CHAPTER-I
INTRODUCTION

1.1 Background

Tuberculosis (TB) remains a major public health problem in Nepal and one of the leading causes of death from communicable diseases among adults. (Ministry of Health and Population, 2011a). *Mycobacterium tuberculosis* (MTB), along with members of the tuberculosis species complex (i.e. genetically closely related group of *Mycobacterium* species) cause TB. Drug resistance in MTB bacteria arises mainly through the acquisition of mutations in the chromosomal sequence.

Conventional methods like sputum smear microscopy are still used in many parts of world to diagnose TB. TB bacteria are observed under a microscope in sputum samples in this method. Diagnosis is made with three such tests within a day however less infectious forms of TB are missed in this method. Diagnosing MDR-TB and HIV-associated TB can be more complex. A new two-hour test has been rolled-out in many countries which is proven as highly effective in diagnosing TB and to identify drug resistances. Diagnosing TB is more complex in children (WHO, 2012).

Effective control of drug-resistant TB relies on timely and accurate identification of cases and the prompt initiation of appropriate therapy (Noordeen and Godal, 1998). TB cases in most resource-limited settings are routinely diagnosed by microscopic examination of sputum specimens stained for acid fast bacilli (AFB) and by chest radiography. Unfortunately, these methods do not provide any data about whether an MTB strain is drug-resistant. Processing of sputum specimen followed by culturing MTB and doing drug-susceptibility testing (DST) if culture grows is essential to diagnose drug resistance (Varma, 2007). After specimen collection, DST results are returned by laboratories in weeks for liquid media methods or months for solid media methods which may also take further extended time

due to delays in transportation and batching of specimens for testing. For this reason, scaling up the laboratory capacity for culture and DST and to evaluate the routine implementation of new, molecular-based assays for diagnosing drug-resistance is being recommended by WHO (WHO, 2008b).

Anti-tuberculosis drugs can act on positive way by destroying pathogenic *M. tuberculosis* as well as on negative way by selecting for drug resistant bacteria against which those drugs are then ineffective. Widespread of drug resistant TB posing threat to tuberculosis control programs in many nations have been shown by global surveillance (WHO, 2003). Understanding of drug resistance in tuberculosis is more rigorously studied and much key information has been discovered by using various molecular methods in recent years. Molecular epidemiological methods are very fundamental for the description of outbreaks of drug resistance in TB.

Molecular genetic tests for detecting drug-resistance are, in general, just a variation of nucleic acid amplification (NAA) tests and can reliably provide information on the presence of mutations associated with drug resistance in one to 2 days. Apart from the impact on morbidity, mortality and transmission of MDR-TB, introduction of these assays in screening and diagnostic algorithms could significantly reduce the need for sophisticated and costly conventional laboratory infrastructure which is still vastly inadequate in low income countries.

Drug resistance can be shown by detection of resistance-conferring mutations in relevant genes, using various techniques. Mutation in the resistance determining region (RDR) of the *rpo*B gene accounts for over 96% of rifampicin (RMP) resistant *M. tuberculosis* strains (Ramaswami *et al.,* 1998; Traore *et al.,* 2000) while mutation in the *kat*G gene accounts for 70–85% of isoniazid (INH) resistant strains with only 20–35% having mutation in *inh*A promoter region (Heym *et al.,* 1997; Piatek *et al.,* 2000). For rapid detection of resistance to RMP and INH within 1–2 days, WHO has recommended the use of polymerase chain reaction (PCR) followed by line-probe assay (LPA) (WHO, 2008b). These reversed hybridization assays can be applied directly on

smear-positive sputum. The Genotype MTBDRplus assay (Hain LifeScience GmbH, Nehren, Germany) is one of the commercially available LPAs. Because of its accuracy and rapidity, genotypic detection of RMP and INH resistance has emerged as an important tool for the diagnosis of multidrug resistant TB (MDR-TB). In areas with limited capacity to perform phenotypic DST, Genotype MTBDRplus assay has been suggested as an alternative for DRS. Safe and easy transportation of sample to reference laboratories is guarranted in genotypic assays by inactivating bacilli at the sampling point since these assays do not require viable bacilli (Drobniewski *et al.,* 2000).

Target gene associated with resistance is amplified using PCR in Genotype MTBDRplus *assay* and the labeled PCR products are hybridized to oligonucleotide probes that are immobilized on a nitrocellulose strip (Barnard, 2008). Accuracy for rifampicin resistance which is a proxy for MDR-TB is observed to be excellent for GenoType MTBDR assays. Therefore, in places where appropriate infection control is a major concern like in settings with high rates of MDR-TB or HIV, it can be utilized as a rapid screening tool. Even though specificity is excellent for isoniazid, sensitivity estimates are modest and highly variable which could be further improved once more data on mutations conferring isoniazid resistance becomes known. However, sensitivity for isoniazid resistance has been improved to 90% in MTBDRplus version of the assay as used in this study. As mutations occuring outside the 81-bp region of the *rpoB* gene are not detected by this assay, sensitivity for RIF resistance may be low in this settings and they may also be responsible for RIF resistance (Bartfai *et al*., 2001). Genotype MTBDRplus *has* advantage over other line probe assays in that it detects mutations associated with both rifampicin and isoniazid resistance. Mutations are detected by lack of binding to wild-type probes and/or by binding to probes that are designed specifically for commonly occurring mutations.

The performance of the Hain MTBDR *(plus)* assays relative to culture-based DS tests was evaluated in one study and found to have the pooled sensitivity of 0.98 and pooled specificity of 0.99 for detecting rifampicin resistance (Morgan *et al.,* 2005; Ling *et al.,* 2008; WHO, 2008b) while in one another

study evaluated for isoniazid resistance pooled sensitivity of 0.85 and a pooled specificity of 0.99 was reported (Lin *et al.,* 2004). This showed excellent sensitivity and specificity for rifampicn and variable accuracy for isoniazid (with modest sensitivity and excellent specificity). Therefore Genotype MTBDRplus assay can be successfully used for rapid drug resistance detection especially in resource poor countries like Nepal.

Effective TB control programs rely on rapid and accurate detection of drug resistance in *Mycobacterium tuberculosis* complex (MTBC) clinical isolates. Very limited drug resistance surveys have been carried out in Nepal and MDR-TB rates are not yet controlled and hence reported every year with higher rate of detection among re-treatment cases than in new cases (WHO, 2009a). Mycobacteria isolates do vary according to the geographical distribution and time interval therefore it is very necessary to understand the genetic diversity of these isolate and their sensitivities towards various drug in frequent basis for the effective control program and timely treatment of patients. Hence this study was carried out using Genotype MTBDRplus assay for rapid confirmation of MDR TB in clinical specimen through the identification of genetic mutations associated with rifampicin and isoniazid resistance in GENETUP, Nepal. Even though this study covers a small sample size but it represents the whole country and hence highlights the current scenario of drug resistant tuberculosis in different geographic area of Nepal which helps to take further step for the control program of disease.

