of age (p=0.531) and sex (p=0.428) groups of pre-XDR-TB among MDR-TB cases (Table 10).

(* By definition of NTP, MTB isolate identified as MDR-TB, which is simultaneously resistant to fluoroquinolones alone or along with injectable aminoglycosides as well is categorized as pre-XDR-TB).

4.1.9 Age/Sex Wise Distribution of Pre-XDR TB Identified by Line Probe Assay

Table 10: Age/sex wise distribution of Pre-XDR TB patterns

Pre-XDR TB identified by Line Probe Assay, n(%)			
	Sex category		
Age category (years)	**Female (F)**	**Male (M)**	**Total**
<15	0(0.0)	0(0.0)	0(0.0)
15-60	6(50.0)	5(41.7)	11(91.7)
>60	0(0.0)	1(8.3)	1(8.3)
Total	6(50.0)	6(50.0)	12(100.0)

Among 159(100.0%) retreatment TB cases enrolled in this study, a total of 128(80.5%) were further analyzed for their time difference of earlier cured TB but currently diagnosed RR/MDR-TB by Gene Xpert and C/DST. The previous history of TB patients was collected from the records of DR TB Treatment Centres in coordination and cooperation with the DR TB focal persons and by direct telephone contact to the patients or their guardians. A format for the information collection was sent to the DR TB focal persons (Appendix K) and the information completely fulfilled only was used for further analysis. Similarly, the complete information collected by direct telephonic conversations with the patients or their guardians (based on the format) only was added in the analysis. The time difference of TB patients who become cured after Cat1/Cat2 treatment but diagnosed as RR/MDR-TB by Gene Xpert alone or along with C/DST and XDR-TB by LPA was divided into 5 groups i.e. <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years groups respectively. Out of 128(100%) cases analyzed; 32(25.0%), 21(16.4%), 32(25.5%), 37(28.9%), 6(4.7%) were falling in <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years group respectively (Table 11). The age wise distribution were; 2(1.6%), 114(89.0%) and 12(9.4%) fell in <15, 15-60 and >60 years age group respectively. Among 128(100%), 45(35.2%) and 83(64.8%) were females and males respectively (Table

13). From the data made available; the analysis of patient's treatment outcome was also done. This is the first analyzed report on retreatment TB patients in Nepal.

Out of 128(100%) patients, 89(69,5%) were culture positive among which, 18(14.1%), 13(10.2%), 25(19.5%), 29(20.7%), 4(3.1%) were diagnosed as RR/MDR-TB by Gene Xpert and C/DST both, visiting DR TB Treatment Centres after <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years respectively. Similarly, 30(23.4%) i.e.14(10.9%), 8(6.2%), 3(2.3%), 4(3.1%), 1(0.8%) were diagnosed RR/MDR-TB by Gene Xpert but culture negative, whereas 8(6.2%) i.e. 1(0.8%), 0(0.0%), 5(3.9%), 2(1.6%) and 1(0.8%) were diagnosed as RR/MDR-TB by Gene Xpert but culture contamination falling in <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years group respectively (Table 11). Shorter the time difference of previously cured TB but currently diagnosed MDR by Xpert and positive yield by C/DST was higher among <1 month to 4 years groups.

4.1.10 Time Difference between Previously Cured TB and Current Diagnosis of MDR-TB by Gene Xpert and C/DST

Table 11: Time difference between previously cured and current diagnosis of MDR-TB

	Time difference					
Description	**<1 month n(%)**	**1-4 months n(%)**	**5-11 months n(%)**	**1-4 years n(%)**	**≥5 years n(%)**	**Total n(%)**
Previously cured and MDR-TB diagnosed by Gene Xpert	32(25.0)	21(16.4)	32(25.0)	37(28.9)	6(4.7)	128(100.0)
Previously cured and MDR-TB diagnosed by Gene Xpert and C/DST	18(14.1)	13(10.2)	25(19.5)	29(20.7)	4(3.1)	89(69.5)
Previously cured and MDR-TB diagnosed by Gene Xpert/C –*	14(10.9)	8(6.2)	3(2.3)	4(3.1)	1(0.8)	30(23.4)
Contamination	1(0.8)	0(0.0)	5(3.9)	2(1.6)	1(0.8)	9(7.0)

*C- : culture negative

Among 128(100.0%), 87(67.9%) i.e. 21(16.4%), 16(12.5%), 21(16.4%), 23(17.9), 6(4.7%) diagnosed MDR-TB by Gene Xpert alone or both Gene Xpert and C/DST after <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years age groups respectively were treated with SLDs (20-24 months) that were found to be cured. Two cases (1.5%) i.e. 1(0.8%) in <1 month and 1(0.8%) in 1-4 years groups completed treatment, whereas 20(15.6%) cases i.e. 1(0.8%), 3(2.3%), 6(4.6%), 8(6.3%), 2(1.6%) diagnosed and treated with SLDs falling in <1 month, 1-4 months, 5-11 months, 1-4 years ≥5 years groups respectively were died. Fourteen (10.9%) cases have been found to be lost to follow up i.e. 1(0.8%), 5(3.9), 4(3.1%), 4(3.1%), 0(0.0%) falling in <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years groups respectively. Two (1.6%) cases i.e. 1(0.8%) in 5-11 months and 1(0.8%) in 1-4 years groups were not evaluated. Three (2.3%) cases i.e. 1(0.8% fell in 1-4 years and 2(1.6% fell in ≥5yrs) groups respectively were found to be treatment failure (Table 12). Most of the

previously cured but diagnosed and treated MDR-TB patients with SLDs within <1 month to 4 years have been found to be cured/completed (higher survival of the patients). Whereas, delayed diagnosis resulted higher deaths and showed possible chances of developing XDR/pre-XDR TB (Figure 2).

4.1.11 Time Difference between Previously Cured TB and Current Diagnosis of MDR-TB Patients with Treatment Outcome

Table 12: Time difference between previously cured TB and current diagnosis of MDR-TB patients with treatment outcome

	Time difference					
Treatment outcome	**<1 month** n(%)	**1-4 months** n(%)	**5-11 months** n(%)	**1-4 years** n(%)	**≥5 years** n(%)	**Total** n(%)
Cured	21(16.4)	16(12.5)	21(16.4)	23(17.9)	6(4.7)	87(67.9)
Completed	1(0.8)	0(0.0)	0(0.0)	1(0.8)	0(0.0)	2(1.6)
Died	1(0.8)	3(2.3)	6(4.6)	8(6.3)	2(1.6)	20(15.6)
Lost to follow up	1(0.8)	5(3.9)	4(3.1)	4(3.1)	0(0.0)	14(10.9)
Not evaluated	0(0.0)	0(0.0)	1(0.8)	1(0.8)	0(0.0)	2(1.6)
Treatment failure	0(0.0)	0(0.0)	1(0.8)	2(1.6)	0(0.0)	3(2.3)
			Grand total			128(100.0)

4.1.12 Kaplan-Meier Survival Curve Summarizing Time Difference of Previously Cured and Current Diagnosis of MDR-TB with Treatment Outcome

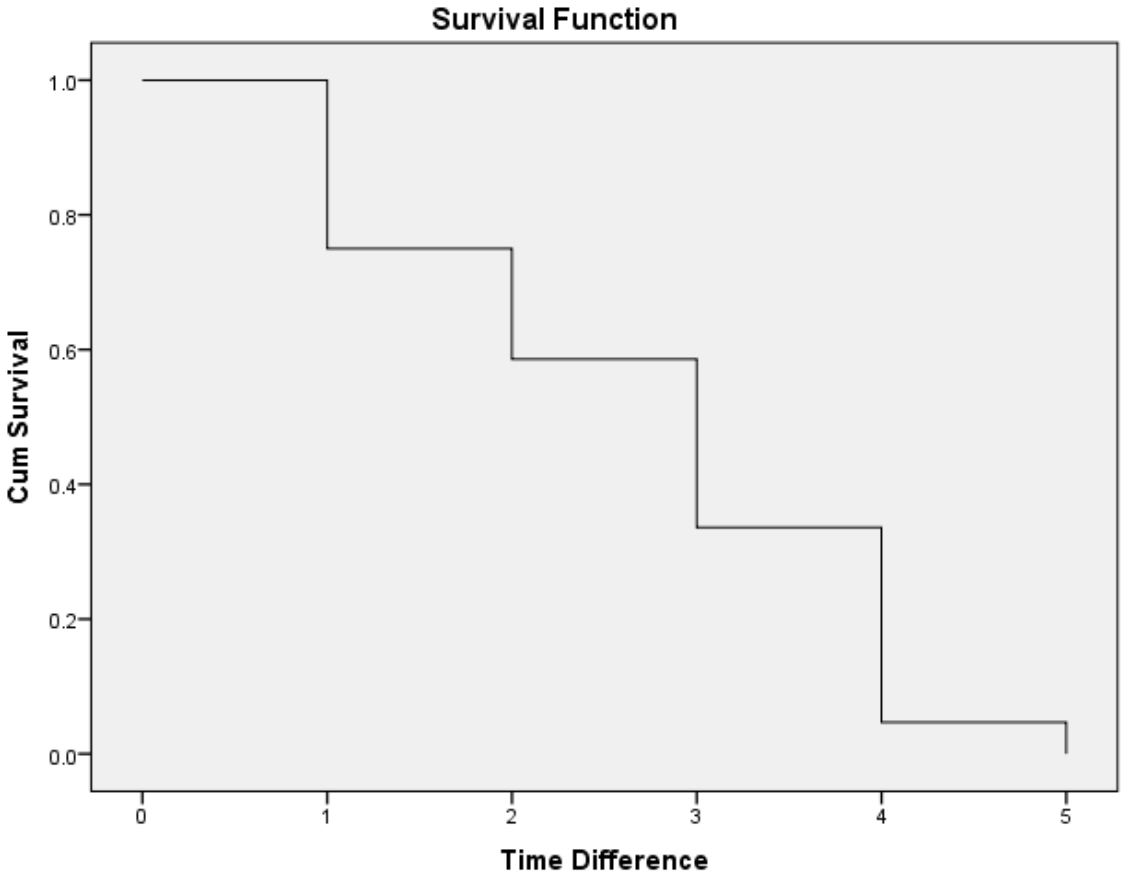


Figure 2 Time difference of previously cured and current diagnosis of MDR-TB with treatment outcome

Y axis of the curve denotes cumulative survival of the patients in percentage. X axis denotes time differences of previously cured and current diagnosis of MDR-TB i.e. 1 = < 1 month, 2 = 1-4 months, 3 = 5-11 months, 4 = 1-4 years, 5 = ≥5 years

The statistical data analysis of the treatment outcome results were categorized into favorable (cured and treatment completed) and unfavorable (died, lost to follow up, not evaluated and treatment failure) groups. The age wise treatment outcome results of 128(100.0%) MDR-TB cases were; 1(1.1%), 80(62.5%), 8(9.0%) were among the favorable group and 1(2.6%), 34(26.6%), 4(10.3%) were among unfavorable groups of <15, 15-60 and > 60 years of age respectively (Table 13). There were no significant differences of age (p=0.897) and sex wise (p=0.357) treatment outcomes. The treatment outcome was favorable for all age groups but higher among males of 15-60 years groups.

4.1.13 Age/Sex Wise Distribution of Previously Cured and Currently Diagnosed MDR-TB Patients with Treatment Outcome

Table 13: Age/sex wise distribution of previously cured and currently diagnosed MDR-TB patients with treatment outcome

Age (years)	**Outcome favorable n(%)**		**Outcome unfavorable n(%)**		**Total n(%)**		**Grand total n(%)**	**P value**
	F	M	F	M	F	M		
< 15	1(0.8)	0(0.0)	1(0.8)	0(0.0)	2(1.6)	0(0.0)	2(1.6)	p=0.897 (age)
15-60	26(20.3)	54(42.2)	15(11.7)	19(14.8)	41(32.0)	73(57.0)	114(89.0)	
>60	2(1.6)	6(4.7)	0(0.0)	4(3.1)	2(1.6)	10(7.8)	12(9.4)	p=0.357 (sex)
Total	29(32.6)	60(67.4)	16(41.0)	23(59.0)	45(35.2)	83(64.8)	128(100.0)	

Favorable: (cured and completed) Unfavorable: (died, lost to follow-up, not evaluated, TF)

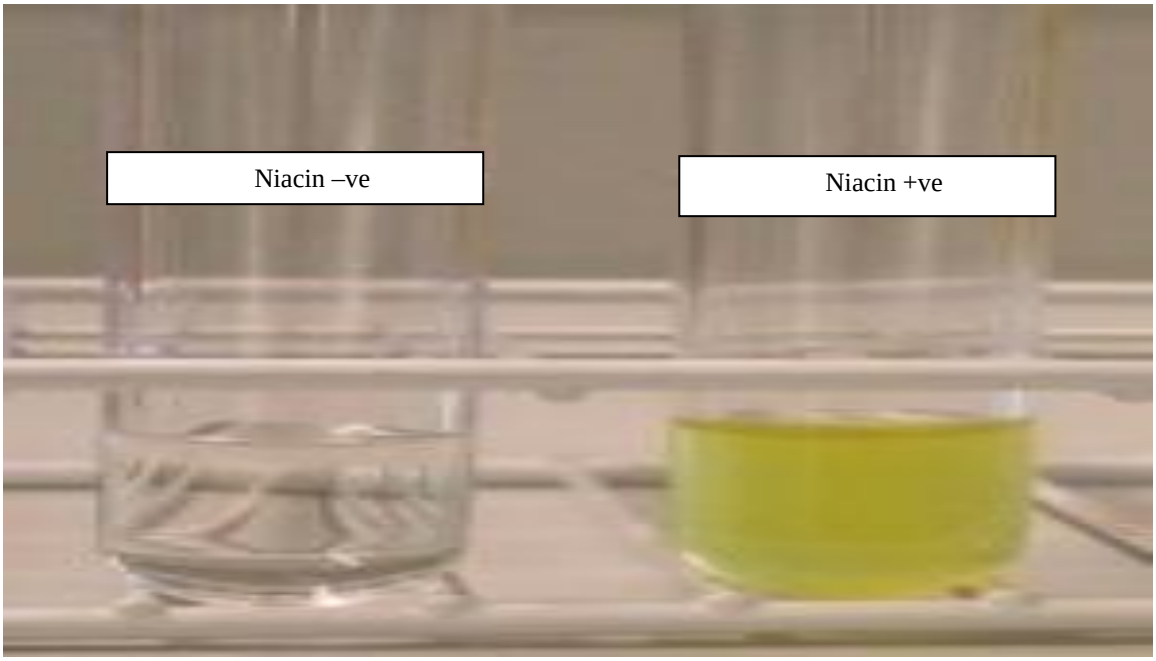
Treatment outcomes were favorable for 89(100%) i.e. 29(32.6%) RR/MDR-TB diagnosed cases by Gene Xpert only and 60(67.4%) were diagnosed by Gene Xpert and C/DST both. Similarly, treatment outcomes were unfavorable for 39(100%) i.e. 14(35.9%) cases were diagnosed by Gene Xpert only and 25(64.1%) by both (Table 14). There was no significant differences between diagnosis of RR/MDRTB by Gene Xpert and C/DST and an outcome for favorable and unfavorable group (p=0.746).
The treatment outcome was high (cured/completed) for MDR-TB cases diagnosed by both Xpert and C/DST than by Xpert only.

4.1.14 Treatment Outcome of Currently Diagnosed MDR-TB Patients by Gene Xpert alone or Along with Culture and Drug Susceptibility Test

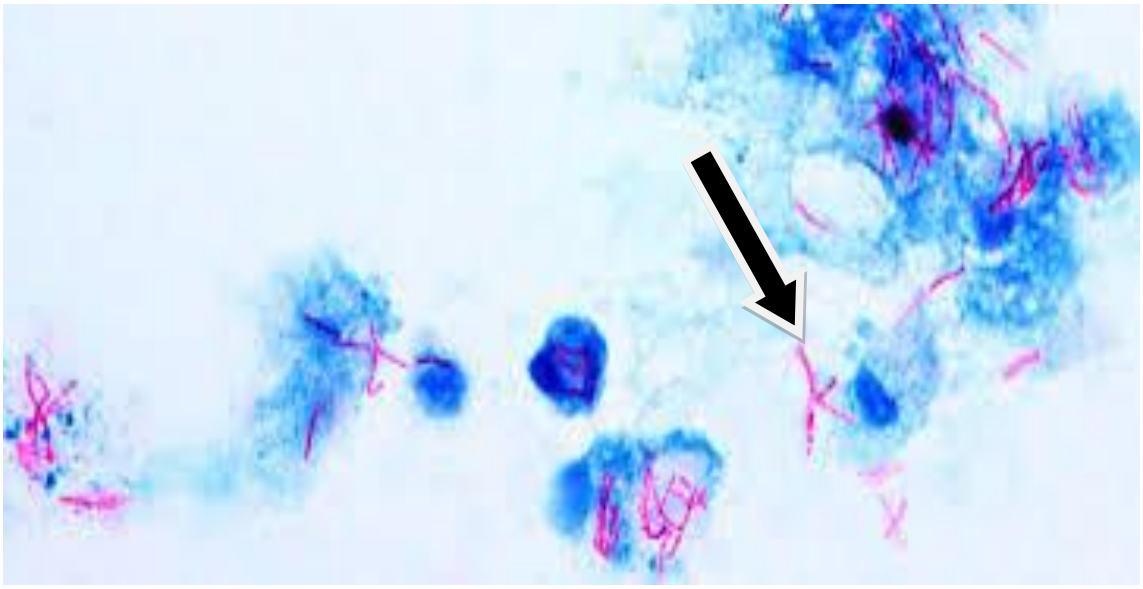
Table 14: Treatment outcome of MDR-TB patients diagnosed by Gene Xpert alone or along with culture and drug susceptibility test

Diagnosis by	Outcome favorable n(%)	Outcome unfavorable n(%)	Total n(%)	P value
Gene Xpert only	29(32.6)	14(35.9)	43(33.6)	
Gene Xpert and C/DST both	60(67.4)	25(64.1)	85(66.4)	p=0.746
Total	89(100.0)	39(100.0)	128(100.0)	

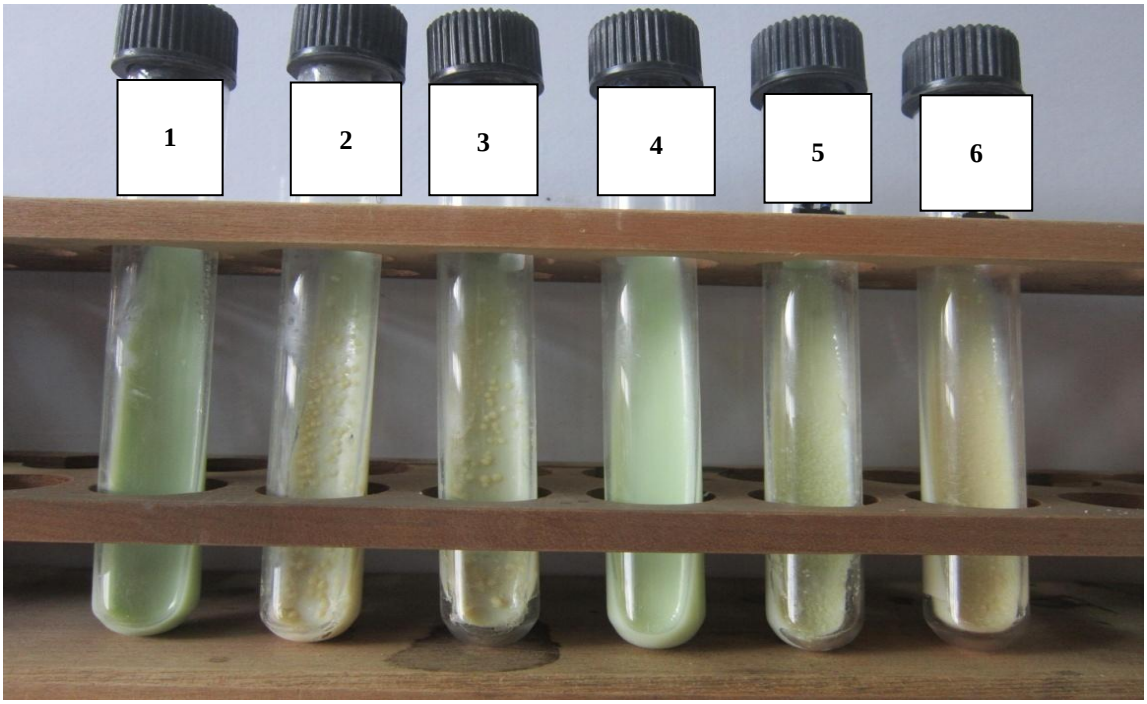
Favorable: (cured and completed)
Unfavorable: (died, lost to follow-up, not evaluated, TF)



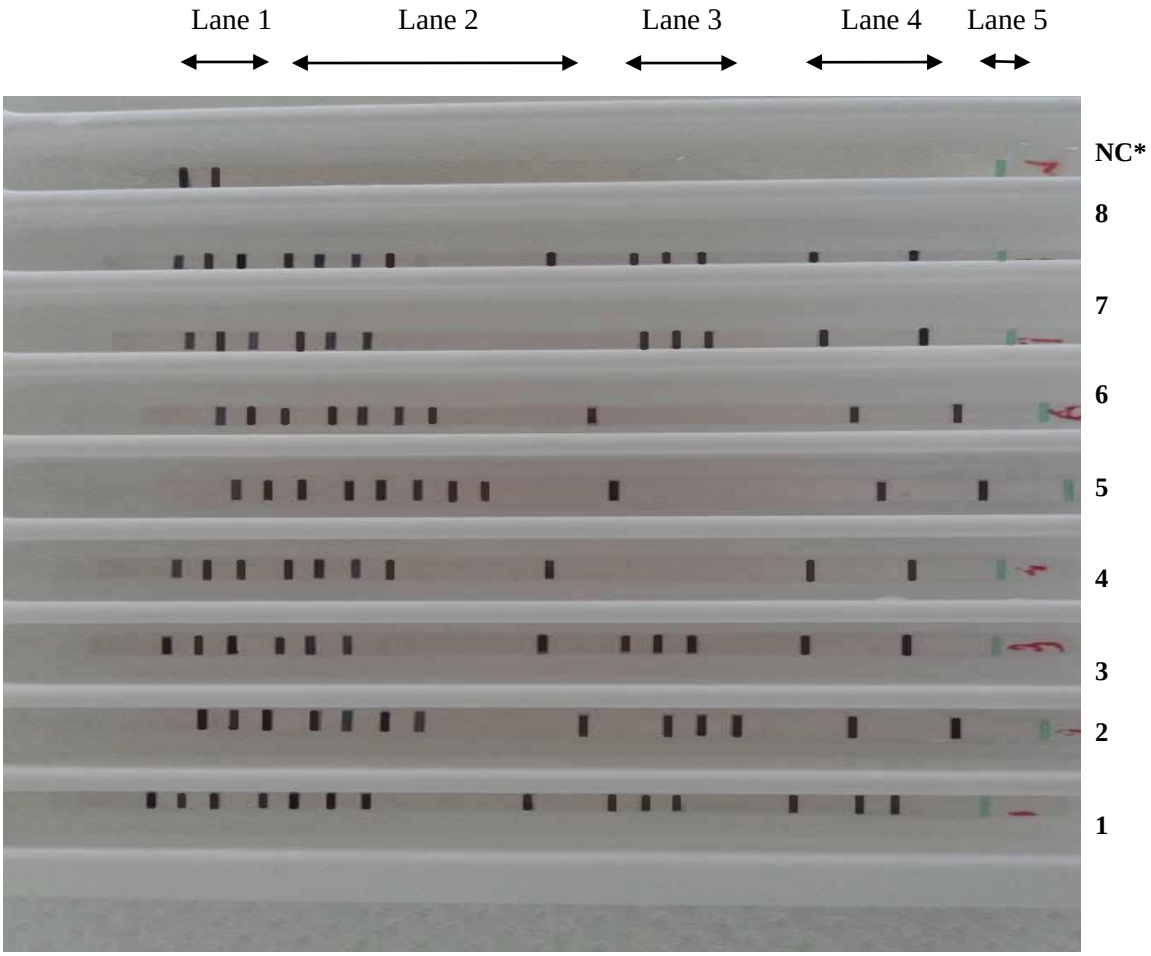
Photograph 1: Tube showing niacin –ve reaction is colorless and yellow colour formation is niacin +ve.



Photograph 2: Microscopic view of Ziehl-Neelsen stained sputum smear and acid fast bacilli (AFB) seen at magnification of 1000X. AFB are stained pink (primary stain: carbol fuchsin) against blue (counter stain: methylene blue) back ground. The presence of AFB on this microscopic visual field is more than 10, so graded as 3+ (WHO grading scale).

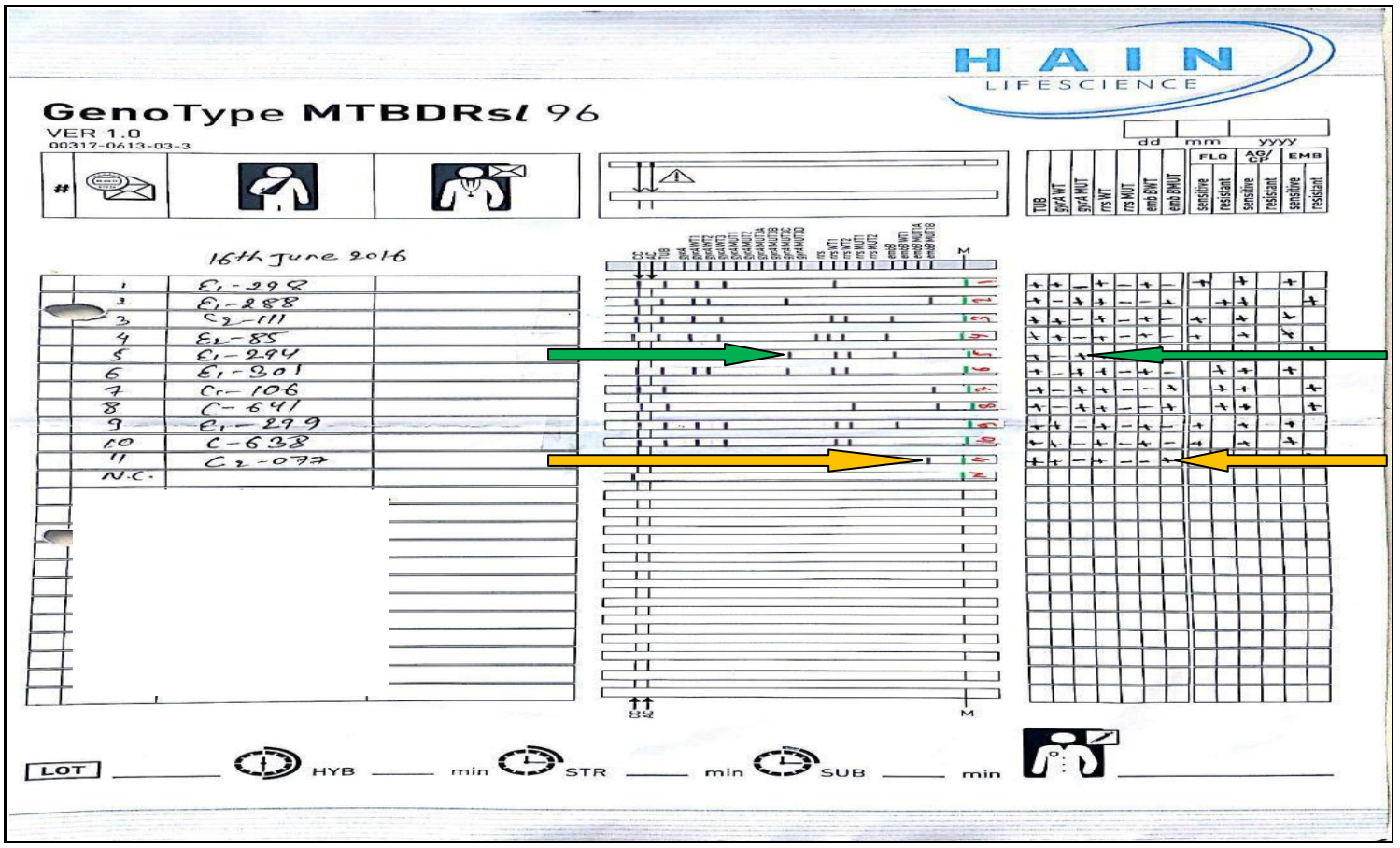


Photograph 3: First-line anti TB drug susceptibility test (FLD-DST) and the resistance pattern of MTBC to SIRE. Tube 1: streptomycin/S-4µg/ml (susceptible strain); 2: isoniazid/I-0.2µg/ml (resistant strain); 3: rifampicin /R-40µg/ml (resistant strain); 4: ethambutol/E-2µg/ml (susceptible strain); 5 and 6: Controls for dilution 10^{-2} and 10^{-4}.



Photograph 4: Hybridization patterns obtained with the Genotype MTBDR*sl* assay.

(NC*: Negative control, 1-8: Test strips)**,** Lane 1: CC/AC/TUB, Lane 2: *gyrA* (WT 1-WT 3/MUT 1-MUT 2, MUT 3A- MUT 3D)**,** Lane 3: *rrs* (WT1/MUT 1-MUT 2), Lane 4: *embB* (WT 1/MUT 1A-MUT 1B), Lane 5: M Marker)



Photograph 5: Post hybridization bench for pasting of the strips and interpretation

- Green arrow (S. No. 5/E1-294, left above) is showing strip band formation in *gyrA* MUT 3C and interpreted as *gyrA* gene mutation (right).
- Orange arrow (S. No.11/C2-077, left above) is showing strip band formation in *embB* MUT 1B and interpreted as *embB* gene mutation (right).

4.2 Discussion

Tuberculosis (TB) remains one of the major public health problems in Nepal. DOTS strategy was started in April 1996 in Nepal to reduce the number of deaths from TB (NTPN, 2012). During 2014, total of 37,025 cases of TB were registered. Among them, 51 % were pulmonary TB. Highest reported cases were among 15-24(20%) years age group though most cases reported among the middle aged (40-60 years) group. A total of 349 MDR-TB and 25 XDR-TB were enrolled for treatment in 2014. According to the WHO estimation, about 4.6 thousand people died from TB in 2014 (WHO, 1997). In Nepal, HIV among TB is 2.4% and TB among HIV 11.6%. Nearly 9.3% of new patient resistant to at least one drug, whereas, MDR-TB is low (2.2%) among new cases while it is comparatively higher (15.4%) among retreatment cases (NTPN, 2015). One of the concerned aspects of drug resistance in Nepal is the high level of resistance to fluoroquinolones (26.4%), which leads to heavy burden (8%) of pre-XDR and XDR-TB among MDR-TB patients (NTPN, 2015).

Microscopy is still familiar as a main diagnostic technique in resource-limited countries. (Peterson, 1999). Due to shortcomings of conventional technique, novel molecular techniques are needed that combine the rapidity of microscopy and the sensitivity of culture. They can identify the mycobacterial species and would help the clinician during the initial treatment of the patient. Though molecular techniques are not used routinely in Nepal, some investigators reported its feasibility (Sapkota, et al., 2007). Despite the expansion and implementation of a much improved NTP through DOTS strategy throughout the country, distribution of TB is still going up. This is all because of delay of diagnosis that leads delaying in treatment. Similarly, the shortage of rapid and accurate techniques in TB diagnosis might have also favored the transmission of the disease (MDR-TB and XDR-TB). This problem of drug resistance might be because of over the counter availability and unregulated use of fluoroquinolones. To combat the excess mortality related to XDR-TB, it is recommended to perform SLD-DST to all MDR-TB cases at the start of treatment. To comply with the above suggestion, it seems important for Nepal to strengthen its capacity to perform SLD-DST, either by culture (solid/liquid) or molecular biology or LPA (NTPN, 2015). In order to overcome such problems, present study has evaluated retreatment TB cases with their microscopic sputum results compared with Gene

Xpert MTB/RIF assay for the rapid diagnosis of MDR-TB. All the MDR-TB cases detected by Gene Xpert MTB/RIF assay were further verified by conventional culture and FLD-DST. Some of the MDR-TB diagnosed by both the methods were randomly selected and analyzed for SLD-DST by LPA for the detection of XDR-TB.

In the present study, age wise sputum smear positivity was high among 15-60 years group and was higher in males 41.5%(66/159) than in females 32.1%(51/159). Similar results were previously reported in which, out of 58 patients (with age range 21-67 years) clinically suspected to have pulmonary retreatment TB, Z–N smear examination was positive for AFB in 49(90.7%). L–J Culture results revealed positive yield in 54 cases (Meawed, 2016).

Despite of smear results (42 sputum smear negative and 117 sputum smear positive), Gene Xpert MTB/RIF assay showed 100% rifampicin resistance (RR-TB or MDR-TB), which was high among males (59.7%) of 15-60 years than in females (40.3%). In previous study, 27 sputum smear positive and 23 sputum smear negative specimens were found to be RR/MDR-TB detected by Gene Xpert MTB/RIF whereas, 8 cases were negative for MTB among sputum smear negative. For pulmonary specimens, the Se and Sp of the MTB/RIF test were 82.3% and 100%, respectively. The sensitivities were 100% and 68.6% for smear-positive and smear-negative specimens respectively (Zeka, et al., 2011). Helb et al. (2010) described in his study that a small clinical validation study of 107 clinical sputum samples from suspected tuberculosis cases in Vietnam detected MTB by Gene Xpert MTB/RIF assay in 29/29(100%) smear-positive and 33/39(84.6%) smear-negative cases respectively were also found to be culture-positive. In addition to positive results for MTBC, detection by Gene Xpert MTB/RIF was in 54 of cases. The study clarified that Gene Xpert detected an extra positive retreatment tuberculosis case as compared with culture on LJ media as the gold standard test. It could detect more five positive TB cases when compared with ZN microscopy indicating a higher sensitivity of 98.15%, specificity was 75% (Meawed, 2016).

A concordance of sputum smear results (sputum smear negative/sputum smear positive) and detection of MTB along with rifampicin resistance by Gene Xpert MTB/RIF assay for all 159 samples were 100%. In the previous study, detection of

MTB with rifampicin resistance was found in 11.53%(37/321) sputum smear positive samples by Gene Xpert MTB/RIF assay. The concordance for sputum smear negative was 88.47%(284/321) and discordance was 8.17%(26/321) (Mahmoud et al., 2015). Whereas, it was reported in the similar study by Helb et al. (2010) from 107 sputum samples in Vietnam that the concordance for sputum smear positive, Gene Xpert MTB/RIF (RR TB) and culture was 100%(29/29). In the same study, it was described that 64 smear-positive sputa from retreatment TB cases in Uganda tested by Gene Xpert MTB/RIF assay detected MTB among 98.4%(63/64) were also found to be culture-positive and 100%(9/9) cases of rifampin resistance. Similar results were obtained out of 62 pulmonary TB cases, 50(80.6%; 23 sputum smear negative, 27 sputum smear positive) cases were Gene Xpert +ve for MTB, but 12(19.4%) sputum smear negative remained Gene Xpert negative. Whereas, for 58(93.5%; 31 sputum smear negative and 27 sputum smear positive) cases were culture +ve. Likewise, 8 sputum smear negative cases not detected MTB by Gene Xpert was culture +ve and, 4 sputum smear negative cases were remained negative by both methods (Zeka, et al., 2011). But in this study, out of 159 cases detected as MDR-TB by Gene Xpert MTB/RIF assay, 115(72.3%; 9 sputum smear negative and 106 sputum smear positive) found to be showing positive growth, 34(21.4%; 29 sputum smear negative and 5 sputum smear positive) showed negative and 10(6.3%; 4 sputum smear negative and 6 sputum smear positive) were found to be contaminated. Sputum smear positive and culture positive results were found higher in males of 15-60 years (even in >60 years). The distribution of sputum smear positive and culture positive results was 106 out of total 115 culture positives, 9 cases were sputum smear negative but culture positive. Five cases were sputum smear positive but culture negative, 29 cases were sputum smear negative and culture -ve that was high in 15-60 years of age group. Ten cases were culture contamination (6 sputum smear positive and 4 sputum smear negative). However, 21.4%(34/159) even detected MDR-TB by GeneXpert method showed no growth that could be due to the prolonged waiting of the sample decontamination with NaOH killing of bacilli more than the usual rate. Next, the samples were taken from the retreatment cases, which might have from the patients currently taking or recently completed anti TB drugs. Since Gene Xpert technique is based on real time PCR that detects DNA/RNA present in the samples and DNA molecules could have remained from the dead bacilli. It should be taken into account that the analytical limit of detection of the Gene Xpert MTB/RIF assay is reported to

be 131 CFU/ml of specimen, based on spiked sputum studies. Culture of concentrated specimens can detect very low concentrations of organisms as low as 10 to100 CFU/ml. The analytical limit of this technique thus mentioned however, should have the presence of live bacilli in the specimens (ATS, 2000).