Even few studies have revealed the range of mutation in clinical samples of MTB from Nepal (Poudel *et al*., 2012 ; Marahatta *et al*., 2011) but lack of broader studies and sufficient data is assisting for the transmission of drug resistance TB and efforts to treat patients and control the spread of TB is also hindered. Additionally, the slow growth rate of MTBC and inherent difficulties associated with conventional drug susceptibility testing (DST) methods often serve as impediments to obtaining timely results. To address these issues, this molecular genetic assay is carried out using Genotype MTBDRplus for the identification of drug resistance associated mutations in isolates of MTBC. This method will allow rapid confirmation of TB through

the identification of genetic mutations associated with rifampicin and isoniazid resistance. About 45% of the total population is infected with TB and cases are reported from all disticts of Nepal. Therefore, to represent the overall situation of Nepal this study has covered all part of the country as its study site. Furthermore, Nepal being situated between China in the north and India in the south which are the two highest MDR burden carrying countries there is higher chance of acquiring drug resistant TB by Nepali patients which is also fostered due to open border with India. Therefore, this study can be useful source of information to track the origin of spread of infection from surrounding two countries whenever data is obtained and can provide insight regarding common mutation pattern of isoniazid and rifampicin drug resistant TB isolates of Nepal.

1.2 Objectives

1.2.1 General objective

To identify *Mycobacterium tuberculosis* complex from other mycobacterial isolates and characterize its gene for identification of rifampicin and/or isoniazid resistance.

1.2.2 Specific objectives

a) To describe socio-demographic characteristics of suspected tuberculosis patients.
b) To identify mycobacterial isolate type responsible for infection.
c) To determine prevalence of multidrug resistance (MDR) and rifampicin/isoniazid monoresistant tuberculosis in Nepal.
d) To assess pattern of gene mutations in resistant *Mycobacterium tuberculosis* strains and correlate drug treatment failure cases and drug resistance pattern of these isolates.

CHAPTER-II
LITERATURE REVIEW

2.1 Mycobacteria

Mycobacteria are Gram-resistant (waxy cell walls), non-motile, pleomorphic rods, related to the Actinomyces. Except few mycobacteria, which are intracellular pathogens of animals and humans, most are found in habitats such as water or soil. Genus *Mycobacteria* has 71 recognized species that produce array of infection and animals (Good and Shinnick, 1998). *Mycobacterium tuberculosis*, along with members of the tuberculosis species complex (i.e. genetically closely related group of *Mycobacterium* species) cause the disease known as tuberculosis (TB). *Mycobacterium tuberculosis* complex includes: *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium bovis BCG*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium pinnipedii* and *Mycobacterium mungi* (Vasconcellos, 2010). Each member of the TB complex is pathogenic, but *M. tuberculosis* is pathogenic for humans while *M. bovis* is usually pathogenic for animals. *M. tuberculosis* is probably a human-specialized form of *M. bovis* developed among milk-drinking Indo-Europeans who then spread the disease during their migration into western Europe and Eurasia and *M. tuberculosis* and pulmonary TB had spread throughout the known world by 1000 BC (Sola *et al.,* 2001).

Many non pathogenic mycobacteria are found most often in dry and oily locales of human body and are components of the normal flora of humans. However, *Mycobacterium tuberculosis* which is pathogenic is a facultative aerobic intracellular parasite, usually of macrophages with longer generation time of 15-20 hours which may be related to its virulence factor (Ismael and Ray, 2004). *Mycoobacterium tuberculosis* is non capsulated, non sporing, straight and slightly curved rod measuring 1-4×0.2-0.5 µm. The colonies of *M. tuberculosis* appear dry, rough and creamy or buff colored on LJ medium (Cheesbrough, 1989).

Their growth requires optimum temperature of 37^0C and p^H of 6.4-7.0 requiring especial enriched media containing potato, serum, egg, asparagine and meat extract (Forbes *et al*., 2002). *M. tuberculosis* has an unusual, waxy coating on its cell surface (primarily mycolic acid), which makes the cells impervious to Gram staining, so Ziehl-Neelsen staining, or acid-fast staining are used, instead. MTB is therefore not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. Since they lack outer cell membrane mycobacteria are classified as acid-fast Gram-positive bacteria (Ismael and Ray, 2004). However, as they do not retain the crystal violet stain they do not seem to fit the Gram-positive category from practical standpoint. Even ability to resist decolorization by weak mineral acid is an important characteristic of them but chemical structure define them more accurately (Minnikin, 1989).

Acid-fast bacilli are also visualized using the histological technique, auramine-rhodamine stain (AR) under fluorescence microscopy, especially for species of *Mycobacterium* genus which display a reddish-yellow fluorescence (Kommareddi *et al.,* 1984). Because auramine-rhodamine stain is more sensitive and affordable it is frequently utilized as a screening tool even though it is not as specific as the Ziehl-Neelsen stain for acid-fast detection.

2.1.1 Genetic diversity

Significant phenotypic differences are observed in clinical isolates of *M. tuberculosis* as it is genetically diverse. Different geographic regions have different strains of *M. tuberculosis* but strain variations are found to have no significance role in the development of new diagnostics and vaccines as suggested by phenotypic studies (Gagneux, 2009).

Typing of *M. tuberculosis* strains were done by pulsed field gel electrophoresis (PFGE) till early 2000s (Zhang *et al.,* 1992; Singh *et al.,* 1999)

which has been later supplanted by variable numbers of tandem repeats (VNTR) that has better discrimating ability and is easy to operate.

VNTR typing for *M. tuberculosis* has three generations. ETR (exact tandem repeat) is the first scheme which uses five loci (Frothingham and Meeker, 1998) nevertheless resolution of these loci are poorer than that of PFGE. MIRU (mycobacterial interspersed repetitive unit) is the second scheme that has good discriminating ability as that of PFGE (Mazars *et al.,* 2001; Hawkey *et al.,* 2003). MIRU2 is the third sheme which has nine more loci giving a total of 24 loci and it is the current standard for typing *M. tuberculosis* since it also provides greater resolution than PFGE (Supply *et al.,* 2006).

Mycobacterial chromosomes were found to have molecular weight of (2.5-5.5) $\times 10^{8}$, with these of the major pathogen *M. tuberculosis* at the lower end of range. Besides main chromosome, some strains have one or more plasmid (Grange, 1990). In 1998 genome of the H37Rv strain was published ("*Mycobacterium tuberculosis*", 2007) whose genome has 250 genes involved in fatty acid metabolism, with 39 of these involved in the polyketide metabolism used to generate the waxy coat. Evolutionary importance of the waxy coat is related to the survival of pathogen because of these large numbers of conserved genes. Two clustered gene families that encode acidic, glycine-rich proteins accounts for 10% of the coding capacity. Deletion of N-terminal motif conserved in these gene families impairs growth in macrophages and granulomas (Glickman and Jacobs, 2001). In *M. tuberculosis* nine noncoding sRNAs have been characterized (Arnvig and Young, 2009) and 56 more noncoding sRNAs are predicted in a screen of bioinformatics (Livny *et al.,* 2006).

2.1.2 Pathogenesis

Cell wall structure of *Mycobacterium tuberculosis* has special importance as it is unique among prokaryotes and is major causal factor responsible for virulence of bacterium. The cell wall complex contains peptidoglycan but

majorly composed of complex lipids that constitutes over 60% of the mycobacterial cell wall is lipid. Mycolic acids, cord factor and wax-D are the three major components of the lipid fraction of MTB's cell wall. Properties like impermeability to stains and dyes, resistance to many antibiotics, resistance to killing by acidic and alkaline compounds, resistance to osmotic lysis via complement deposition and resistance to lethal oxidations and survival inside of macrophages are associated with the high concentration of lipids in the cell wall of *Mycobacterium tuberculosis* (Fox, 2010).