Contamination rates with plain LJ method is recommended as the threshold of 5% for laboratories that receive freshly collected sputum samples or 5 to 10% for settings where samples take several days to reach the laboratory (Narvaiz de Kantor et al., 1998). From this study, the contamination rate has been found to be 6.3% for ten samples only. This lower rate of contamination is within the acceptable range of maintaining the good quality of culture laboratory performance. Such low rate of contamination has been obtained due to the precaution taken during washing/sterilization and inoculation into the culture tubes as well. Each step of further processing each of the newer batches of the samples was seriously taken to maintain the acceptable rate of contamination. From 10 MDR-TB cases (60% were sputum smear positive) detected by Gene Xpert MTB/RIF assay were found to be contaminated and were requested for repeated samples, but none of the case submitted, so not included in this study. In previous study, the average contamination rates for MGIT and LJ throughout the three year period was 6.5% and 3%. Out of 313 Xpert positive specimens, three patients were not recovered by culture (MGIT and LJ combined). They were lost to contamination however two out of these three specimens had smear positive results from their processed concentrated specimens. The culture had a sensitivity that was generally better than Xpert MTB/RIF assay. Concentrated smear microscopy was inferior to Xpert MTB/RIF assay (Jones-Lopez et al., 2014).

In this study, the Se, Sp, PPV and NPV of Gene Xpert and smear microscopy and culture results were evaluated with reference to culture and found to be 92.2% (106/115) at 95% confidence interval (CI) within lower limit 85.1 and upper limit 96.1, 85.3% (29/34) at 95% CI; 68.2 and 94.5), 95.5% (106/111) at 95% CI; 89.3 and 98.3, 76.3% (29/38) at 95% CI; 59.4 and 88.0 respectively. Similar results were reported in previous study in which; according to culture results, the Se of the MTB/RIF test was 79.7%(71/89), the Sp was 98.2%(334/340), NPV was

94.8%(334/352), and PPV 92.2%(71/77). The Se of the MTB/RIF test was 100%(27/27) for smear-positive pulmonary specimens and 74.2%(23/31) for smear-negative pulmonary specimens (Zeka, et al., 2011). In another study completed previously, the Se of the GeneXpert system in detecting smear positive specimens has been reported to be 98 to 100% and detection of RR to be 98% in populations outside the United States. With regard to Sp, four specimens that were GeneXpert positive and culture negative were true positives. Three of four were from patients treated for tuberculosis and likely represented the detection of nonviable organisms (Boehme et al., 2010; Helb et al., 2010).

Out of 115 culture positive samples, 109(94.8%; 9 sputum smear negative 100 sputum smear positive) on DST showed both INH and RIF resistance, whereas, 1 case each (1.8%) showed mono resistance to isoniazid and rifampicin respectively, whereas, 1(0.9%) showed resistance to streptomycin and isoniazid and 4 (3.5%) were susceptible to all 4 first line drugs. The age wise distribution of MDR-TB was high in 15-60 years group (even detected in below 15 and above 60 years). Sex wise detection of MDR-TB was high in males compared to females. FLD-DST results showed that the majority of the cases fell in the age group of 15-60 years. Three cases (all females) were MDR-TB among below 15 years and 5 cases above 60 years age groups. FLD-DST results of 4 cases showed all 4 first line drugs susceptible. One of the previous study has reported that MDR TB among previously treated patients was 19.25% (n=161) irrespective of age and sex variation (Rijal, 2005).

Mboowa, et al. (2014) stated that the resistance was conferred by four different *rpoB* gene mutations in the 81 bp rifampicin resistance detection region (RRDR) of MTBC, which were detected by probes A, B, D, and E. It has also been mentioned in previous study that 96.1% *rpoB* gene mutations located in a region of 426-452 amino acid residues (81bp) of MTB *rpoB* gene (RRDR) detected by probes A-E using Gene Xpert MTB/RIF assay (Andre et al., 2017). This study also revealed that all the MDR-TB identified by Gene Xpert MTB/RIF assay has detected 100% *rpoB* gene mutations in 81bp RRDR of MTBC. Majority of the MDR-TB identified by both Xpert and conventional FLD-DST were males. Zeka, et al. (2011) had also described male dominated MDR-TB results in a similar study. There was only one rifampicin mono resistance found in this study as it is very rarely occurring. Similar study has revealed

that 95%-98% of rifampicin resistance was caused by mutations in *rpoB* gene of β-sub unit of RNA polymerase (Osman, et al., 2014). Mahmoud, (2015) also reported that the rate of rifampicin resistance was found to be 11.43% among pulmonary cases. Previous study has concluded the GeneXpert method is highly sensitive and simple to use system for the detection of *M. tuberculosis* directly from sputum in less than 2 hours (Helb, et al., 2010).

In the present study, among 109 MDR-TB cases, 29(26.6%; 1 sputum smear negative, 28 sputum smear positive) were performed on SLD-DST by LPA to identify how many of the randomly selected isolates could have developed XDR-TB. Among 29 MDR-TB isolates, 17(58.6%) for *gyrA*, 100% for *rrs* and 13(44.8%) for *emb B* genes were found to be wild type. Whereas, 12(41.4%) were mutants for *gyrA*, 16(51.2%) for *embB* and none were for *rrs* genes. As in SLD-DST, it was mandatory for the two negative controls must be positive only at CC and AC bands in the strips that were clearly formed in the strips used in this study, so the test process was valid. It has been revealed by the previous findings in which, the amplicon could not bind to the corresponding WT probe on the strip when a mutation occurred because of a mismatch between the amplicon and the probe. The mutation corresponding to one of the common resistance-associated mutations that was included in the MUT probes on the strip, the amplicon recognized and bound to this probe, predicting that the strain was resistant to the specific antibiotic (Barnard, et al., 2012).

All 29 selected MDR-TB cases for SLD-DST by Genotype MTBDR*sl* showed TUB (MTBC). It was found that the MTB probe was 100% specific. The mutation of *gyrA* gene was detected by the formation of positive band in 3(3.4%) cases. Similarly, gene mutations of *gyrA* together with *embB* gene regarded as fluoroquinolones and ethambutol resistance were found in 9(31.0%) cases. Any case showing single *gyrA* gene mutation or *gyrA* gene mutation together with *embB* gene was interpreted as pre-XDR TB. Out of 29 isolates, 7(24.1%) were found to be susceptible to all SLDs. None of the cases were identified as XDR-TB.

The study results showed the detection of *gyrA* gene mutation that was high among 15-60 years group and equally distributed among females and males. The result

showed that one case of pre XDR-TB in male, which was sputum smear negative. Likewise, the distribution of pre-XDR-TB in female was found to be higher in 15-60 years group. All the 6 female pre-XDR-TB cases thus identified were sputum smear positive, MDR-TB diagnosed by Gene Xpert MTB/RIF and conventional FLD-DST method. The sex wise pre-XDR-TB was identified similar in females/males (F6/M6).

Among the total of 29 MDR-TB, 12(41.4%) were found to be *gyrA* gene mutations associated with fluoroquinonlones resistance (FQ^r) resulting pre-XDR-TB, but no XDR-TB. The age/sex wise distribution of the SLD-DST pattern showed maximum number of pre-XDR TB among 15-60 years of age groups. None were identified as pre-XDR-TB among below 15 years but one case above 60 years group. All the 29 MDR-TB cases were found to be *rrs* gene wild type. All the 12 pre-XDR-TB cases identified by LPA method were found to be due to FQ^r. It has been revealed by the previous similar study that the mutations known to be associated to FQ^r were found in 14(14%) out of 101 MDR-TB isolates and 1 out of the 65(1.5%) non MDR isolates (Surcouf et al., 2011).

In the present study, among 159 RR/MDR-TB cases, 128(80.5%) were analyzed for time difference of previously cured TB, currently diagnosed and treated MDR-TB with SLDs and outcome. Out of 128 cases i.e. 21(16.4%), 16(12.5%), 21(16.4%), 23(17.9), 6(4.7%) were cured, 2(1.5%) i.e. 1(0.8%), 0(0.0%), 0(0.0%), 1(0.8%), 0(0.0%) completed treatment after <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years respectively. Whereas, 20(15.6%) i.e. 1(0.8%), 3(2.3%), 6(4.6%), 8(6.3%), 2(1.6%) were died, 14(10.9%) i.e. 1(0.8%), 5(3.9%), 4(3.1%), 4(3.1%), 0(0.0%) were lost to follow up among those visited the DR TB Treatment Centres after <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years respectively. Similarly, 2(1.6%) i.e. 1(0.8%) in 5-11 months and 1(0.8%) in 1-4 years groups were not evaluated, 3(2.3%) i.e. 1(0.8% fell in 1-4 years and 2(1.6% fell in ≥5yrs) groups respectively showed treatment failure. Shorter the gap of diagnosis and appropriate treatment saved many MDR-TB patients, whereas longer the gap resulting higher the death rate. This is the first analyzed report on retreatment TB patients in Nepal. But in a previous study, out of 53 MDR-TB patients, successful outcome was seen in 25(47.2%), 25(47.2%) were lost to follow up and 3(5.6%) were treatment failure. No death occurred in patients who continued treatments and the outcome of defaulted patients was not evaluated.

The time difference for majority MDR-TB patients was 4-6 months (Waheed et al., 2011).

Previously, Kefyalew, et al. (2016) reported that out of 242 MDR-TB patients, 131(54%) were cured, 23(9%) completed treatment, 31(13%) died, 4(2%) treatment failure, 27(11%) were lost to follow-up and 20 (8%) not evaluated. In the similar report, out of 130 MDR-TB patients, 51(39%) were cured, 7(5%) completed treatment, 25(19%) died, 17(13%) treatment failure and 30(23%) lost to follow up (Jain et al., 2014).

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5. 1 Conclusion

Taking the findings of the study into consideration, it can be concluded that;

- Gene-Xpert is more sensitive, rapid and easy technique for the diagnosis of drug resistant TB in Nepal.
- The method of culture and proportional drug susceptibility testing still stays as gold standard for the detection of drug resistant TB.
- Line probe assay is useful for early detection of XDR-TB
- Testing all the re-treatment TB cases by Gene Xpert and line probe assay in sequence or parallel provides credible data for MDR/XDR, making it possible for appropriate treatment at the earliest and also containing its further transmission.
- Treatment outcome should be based on clinical plus laboratory results, including Gene-Xpert, as almost 95% of the cases previously cured have been found to be relapse/re-infection within 1 month to 4 years.

5.2 Recommendations

On the basis of the study findings, some recommendations for the programme as below have been put forward:

1. All retreatment TB cases should be comprehensively evaluated for laboratory diagnosis and drug resistance following microscopy, culture/DST and molecular testing.
2. All the Cat 1 and Cat 2 treatment failure cases should be tested for RR/MDR-TB using Gene Xpert.
3. Expansion of Gene-Xpert based TB diagnosis to all TB treatment centers at the earliest is imperative for early detection, appropriate treatment and containment of the TB drug resistance spread in the country.
4. WHO in 2017 recommended to use Gene Xpert MTB/RIF Ultra cartridges having higher sensitivity and less testing time (16 CFU/ml, 1 hour 15 minutes)

than current Xpert MTB/RIF (131 CFU/ml, nearly 2 hours). Xpert Ultra cartridge is less expensive than and suitable for the existing Xpert machine. Xpert Ultra cartridge should be another priority of NTP Nepal.

5. Gene Xpert MTB/RIF Omni is under development, which will be single cartridge based, portable, battery operated (4 hours), suitable for existing Xpert machine, less expensive and point of care diagnostic. Once the Xpert Omni would be completely developed and WHO recommended, should be taken as testing friendly to the local level health facilities in our settings.
6. Culture and proportional DST should be made available in province level laboratories for the detection of DR-TB and making isolates available for further characterization, as required.
7. All the MDR-TB cases should be subjected to second line DST, where LPA Genotype MTBDR*sl* may be taken as the choice of method.
8. Line probe assay needs to be available in province level laboratories for the detection of XDR-TB.
9. National TB Programme should be vigilant in conducting drug resistance surveillance on a regular basis generating evidence for the policy changes as indicated by own programmatic surveillance data.

CHAPTER 6

6. SUMMARY

6.1 Summary

TB remains one of the major global public health problems and in Nepal too. In recent days, the increasing trend of MDR and XDR-TB has become a big challenge to NTP Nepal. MDR-TB is mainly caused by resistance of two major anti-TB drugs; isoniazid and rifampicin, the later one is the surrogate marker of the disease. Rifampicin resistance is caused by mutation of *rpoB* gene, whereas fluoroquinolones and injectable aminoglycosides resistance is due to *gyrA* and *rrs* gene mutations of MTBC respectively. MDR-TB has been found to be higher among retreatment cases and XDR-TB is arising among the MDR-TB treatment failures. Sputum microscopy methods of diagnosing TB is the fastest, easiest, least expensive and accessible nationwide, but has lower sensitivity (50-80% compared to culture). Culture and DST is considered as the gold standard method because of its high degree of sensitivity and specificity, which yields live organisms, but is time consuming (usually 6 to 8 weeks), requires sophisticated equipments and larger spaces. So, there is a need of more rapid and reliable methods of laboratory diagnosis for case management and treatment of all kinds of TB. In this analytical study, samples from retreatment TB cases were investigated using Gene Xpert MTB/RIF assay, despite of positive or negative sputum microscopy results to identify *rpoB* gene mutation with reference to conventional culture and FLD-DST to detect MDR-TB. Similarly, Genotype MTBDR*sl* (for SLD-DST by LPA) identifying *gyrA* and *rrs* gene mutations was used to detect pre-XDR/XDR-TB among these confirmed MDR-TB isolates.

Out of 159 retreatment TB patients enrolled in this study, 100% were detected as RR/MDR-TB by Gene Xpert MTB/RIF assay irrespective of sputum smear results. Of the total of 159 RR/MDR TB cases, 115(72.3%) were culture positive, 34(21.4%) cultures negative and 10(6.3%) were contaminated. Out of 115 culture positive isolates, 109(95%) were identified resistant to isoniazid and rifampicin with or without streptomycin/ethambutol resistance. Out of the total 109, MDR-TB isolates,

29(26.6%) randomly selected isolates were tested for SLD-DST using LPA (Genotype MTBDR*sl*), in which, 12(41.4%) MDR-TB isolates were found to be pre-XDR-TB. The prevalence of MDR and/or pre-XDR-TB was higher in the 15-60 years age group and equally distributed in both the females and males. Out of 159 cases enrolled in this study, a total of 128(80.5%) retreatment TB cases with details of each sector of analysis requirements, were further analyzed for their previous history of TB and time difference of diagnosing RR/MDR-TB by Gene Xpert and C/DST. Treatment outcomes for favorable (cured and treatment completed) and unfavorable (died, lost to follow up, not evaluated and treatment failure) groups were calculated. Out of 128 cases analyzed to understand relapse or re-infection, treatment outcomes were favorable for; 23(25.8%), 13(14.6%), 21(23.6%), 26(29.2%), 6(6.7%) falling in <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years groups respectively. Similarly, treatment outcomes were unfavorable for; 9(23.1%), 8(20.5%), 11(28.2%), 11(28.2%), 0(0.0%) falling in <1 month, 1-4 months, 5-11 months, 1-4 years and ≥5 years groups respectively.

In our study, Gene Xpert a rapid molecular technique was used to diagnose MDR-TB (*rpoB* gene detection) from sputa of retreatment cases. The results obtained from GX were compared with culture and DST as gold standard. Few MDR confirmed cases by both the methods were tested by line probe assay for XDR-TB. All these data were used to analyze time difference between previously cured and currently diagnosed MDR-TB cases with their treatment outcome (<1 month to ≥5years). Our study results showed that the majority of cases diagnosed MDR-TB and treated appropriately within shorter time difference were cured or completed treatment and survived, whereas, longer the time difference, higher the death and greater could be the disease transmission. This is the first analytical report for Nepal NTP.

By Gene Xpert, MDR-TB was diagnosed in children less than 15 years also in this study. It has become necessary to screen contact cases or children less than 15 years living with TB/DRTB patients in any household or in the community or educational institutes for TB/MDR-TB as soon as possible. Simultaneously, effective dissemination of information on TB disease seems urgent to the remote and hard to reach areas (yet to reach through different media) in a joint collaboration of local

leaders, health workers, teachers, community volunteers, other stakeholders and also cured TB patients without delay.

Taking the results into consideration; internationally recommended molecular diagnostics should be the tools of choice for prompt diagnosis of the suspected drug resistant tuberculosis in Nepal. By implementing of such diagnostic tools, it can also be expected that the challenges of tuberculosis in Nepal might be addressed to a greater extent.

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APPENDIX A

Sample size calculation

The sample size is calculated by using the formula recommended by WHO (Lwanga and Lemeshow, 1991) i.e. $n = Z^2 (p) (1-p/d^2)$

Where,

n = sample size

Z = 1.96 (CI 95%)

p = anticipated prevalence [#] (10%)

d = absolute precision (5%)

While applying equation/formula; $(n = Z^2 (p) (1-p)/d^2)$

$n = (1.96)^2 * (0.1) (1- 0.1) / (0.05)^2$

$= 3.84 * (0.1) (0.9) / 0.005$

= 138

The sample size thus calculated was 138 using the formula, but to minimize the sample processing errors and other errors during study, the sample size was taken as 159.