Virulence of the *Mycobacterium* genus and nature of host's immune response determines the clinical manifestation and consequent outcome of TB (Grange, 1998). Secreted mycobacterial products, heat shock proteins, lipoarabinomannans and hemolysin can induce production of inflammatory products or cytokines by host cell that help to progress tissue damage in host (Quinn, Newmann *et al.*, 1996). Besides survival within macrophage and exhibition of metabolic inactivity for long period, factors like cord factor, pthiocerol dimicocerosate are also associated with virulence of the bacteria.

Alveolar macrophages take up *M. tuberculosis* when in lungs but can't digest them since cell wall prevents the fusion of the phagosome with a lysosome. Early endosomal autoantigen 1 (EEA1) is blocked by the *M. tuberculosis* which is responsible to prevent this fusion but does not prevent fusion of vesicles filled with nutrients. Accordingly, the bacteria keep multiplying within the macrophage. Besides this, *UreC* gene carried by bacteria prevents acidification of the phagosome (Bell, 2005). Macrophage-killing is further prevented by neutralizing reactive nitrogen intermediates (Joanne and John, 2003).

2.2 Tuberculosis and its epidemiology

Tuberculosis is caused by various strains of mycobacteria usually *Mycobacterium tuberculosis* (Kumar *et al.,* 2007) and it's a common infectious disease in many instances being fatal. In individuals aged fifteen to

forty nine years, tuberculosis is the single most common cause of death (Enarson *et al*., 1996). TB may be of pulmonary, extrapulmonary and disseminated type depending upon the site of involvement of infection and host response. Tuberculosis transmission occurs via droplet and droplet nuclei that contain viable virulent organism, which generates during coughing, sneezing and vocalizing of sputum positive patients with pulmonary tuberculosis. Sneezing, coughing or transmission of saliva of actively TB infected people through the air help to spread the disease (Konstantinos A, 2010). About 3000 to 5000 droplet nuclei are produced during a single cough and patient excreting 10000 or more tubercle bacilli per ml of sputum are infectious (Park, 2005). Even though most infectious patients are identified by their AFB smear positivity but transmission occuring from smear negative patients is possible in theoretical consideration and empirical observations (Groothuis and Yates, 1991).

Most infections are asymptomatic and latent but 10% of these latent infections eventually progresses to active disease which has fatality rate of 50% if not treated. General symptoms of active TB infection are a chronic cough with blood-mixed sputum, fever, night sweats, and weight loss . Symptoms are varied when other organs are infected (Van *et al.,* 1997). Mostly lungs are infected by the disease due to dissemination via lympho, hematogenous route even though there is always a chance of every organ to be infected (Haas, 2000).Hematogenous spread of the organism from primary focus results in the development of extrapulmonary lesion (Chakraborty, 2003).

New infections occurring at a rate of about one per second, one third of the world's population are infected with *M. tuberculosis.* There were 13.7 million chronic active cases in world in 2007 (WHO, 2009b) and there were 8.8 million new cases and 1.5 million (including 0.35 million people with HIV) associated deaths in 2010. Most cases were reported from developing countries. In the same year, 5.7 million new and recurrent TB cases were treated. The absolute number of new cases have declined since 2002 and tuberculosis cases has been falling since 2006 (WHO, 2011). Distribution of tuberculosis is uneven in world as only 5–10% of the United States population

tests positive in comparison to 80% of the population in many Asian and African countries (Kumar *et al.,* 2007). High rates of HIV infection and the corresponding development of AIDS giving rise to compromised immunity of these people in developing countries led to transmit tuberculosis higher in these nations (Lawn and Zumla, 2011).

Figure 1: Estimated TB incidence rates (WHO, 2010).

2.2.1 Tuberculosis in Nepal

Tuberculosis (TB) is a major public health problem in Nepal. Introduction of treatment by directly observed treatment short course (DOTS) have already reduced the number of deaths however 5000-7000 people continue to die every year from this disease (NTC, 2004/2005). Incidence of all forms of TB is 173 cases per 1, 00,000 population in Nepal (NTC, 2009). About 45% of the total population is infected with TB, of which 60% are adult. Every year, 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. These 20,000 are able to spread the disease to others (Ministry of Health and Population, 2011a).

2.3 Tuberculosis diagnosis

Culturing of *Mycobacterium tuberculosis* organisms from a specimen taken from the patient that may include sputum or pus, CSF, tissue biopsies etc. can only help to diagnose tuberculosis definitively. For patient producing sputum, smears and cultures should be done for acid-fast bacilli (Kumar *et al.,* 2007). Fluorescence microscopy (truant staining) has more sensitivity than conventional Ziehl-Neelsen staining and is the preferred method for sputum smear preparation (Steingart *et al.,* 2006).

Traditionally, cultures have used the Lowenstein-Jensen (LJ), Middlebrook media (7H9, 7H10, and 7H11) which are egg based media and now many types of cultures are available for culture growth detection.

2.4 Treatment of tuberculosis and mode of action of antitubercular drugs

TB disease can be treated by taking several drugs for 6 to 9 months. There are 10 drugs currently approved by the U.S. Food and Drug Administration (FDA) for treating TB. Of the approved drugs, the first-line anti-TB agents that form the core of treatment regimens include: isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) (CDC, 2003). With an initial two month regimen comprising multiple antibiotics- isoniazid, rifampicin pyrizinamide and ethambutol (HRZE) that insure to prevent development of mutants resistant to any single drug following another four months with administration of isoniazid and rifampicin that eliminate any persisting tubercle bacilli are used to treat tuberculosis (NTP, 2009).

Antituberculosis chemotherapy is primarily given to rapidly kill tubercle bacilli, prevent drug resistance and relapse (Mitchison, 1985; Maher *et al*., 1997). Multiple antituberculosis drugs must be taken for a sufficiently long time (combination chemotherapy) to fulfill these goals. For all forms of drug susceptible tuberculosis isoniazid (INH) and rifampicin (RIF) are given as

first-line agent for treatment. Early bactericidal activity against rapidly dividing cells is achieved through use of isonoiazid (Jindani *et al.,* 1980; Hafner *et al.,* 1997). Both semidormant bacterial cells and rapidly dividing cells are killed by rifampicin giving sterilizing activity (Dickinson et.al., 1981). All short-course regimens constitute rifampicin as an essential component.

2.4.1 Mode of action of antitubercular drugs - isoniazid and rifampicin

For the action of prodrug isoniazid it need to be activated by *KatG* which is a catalase-peroxidase enzyme of *M. tuberculosis* (Suarez *et al.,* 2009). Isonicotinic acyl-NADH complex is formed when *KatG* couples the isonicotinic acyl with NADH and this complex tightly binds to *InhA*, enoyl-acyl carrier protein reductase that blocks the natural enoyl-AcpM substrate and the action of fatty acid synthase. Synthesis of mycolic acid, required for the mycobacterial cell wall is inhibited by this action. A range of radicals including nitric oxide are produced by *KatG* activation of isoniazid (Timmins *et al.,* 2004) which is important for the action of another prodrug PA-824 which is also antimycobacterial (Singh *et al.,* 2008). Resistance to isoniazid is also caused by *kas*A encoding -ketoacyl ACP synthase and *ahp*C encoding alkyl-hydroperoxide reductase (Marahatta *et al*., 2011).