APPENDIX B

Equipments and Materials used during the study

A. Equipments

Autoclave	Centrifuge
Weighing machine	Refrigerator
Incubator	Vortex mixer
Microscope	Water bath
Hot air oven	Distillation plant
Gas burner	Biosafety cabinet Class II A
GeneXpert MTB/RIF assay (full set)	
Microfuge	Refrigerator
Digital water bath	Freezer -85^{o}C
PCR safety cabinet	Vortex mixer
Thermal Cycler	
Hybridization oven/Twincubator	

B. Materials

Chemicals and media base

Alcohol, 95%	Magnesium citrate
Sodium glutamate	Barium chloride
Fuchsin, basic	Glycerol, reagent, grade
Potassium dihydrogen phosphate	Malachite green
Sodium hydroxide, pellets	Sodium chloride
Immersion oil	Eggs
Monopotassium phosphate, anhydrous	Methylene blue
Disodium phosphate, anhydrous	Sodium nitrate

C. Miscellaneous

Glass beads, 3mm diameter	Cotton wool
Tube racks	Aluminium foil
Staining racks	Diamond pencil
Forceps	Protective clothing
Blotting papers	Discard pans
Marker	Pipettewasher
Homogenizer, for eggs	Gasburner
Inoculating loop & wires	Glasswares

Materials Required For Primary Culture

4% NaOH	Glaxo Smith Kline Pharmaceuticals Ltd.
5% Phenol	Arora & Co.
70% Alcohol	Bengal Chemical & Pharmaceuticals Ltd
Apron	
Beaker	Borosil
BSC II	ESCO
Digital clock timer	Electronic Manufacture Co. Ltd
Disodium hydrogen orthophosphate	SD fine chem. Limited, Mumbai
Falcon tube	Tarsons products Pvt. Ltd
Gloves	Supermax Glove Manufacturing Sdn.Bnd
Heating Block	LAB Companion
Inoculating loop	Nichrome wire
LJ media	Tulip Diagnostic Pvt Ltd
Microscopic slides	Safe Co.
N95 Mask	Handan Hengyong Protective and clean products Co, Ltd. China
Sterilized Droppers	Cepheid. France
Sonicator	Elma
Vortex mixer	Sonar, India

Permanent Marker	Fabrique Au Japon (Japan)
Phosphate buffer	
Potassium dihydrogenphosphate	Wako pure chemical Industries Ltd
Wooden Racks	Locally made
Wooden Tray	Locally made

Materials Required For Drug susceptibility test (Xpert, proportion, LPA)

1. Equipments

Autoclave	Panasonic, Japan
Balance top loading and analytical	Ohaus , Switzerland explorer
Coagulator	Hirasawa, Japan
Digital Water bath	LAB TECH
Distillation apparatus	Japan
Freezer -85^{o}C	SANYO, Japan
Gene Xpert MTB/RIF Machine	CEPHIED, France
GT BLOT 48	HAIN, Germany
GTQ Cycler 96	HAIN, Germany
Hybridization oven/Twincubator	HAIN, Germany
Hot air oven	Kayagaki, Japan
Hot water bath	York, India
Incubator	Memmert (ATMO safe, Germany)
Microscope (light binocular)	Olympus, Japan
Microfuge	KINTARO
Refrigerated Centrifuge	Kubota 6200, Japan
Refrigerator	Haier, China
Refrigerator	SANYO, Japan
PCR cabinet/LPA Safety Hood	LAB Companion
Thermal Cycler	USA
Ultrasonic water bath	
Vortex shaker	Wisestir/Sonar

Vortex mixer	Sonar, India

2.Glass wares

Beaker	Borosil
Conical flask	Borosil
Culture tubes	Borosil
Funnel	Borosil
Measuring Cylinder	Borosil
Microscopic slides	Sonar, India

Pasteur Pipettes

MTBDR*sl* 96 version 1.0 (HAIN Life Science, GmbH Germany)

3. Safety

Biological safety cabinet II	Microflow, UK/Air Tech, Japan

Disinfectant (5% phenol, 0.5% sod hypo, 70% ethanol)

Gloves (nitrile/latex free)

Laboratory shoes

Micropipettes

N95 masks

Safety pipetting device

White/blue/green coats/apron

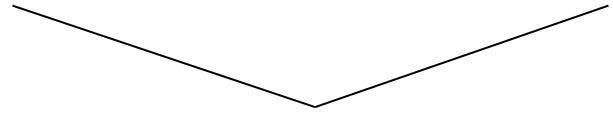
APPENDIX C

Composition and preparation of reagents

1. Ziehl-Neelsen staining reagent

i. 1% basic fuchsin (in ethanol) (Solution I) — 10.0 ml

5% phenol solution (Solution II) — 90.0 ml



Working Ziehl Neelsen solution

ii. Decolorizing solution (25% Sulphuric acid)

iii. Counter stain solution (0.1% Methylene blue)

Methylene blue 1.0g was added in 1000.0 ml distilled water and mixed well.

2. Composition and preparation of digestion and decontamination reagents

i. 4% Sodium hydroxide solution

NaOH 4.0 g was dissolved in 100.0 ml distilled water with frequent shaking and sterilized by autoclaving at 121°C for 15 minutes.

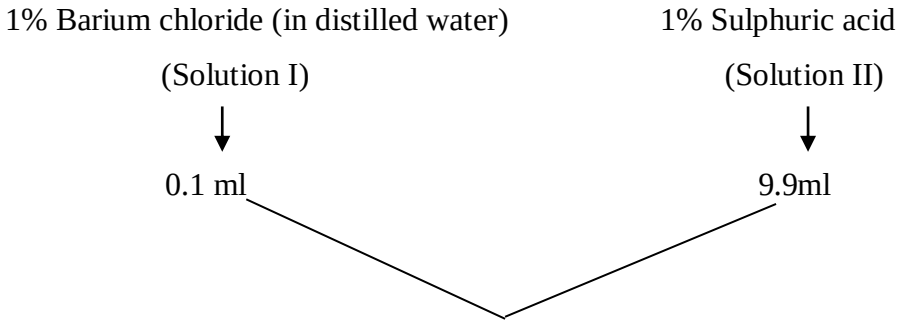
ii. Sterile normal saline

NaCl 0.85 g was dissolved in 100.0 ml distilled water with frequent shaking and sterilized by autoclaving at 121°C for 15 minutes.

3. McFarland Nephelometer Standard No. 1 (McFarland, 1907)

- Prepare 1% aqueous barium chloride and 1% aqueous VII sulphuric acid (AR) solutions. (100 mg of Barium chloride (anhydrous) in 10 ml of SDW and 0.1 ml of sulphuric acid (AR) in 10 ml of SDW).
- Add 0.1 ml of 1% Barium Chloride to 9.9 ml of 1% Sulphuric acid to obtain the McFarland standard, which matches with 1 mg/ ml (approximately $3*10^8$/ml of bacterial suspension) of *M. tuberculosis*.
- Seal the tubes and label as McFarland standard No. 1 tube with date of preparation.

During preparation of neat bacterial suspension, the comparison is done against a white background.



McFarland standard No. 1: Working solution (kept in a refrigerator)

APPENDIX D

Procedure of Z-N Staining

1. The heat-fixed slides were placed on a staining rack with smears facing up.
2. The slides were flooded with carbol fuchsin covering the whole smear.
3. The slides were heated from downside with burning cotton spirit till steam comes off and slides were not allowed to dry.
4. Slides were left for about 5 minutes.
5. The slides were washed with gentle stream of running water.
6. Slides were titled to drain off excess water and then decolorized with 20% sulfuric acid, until the smear turns clear.
7. Slides were then washed with distilled water and again tilted to drain off excess water.
8. Methylene blue 0.1% was poured to cover whole surface of the slide and left for 2-3 seconds.
9. Slides were then washed with gentle stream of running water.
10. The slides were titled and water drained and then placed on slide rack to dry.

Then, slides were examined with 100X objective (oil immersion) and 10X eye piece lenses under microscope.

APPENDIX E

A. Grading of smear microscopy (Based on WHO/IUATLD grading scale)

No. of AFB	***Reporting scale***
No AFB found per 300 *VF	Negative
1 to 9 AFB found per 100 VF	Record exact no. of AFB / 100VF
10 to 99 AFB found per 100 VF	1+
1 to 10 AFB found per VF	2+
More than 10 AFB found per VF	3+

* VF=Visual field (one microscopic field seen for one time through the eye piece)

Note: WHO: World Health Organization, IUATLD: International Union Against TB and Lung Diseases/ The Union.

B. Grading of Primary Culture

Number of colonies	***Reporting scale***
No growth	NEG
1-19 colonies	Record exact figure
20-100 colonies	1+
>100 colonies	2+
Confluent growth	3+
Contaminated	Contamination

C. Grading for Drug Susceptibility Tests

Number of colonies	***Reporting scale***
No growth	NEG
1-100 colonies	Record exact figure
>100 colonies	2+
Confluent growth	3+

APPENDIX F

1. Preparation of Lowenstein-Jensen (LJ) medium

i. Mineral salt solution

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	2.4g
Magnesium sulphate ($MgSO_4$. $7H_2O$)	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol (reagent grade)	12ml
Distilled water	600ml

The ingredients were dissolved in distilled water and sterilized by autoclaving at 121° C for 15 mins.

ii. Malachite green solution, 2%

Malachite green dye	2.0g
Sterile distilled water	100ml

Using aseptic techniques, the dye was dissolved in sterile distilled water by placing the solution in the incubator for 1-2 hours.

iii. Homogenized whole eggs

Fresh hen's eggs were cleaned with brush and soap, and then run with tap water. Then dried and wiped the outer surface with spirit cotton. After drying, the eggs were cracked with a sterile forceps into a Petri dish to check the freshness of the egg. The chalazae attached to the egg yolk were removed and the eggs were transferred to sterile blender. The whole egg homogenate was then filtered through two layers of sterile gauze into a sterile cylinder.

Preparation of complete medium

The following ingredients were aseptically pooled in a large, sterile flask and mixed well:

Mineral salt solution	600 ml
Malachite green solution	20 ml
Homogenized eggs (20-25 eggs, depending on size)	1000 ml

Five (5.0) ml of raw medium were distributed in each tube and the tubes were placed in an inspissator which has been set at 85^0C beforehand. The tubes were left in the inspissator at 85˚C for 50 minutes. The tubes were cooled, sterility checked by incubating at 37˚C 24 hours and stored in the refrigerator.

2. Preparation of 0.5 mg/ml para-nitrobenzoic acid (PNB) containing medium

PNB	250.0 mg
Propylene glycol	10.0 ml

PNB was dissolved in propylene glycol by vortexing till clear solution was obtained.

Complete PNB containing medium (0.5mg/ml)

PNB solution (25mg/ml) 2.0 ml of was added into 98.0 ml of complete raw L-J medium and mixed well. PNB containing L-J medium 5.0 ml was dispensed in each tube and then inspissated all the tubes by keeping on a slanted bed, at 85˚C for 50 minutes. The tubes were cooled, sterility checked and stored in the refrigerator.

3. Preparation of Drug solutions and Drug containing media

Name of drugs	***Code***	***Manufacturing Co.***
Dihydrostreptomycin sulphate	SM	SIGMA-ALDRICH
Isonicotinic acid hydrazide	INH	SIGMA-ALDRICH
3-(4-Methyl piperazinyliminomethyl) rifamycin	RMP	SIGMA-ALDRICH
Ethambutol dihydrochloride	EMB	SIGMA-ALDRICH

I. Dihydrostreptomycin (S)

For dry and pure S, the correction factor is 1.251. For S sulphate, dry and 98% pure, the factor is 1.277.

The solutions were prepared as follows to obtain the desired concentrations.

Factor 1.251:

Solution I: 12.51mg S was dissolved in 25ml of SDW. 400µg/ml

Solution I: 12.77mg S was dissolved in 25ml of SDW. 400µg/ml

Solution II: 5ml of solution I was added in sterile conical flask and was made up to 10ml of sterile distilled water. 200µg/ml

Stock solution I (400µg/ml) can be aliquoted into sterile cryovial and stored frozen at -20˚C.

	Final drug concentrations in drug media (µg/ml)			
	4µg/ml	2µg/ml	1 µg/ml	0.5 µg/ml
Medium(ml)	247.5ml	19.8	19.8	19.8
Solution I(ml)	2.5	-	-	-
Solution II (ml)	-	0.2	0.10	0.05*
SDW(ml)	-	-	0.10	0.15*
Final volume(ml)	250	20	20	20

*Single channel adjustable micropipettes were used with sterile tips for accuracy

Number of sets required to be prepared	Number of bottles of 'S' drug media required	MI of working solution of S(4000 µg/ml)	Amount of LJ to be added (ml)	Final concentration of S in LJ (µg/ml)
5	10	0.5	49.5	4
10	20	1	99	4
15	30	1.5	148.5	4
20	40	2	198	4
25	50	2.5	247.5	4
30	60	3	297	4

II. Isoniazid (H)

For dry and pure H, the correction factor is 1.

The solutions were prepared as follows to obtain the desired concentrations.

Solution I: 10mg H was dissolved in 50ml of sterile distilled water (SDW). 200µg/ml

Solution II: 2.5ml Solution I was added in sterile conical flask and was made up to 25ml of sterile distilled water.

20µg/ml

Solution III: 5ml of solution II was added in sterile conical flask and was made up to 10ml of sterile distilled water.

10µg/ml

Stock solution I (200µg/ml) can be stored in a sterile cryovials at -20°C.

	Final drug concentrations in drug media (µg/ml)			
	0.2µg/ml	0.1µg/ml	0.05 µg/ml	0.025 µg/ml
Medium(ml)	247.5ml	19.8	19.8	19.8
Solution II(ml)	2.5	-	-	-
Solution III (ml)	-	0.2	0.10	0.05*
SDW(ml)	-	-	0.10	0.15*
Final volume(ml)	250	20	20	20

*Single channel adjustable micropipettes were used with sterile tips for accuracy.

Number of sets required to be prepared	Number of bottles of 'H' drug media required	MI of working solution of H(20 µg/ml)	Amount of LJ to be added (ml)	Final concentration of H in LJ (µg/ml)
5	10	0.5	49.5	0.2
10	20	1	99	0.2
15	30	1.5	148.5	0.2
20	40	2	198	0.2
25	50	2.5	247.5	0.2
30	60	3	297	0.2

III. Rifampicin (R)

For dry and pure R, the correction factor is 1 and for the sodium salt the correction factor is 1.03.

The solutions were prepared as follows to obtain the desired concentrations.

Solution I:

Factor 1:40mg R was dissolved in 10ml of Dimethylsulphoxide (DMSO). 4000µg/ml

Factor 1.03: R 41.2mg was dissolved in 10ml of Dimethylsulphoxide (DMSO). 4000µg/ml

Solution II: Solution I 2.5ml was made up to 10ml SDW. 1000µg/ml

	Final drug concentration in drug media (µg/ml)			
	40µg/ml	10µg/ml	5 µg/ml	2.5 µg/ml
Medium(ml)	247.5ml	19.8	19.8	19.8
Solution I(ml)	2.5	-	-	-
Solution II (ml)	-	0.2	0.10	0.05*
SDW(ml)	-	-	0.10	0.15*
Final volume(ml)	250	20	20	20

*Single channel adjustable micropipettes were used with sterile tips for accuracy.

Number of sets required to be prepared	Number of bottles of 'RMP' drug media required	MI of working solution of RMP(4000 µg/ml)	Amount of LJ to be added (ml)	Final concentration of RMP in LJ (µg/ml)
5	10	0.5	49.5	40
10	20	1	99	40
15	30	1.5	148.5	40
20	40	2	198	40
25	50	2.5	247.5	40
30	60	3	297	40

IV. Ethambutol (E)

For Ethambutol dihydrochloride, the correction factor is 1.36.

The solutions were prepared as follows to obtain the desired concentrations.

Factor 1.36:

Solution I: 13.6mg E was dissolved in 50ml of SDW. 200µg/ml

Solution II: 5ml of solution I was added in sterile conical flask and was made up to 10ml of SDW. 100µg/ml

Stock solution I (200µg/ml) can be aliquot into sterile cryovial, stored frozen at -20°C.

	Final drug concentration in drug media (µg/ml)			
	2µg/ml	1µg/ml	0.5 µg/ml	0.25 µg/ml
Medium(ml)	247.5ml	19.8	19.8	19.8
Solution I(ml)	2.5	-	-	-
Solution II (ml)	-	0.2	0.10	0.05*
SDW(ml)	-	-	0.10	0.15*
Final volume(ml)	250	20	20	20

*Single channel adjustable micropipettes were used with sterile tips for accuracy.

Number of sets required to be prepared	Number of bottles of 'E' drug media required	MI of working solution of E(4000 µg/ml)	Amount of LJ to be added (ml)	Final concentration of E in LJ (µg/ml)
5	10	0.5	49.5	2
10	20	1	99	2
15	30	1.5	148.5	2
20	40	2	198	2
25	50	2.5	247.5	2
30	60	3	297	2

All the media tubes were left in the inspissator at 85˚C for 50 minutes. The tubes were cooled, sterility checked and stored in the refrigerator with batch number and date.

APPENDIX G

Procedure for modified Petroff's method

Double the volume of sterile 4% NaOH solution was added aseptically to 2-5 ml of sputum specimens collected in a 50 ml falcon tube. The caps of the falcon tubes were tightened and mixed well by inverting the tubes. The tubes were inverted to mix to ensure that NaOH solution contacted all the side s and even inner portion of the caps. The tubes were then allowed to stand at room temperature for 15 minutes with periodical agitation. The phosphate buffer solution was then added to the mixture in the falcon tube as to the mark of 50 ml, vortex mixed and centrifuged at 4°C for 15 minutes (keeping in centrifuge bucket with safety sealing cap). Then the supernatants were decanted through funnel into a discard bottle containing 5% phenol and the deposit was resuspended with 1 ml of phosphate buffer and gently vortexed.

APPENDIX H

Preparation of Bacillary Suspension

Inoculums: 4mg approximately + 1ml Sterile Distilled water (SDW) in Bijou bottle

↓

Vortex and allow to stand for 15 minutes

↓

Supernatant to be transferred into another Mc Cartney bottle

↓

Turbidity to be compared with McFarland Standard No.1

↓

Add water if required to achieve the requisite turbidity

↓

Make 100 fold dilutions from the bacillary suspension equivalent to McFarland Standard No.1

(10^6-10^8 CFU/ml)

↓

1 loopful 3mm (0.01ml) in 1ml of SDW in Bijou bottles is taken and vortexed

(10^{-2}: 10,000 CFU/ml)

↓

1 loopful (10^{-2}) in 1ml of SDW in Bijou bottles is taken and vortexed

(10^{-4}: l00 CFU/ml)

↓

1 loopful of each dilution is inoculated on the LJ slopes

Inoculation for DST – Loop method

The loop should be of Nichrome wire (24 SWG) and should have an internal diameter of 3 mm, which delivers 0.01 ml.

Delivery volume must be verified by weighing 10 loopful of distilled water deposited on a filter paper.

The two bacterial dilutions required for inoculation with the loop are 10^{-2} and 10^{-4} from the neat prepared as above. The dilution 10^{-2} is produced by discharging two

loopful of the bacterial suspension, standardized at 1 mg/ml, into a small tube containing 2 ml of distilled water, and shaking. Similarly, the dilution 10^{-4} is produced by discharging two loopfuls of the dilution 10^{-2} into a small tube containing 2 ml of distilled water, and shaking. Two slopes of medium without drug and one slope of medium with drug for each of the four drugs are inoculated with a loopful of each dilution.

Precautions:

- Avoided touching the water of condensation while inoculation, and do not tilt the LJ slope so much while inoculation
- Inoculated uniform suspension into all the L-J slopes.

Reading of the tests

The results are read for the first time on the 28th day. The colonies are counted only on the slopes seeded with the inoculums that have produced exact readable counts or actual counts (up to 100 colonies on the slope). These inoculums may be the same for the control slopes and the drug-containing slopes, or it may be the low inoculums (10^{-4} dilution) for the control slopes and the high inoculums (10^{-2} dilution) for the drug-containing slopes.

The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculums. Dividing the number of colonies in drug containing slopes by that in drug free slopes gives the proportion of resistant bacilli existing in the isolate. Below a certain value – the critical proportion – the isolate is classified as susceptible; above that value, it is classified as resistant. The proportions are reported as percentages. If, according to the criteria indicated below, the result of the reading made on the 28th day is "resistant", no further reading of the test for that drug is required: the isolate is classified as resistant. If the result at the 28th day is "susceptible", a second reading is made on the 42nd day only for the susceptible strain. The final definitive results for all the four drugs should be reported on 42nd day. *Incomplete reports should not be given before 42nd day*. If the isolate is resistant for all the four drugs on 28th day, then the report can be given on the same day.