Isoniazid is bacteriostatic for the mycobacteria that are slow-growing but it is bactericidal to rapidly dividing mycobacteria. P450 system is inhibited by isoniazid.

DNA-dependent RNA synthesis is inhibited by rifampicin through the inhibition of bacterial DNA-dependent RNA polymerase. Rifampicin binds to RNA polymerase near the active center of RNA polymerase that blocks RNA synthesis by physically preventing extension of RNA products. Mutations that change residues of the rifampicin binding site on RNA polymerase give rise to rifampicin resistance decreasing affinity for rifampicin at this site. rpo*B* gene

is being mapped for resistant mutations which encode RNA polymerase beta subunit (Feklistov, 2008).

Rifampicin inhibits DNA-dependent RNA polymerase activity in susceptible cells. Bacterial RNA polymerase has ability to interact with rifampicin but not the the mammalian enzyme keeping them to work. Rifampicin has bactericidal activity against both intracellular and extracellular *Mycobacterium tuberculosis* organisms at therapeutic levels. Slow and intermittently growing *M. tuberculosis* organisms are killed by rifampicin. Only rifamycins have shown cross resistance to rifampicin (Hoechst, 2000).

2.5 Drug resistance

2.5.1 Development of antituberculosis drug resistace

Improper use of drugs in chemotherapy of drug-susceptible TB patients gives rise to drug resistance. Mutations in the chromosomal sequence lead to develop drug resistance in *Mycobacterium tuberculosis* bacteria which encode changes by blocking the activity of a drug (e.g.mutations in *rpoB* prevent binding of rifampicin to RNA polymerase inhibiting transcription process), blocking the activation of a prodrug (e.g., mutations in *katG* lead function of catalase to be unabled to activate the prodrug isoniazid to its active form) or producing an action to destroy or bind the drug (e.g., increased amount of *InhA* protein due to mutation in *inhA* binds most of the isoniazid decreasing level of required inhibitory concentration in the bacterium) (Zhang and Telenti, 2000; Johnson *et al.,* 2006).

Random mutation rate of 3 x 10-7 to 1 x 10-9 per organism per generation is natural for first-line anti-tuberculosis drugs in *M. tuberculosis* that gives drug resistance (Gillespie, 2002). Therefore, a small proportion of naturally occurring drug resistant mutants in the *M. tuberculosis* population present inside a any human host is a normal phenomenon but this number rapidly rises when inaccurate or incomplete chemotherapy is administered in an individual.

Telenti *et al*. (1993) reported molecular mechanism of rifampicin resistance in *M. tuberculosis*. Consequently, various genes located in different region of chromosome were identified to be responsible for isoniazid resistance using DNA sequencing. Several factors can give isoniazid resistance that includes pro-drug activation by *KatG* (encoded by the *katG* gene), increased expression of the target *InhA* or prodrug binding to its *InhA* target (Brossier *et al.,* 2006; Miotto *et al.,* 2008).

Of all isoniazid resistant strains 10-15% of strains were found to have mutations in ahpCoxyR intergenic region (including mutation in other regions of *katG* gene), while 20-35% were reported having mutations in the *inhA* regulatory region and 50-90% have had mutations in codon 315 of the *katG* gene (Gillespie, 2002; Drobniewski and Wilson, 1998; Brossier *et al.,* 2006; Miotto *et al.,* 2008). High-level isoniazid resistance is associated with inactivity of catalase. Single point mutations involving a serine-to-threonine amino acid substitution at codon 315 are the most common mutations of *katG* gene however, other mutation may occur in least rate in *katG*. Low-level isoniazid resistance is most commonly related with mutation of ahpC-oxyR and *inhA* gene (Miotto *et al.,* 2008). Mutations in 81-base pair region (codons 507 - 533) of the *rpoB* gene has been linked with rifampicin resistance in more than 95% of rifampicin resistant strains (Hirano *et al.,* 1999; Cavusoglu *et al.,* 2002; Bartfai *et al.,* 2001). Automated DNA sequencing have revealed more than 50 mutations within region of rpo*B* gene with codons 516, 526, or 531 being the most common region with point mutations (Gillespie, 2002; Drobniewski and Wilson, 1998). Moreover, less common and few silent mutation also occur less commonly but they don't seem to give rise to rifampicin resistance. Mutations in other regions of the *rpoB* gene are very rare.

When a patient is infected with a resistant strain it is known as primary resistance while infection caused during treatment due to selected drug resistant mutants is known as acquired resistance in tuberculosis. Both of these resistances occur due to mutations in the genome of *M. tuberculosis* that can also occur spontaneously with approximated mutation rate of 3.5×10^{-6} for

INH and 3.1×10^{-8} for RIF. However there is chance of double spontaneous mutation at a frequency of 9×10^{-14} for both INH and RIF but this rarely occurs as chromosomal loci that give these resistances are not linked (Dooley and Simone, 1994).

2.5.2 Multidrugresistant TB

Bacteria that do not respond to, at least, isoniazid and rifampicin, the two most powerful, first-line (or standard) anti-TB drugs give rise to a form of TB which is termed as multidrug-resistant tuberculosis (MDR-TB). The main cause of MDR-TB is inappropriate treatment. Frequency of mutation in *kat*G gene that accounts for isoniazid resistance is 100 times greater than in *rpo*B gene that accounts for rifampicin resistance (de Siqueira *et al*., 2009) and considered as proxy for MDR-MTB (Jindani *et al*., 1980; Tracevska *et al*., 2002; Brimacombe *et al*., 2007; Hu *et al*., 2010) producing chances for other genes to acquire mutation. Some INH resistant strains, specifically *kat*G Ser315Thr mutated isolates, develop MDR-TB due to combination of sustained virulence and INH resistance developed in them (Hu *et al*., 2010). Multidrug resistant tuberculosis is not due to emergence of novel resistance mechanism (Rattan, 1998) rather because of man made amplification of natural phenomenon (Pfyffer, 2000; NTP, 2009).

Second-line drugs can be used to treat and cure MDR-TB (Iseman, 1993). However, second-line treatment options are limited and recommended medicines are not always available. In some cases more severe drug resistance called extensively drug-resistant TB (XDR-TB) can develop which is a form of multi-drug resistant tuberculosis that responds hardly a few medicines that are available (WHO, 2012). XDR TB is more importantly a big problem for persons who are immunocopmpromised like with HIV patients (Gandhi, 2006).

Figure 2: Proportion of MDR TB among new TB cases (WHO, 2009b).

2.5.3 Drug resistance tuberculosis in Nepal

Four drug resistance surveys have been carried out in Nepal since 2005. MDR-TB rates of 2.9% (1.8%-3.2%) among new cases and 11.7% (7.1%-18.3%) among re-treatment cases were reported at the end of the fourth survey (WHO, 2009a).

In one of the survey reported by WHO from 2007 to 2010, notified TB cases rises by two thousand from 2007 to 2009 and the number keep almost constant at 35000 cases from 2009 to 2010 while notified MDR TB decreases gradually from 163 in 2007 to 69 in 2009 but abruptly reaching 220 in 2010 in Nepal. Treatment success rate of MDR in the country dropped from 73% to 64% and mortality rate of MDR TB decline from 9% to 7% from the year 2007 to 2008 (WHO, 2010c).

2.6 Detection of drug resistant TB

2.6.1 Drug susceptibility testing

For appropriate management of patient susceptibility testing of *M. tuberculosis* is of utmost importance from initial isolate of these patients (ATS, 2000). Susceptibility testing also differentiates primary resistant strains from secondary resistant strains and hence helps to decide the treatment strategy accordingly. Patient with positive culture result after 3 months of therapy or those developing positive cultures after a period of negative cultures should be repeated for susceptibility testing (Woods, 2000). Standard methodology, such as that recommended by the National Committee for Clinical Laboratory Standards should be adopted for antimicrobial susceptibility testing (NCCLS, 2000).

Drug susceptibility testing is carried out on sub-cultured bacteria after the initial positive culture is obtained for diagnosis. It usually takes 3–6 weeks to obtain the initial positive culture with an additional 3 weeks for susceptibility testing (reduced to about 15 days when using the BACTEC system) (Siddiqi *et al*., 1985; Snider, Jr. *et al*., 1981; Tarrand and Groschel, 1985; Rastogi *et al*., 1989). Thus, susceptibility testing is time consuming and pric, and there are numerous problems associated with the standardization of tests and the stability of the drugs in different culture media (Martin-Casabona *et al*., 1997; Victor *et al*., 1997). The slow diagnosis of drug resistance may be a major contributor to the transmission of MDRTB (Victor *et al*., 2002). The WHO recommended that drug susceptibility testing is done by the proportion method on Löwenstein-Jensen medium, but other media, such as Middlebrook 7H10, 7H11, 7H12 (BACTEC460TB) and other methods, including the absolute concentration and resistance ratio methods, may also be used (WHO, 2001).

2.6.2 Molecular detection technique

Acid-fast bacilli (AFB) smear microscopy which gives results in 24 hours and culture that gives result in 2-6 weeks are used conventionally for laboratory confirmation of TB (CDC, 2005; ATS, 2000). Even though AFB smear microscopy is quick and cost effective the technique is limited by its poor positive predictive value (50%-80%) and its poor sensitivity (45%-80% with

culture-confirmed pulmonary TB cases) for tuberculosis in circumstances where nontuberculous mycobacteria are commonly isolated (CDC, 2000; ATS, 2000; Guerra *et al.,* 2007). Nucleic Acid Amplification (NAA) tests are the best choice for reliable and fast result that can provide results within 24-48 hours.

While resistance to INH is mainly associated with mutations in the *katG*, *inhA*, and *ahpC* genes (Hillemann *et al.,* 2005), RIF resistant strains are predominantly linked to mutations in the *rpoB* gene (Hillemann *et al.,* 2007; Vattanaviboon *et al.,* 1995). MDR-TB is detected targeting genes *rpoB* (Rifampicin) and *inhA* and *katG* (Isoniazid) of first-line drugs and XDR-TB is detected targeting genes *gyrA* (Fluoroquinolones), *rrs* (Kananmycin, Amikacin, Capreomycin), *eis* (Kanamycin), *tlyA* (Capreomycin), *pncA* (Pyrazinamide) and *embB* (Ethambutol) of second line drugs (Driscoll, 2010).

Detection of drug resistance in clinical specimens within a day have been demonstarated using rapid molecular assays like line probe assays and real-time PCR (Cavusoglu *et al.,* 2002; Hillemann *et al.,* 2005; Cirillo *et al.,* 2004; Makinen *et al.,* 2006; Mani *et al.,* 2001). Easier referral of specimens to central laboratories and large-scale drug-resistance surveillance studies can be accomplished because of simplified specimen processing needed for molecular testing (Yagui, 2006).

2.6.3 Line probe assays for tuberculosis drug resistance

Line probe assay technology involves the following steps: First, DNA is extracted from *M. tuberculosis* isolates or directly from clinical specimens. Next, polymerase chain reaction (PCR) amplification of the resistance-determining region of the gene under question is performed using biotinylated primers. Following amplification, labelled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip by colorimetric development, enabling detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. If a

mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations. The post-hybridization reaction leads to the development of colored bands on the strip at the site of probe binding and is observed by eye (Brossier *et al.*, 2006).

Genotype MTBDRplus assay is based on multiplex PCR, which is a variant of PCR enables simultaneous amplification of many targets of interest in a single reaction employing more than one pair of primers. The GenoType MTBDR assay identifies *M. tuberculosis* complex and consequently detects mutations in the *rpoB* gene and mutations in the *katG* gene for high-level isoniazid resistance and this assay was introduced in 2004.

Figure 3: Detection of mutations through missing of wild type signals and detection of mutations through presence of mutation signals. *rpoB* wild type probes: WT 1 to WT 8, *rpoB* mutation probes: MUT D516V, H526Y, H526D and S531L.

Genotype MTBDRplus is the second-generation assay that replaces universal control and includes an amplification control along with three *rpoB* probes to increase the sensitivity for detecting mutations that confer RIF-resistance and *inhA* probe to detect mutations in the *inhA* gene which confers low-level isoniazid resistance. Four specific *rpoB* mutations D516V, H526Y, H526D and S531L are detected for rifampicin resistance, two *katG* mutations S315T1 and S315T2 for high level isoniazid resistance and four *inhA* mutations C15T, A16G, T8C and T8A for low level isoniazid resistance are detected by the method. Both direct use on smear-positive pulmonary specimens and on isolates of *M. tuberculosis* grown on liquid medium or in solid medium has

been validated for use by this method however specimen processing by NaOH-NALC is necessary for both ways (WHO, 2008b).

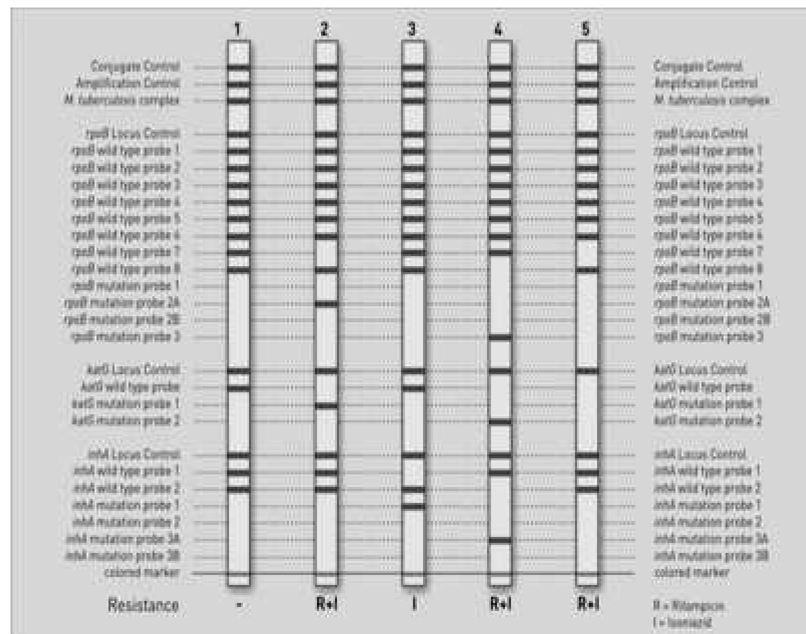


Figure 4: The Genotype MTBDRplus kit showing various mutation and wild type probes and respective gene band appearance depending on the drug resistance pattern given by *M. tuberculosis* complex strains.