Criteria of resistance

Any isolate with 1% (the critical proportion) of bacilli resistant to any of the four drugs (streptomycin, isoniazid, rifampicin and ethambultol) 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 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd day).*

APPENDIX I

Identification of *Mycobacterium tuberculosis*

1. PNB identification test

A loopful of neat bacterial suspension (McFarland standard No. 1) was inoculated into one slope of LJ medium and one slope of p-nitrobenzioc acid (PNB) at a concentration of 500 μg/ml and incubated at 37°C for each set. Read on 28th day.

Results and interpretation

M. tuberculosis does not grow on PNB medium.

All other mycobacteria are resistant to PNB.

2. Niacin production test

BBL Taxo TB Niacin test strips (Becton and Dickinson, USA), absorbent paper strips and TB niacin positive test control paper discs were used according to the manufacturer's instruction. In brief, with the help of sterile transfer pipette, approximately 0.6 ml of the positive culture broth extract was transferred to the bottom of 20 mm × 125 mm screw cap test tube. Negative control was also prepared. The strips were dropped with arrow downward into the tubes: Positive and negative controls, test culture and stopper immediately. The colors of the extracts were then compared after 15 minutes. Niacin accumulation was indicated by vivid appearance of a yellow color in the extract. (The niacin test strip is typically composed of potassium thiocyanate, chloramine-T, citric acid, and 4-Aminosalicylic acid. In the presence of citric acid, chloramine-T and potassium thiocyanate will react to form cyanogen chloride. This chemical will break apart the pyridine ring of niacin to produce y-carboxy glutaconic aldehyde and joins an aromatic amine to form a yellow color). The test is processed inside a Biosafety cabinet-II with careful precautions maintained.

Controls

The reagents were checked by testing extract from an un-inoculated tube of medium (negative control) and an extract from a culture of *M. tuberculosis* $H_{37}Rv$ (positive control).

Interpretation

M. tuberculosis	Niacin +ve
M. bovis	Niacin -ve

APPENDIX J

Quality control

Quality control of drug susceptibility tests is best performed by titrating the standard $H_{37}R_v$ strain of *M. tuberculosis* for each newly produced batch of drug susceptibility testing media. Minimum, median, and maximum numbers of resistant bacilli by 10^6 CFU/ml are given in the table below. If these values are exceeded then the batch is considered to be inadequate. This circumstance invalidates all results obtained with that particular batch. 0.1 ml of 1mg/ml $H_{37}R_v$ suspension (equivalent to McFarland standard No. 1), approximately 10^6 bacilli/ml is inoculated onto each drug containing medium then incubated at 37˚C for 6 weeks. The number of colonies for natural resistant mutants is observed.

Minimum, median and maximum numbers of bacilli resistant to anti-tuberculosis drugs for *M. tuberculosis* $H_{37}R_v$ (10^6 bacilli/ml)

Drug	Concentration (µg/ml)	Minimum	Median	Maximum
SM	4.0	0	7	300
INH	0.2	0	4	32
RFP	40.0	0	0.02	..
EMB	2.0	100	…	1,000

Note: SM: streptomycin INH: isoniazid RFP: rifampicin EMB: ethambutol

APPENDIX K

Reagents and instruments for Gene Xpert MTB/RIF assay

Materials Provided

The Xpert MTB/RIF Assay kit contains sufficient reagents to process 10 specimens or quality control samples. The kit contains the following: **Notes**: Material Safety Data Sheets (MSDS) are available at; http://www.cepheid.com/tests/reagents/literature/msds, or http://www.cepheidinternational.com/testsreagents/literature/msds. The bovine serum albumin (BSA) in the beads within this product was produced and manufactured exclusively from bovine plasma sourced in the United States. No ruminant protein or other animal protein was fed to the animals; the animals passed ante- and post-mortem testing. During processing, there was no comingling of the material with other animal materials. The transfer pipettes have a single mark representing the minimum volume of sample necessary to transfer to the GX cartridge. Use only for this purpose. All other pipettes must be provided by the laboratory.

Xpert MTB/RIF Assay Cartridges with Integrated Reaction Tubes 10

Bead 1: (freeze-dried) 2 of each per cartridge

- Polymerase
- dNTPs (deoxynucleoside triphosphates)
- Probe
- BSA (Bovine serum albumin)

Bead 2: (freeze-dried) 2 of each per cartridge

- Primers
- Probes
- BSA (Bovine serum albumin)

Bead 3: (freeze-dried) 1 per cartridge

- Sample Processing Control (SPC) ~6,000 non-infectious *B. globigii* spores

Reagents provided

Reagent 1: 4 ml per cartridge

• Tris Buffer

• Surfactants

• EDTA (ethylenediaminetetraacetic acid)

Reagent 2: 4 ml per cartridge

• Tris Buffer

• Surfactants

• EDTA (ethylenediaminetetraacetic acid)

Sample Reagent: 8 ml per bottle

• Sodium Hydroxide

• Isopropanol

Disposable Transfer Pipettes: 12 CD 1 per kit

• Assay Definition File (ADF) – ADF for use with both the GeneXpert Dx and Infinity Systems

• Instructions to import ADF into GX software

• Package Insert For Information

APPENDIX L

S. No.	Name of Patients	Age/Sex	TB Regd. No.	Lab No.	Date of Cat 1 or Cat 2 treatment completed/CURED	Date of visiting DR TB Treatment Centre	Date of MDR TB Diagnosis (by Gene Xpert/culture DST)	Gap of previous treatment completed or CURED and MDR TB diagnosis	Treatment outcome/ Remarks
1.									
2.									
3.									
4.									
5.									
6.									
7.									

- Gene Xpert MTB/RIF test positive (mutation in rpoB gene of *M. tuberculosis* detected and rifampicin resistance) along with mycobacterial culture and DST patterns for the diagnosis of patients as MDR-TB
- Time difference of Cat 1/Cat 2 treatment failures or others (all the criteria fulfilled to be categorizing as **Retreatment TB cases**) and development of **MDR TB/XDR TB**
- When had the patient/s completed Cat 1/Cat 2 regimens and registered as **CURED.**
- After how many years the same patients visited to DR TB Treatment Centre/s and bacteriologically **diagnosed as MDR/XDR TB**

IDENTIFICATION OF *rpoB, gyrA* AND *embB* GENE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM RETREATMENT TUBERCULOSIS PATIENTS IN NEPAL

Dhruba Kumar Khadka, Rajendra Prasad Pant, Bikash Lamichhane, Sharat Chandra Verma, R. P. Bichha, Prakash Ghimire, Anjana Singh

ABSTRACT

Introduction:

Tuberculosis remains one of the major public health problems in Nepal and increasing trend of multi drug resistant and extensively drug resistant tuberculosis (MDR /XDR TB) is a big challenge. Rapid diagnosis and appropriate treatment of MDR/XDR TB is crucial. Identification and comparison of MDR TB using rapid molecular techniques (for *rpob, gyrA, rrs* and *embB* gene mutations) with reference to drug susceptibility test (DST) were the main objectives of this study.

Methodology:

A cross sectional study was carried out in National TB Centre (NTC). Gene Xpert, proportion method and Line Probe Assay (LPA) were used for first and second line drugs susceptibility testing (FLD-DST and SLD-DST). A total of 29 mucopurulent sputum samples were freshly collected from retreatment TB patients (Female 41.4%, Male 58.6%) with median age of 40 years attending to the four MDR TB treatment centres of eastern and central Nepal (via private courier and directly to National TB Reference Laboratory (NRL) at NTC from April 2013 to October 2017.

Results:

Among 29 sputum samples (Female 41.4%; all smear+ve, Male 58.6%; 16 smear+ve and 1 smear-ve), Gene Xpert MTB/RIF assay detected 100% *M. tuberculosis* and rifampicin resistance (*rpoB* gene resistant) of which, 100% were culture positive by conventional Lowenstein–Jensen (LJ) method. FLD-DST was performed on all culture positives of which, 96.6% showed MDR TB and 3.4% showed mono resistance to isonizid only. SLD-DST on 29 MDRTB strains by LPA showed 100%, 58.6%, 44.8% wild type for *rrs*, *gyrA* and *emb B* genes respectively. Mutation for *gyrA* and *emb B* genes was 41.4% and 51.2% respectively, *rrs* genes none. Twelve (Female 6, Male 6) MDR TB strains were identified as pre-XDR-TB. Chi square (χ^2) for trend was used to analyze Gene Xpert, smear, FLD-DST and LPA results.

Conclusion:

From this study, 29(100%) MDRTB were detected from retreatment TB cases by Gene Xpert and FLD-DST. Almost 41.4% MDR TB strains were detected as pre-XDR TB by LPA, which were found to be higher in 15-60 years group of females and males. Samples from retreatment TB patients need to be tested by rapid molecular techniques with reference to culture and DST.

Key words:

Mycobacterium tuberculosis, Gene Xpert, Line Probe Assay, Multi and Extensively Drug Resistance.

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INTRODUCTION

Tuberculosis (TB) is a top infectious killer disease worldwide. Over 95% of TB deaths occur in low- and middle-income countries, and it is among the top 5 causes of death for women aged 15 to 44. Globally in 2014, an estimated 480, 000 (an estimated 3.3% of new TB cases and 20% of previously treated cases) people developed multidrug-resistant TB (MDR-TB) and 190, 000 people died of MDR-TB. An estimated 43 million lives were saved through TB diagnosis and treatment between 2000 and 2014. *M. tuberculosis* strains identified as MDR TB (resistant to isoniazid and rifampicin with or without other first line anti-TB drugs plus any fluoroquinolone and at least one of three injectable second-line drugs is defined XDR TB. MDR TB resistant to either of fluoroquinolones or injectable aminoglycosides is categorized as pre-XDR-TB. An estimated 9.7% of people with MDR-TB have XDR-TB.[1] The SAARC region, with 34% of the global burden of TB, a total 81,142 estimated cases of MDR-TB among notified cases were notified in 2013, of which 59% were previously treated cases.[2]

Tuberculosis (TB) remains one of the major public health problems in Nepal. In 2014, total of 37,025 cases of TB were registered. Most cases were reported among the middle aged group with the highest among 15-24 years of age (20%). TB-HIV co-infection rate in Nepal is 2.4% (HIV among TB) and 11.6% (TB among HIV) based on the sentinel survey, 2011/12. Nationwide, the proportion of multidrug-resistant TB (MDR-TB) was 2.2% among new cases and 15.4% among retreatment cases based on survey carried out in 2011/12. In 2014, total of 349 MDR TB and 25 XDR TB were enrolled for treatment. WHO estimated 4.6 (2.1-7.5) thousand people died from TB in 2014 (NTP Annual report Nepal 2070/71 or 2014).[3] One of the concerned aspects of drug resistance in Nepal is the high level of resistance to fluoroquinolones (26.4%), which leads to heavy burden of pre-XDR and XDR-TB among MDR-TB patients (8% of the cases were found to be XDR among MDR cases in the same survey). To combat the excess mortality related to XDR-TB, it is recommended to perform DST for second line-drugs to all MDR-TB cases at the start of treatment.[4]

Isoniazid (INH) with rifampicin (RIF) forms the cornerstone of short course chemotherapy for tuberculosis and resistance to either drug hampers the complete cure of patients. *M. tuberculosis* strains resistant to at least these two major frontline drugs (INH and RIF) develop multi-drug resistant tuberculosis (MDR-TB).[5]

More than approximately 95% RIF resistant *M. tuberculosis* strains have mutations in an 81 bp hot spot region (codon 507-533) of *rpoB* gene that encodes RNA polymerase beta subunit.[6,7,8,9]

Globally, more than half of all TB cases are not detected the result of health care system weakness and the inadequacy of available technology. If a diagnosis is absent, patients are not treated, transmission may continue, patients suffer needlessly and may eventually die.[10]

Cepheid (Cepheid, Sunnyvale, CA) has recently introduced the GeneXpert MTB/ RIF assay for research use only.[11] The GeneXpert assay is a real-time PCR test that will simultaneously identify *M. tuberculosis* and detect rifampin resistance directly from clinical specimens.[12,13,14] Rifampin resistance can serve as a marker for multidrug-resistant tuberculosis (MDR-TB) and has been reported in 95% of the multidrug-resistant *M. tuberculosis* isolates. The GeneXpert assay detects an 81-bp "core" region of the *rpoB* gene.

The suggested target sites of first line anti-TB drugs and the sites for most frequent mutations occur; for streptomycin, isoniazid, rifampicin, ethambutol, pyrazinamide were described. [15, 16, 17, 18, 19, 20, 21, 22] Similarly, the target sites of second line anti-TB drugs and the most frequent mutations occur for fluoroquinolone, injectable capreomycin and kanamycin were suggested.[23, 24]

The Gene Xpert MTB/RIF assay, conventional culture and FLD-DST and LPA (Genotype MTBDR*sl)* are the choice of DR/MDR TB and XDR TB diagnostics tools. Culture and FLD-DST method takes usually longer time but always being considered as the gold standard that gives the viable organisms and can be used for various research purposes.

The occurrence of MDR TB among retreatment (Cat1 and Cat 2 treatment failures) cases alerts the NTP managers for prompt diagnosis of TB/DR/MDR/ XDR TB using reliable and rapid diagnostics tools based on molecular biological techniques. The main objective of this study was to identify and compare the findings of DR/MDR TB using Gene Xpert MTB/ RIF assay (*rpoB* gene mutations) with reference to culture and drug susceptibility test (DST) and XDR TB (gyrA, rrs and embB gene mutations) by line probe assay (LPA).

METHODOLOGY

The study was descriptive type cross sectional study to identify rifampicin resistant or multidrug resistant tuberculosis (RR/MDR TB) among retreatment TB cases using Gene Xpert MTB/RIF assay and to compare the prospective data obtained with reference to conventional culture and FLD-DST as

well as to identify XDR TB among those MDR TB cases by LPA (Genotype MTBDRs*l* for SLD-DST).

Study site

This study was carried out from April 2013 to October 2017 in NRL at NTC, Thimi, Bhaktapur, Nepal.

Sample size

Twenty nine (29) retreatment tuberculosis patients (Female 41.4%; all smear+ve, Male 58.6%; 1 smear-ve, 16 smear+ve) with median age of 40 years were involved in this study before they were registered for starting second line anti-tuberculosis treatment.

Study population

Retreatment pulmonary TB cases (relapse, treatment after failure, and treatment after loss to follow-up) previously treated with Cat I and Cat II treatment regimen as per National TB Programme (NTP) guidelines based on WHO recommendations were enrolled in this study.

Inclusion/exclusion criteria

Retreatment TB cases (Cat I and Cat II failure) visiting for further diagnosis and diagnosed as sputum smear positive or negative before being registered for and started MDR treatment were included in this study. But the cases already registered and recently undergoing second line anti tuberculosis treatment, blood stained sputum, sputum with food particles, with saliva in greater amount, leaking, dried or if not freshly collected and patients suspected of extra-pulmonary tuberculosis were excluded from this study. The samples showing contamination during culture were not further included in the study.

Sample collection

Twenty nine early morning sputum samples (stuffy and mucopurulent, 3-5ml each) were collected from retreatment TB patients in leak proof, wide mouthed, transparent and sterile 50 ml disposable plastic centrifuge tube (Falcon BD, USA); then were appropriately labeled and stored at refrigerated temperature (2-8^{o}C) until dispatched or processed. Out of total 29 samples 16 were received; 11(F3/M8) from Nepal Anti TB Association (NATA) Biranagar Morang, 5(F4/M1) from BP Koirala Institute of Health Sciences (BPKIHS) Dharan Sunsari of the eastern development region. Similarly, the remaining 13 samples were received; 3(F2/M1) from National TB Centre (NTC) Thimi Bhaktapur, 3(F1/M2) from United Mission to Nepal Hospital (UMN) Lalgarh Janakpur and 7(F2/M5) from National Medical College (NMC) Birgunj Parsa of central development region. The samples from the centres other than NTC were transported through private courier and the duration of sample transportation was not more than 48 hours. Some patients being treated at National TB Centre (one of the MDR TB treatment centres in central development region) submitted fresh samples directly to NRL/NTC.

Sample processing

Sputum samples were processed inside a Biological Safety Cabinet class II (BSC-II AIRTECH, Japan) directly by adding twice the volume of 4.0% NaOH digestion method (modified Petroff's method), vortex mixed and left for 15 minutes at room temperature with occasional shaking. Then phosphate buffer (pH 6.8) were added up to level of 45 ml graduation mark, vortex mixed and centrifuged at 3000x g for 15 minutes in a refrigerated centrifuge at 4^{o}C (KUBOTA, Japan).

Culture on Lowenstein Jensen (LJ) medium

The supernatants were discarded and pellets were used for culture; 0.2ml of pellet was inoculated on duplicate LJ media, incubated at 37^{o}C for 4-8 weeks in an incubator (MEMMERT, Germany). The tubes were examined on 7th day for rapid growers and were checked for growth at 2, 3, 4, 5, 6, 7, 8 weeks until negative. If there was any contamination in the culture tube, was recorded.

Gene Xpert MTB/RIF Assay

From the remaining of the pellets, Gene Xpert MTB/RIF tests were performed by following the procedures provided by the manufacturers (Cepheid Sunnyvale, CA, USA, and Gene Xpert IV Cephied, France). Sputum pellets were decontaminated and treated with sample reagent (SR); a mixture of NaOH and iso-propanol. The SR was added at a 3:1 ratio to the sputum pellets and left for 15 minutes at room temperature; 2 ml of the treated samples were transferred to the Gene Xpert cartridges (Cephied, France), which were then loaded into the programmed Gene Xpert modules. Gene Xpert device was kept on and results were observed after the whole process completed (within about 2 hours).

Smear microscopy

A smear of the processed pellets was prepared (size of 2*3 cm), air dried at room temperature (RT), heat fixed, stained by Ziehl-Neelsen method and read under binocular light microscope at the total magnification of 1000X (Olympus, Japan) and reported according to NTP Nepal grading scale that is adopted exactly same scale from WHO/IUATLD grading scale.