2.7 Treatment of MDR-TB

Even though there is no intermittent regimen validated for use in MDR-TB, extended use of second-line anti-TB drugs that are less effective and less tolerated but more toxic and costly than first-line anti-TB drugs is required for the treatment of MDR TB (WHO, 2006). Irrespective of *in vitro* tests showing susceptibility, previous therapy with a drug for more than a month has been reported to have diminished efficacy (Goble *et al*., 1993) which necessitates to trace careful treatment history of a tuberculosis patient. Directly observed therapy should be considered an integral part of the treatment of MDR-TB that helps to improve outcomes in MDR-TB (Leimane *et al*., 2005).

2.8 Tuberculosis prevention

Tuberculosis is curable and preventable. Prompt detection and treatment of people who have suspected or confirmed TB disease, adopting airborne precautions, reduce exposures when a person is known to be infectious, avoid enclosed environments can prevent tuberculosis. Prevention also relies on screening programs and vaccination with the Bacillus Calmette–Guerin vaccine.

CHAPTER-III

MATERIALS AND METHODS

This study was conducted from September 2011 to March 2012 using descriptive cross sectional study design. Along with Tribhuvan University Teaching Hospital, Norvic Hospital, Bir Hospital and GENETUP; clinical samples of tuberculosis patients were collected from nine of the MDR treatment centres of Nepal as recognized by National Tuberculosis Centre (NTC) that includes National Tuberculosis Centre, Thimi; Lalgadh Hospital; NMCTH, Birgunj; NATA, Morang; BPKIHS Dharan; RTC, Pokhara; Bhim Hospital, Bhairawa; Butwal Hospital; INF, Nepalgunj and Seti Zonal Hospital, Kailali.

Molecular testing of this study was carried out in GENETUP, Kathmandu where samples used for the assay were randomly choosen. Samples included in the study were sputum samples (56), bronchoscopic aspirates (1), bronchoalveolar lavage (1), deep tracheal aspirate (1), lymph aspirate (1), gastric juice (1) and urine sample (1). CAT I failure patients (Patients who are not sensitive to standard CAT I drug regimen) were also included in this study. Sample size was obtained on the basis of 2.9% MDR prevalence within 95% confidence interval using $n = Z^2 P(1 - P)/d^2$ where n, Z, P and d are sample size,Z statistic for a level of confidence, expected prevalence or proportion and precision respectively (Daniel, 1999).

3.1 Sample collection

Clinical samples were collected depending upon the tuberculosis patient's disease status and its relevance to collect specimen by medical lab technician. No ethical approval and informed consent were taken as the samples were send to referral centre for treatment process. Specimens requiring decontamination includes sputum, bronchial wash, skin soft tissue, gastric lavage and urine. These samples were studied for AFB microscopy in the same centre and those which were identified as positive by the assigned

treatment centre were send to GENETUP which is providing both culture and molecular DST facility. Those AFB positive samples were only included in the study while other were excluded. Specimens were transported to the GENETUP laboratory in refrigerated ice box.

3.2 Specimen decontamination

Clinical specimens obtained in GENETUP were further processed in the same laboratory using conventional *N*-acetyl-L-cysteine–NaOH method for digestion decontamination purpose. In a biological safety cabinet 2, using a sterile, aerosol-free 50 ml centrifuge tube with screw cap equal amounts of specimen and activated NALC-NaOH solution (approximately 10 ml of each) was added. Centrifuge tube was capped and mixed on a Vortex-type mixer until specimen is liquefied. For specimens which are especially viscous more NALC-NaOH solution was added and mixing was repeated. Mixture was allowed to stand at room temperature for 15 min with occasional gentle shaking. Over-treating the specimen was avoided. Prepared phosphate buffer was added to the 50 ml mark on the centrifuge tube and mixed. It was centrifuged for 15 - 20 min at 3000 x *g*. and all of the supernatant fluid was carefully decanted. 1ml of phosphate buffer of pH 6.8 was added and sediment was resuspended. This suspension was used for the preparation of smears and culture while the left over sediment was stored at -20°C.

3.3 Truant's staining method

Smears were prepared using above suspension by the auramine-rhodamine staining method for the reassurance of AFB samples. For this purpose, heat fixed slides were flooded with auramine rhodamine stain and allowed to stain for 20 minutes. It was then followed by flooding with 0.5% acid alcohol allowing it to decolorize for 5 minutes and was rinsed off with water. Finally, it was flooded with potassium permanganate and left for 1 minute which was washed off with water and left to dry. It was then examined with 25X objective using a fluorescence microscope.

3.4 Culture

Each slope of Lowenstein-Jensen medium was inoculated with 0.2-0.4 ml of the above prepared decontaminated suspension using pipette and it was distributed over the surface of slope. Two slopes of LJ medium were inoculated per specimen. Inoculated media were incubated in a slanted position for at least 24 hours to ensure even distribution of inoculum. Tops of the tubes of medium were tightened to minimise evaporation and drying of media. All inoculated cultures were incubated at 35°-37°C until growth is observed or discarded as negative after eight weeks. All cultures were examined 72 hours after inoculation to check if liquid had completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants. Thereafter cultures are examined weekly and at least be incubated for three to four weeks to detect positive cultures of *M. tuberculosis.*

These cultured samples were then used for molecular study after growth is observed. For the identification of *M. tuberculosis* complex and its resistance to isoniazid and/or rifampicin, these cultured specimens were further processed by molecular assay- Genotype MTBDRplus. All the genetic analysis in this study were performed from cultivated sample only and not from clinical specimen.

3.5 DNA extraction

All steps were performed in processing laboratory. The grown colonies were harvested with a sterile sharpened applicator stick by gently removing the cells from the surface of the medium without any cell crop in the medium and these colonies were suspended in approximately 300 µl of water (molecular biology grade). It was then incubated for 20 mins at 95°C in a water bath and further 15 minutes in an ultrasonic bath. It was then spinned down for 5 minutes at full speed of 12000 rpm and 5 µl of the supernatant was directly used for PCR. All these steps were performed in processing laboratory which is biosafety level 2 (BSL2) laboratory.

3.6 PCR of DNA

A volume of 35 µl of polynucleotide mix (PNM), 5 µl 10 x polymerase incubation buffer, 2 µl 2.5mM $MgCl_2$ solution, 1-2 unit thermostable DNA polymerase and remaining volume (in µl) molecular biology grade water added to obtain a 45 µl of amplification mix was made in pre PCR/amplification room which is a DNA free room. Exactly 5 µl of DNA solution/sample was added to this amplification mix in separate area (inside BSL-2 cabinet) to make a final volume of 50 µl (not considering volume of enzyme). These sample mix along with a negative control mix which contains the same amplification mix except it has 5 µl of water instead of DNA solution were then kept in thermocycler and PCR was run. Amplification product not immediately processed were stored at +4 to –20°C.

Table 1: Amplification profile used during PCR for culture samples

Time and temperature	Number of cycles
15 min 95°C	1
30 sec 95°C ; 2 min 58°C	10
25 sec 95°C ; 40 sec 5 ^{o}C ; 40 sec 70°C	20
8 min 70°C	1

3.7 Hybridization

All hybridization steps were performed in post-amplification room. hybridization buffer (HYB) and stringent solution (STR) was prewarmed to 45°C in water bath for 15 minutes. Twincubator was also prewarmed to 45°C. Twenty microliter of DEN (denaturing solution) was pipetted to each well of tray to be used. For each repetitive addition step, same pipette tip was used unless wells/samples not touched. Twenty microliter of corresponding amplified DNA sample was added to each well and mixed well by pipetting up and down several times and incubated for 5 minutes at room temperature. DNA strips were removed from tube (shaking strips down to end of tube then removing carefully holding the end of the strip with forceps) and they were

marked with provided pencil. One milliliter of HYB was added to each well and gently shaked to homogenize solution. Single strip was added to each well with colored marker facing up. If strips were turned over, they were re-positioned with a fresh pipette tip. Tray was placed on twincubator incubated for 30 minutes at 45°C. HYB was completely poured off into the sink, holding it low and close to the drain to avoid amplicon transmission. Remaining solution was removed by forcefully tapping tray against paper towels on benchtop, membranes doesn't fall out doing so.

Condensation that forms on lid was wiped off before every incubation step. One milliliter of STR (stringent buffer) was added per well and incubated for 15 minutes in twincubator at 45°C. Diluted conjugate and substrate was prepared from respective conjugate concentrate (CON-C) and substrate concentrate (SUB-C) in 15 ml conical vials by diluting 1:100 with corresponding concentrate buffer (Con-D) and substrate buffer (Sub-D). Colors of concentrates corresponded to colors of dilution buffer tubes. Substrate dilution was wrapped in aluminum foil. Fresh conjugate and substrate dilutions was prepared every day, but old conical tubes were re-used after washing thoroughly in distilled water. STR was removed completely as previously described for HYB removal. One milliliter of RIN (rinse solution) was added per well and incubated for 1 minute on twincubator. RIN was removed and 1 ml of diluted conjugate added per well and incubated for 30 minutes on twincubator. Solution was removed and washed for 1 minute with 1 ml RIN per well on twincubator. Solution was poured out and rinse was repeated with 1 ml RIN per well for 1 min. RIN was removed and washed with 1 ml distilled water per well on twincubator. Water was removed and 1 ml of diluted substrate added per well and incubated on twincubator under aluminum foil to protect from light without shaking for a maximum of 10 minutes. Color reaction was noted to indicate reaction completion after 4-5 minutes. Color reaction if found to be too weak, foils were replaced and re-incubated for several more minutes, up to a maximum of 10 minutes. It was then washed twice for 1 minute each with distilled water. Strips were then removed from the tray using tweezers and were dried between two layers of absorbent layer. All these steps were performed in post-amplification room.

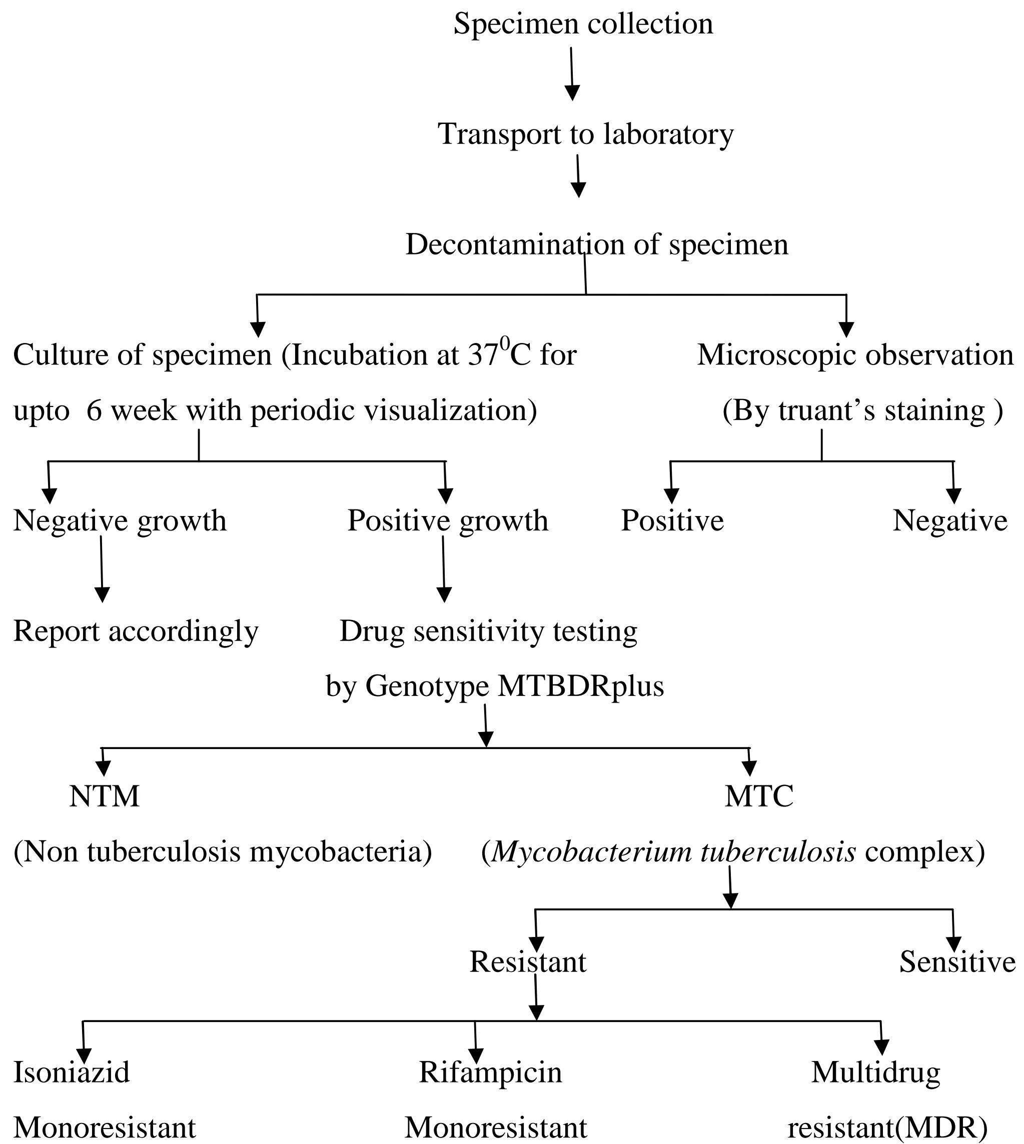
3.8 Interpretation of results

Strips were transferred to the Genotype MTBDRRplus results sheet provided with the kit using forceps for interpretation. Results were read by matching lining strips to code provided with kit. In order for results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that *M. tuberculosis* complex is present in the sample. A mutation in the relevant gene (and resistance to the relevant drug) is signified by either an absent wild type band and/or the presence of a mutant band for each gene cluster. The *rpoB, katG* and *inhA* each have a control band which must be present in order to interpret the results where *rpoB* predicts RIF resistance, *katG* predicts high level INH resistance, *inhA* predicts low level INH resistance. For results to be valid the bands must be of intensity equal to or greater than the intensity of the AC band. In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible. Weak or no signal except for CC zone may appear if quality and/or quantity of extracted DNA do not allow an efficient amplification or incubation temperature is too high. Overall weak or no signal in any zone may appear which may be due to too low room temperature or reagents not equilibrated to room temperature as well as due to no or little amount of CON-C and/or SUB-C used. If a positive result appears in negative control, measures are taken to remove amplicon contamination from all rooms and equipment and process are repated for whole batch.