Preparation of Bacillary Suspension and inoculation for DST

One looful (4mg approximately) of mycobacterial colonies grown on LJ media was harvested and emulsified with 1ml of sterile distilled water (SDW) in a sterile bijou bottle, vortex mixed and allowed to stand for 15 minutes, supernatant was transferred into a McCartney bottle. Turbidity of supernatant was compared with McFarland Standard No.1 Nephelometer (standardized at 1 mg/ml equivalent to 10^6-10^8 CFU/ml). Made 100 fold dilutions from McFarland Standard No.1 suspension; 1 loopful (nichrome wire loop 24 SWG and 3mm diameter delivering 0.01ml) of bacillary suspension was transferred to 1ml of SDW in bijou bottles and vortexed (10^{-2}: 10,000 CFU/ml), similarly 10^{-4}(l00 CFU/ml) was prepared. One loopful of each dilution (10^{-2} and 10^{-4}) was inoculated on two slopes of plain LJ medium (controls) and one set each of slopes with 4 drugs (streptomycin, isoniazid, rifampicin, ethamputol), incubated at 37°C, read on 4th and 6th week for resistant (growth on drug medium ≥ 1% colonies on control) and final susceptible (no or <1% colonies on control) patterns respectively.

Biochemical identification tests

From the positive growth, identification tests were performed by biochemical methods i.e. growth on PNB containing LJ medium and niacin production tests.

1. *Growth on PNB containing media*

A loopful of neat bacterial suspension (McFarland standard No. 1) was inoculated into one slope of LJ medium and one slope of p-nitrobenzioc acid (PNB) at a concentration of 500 g/ml and incubated at 37°C for each set. Read on 28th day.

M. tuberculosis does not grow but all other mycobacteria are resistant to PNB.

M. tuberculosis $H_{37}Rv$ as negative control (PNB susceptible) and *M. kansasii* as positive control (PNB resistant) were used.

Results and interpretations

No growth on PNB medium: *M. tuberculosis*

Growth on PNB medium: *M. kansasii*

2. *Niacin production test*

BBL Taxo TB Niacin test strips (Becton and Dickinson, USA), absorbent paper strips and TB niacin positive test control paper discs were used according to the manufacturer's instruction. With a sterile transfer pipette, approximately 0.6 ml of the positive culture broth extract was transferred to the bottom of 20 mm × 125 mm screw cap test tube. Negative control was also prepared. The strips were dropped with arrow downward into the tubes. Positive controls, negative controls and test culture tubes were recapped immediately. The colors of the extracts were then compared after 15 minutes.

M. tuberculosis $H_{37}Rv$ as positive control and *M. kansaii* as negative control were used. Niacin accumulation was indicated by vivid appearance of a yellow color in the extract.

Results and interpretation

M. tuberculosis $H_{37}Rv$: yellow colour (niacin positive)

M. kansaii: colourless (niacin negative)

All the positive cultures have shown PNB negative and niacin positive.

Drug Susceptibility Test on First Line Drugs (proportion method)

Drug susceptibility test (DST) on first line anti tuberculosis drugs (FLD); streptomycin(4.0 µg/ ml), isoniazid(0.2 µg/ ml), rifampicin(40.0 µg/ml), and ethambutol(2.0 µg/ml) (SIRE; manufactured by SIGMA-Aldrich, USA) was performed on all the culture positive samples in duplicated drug tubes as well as two LJ slopes without drug (control) using 1% proportion method. Internal quality control was routinely performed (for each batch of new drug media) using the reference strain *M. tuberculosis* H37Rv (ATCC-27294), which was susceptible to all the 4 drugs. All the inoculated tubes were then incubated at 37°C, resistance pattern of the SIRE was checked at 4th week and 6th week.

Drug Susceptibility Test on Second Line Drugs by Line Probe Assay

The whole process of the LPA was followed as per the guideline provided by the manufacturer-MTBDR*sl* 96 version 1.0 (HAIN Life Science, GmbH Germany) following the steps of DNA extraction, PNM mix, amplification by PCR, hybridization and detection as mentioned below:

1. DNA extraction

Homogenized bacterial suspension was prepared by harvesting 1-2 colonies of organisms from LJ tube with sterile inoculating wire loop inside a BSC-IIA (Micro Flow, Bioquell, UK), re-suspended in 300 µl of molecular grade water in a cryovial (1.5-2ml), mixed by vortexing (SONAR, India), heat inactivated at 95°C for 20 minutes, incubated in an ultrasonic water bath (LABTECH, India) for 15 minutes, centrifuged for 5 minutes at 13000*g (Microfuge, KINTARO) and supernatant containing DNA was transferred to another cryotube and stored at 4°C to -20°C until processed in a refrigerator (SANYO, Japan).

2. Primer nucleotides mix (PNM)

With micropipettes 35µl of PNM, 5µl of 10x buffer (15mM MgCl2), 2 µl MgCl2 (25mM), 3 µl H_2O, 0.2 µl Taq polymerase (Hot star Thermis aqaticus) were added into a cryotube and mixed well carefully. Prepared a master mix for the determined number of samples, mixed and aliquoted (45 µl) in 1.5ml PCR tubes. PNM process was completed inside a LPA Safety Hood (LAB COMPANION). The molecular grade water to bring the master mix to volume was used as conjugate control (CC) and the LPA strip (functions as both the internal "PCR positive control" and the "inhibition positive control) was used as amplification control (AC).

3. Amplification by PCR (Thermal Cycler, USA)

To the aliquoted 45 µl master amplification mix, 5 µl DNA was added (inside a BSC-IIA), gently vortexed to mix, placed into the thermal cycler (Genotype Hot 30 specific progamme). The DNA amplification was performed for 30 cycles following an initial denaturation; 10 cycles of initial denaturation followed by denaturation at 95°C for 30 seconds, chain elongation at 58°C for 2 minutes followed by additional 20 cycles of denaturation at 95°C for 25 seconds, primer annealing at 53°C for 40 seconds and elongation at 70°C for 40 seconds and final extension at 70°C for 8 minutes.

4. Hybridization and Detection

The hybridization buffer (HYB) and stringent wash solution (STR) were prewarmed at 45°C to dissolve the undissolved precipitates, rinse solution (RIN) and DW were prewarmed at RT, freshly diluted Con-C and Sub-C 1:100 in the respective dilution buffer and protected from light. Twincubator set with P1 programme was used for Hybridization and Detection.

4.1 Hybridization probe

Denaturing buffer (DEN) 20 µl was added with 20 µl amplicon, mixed well and incubated for 5 minutes at RT on the shaking platform, 1ml HYB was added using micropipette and filter tips, mixed by tilting the tray back and forth carefully (purple DEN and green HYB mixed well), tray was then placed on the TwinCubator (HAIN Life Science, GmbH Germany). DNA strip was placed in each well (with a forceps) of TwinCubator tray and covered by the liquid. When the temperature reached to 45°C, tray cover was closed and incubated for 30 minutes at 45°C.

4.2 Detection probe

HYB was aspirated completely and 1 ml STR was added and incubated for 15 seconds at 45°C, STR removed completely, 1ml RIN added, incubated for 1 minute at RT, RIN removed completely, 1ml diluted conjugate (10 µl Con-C /conjugate C and 990 µl Con-D/conjugate D) were added and incubated for 30 minutes at RT and conjugate was removed completely. Added 1ml RIN, incubated for 1minute at RT, removed RIN completely and rinsed with H_2O for 1 minute, 1ml diluted substrate added (10 µl Sub-C and 990µl Sub-D), incubated 2-10 minutes at RT and removed substrate completely. Stopped reaction by rinsing twice with H_2O for 1 minute then removed DNA strip from tray and dried it on absorbent paper. Detection process was completed using a TwinCubator (HAIN Life Science, GmbH Germany).

Individual strip after colour development was adhered to the corresponding column of the HAIN Life Science, GmbH Germany provided format and resistance pattern was identified. The original strips showing positive bands were kept for NRL record after being scanned for the present study purpose (Figure 1).

Statistical data analysis

The statistical analysis of the study data were analyzed using SPSS version 16.0 software. The Chi square test was used to compare age and sex wise distribution of negative and positive sputum smear results, smear and culture results, MDR TB identified by Gene Xpert MTB/RIF assay and conventional culture and FLD-DST results, identification of XDR/ pre XDR TB cases by LPA (MTBDR*sl*). The *P*-value <0.05 was considered statistically significant.

RESULTS

The sputum smear microscopy results for female (12/41.4%) were all +ve, whereas for male (17/58.6%); 16 +ve and 1-ve. The age wise distribution of smear results was; 3.4% was smear-ve in 15-29 years and 51.7%, 20.7%, 13.8% and 10.3% in 15-29, 30-45, 46-60 and above 60 years of age groups respectively were smear positives (Table 1).

Table 1: Age (years)/sex wise distribution of retreatment TB cases from different treatment centres

Treatment centre	15-29		30-45		46-60		>60		Total		Remarks
	F	M	F	M	F	M	F	M	F	M	
E+E1	3	2		2		2		2	3	8	*NATA Morang*
E2	2		2			1			4	1	*BPKIHS*
C	2	1							2	1	*NTC*
C1	1	2							1	2	*UMN Lalgarh*
C2	1	2	1	1		1		1	2	5	*NMC Parsa*

A total of 29 freshly collected good quality sputum samples from 5 DR/MDR treatment centres; E+E1: Nepal Anti TB Association (NATA) Morang, 11 (F3/M8) samples and E2: BP Koirala Institute of Health Sciences (BPKIHS) Sunsari Eastern Nepal; 5 (F4/M1) samples. C: National TB Centre (NTC) Kathmandu; 3 (F2/M1) samples, C1: United Mission to Nepal Hospital (UMN) Lalgarh Dhanusha; 3 (F1/M2) samples and C2: National Medical College (NMC) Birgunj Parsa Central Nepal; 7 (F2/M5) samples. There was no significant difference of age and sex wise smear results (p>0.05). All 29 sputum specimens showed rifmapicin resistance (RR/MDR) by Gene Xpert MTB/RIF assay in which 55.2%, 20.7%, 13.8% and 10.3% in 15-29, 30-45, 46-60 and above 60 years age group and 41.4%, 59.6% females and males respectively (Table 2).

Table 2: Age (years)/sex wise distribution of MDR TB cases diagnosed by Gene Xpert from different treatment centres

	15-29		30-45		46-60		>60		Total		Grand total	Remarks
	F	M	F	M	F	M	F	M	F	M		
	3	2		2		2		2	3	8	11	NATA Morang
	2		2			1			4	1	5	BPKIHS
	2	1							2	1	3	NTC
	1	2							1	2	3	UMN Lalgarh
	1	2	1	1		1		1	2	5	7	NMC Parsa
Total	9	7	3	3		4		3	12	17	29	

The age wise distributions of MDR TB by Gene Xpert MTB/RIF assay for 15-29 years, 30-35 years, 46-60 years and above 60 years of age group were 55.2%, 20.7%, 13.8% and 10.3% respectively. Similarly, sex wise MDR TB detection by Gene Xpert was 41.4% and 59.6% for females and males respectively. Similarly, all 29 specimens showed positive growth for all age and sex groups on LJ culture media (Table 3). There was no significant difference of age and sex wise smear and culture results (p>0.05).

Table 3: Age (years)/sex wise Comparison of smear and culture results

Results	Age and sex distribution								Total culture positive		Grand Total culture positive
	15-29		30-45		46-60		>60				
	F	M	F	M	F	M	F	M	F	M	
S+ C+	9	7	3	3	0	4	0	3	12	16	28
S+ C-	0	0	0	0	0	0	0	0	0	0	0
S- C+	0	1	0	0	0	0	0	0	0	1	1
S- C-	0	0	0	0	0	0	0	0	0	0	0
Contamination	0	0	0	0	0	0	0	0	0	0	0

A. χ^2 for trend of age wise smear results=0.842, df=3, P value=0.840 (>0.05), so there was no significant difference of age wise smear results.

B. χ^2 for trend of sex wise smear results=0.731, df=1, P value=0.393 (>0.05), so there was no significant difference of sex wise smear results.

The culture positives were biochemically (PNB-ve, niacin test+ve) identified as *M. tuberculosis* which were then processed for FLD-DST following proportion method. Twenty eight (96.6%; 11F/17M; ss-ve 1M, 11 F and 16M ss+ve) of 29 FLD-DST) showed both INH and RIF resistance, 1 case (3.4%/M) detected as RRTB by Gene Xpert MTB/RIF showed mono resistance to isoniazid only (Table 4).There was no significant difference of age and sex wise MDR TB cases identified by conventional FLD-DST for smear results (p>0.05).

Table 4: Age (years)/sex wise distribution of FLD-DST patterns

DST patterns of FLDs by conventional proportion method											
	15-29		30-45		46-60		>60		Total		Grand total
	F	M	F	M	F	M	F	M	F	M	
Total Tested	9	7	3	3	0	4	0	3	12	17	29
Fully Susceptible	0	0	0	0	0	0	0	0	0	0	0
Any Resistance	1	0	0	0	0	0	0	0	1	1	
Mono Resistance	1	0	0	0	0	0	0	1	1	1	1
S	0	0	0	0	0	0	0	0	0	0	0
I	1	0	0	0	0	0	0	0	1	0	1
R	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0
Total I+ R Resistance (MDR)	8	7	3	3	0	4	0	3	11	17	28
IR	2	2	0	2	0	0	0	0	2	4	6
IRE	1	0	0	0	0	0	0	1	1	1	2
SIR	1	3	2	0	0	2	0	1	3	6	9
SIRE	4	2	1	1	0	2	0	1	5	6	11

S: streptomycin I: isoniazid R: rifampicin E: ethambutol

A. χ^2 for trend of age wise MDR TB detected by culture and DST results=0.842, df=3, P value=0.840 >0.05, so there was no significant difference of age wise MDR TB detection by culture and DST.

B. χ^2 for trend of sex wise MDR TB detected by culture and DST results=1.467, df=1, P value=0.226 >0.05, so there was no significant difference of sex wise MDR TB detection by culture and DST.

All the MDR TB cases detected by Gene Xpert MTB/RIF assay and/or FLD-DST were performed for DST on second line anti-TB drugs (SLD-DST) by LPA. During the process of LPA, negative controls were developed at CC and AC bands in the strips. MDR TB strains showed TUB bands formation in the strips. Similarly, 17(58.6%, F5/M12) for *gyrA* WT1, WT2, WT3 probes located in regions from codons 85 to 97 (binding sites for fluoquinonlones / ofloxacin or levofloxacin), 29(100%, F12/M17) for *rrs* WT1, *rrs* WT2 probes located in regions for nucleotides 1401,1402 and 1484 (binding sites for injectable aminoglycoside / capreomycin), and 13(44.8%, F4/M9) for *emb B* WT1 (binding site for ethambutol) gene probes located in regions from codons 306 were found to be susceptible sites for the corresponding drugs. Whereas, the DNA matched with the mutant probes; 12(41.4%, F6/M6) were mutants for *gyrA* gene (*gyrA* MUT1, MUT2, MUT3A to 3D) conferring most frequently mutation occurring codons (A90V, S91P, D94A, D94N/Y, D94G, and D94H), 16(51.2%, F8/M8) for *emb B* (*embB* MUT1A, MUT1B) probes conferring mutations M306V and M306I and none were for AG/CP or *rrs* genes (*rrs* MUT1, MUT2) probes conferring mutations for A1401G and G1484T. Similarly, *gyrA* gene mutations together with *embB* gene that were regarded as fluoroquinolones and ethambutol resistance (*embB* MUT1B) were found in 9 cases (31.0%, F5/M4). Any case showing single *gyrA* gene mutation or *gyrA* gene mutation together with or without other gene mutations was interpreted as pre-XDR TB (Figure 1).

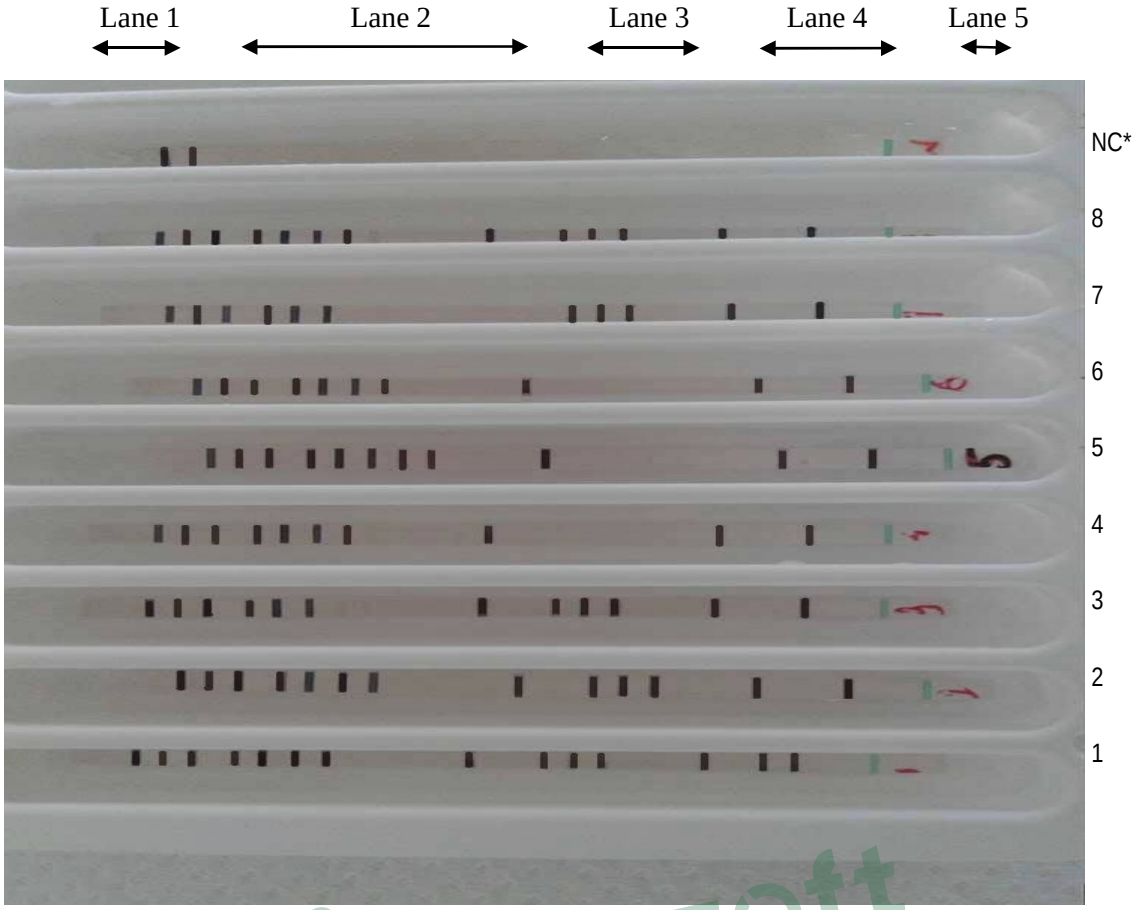

Figure 1: Hybridization process and development of different bands on MTBDR*sl* strips

Lane 1: CC/AC/TUB *(CC: conjugate control, AC: amplification control, TUB: tuberculosis)*

Lane 2: *gyrA* (WT 1-WT 3/MUT 1- MUT 2, MUT 3A- MUT 3D) (*WT: wild type, MUT: mutation)*

Lane 3: *rrs* (WT1/MUT 1-MUT 2)

Lane 4: *embB* (WT 1/MUT 1A- MUT 1B)

Lane 5: M *(Marker)*

A total of 12(43.4%; F6/M6) among 29 MDR confirmed *M. tuberculosis* strains were found to be pre-extensively drug resistant (pre-XDR-TB), but no XDR TB. The age/sex wise distribution of the SLD-DST pattern showed maximum number of pre-XDR TB among 15-45 years of age groups and similar for both females and males (50%; 4F and 2M in 15-29, 33.3%; F2 and 2M in 30-45, 8.3%; M1 in 46-60 and 8.3%; M1 above 60 years). The mutations detected for *emb B* (ethambutol) genes were also similar for both females and males (F8/M8). Twelve cases thus identified as pre-XDR TB were from the MDR TB treatment centres providing treatment to the MDR TB patients were; 4 (F1/M3) out of 11 (F3/M8) from Nepal Anti TB Association (NATA) Biranagar Morang, 1 (F1) of 5 (F4/M1) from BP Koirala Institute of Health Sciences (BPKIHS) Dharan Sunsari of the eastern development region. Similarly, 1(F1) of 3 (F2/M1) from National TB Centre (NTC) Thimi Bhaktapur, 2 (F1/M1) of 3 (F1/M2) from United Mission to Nepal Hospital (UMN) Lalgarh Janakpur and 4 (F2/M2) of 7 (F2/M5) from National Medical College (NMC) Birgunj Parsa of central development region, were found to be pre-XDR TB (Table 5). There was no significant difference between XDR TB detection by LPA on MDR TB identified by Gene Xpert and FLD-DST (p>0.05).

Table 5: Age (years)/sex wise distribution of SLD-DST patterns by Line Probe Assay on MDR TB detected by Gene Xpert and FLD-DST

Description	15-29		30-45		46-60		>60		Total		Remarks
	F	M	F	M	F	M	F	M	F	M	
TUB	9	7	3	3		4		3	12	17	29
gyrA WT	4	5	1	1		3		3	5	12	17
gyrA MUT	4	2	2	2		1		1	6	6	12
rrs WT	9	7	3	3		4		3	12	17	29
rrs MUT											0
emb BWT	4	4		1		2		2	4	9	13
emb BMUT	5	3	3	3		1		1	8	8	16
Treatment centre	Patients tested for SLDST										Remarks
*E+E1	3	2		2		2		2	3	8	NATA Morang
E2	2			2		1			4	1	BPKIHS
**C	2	1							2	1	NTC
C1	1	2							1	2	UMN Lalgarh
C2	1	2		1		1		1	2	5	NMC Parsa
Treatment centre	Patients diagnosed as Pre-XDR TB										Remarks
E+E1	1			1		1		1	1	3	NATA Morang
E2			1						1		BPKIHS
C	1								1		NTC
C1	1	1							1	1	UMN Lalgarh
C2	1	1	1	1					2	2	NMC Parsa

** E+E1, E2: NATA Morang and BPKIHS Eastern Nepal. ** C, C1, C2: NTC, UMN and NMC of Central Nepal.*

From NATA Morang 4 (F1/M3), BPKIHS 1 (F1), NTC 1 (F1), UMN Lalgarh 2 (F1/M1) and NMC Parsa 4 (F2/M2) were found to be pre-XDR TB. χ^2 for trend of MDR TB by Gene Xpert, culture and DST and XDR TB detection by LPA=0.73, df=1, P value=0.393 (>0.05), so there was no significant difference between XDR TB detection by LPA on MDR TB by Gene Xpert and culture and DST.

The age wise distributions of pre-XDR TB by LPA on MDR TB by Gene Xpert MTB/RIF assay and FLD DST for 15-29 years, 30-35 years, 46-60 years and above 60 years of age group were 50.0%, 33.3%, 8.3% and 8.3% respectively. Similarly, sex wise MDR TB detection by Gene Xpert was 50.0% and 50.0% for females and males respectively.

DISCUSSION

Microscopy is still familiar as a main diagnostic technique of diagnosing tuberculosis in resource-limited countries including Nepal. Due to shortcomings of conventional technique, novel molecular techniques are needed that combine the rapidity of microscopy and the sensitivity of culture. They can identify the mycobacterial species, and would help the clinician during the initial treatment of the patient. Though molecular techniques are not used routinely in Nepal, some investigators reported its feasibility (Sapkota et al 2007).[26]

To combat the excess mortality related to XDR-TB, it is recommended to perform DST for second line-drugs to all MDR-TB cases at the start of treatment. To comply with the above suggestion, it seems important for Nepal to strengthen its capacity to perform SLD-DST, either by culture (solid/liquid) or molecular biology e.g. Line Probe Assay (NTP Annual report Nepal 2015).[4] In order to overcome such problems, present study has evaluated a study of sputum specimens collected from retreatment TB cases by Gene Xpert MTB/RIF assay for the rapid diagnosis of DR/MDR TB. All the MDR TB cases detected by Gene Xpert MTB/RIF assay were further verified by conventional culture and DST for first line anti-tuberculosis drugs (FLD-DST). MDR TB diagnosed by both the methods were analyzed for second line drugs (SLD-DST) LPA.

In the present study, despite of smear results (1 ss-ve and 28 ss+ve), Gene Xpert MTB/RIF assay

showed 100% rifampicin resistance (RR/MDR TB), which was high among 15-60 years and in males (58.6%) than in females (41.4%). In previous study, 27 ss+ve and 23 ss-ve specimens were found to be RR/MDR TB detected by Gene Xpert MTB/RIF, whereas 8 cases were negative for MTB among ss-ve. The study report published previously for smear microscopy and Gene Xpert has revealed the similar results.[27]

In this study, concordance of sputum smear results and detection of *M. tuberculosis* along with rifampicin resistance by Gene Xpert MTB/RIF assay for all 29 samples was 100%.

The assay was successful in rapidly detecting *M. tuberculosis* as well as rifampicin susceptibility pattern. Whereas, it was reported in the similar study by Helb et al (2010)[12] from 107 sputum samples in Vietnam that the concordance for ss+ve, Gene Xpert MTB/RIF (RR TB) and culture was 100%(29/29). In the same study, it was described that 64 smear-positive sputa from retreatment tuberculosis cases in Uganda tested by Gene Xpert MTB/RIF assay detected *M. tuberculosis* among 63/64(98.4%) were also found to be culture-positive and rifampin resistance. Similar results were obtained out of total 62 pulmonary TB cases in previous study.[28] But in this study, out of 29 cases detected as MDR TB by Gene Xpert MTB/RIF assay, all the samples were found to be showing positive growth, none were contaminated. Sputum smear +ve and culture positive results were found higher in 15-60 years (even in >60 years) and in males (16) than in females (12). The distribution of ss+ve and culture results was 28(12 F/16M) and 1 male case was ss-ve out of total 29 culture positives.

Twenty eight of 29(96.6%) culture positive samples on drug susceptibility test (DST) showed both INH and RIF resistance, 1 case (3.4%) detected as RRTB by Gene Xpert MTB/RIF showed mono resistance to isoniazid only. Marlowe et al (2011)[14] has reported similar results previously. The age and sex wise distribution of MDR TB by FLD-DST was high in 15-60 years group (even in above 60 years) in males compared to females. It has been reported in one study by Rijal et al (2005)[29] that the MDR TB among previously treated patients was 19.25% (n=161) irrespective of age and sex variation.

Mboowa et al (2014)[30] stated that the resistance was conferred by four different *rpoB* gene mutations in the 81 bp rifampicin resistance detection region (RRDR) of MTB. These were detected by probes A, B, D, and E. It has also been mentioned in previous study that 96.1% *rpoB* gene mutations located in a region of 426-452 amino acid residues (81bp) of MTB *rpoB* gene (RRDR) detected by probes A-E using Gene Xpert MTB/RIF assay.[31] In this study also it can be revealed that all the MDR TB identified by Gene Xpert MTB/RIF assay has detected 100% *rpoB* gene mutations in 81bp RRDR of MTB. Majority of the MDR TB identified by both Xpert and conventional FLD-DST were males. Male dominated MDR-TB results had been described in a similar study previously.[28]

In the present study, all 29 MDR TB cases were performed for DST on second line anti-TB drugs (SLDs) by LPA. As it was mandatory for the two negative controls must be positive only at CC and AC bands in the strips that were clearly formed in the strips used in this study, so the test process was valid. Among 29 *M. tuberculosis* strains confirmed as MDR TB, all were (100%) showing TUB (*M. tuberculosis* complex). It was found that the *M. tuberculosis* probe was 100 per cent specific. All were found to be wild type for *rrs* WT1, *rrs* WT2, 17(58.6%, F5/M12) for *gyrA* WT1, WT2, WT3 and 13(44.8%, F4/M9) for *emb B* WT1 genes, whereas 12 (41.4%, F6/M6) were mutants for *gyrA,* 16(51.2%, F8/M8) for *emb B*) and none were for *rrs* genes. The mutation of *gyrA* gene was detected by the formation of positive band on the nitrocellulose membrane strip (*gyrA* MUT3A and MUT3C) alone and interpreted as fluoroquinolone/FLQ resistance in 12 cases (F6/M6). Similarly, gene mutations of *gyrA* together with *embB* gene in 9 cases (F5/M4). Any case showing single *gyrA* gene mutation or *gyrA* gene mutation together with *embB* gene was interpreted as pre-XDR TB. Seven out of 29 strains (24.1%) were found to be susceptible to all drugs (F3/M4) but none were XDR TB.

There were 12 *gyrA* mutations identified was high among 15-60 years group that were equally distributed among females and males (F6/M6) and 9 *gyrA* and *embB* gene mutations found to be high among 15-60 years group. The result showed that the distribution of pre XDR TB was higher in the male age group of 15-60 years, among which 1 case was ss-ve, which may suggest us to screen ss –ve retreatment TB cases frequently. Whereas, age wise distribution of pre-XDR TB in female was found to be higher in 15-45 years group (6 cases; all ss+ve). The sex wise pre-XDR TB was identified similar in females/males (F6/M6). None of the age or sex group showed pre-XDR TB below 15 years.

Four pre-XDR TB cases identified were from NATA Morang, 1from BPKIHS Sunsari, 1 from NTC, 2 from UMN Hospital Lalgargh and 4 from NMC Parsa. All the 12(41.4%: F6/M6) among 29 MDR confirmed *M. tuberculosis* strains were found to be *gyrA* gene mutations (pre-XDR-TB). The age/sex wise distribution of the SLD-DST pattern showed maximum number of pre-XDR TB among 15-45 years of age groups and was similar for both sexes.

All the 29 MDR TB cases were found to be *rrs* gene wild type. It has been revealed by the previous similar study.[32] It is now clear that the pre-XDR TB is prevalent and scattered in all the MDR TB treatment centres in Nepal.

CONCLUSIONS

Gene Xpert MTB/RIF assay as it is a useful method of simultaneous detecting MTB and rifampicin resistance (surrogate marker of MDR-TB) in both the sputum negative and positive samples along with the culture and DST as reference gold standard.

From this study, out of 29 retreatment TB patients enrolled, 100% were detected RR/MDR TB by Gene xpert MTB/RIF assay irrespective of sputum smear results and all were found to be culture positive. All the culture positive strains were identified as to resist isonoazid and rifampicin with or without remaining drugs resistance. All the 29 MDR TB strains were tested for SLD-DST using LPA (Genotype MTBDR*s*/) and 12 out of 29 MDR TB strains were found to be pre-XDR TB as well. The prevalence of MDR and/or pre-XDR TB was higher in the 15-60 years age group and distributed in both the females and males.

RECOMMENDATIONS

It is recommended that all the samples submitted for microscopic examination should be further processed for culture and DST, if the laboratory setting is capable to do. If not so, all the Cat1 treatment failures as well as Cat 2 treatment failure cases should be tested for RR/MDR TB using Gene Xpert MTB/RIF assay and culture and FLD-DST. Simultaneously, all the MDR-TB cases confirmed by Gene Xpert MTB/RIF assay and culture and FLD-DST should be further tested for SLD-DST by various culture/DST methods and Genotype MTBDR*s*/ to identify pre-XDR and/or XDR TB. This way, prompt diagnosis of TB/DR-MDR TB/XDR TB can be made possible and patient's treatment management as well. Further study should be frequently performed at the national, regional or provincial level on higher samples.

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Original Article

Gene Xpert based detection of drug resistant tuberculosis among retreatment patients visiting National Tuberculosis Centre, Nepal

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Abstract

Introduction: Tuberculosis is still one of the major public health problems in Nepal and multi drug resistant and extensively drug resistant tuberculosis (MDR /XDRTB) additionally has become serious issue. Prompt diagnosis and effective treatment of MDR/XDRTB is urgently needed. The main objective of this study was to detect MDR TB using novel molecular techniques (*rpob* gene mutations) in reference with drug susceptibility test (DST).

Methods: A cross sectional study was carried out identifying MDRTB among retreatment patients using Gene Xpert, culture and DST on first line drugs (FLD-DST). A total of 159 sputum samples were collected from retreatment TB patients (Female 40.3%, Male 59.7%) with median age of 30 years visiting to the DR TB treatment centres of eastern and central Nepal (via private courier and directly to National TB Reference Laboratory (NRL) at NTC from April 2013 to August 2017.

Results: *M. tuberculosis* and rifampicin resistance were detected on all 159 (100%) samples by Gene Xpert of which, 73.3%, 21.4% and 6.3% were positive, negative and contaminated respectively by culture. FLD-DST was performed on 115 cultures positives of which, 94.78% showed MDRTB, 1.74% showed mono resistance to isoniazid or rifampicin, 0.87% to streptomycin and isoniazid and 3.47% were pan susceptible.

Conclusion: One hundred fifteen of 159 cases detected rifampicin resistances (RR) by Gene Xpert were culture positive and almost 95% strains were MDRTB by FLD-DST, which was found to be higher in 15-60 years group. Sputa from retreatment TB patients required to be tested by rapid diagnostics with reference to culture and DST.

Key words: *Gene Xpert, Culture, Drug Susceptibility Test, Multi Drug Resistant Tuberculosis, Sputa.*

Introduction

Tuberculosis is a global threat because nearly two billion people (one third of the world's population) harboring latent infection. In 2014, 9.6 million people fell ill with TB and 1.5 million died from the disease. An estimated 9.7% of people with MDRTB have XDRTB.[1] The SAARC region, with 34% of the global burden of TB, where a total of 81,142 estimated cases of MDRTB among notified cases of which, 41% were new pulmonary cases and 59% were previously treated cases.[2]

TB remains one of the major public health problems in Nepal. In 2014, total of 37,025 cases of TB were registered. Most cases were reported among the middle aged group with the highest among 15-24 years of age (20%). Nationwide, the proportion of new cases with MDRTB was 2.2% among new cases and 15.4% among retreatment cases based on survey carried out in

2011/12. In 2014, total of 349 MDRTB and 25 XDR TB were enrolled for treatment. WHO estimated 4.6 (2.1-7.5) thousand people died from TB in 2014.[3]

Isoniazid (INH) with rifampicin (RIF) forms the cornerstone of short course chemotherapy for tuberculosis and resistance to either drug hampers the complete cure of patients. *M. tuberculosis* strains resistant to at least these two major frontline drugs (INH and RIF) develop MDRTB.[4]

More than 95% RIF resistant *M. tuberculosis* strains have mutations in an 81 bp hot spot region (codon 507-533) of *rpoB* gene that encodes RNA polymerase beta subunit.[5] This region is therefore an ideal target for molecular tests for RIF resistance.[6,7,8]

If a diagnosis is absent, patients are not treated, transmission may continue, patients suffer needlessly and may eventually die. Sputum smear microscopy, as a diagnostic tool of tuberculosis, is varying between 30% and 70% depending on a number of factors relating to how the test is implemented.[9]

Cepheid (Cepheid, Sunnyvale, CA) has recently introduced the GeneXpert MTB/ RIF assay, which is a real-time PCR test that will simultaneously identify *M. tuberculosis* and detect rifampin resistance directly from clinical specimens.[10,11] Rifampin resistance can serve as a marker for MDRTB and has been reported in 95% of the MDR TB isolates.[12] The GeneXpert assay detects an 81-bp "core" region of the *rpoB* gene.[13]

The Gene Xpert MTB/RIF assay, conventional culture and FLD-DST are the choice of DR/MDR TB diagnostics tools. Culture and FLD-DST method takes usually longer time but always being considered as the gold standard that gives the viable organisms and can be used for various research purposes. So, the present study has been giving priority to identify MDRTB among retreatment cases using Gene Xpert MDR/RIF assay with reference to conventional culture and FLD-DST.

Methodology

The study was a cross sectional study design to identify RR/MDRTB among retreatment TB cases using Gene Xpert MTB/RIF assay and to compare the prospective data obtained with reference to conventional culture and proportional FLD-DST.

Study site: This study was carried out from April 2013 to November 2017 in National Tuberculosis Reference Laboratory at National Tuberculosis Centre (NRL/ NTC), Thimi, Bhaktapur, Nepal.

Sample size: One hundred and fifty nine (159) retreatment tuberculosis patients were involved in this study before they were registered for starting second line anti-tuberculosis treatment.

Study population: Retreatment pulmonary TB cases (relapse, treatment after failure, and treatment after loss to follow-up) previously treated with Cat I and Cat II regimen were enrolled in this study.

Patient's consent: The samples were collected and examined at NRL/NTC regularly from the same selected sites before starting this study.

Inclusion/exclusion criteria: Retreatment TB cases visiting for further diagnosis and diagnosed as sputum smear positive or negative before being registered for and started MDR treatment were included in this study. The cases already registered and recently undergoing second line anti tuberculosis treatment, blood stained sputum, sputum with food particles, with saliva in greater amount, leaking, dried or if not freshly collected and patients suspected of extra-pulmonary tuberculosis were excluded from this study. The samples showing contamination during culture were excluded from the study.

Sample collection

Early morning sputum samples (stuffy and mucopurulent, 3-5ml each) were collected from 159 retreatment TB patients out of which, 76 (F23/M53) from Nepal Anti TB Association (NATA) Biratnagar Morang and 19 (F8/M11) from BP Koirala Institute of Health Sciences (BPKIHS), Dharan, Sunsari of the eastern Nepal, 24 (F11/M13) from United Mission to Nepal Hospital (UMN), Lalgarh, Janakpur, 18 (F7/M11) from National Medical College (NMC), Birgunj, Parsa, and 22 (F15/M7) from National TB Centre (NTC), Thimi, Bhaktapur of central Nepal) in leak proof, wide mouthed, transparent and sterile 50 ml disposable plastic centrifuge tube (Falcon BD, USA), appropriately labeled and stored at 2-8^{o}C until dispatched or processed. The samples were transported through private courier and the duration of sample transportation was not > 48 hours to reach to NRL, but patients being treated at NTC had submitted fresh samples directly to NRL. The age, sex and smear result wise data are shown in table 1.

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Table 1: Age /sex wise distribution of retreatment TB cases enrolled in this study

Age (years)	Sex		Total N (%)
	Female N (%)	Male N (%)	
<15	3 (1.9)	0 (0.0)	3 (1.9)
15-29	34 (21.4)	41 (25.8)	75 (47.2)
30-45	14 (8.8)	21 (13.2)	35 (22.0)
46-60	10 (6.3)	22 (13.8)	32 (20.1)
>60	3 (1.9)	11 (6.9)	14 (8.8)
Total	64 (40.3)	95 (59.7)	159 (100)
Age range (Min-Max)	11-72		
Mean age (years) ± SD	35.86 ±15.95		
Median age (years)	30		

Sample processing and culture on Lowenstein Jensen (LJ) medium

Sputum samples were processed inside a Biological Safety Cabinet class II (BSC-II AIRTECH, Japan) directly by adding twice the volume of 4.0% NaOH digestion method (modified Petroff's method), vortex mixed and left for 15 minutes at room temperature with occasional shaking. Then phosphate buffer (pH 6.8) was added up to level of 45 ml graduation mark, vortex mixed and centrifuged at 3000x g for 15 minutes in a refrigerated centrifuge at 4°C (KUBOTA, Japan).

The supernatants were discarded and pellets were used for culture; 0.2ml of pellet was inoculated on duplicate LJ media, incubated at 37°C for 4-8 weeks in an incubator (MEMMERT, Germany). The tubes were examined on 7th day for rapid growers and checked for growth at 2, 3, 4, 5, 6, 7, 8 weeks until negative. If any contamination seen in the culture tube, that was recorded.

Gene Xpert MTB/RIF assay

Gene Xpert MTB/RIF tests were performed as per the instructions provided by the manufacturers (Cepheid Sunnyvale, CA, USA). Sputum pellets were decontaminated and treated with sample reagent (SR; a mixture of NaOH and iso-propanol) as to make 3:1 ratio to the pellets and left for 15 minutes at room temperature; 2 ml of the treated samples were transferred to the Gene Xpert cartridges (Cephied, France), and then loaded into the programmed Gene Xpert modules. Gene Xpert device was kept on and results were observed after the whole process completed (within about 2 hours).

Microscopy observation

A smear of the processed sediment was prepared (size of 2 x 3 cm), air dried, heat fixed, stained by Ziehl-Neelsen method and read under binocular light microscope at the total magnification of 1000X (Olympus, Japan) and reported according WHO/IUATLD grading scale.

Preparation of bacillary suspension and inoculation for DST (1% proportion method)

One loopful (4mg approximately) of mycobacterial colonies from LJ media was harvested and emulsified with 1ml of sterile distilled water (SDW) in a sterile Bijou bottle, vortex mixed and allowed to stand for 15 minutes, transferred to a McCartney bottle; turbidity was compared and adjusted with McFarland standard no.1 Nephelometer (1 mg/ml or 10^6-10^8 CFU/ml of bacteria). Made 100 fold dilutions from McFarland standard no.1 bacillary suspension; 1 loopful (nichrome wire loop 24 SWG and 3mm diameter delivering 0.01ml) was transferred to 1ml of SDW in Bijou bottles and vortexed to make 10^{-2} dilution or 10,000 CFU/ml, from which 10^{-4} dilution (l00 CFU/ml) was prepared.

One loopful of each dilution (10^{-2} and 10^{-4}) was inoculated on two plain LJ slopes (controls) and one set each of slopes with 4 drugs (streptomycin (S); 4.0 µg/ml, isoniazid (I); 0.2 µg/ml, rifampicin (R); 40.0 µg/ml, ethambutol (E); 2.0 µg/ml or SIRE; manufactured by SIGMA-Aldrich, USA), incubated at 37°C, read on 4th and 6th week for resistant (growth on drug medium ≥ 1% colonies on control) and final susceptible (no or <1% colonies on control) patterns respectively. Internal quality control was routinely performed (for each batch of new drug media) using the pan-susceptible reference strain *M. tuberculosis* H37Rv (ATCC-27294).

Biochemical Identification tests

From the positive growth, identification tests were performed by biochemical methods i.e. growth on PNB containing LJ medium and niacin production tests.

1. *Growth on p-nitrobenzioc acid (PNB) containing media*

A loopful of neat bacterial suspension (McFarland standard No. 1) was inoculated into one plain and other slope of LJ with PNB at a concentration of 500 µg/ml and incubated at 37°C for each set and read on 28th day. *M. tuberculosis* H_{37}Rv as negative (PNB susceptible) and *M. kansasii* as positive control (PNB resistant) were used.

Results and interpretation No growth on PNB medium: *M. tuberculosis;* growth on PNB medium: *M. kansasii*

2. *Niacin production test*

Niacin test was performed according to the manufacturer's instruction. With a sterile transfer pipette, approximately 0.6 ml of the positive culture extract was transferred to the bottom of 20 mm × 125 mm screw cap test tube along with *M. tuberculosis* H_{37}Rv as positive and *M. kansaii* as negative control. The niacin test strips (BBL Taxo TB strips, Becton and Dickinson, USA), were dropped with arrow downward into the tubes. The colors of the extracts were then compared after 15 minutes.

Results and interpretation: *M. tuberculosis* H_{37}Rv: yellow colour (niacin positive); *M. kansaii:* colourless (niacin negative). All the cultures have shown PNB negative and niacin positive.

Statistical data analysis

The statistical analysis of the study data were analyzed using SPSS version 16.0 software. The χ^2 test was used to compare age and sex wise distribution of smear and culture, Gene Xpert and culture and FLD-DST results. The *P*-value <0.05 was considered statistically significant. The sensitivity, specificity, PPV, and NPV of the GeneXpert and FLD-DST were calculated using MedCalc software and 95% confidence intervals were estimated.

Results

Out of 159 (64 F; 40.3%, 95 M; 59.7%, Male:Female ratio 1.48:1), 42 (13F/29M; 26.4%) and 117 (51F/66M; 73.6%) were sputum smear negative (ss -ve) and sputum smear positive (ss +ve) respectively. The grading was high positives for 111 cases (1+, 2+, 3+) and 6 were scanty positives (2-6 AFB) as shown in figure 1. There was no significant difference in age and sex wise smear results (p >0.05). Inconclusive results for 10 (15.9%) cases (4 ss–ve; F1/M3, and 6 ss+ve; F1/M5) were found to be contaminated, so excluded from the study.

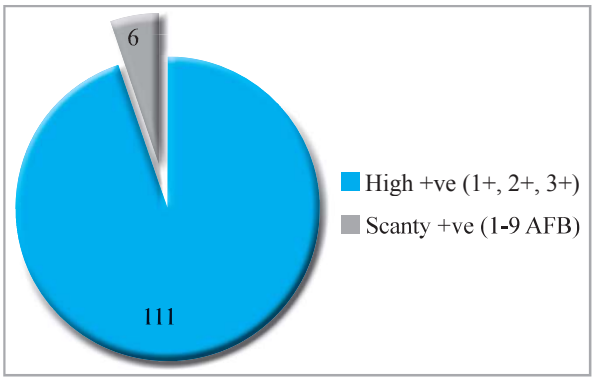

Figure 1 Grading distributions of smear positive results

Table 2 shows age and sex wise distribution of smear and culture results, in which 115 (72.3%) of 159 cases (F48/M67; ss -ve 9/ss +ve 106) were culture positive (F45/M70); s+/c+ (F44/M62), s+/c- (F2/M3), s-/c+ (F1/M8) and s-/c- (F12/M17). There was no significant difference in gender wise culture positivity (p = 0.213). The culture positives were high among smear positive cases and majority fell within 15-60 years age group and among males. There was an association between smear and culture positive results (p < 0.001).

Table 2: Age/sex wise Comparison of smear and culture results

Results	Age & sex distribution												Grand Total
	<15 years		15-29 years		30-45 years		46-60 years		>60 years		Total culture positive		
	F	M	F	M	F	M	F	M	F	M	F	M	
S+ C+	3	0	26	29	8	16	5	12	2	5	44	62	106
S+ C-	0	0	1	2	1	0	0	0	0	1	2	3	5
S- C+	0	0	1	5	0	2	0	1	0	0	1	8	9
S- C-	0	0	4	5	4	3	4	4	0	5	12	17	29
Contamination	0	0	0	2	0	2	1	2	1	2	2	8	10

F = female M = male S + = smear +ve S - = smear -ve, C+ = culture +ve

Detection of *M. tuberculosis* with RR/MDR was found in all 159 samples (40.3% F, 59.7% M) by Gene Xpert MTB/RIF assay (figure 2) and was occurred in all age categories (<15, 15-29, 30-45, 46-60 and >60 years), were 1.9%, 47.2%, 22.0%, 20.1% 8.8% respectively but the majority in 15-60 years and even above (14 cases). There was a significant difference in distribution of MDR TB among age groups ($p = 0.050$), but no significant difference of MDR TB between females and males ($p = 0.225$).

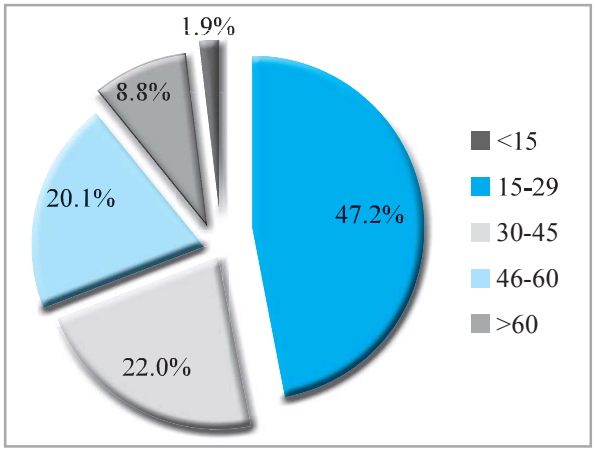

Figure 2 Age wise distribution of MDR TB case

The sensitivity (106/115; 92.2% at 95% CI; 85.1, 96.1), specificity (29/34; 85.3% at 95% CI; 68.2, 94.5), predictive value of positive test (106/111; 95.5% at 95% CI; 89.3, 98.3), predictive value of negative test (29/38; 76.3% at 95% CI; 59.4, 88.0) of Gene Xpert and smear microscopy results were evaluated with reference to culture using standard formula (Table 3).

Table 3: Comparison of smear and *R^r by Gene Xpert results with reference to culture

Smear	* Gx R^r	Culture +ve	Culture -ve	Sensitivity 95% CI	Specificity 95% CI	PPV 95% CI	NPV 95% CI
Negative n (%)	42 (28.9)	9 (5.6) (c)	29 (18.3) (d)	**a/a+c*100**	**d/b+d*100**	**a/a+b*100**	**d/c+d*100**
Positive n (%)	117 (71.1)	106 (a) (66.7)	5 (3.1) (b)	**106/115 = 92.2 % (85.1, 96.1)**	**29/34 = 85.3 % (68.2, 94.5)**	**106/111 = 95.5 % (89.3, 98.3)**	**29/38 = 76.3 % (59.4, 88.0)**
Total n (%)	**159 (100)**	**115 (72.3)**	**34 (21.4)**				

***GxR^r: rifampicin resistant by Gene Xpert MTB/RIF assay**

Formula: Sensitivity (Se) = a/(a+c)* 100, Specificity (Sp) = d/(b+d)* 100, Positive predictive value (PPV) = a/(a+b)* 100, Negative predictive value (NPV) = d/(c+d)* 100

Where, a = true positive, a+c = total positive (positive test): a, c = disease

d = true negative, b+d = total negative (negative test): b, d = no disease

PPV = positives among total positive predicted

NPV = negatives among total negative predicted

FLD-DST was performed on 115 culture positive isolates; of which 109 strains were identified as MDR TB. A total of 109 (94.78% 48F/61M; ss –ve 9: all M & ss +ve 100: 43F/57M) out of positives showed resistance to both I & R, 1 case each (1.74% 1F/1M) only to I and R,1 (0.87% M) to S and I and 4 (3.7% 3F/1M) were susceptible to all SIRE. There was significant difference of culture positive results and MDRTB detection ($p = 0.001$).

Out of 109 MDR TB cases; 11.0% (F2/M10) were resistant to IR which were high among 15-45 years age group, 36.7% to IRE (F3/M4 & high in 15-45 years), 30.3% to SIR (F16/M17 were high among 15-60 years group respectively) & 50.5% (F23/M32) were resistant to SIRE that was high among 15-60 years group & even above (F2/M4). Three cases (2 resistant to SIR &1 to SIRE) were all females below 15 years age group. The age wise distribution of MDR TB by Gene Xpert and FLD-DST was 2.7%, 49.5%, 23.9%, 19.3% and 4.6% for <15, 15-29, 30-45, 46-60 and >60 years group. There was no significant difference of age wise MDR TB cases identified by Gene Xpert and conventional C/DST ($p = 0.532$), the reference strain *M. tuberculosis* H37Rv was pan susceptible. The sex wise distribution of FLD-DST patterns was higher among males (43F/64M). There was no significant difference of sex wise distribution of MDR TB cases identified by Gene Xpert and conventional C/DST ($p = 0.775$) as shown in table 4.

Table 4: Age/sex wise distribution of FLD-DST patterns

DST patterns of FLDs by conventional proportion method													
	<15		15-29		30-45		46-60		>60		Total		Grand total
	F	M	F	M	F	M	F	M	F	M	F	M	
Total Tested	3	0	29	31	9	17	5	13	2	6	48	67	115
Fully Susceptible	0	0	2	0	1	0	0	1	0	0	3	1	4
Mono Resistance	0	0	1	0	0	0	0	0	0	1	1	1	2
S	0	0	0	0	0	0	0	0	0	0	0	0	0
I	0	0	0	0	0	0	0	0	0	1	0	1	1
R	0	0	1	0	0	0	0	0	0	0	1	0	1
E	0	0	0	0	0	0	0	0	0	0	0	0	0
Total I+ R Resistance /MDR	3	0	26	28	7	19	10	11	2	3	48	61	109
IR	0	0	1	5	0	5	1	1	1	0	3	11	14
IRE	0	0	3	0	0	2	0	1	0	1	3	4	7
SIR	2	0	8	8	4	6	4	3	0	0	18	17	35
SIRE	1	0	14	15	3	6	5	6	1	2	24	29	53

S: streptomycin I: isoniazid R: rifampicin E: ethambutol

Discussion

Due to shortcomings of conventional technique, novel molecular techniques are needed that combine the rapidity of microscopy and the sensitivity of culture. Though molecular techniques are not used routinely in Nepal, some investigators reported its feasibility.[14] In order to overcome such problems, present study has evaluated a study of sputum specimens collected from retreatment TB cases by Gene Xpert for the rapid diagnosis of DR/MDR TB, which were further verified by FLD-DST) as gold standard.

In the present study, age wise sputum smear positivity was high among 15-60 years of age group and in males (66/159) than in females (51/159). Similar results were reported previously, in which, out of 58 patients (with age range 21–67 years) clinically suspected to have pulmonary retreatment TB, Z–N smear examination was positive for AFB in 49 (90.7%). L–J culture results revealed positive yield in 54 cases.[15]

In the present study, despite of smear results (42 ss-ve and 117 ss +ve), Gene Xpert showed 100% RR/MDR TB, which was high among 15-60 years and in males (58.6%) than in females (41.4%), whereas, 115 (72.3%) of 159 cases (F45/M70; 9 ss -ve and 106 ss +ve) were culture positive (F39.1%, M60.9%). From this study, sensitivity (92.2% at 95% CI; 85.1, 96.1), specificity (85.3% at 95% CI; 68.2, 94.5), PPV (95.5% at 95% CI; 89.3, 98.3) and NPV at 95% CI (76.3% at 95% CI; 85.1, 96.1), of Gene Xpert and smear microscopy results were evaluated with reference to culture. Similar results for sensitivity, specificity, PPV and NPV respectively were described in previous study in which, sensitivity, specificity, PPV, & NPV was 70% at 95% CI; 60.8, 77.8, 100% at 95% CI; 98.8, 100, 100% at 95% CI; 95.2, 100, and 90.6% at 95% CI; 87.1, 93.2 respectively.[16]

In our study, 109 of 115 (94.8%) culture positives on FLD-DST showed both IR resistances, 1 case each (1.74%) detected as RRTB by Gene Xpert showed I mono resistance only. The assay was successful in rapidly detecting *M. tuberculosis* as well as rifampicin susceptibility pattern. It was reported in the similar studies in Vietnam and Uganda that the concordance for ss +ve, Gene Xpert (RR TB) and culture was 100% (29/29) and 63/64 (98.4%) respectively. [12, 13,17]

In this study, no repeated culture/FLD DST or Gene Xpert was performed. The age and sex wise distribution of MDR TB was high in 15-60 years group (even above 60 years) and in males than females by both the methods. It has been reported in one study that the MDR TB among previously treated patients was 19.25% (n=161) irrespective of age and sex variation.[18]

The resistance was conferred by four different rpoB gene mutations in the 81 bp rifampicin resistance detection region (RRDR) of MTB by probes A, B, D, and E. It has also been mentioned in previous study that 96.1% rpoB gene mutations located in a region of 426-452 amino acid residues (81bp) of MTB rpoB gene (RRDR) detected by probes A-E using Gene Xpert MTB/RIF assay.[19,20] In this study also it can be revealed that all the RR/MDR TB identified by Gene Xpert has detected 100% rpoB gene mutations in 81bp RRDR of MTB. Majority of the MDR TB identified by both Xpert and conventional FLD-DST were males. Male dominated MDRTB results had been described in a similar study.[17] There was not a single rifampicin mono resistance by conventional FLD-DST in this study as it is very rarely occurring.

Conclusions

Gene Xpert MTB/RIF assay as it is a useful method of simultaneous detecting MTB and rifampicin resistance (surrogate marker of MDRTB) in both the sputum negative and positive samples along with the culture and DST. From this study, out of 159 retreatment TB patients enrolled, 100% were detected RR/MDR TB by Gene Xpert irrespective of smear results and 72.3% were culture positive, among which, 94.8% were MDR TB by FLD-DST. The prevalence of MDR TB was found to be high among 15-60 years age group and distributed in both the females and males by both methods. Results of this study has given a very good example that all the Cat1 treatment failures as well as Cat 2 treatment failure cases should be tested for RR/MDR TB using Gene Xpert MTB/RIF assay (prompt diagnosis) and culture and FLD-DST (gold standard method).

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Conflict of interest: None declared.

Ethical approval: This study was conducted with the approval of the Nepal Health Research Council (NHRC) Ethical Review Board (Registration No. 308/2017).

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