3.9 Data analysis

Data was entered into the SPSS and analyzed and various mutated gene percentages was calculated for drug resistant MTC.

Flow chart of the Procedures



CHAPTER–IV

RESULTS

A total of 62 samples were cultured in LJ medium for the identificaton of *Mycobacterium* species and of these all the samples were found to give a positive culture result.

Out of all culture positive isolates, nearly 88% of the samples were found to be *Mycobacterium tuberculosis* complex by Genotype MTBDRplus assay of which only slightly more than a quarter of them were drug sensitive while remaining samples were drug resistant. In contrast, only about 13% samples were non *Mycobacterium tuberculosis* isolates.

Table 2: Performance of Genotype MTBDRplus for identification of *Mycobacteria* type and drug susceptibility result among *Mycobacterium tuberculosis* complex isolates

Mycobacteria isolates	No. of isolates (%)
MTC(Total)	54 (87.1)
Drug resistant MTC	39 (72.2)
MDR	28 (71.7)
Rifampicin monoresistant	6 (15.3)
Isoniazid monoresistant	5 (12.8)
Drug sensitive MTC	15 (27.7)
NTM	8 (12.9)

Female with more than double the number of male patient were found to be infected with tuberculosis. Both drug susceptible as well as drug resistant tuberculosis was more in females. Drug susceptible tuberculosis was double in females than in males and drug resistant tuberculosis was almost three times in females than in males.

Table 3: Sexwise distribution of resistant tuberculosis

Tuberculosis type	Genotype MTBDRplus		
	Male (%)	Female (%)	Total (%)
Monoresistance			
Rifampicin	1 (6.6)	5 (12.8)	6 (11.1)
Isoniazid	0 (0)	5 (12.8)	5 (9.2)
MDR	9 (60)	19 (48.7)	28 (51.8)
Drug susceptible	5 (33.3)	10 (25.6)	15 (27.7)
Total(%)	15 (27.7)	39 (72.2)	54 (100)

Highest number of multidrugresistance isolates, rifampicin monoresistant isolates and drug susceptible isolates were all obtained from central region of the country while highest number of isoniazid monoresistant isolates were obtained from midwestern region of the nation.

Table 4: Regionwise distribution of resistant tuberculosis

Geographic Region	Genotype MTBDRplus				
	Susceptible n = 15 (%)	Rifampicin monoresis-tant n = 6 (%)	Isoniazid monoresist-ant n = 5 (%)	MDR n = 28 (%)	Total n = 54 (%)
Eastern	0 (0)	1 (16.6)	0 (0)	4 (14.2)	5 (9.2)
Western	1 (6.6)	0 (0)	0 (0)	6 (21.4)	7 (12.9)
Central	11 (73.3)	3 (50)	2 (40)	10 (35.7)	26 (48.1)
Mid western	3 (20)	1 (16.6)	3 60)	4 (14.2)	11 (20.3)
Far western	0 (0)	1 (16.6)	0 (0)	4 (14.2)	5 (9.2)
Total (%)	15 (27.7)	6 (11.1)	5 (9.2)	28 (51.8)	54 (100)

Tuberculosis was found to be highest in patients with age group 20 to 45 along with highest MDR rate in the same group. Majority of drug resistant tuberculosis was reported in age group 20 to 45.

Table 5: Age wise distribution of resistant tuberculosis

Age group	Genotype MTBDRplus				
	Susceptible (%)	Rifampicin monoresistant (%)	Isoniazid monoresistant (%)	MDR (%)	Total (%)
< 20	1 (6.6)	0 (0)	0 (0)	3 (10.7)	4 (7.0)
20-45	5 (33.3)	4 (66.6)	4 (80)	23 (82.1)	36 (66.6)
> 45	9 (60)	2 (33.3)	1 (20)	2 (7.1)	14 (25.9)
Total	15 (27.7)	6 (11.1)	5 (9.2)	28 (51.8)	54 (100)

Most CAT I failure patients have MDR tuberculosis while very least of them have isoniazid resistance. One fifth of these patient have drug susceptible tuberculosis and a half of this number have rifampicin monoresistant isolates.

Table 6: Genotype MTBDRplus test for detection of rifampicin and isoniazid susceptibility pattern among Cat I failure patients

Drug susceptibility testing using Genotype MTBDRplus	Number of isolates (%)
Rifampicin monoresistant	3 (11.5)
Isoniazid monoresistant	2 (7.4)
MDR-TB (Resistant to RIF & INH)	16 (61.5)
Susceptible (Susceptible to RIF & INH)	5 (19.2)

Among various gene mutations observed, mutations in the 530-533 regions was the most common mutation for detection of rifampicin resistance with 61.7% (21/34) as detected by the lack of binding to various wild type probes in the presence of D516V and S531L mutation. A significantly higher proportion of RIF-monoresistant strains (16.6%) had a D516V mutation (MUT1 band) compared with MDR strains (3.5%). Of all INH resistant strains, 96.9% (average of 96.5% of MDR strains and 80% of INH-monoresistant strains) had a mutation in the *katG* gene and 3.03% (1/33) (100% MDR strains) had a mutation in the *inhA* gene. None of the strains had mutations in both the *katG* and *inhA* genes. Only one strain had mutaion in *inhA* genes.

Table 7: Pattern of *rpoB* gene mutations in resistant *Mycobacterium tuberculosis* strains

Gene Band	Gene region of mutaton	RIF monore-sistant n = 6 (%)	MDR n = 28 (%)
rpoB MUT1	D516V	1 (16.6)	1 (3.5)
rpoB MUT2A	H526DY	0 (0)	0 (0)
rpoB MUT2B	H526D	0 (0)	0 (0)
rpoB MUT3	S531L	0 (0)	1 (3.5)
rpoB WT1		0 (0)	0 (0)
rpoB WT2		0 (0)	0 (0)
rpoB WT3		0 (0)	0 (0)
rpoB WT4		0 (0)	0 (0)
rpoB WT5		0 (0)	0 (0)
rpoB WT6		0 (0)	0 (0)
rpoB WT7		1 (16.6)	2 (7.1)
rpoB WT8		0 (0)	2 (7.1)
rpoB WT1, *rpoB* WT6, *rpoB* WT7, *rpoB* WT8		1 (16.6)	0 (0)
rpoB WT6, *rpoB* WT7 *rpoB* WT8		1 (16.6)	0 (0)
rpoB MUT1, *rpoB* WT3, *rpoB* WT4		0 (0)	5 (17.8)
rpoB WT3, *rpoB* WT4		0 (0)	1 (3.5)
rpoB MUT3, *rpoB* WT8		1 (16.6)	15 (53.5)
rpoB WT2, *rpoB* WT3		0 (0)	1 (3.5)
rpoB MUT1, *rpoB* WT8		1 (16.6)	0 (0)

Abbreviations: RIF = rifampicin; MDR = multidrug resistance; MUT= mutation probe; WT= failing wild type probe. D= aspartate; V=valine; H=Histidine; Y= tyrosine; S= Serine; L= leucine.Values are numbers, with percentages in parentheses.

Table 8: Pattern of *katG* gene mutations in resistant *Mycobacterium tuberculosis* strains

Gene Band	Gene region of mutaton	INH-monoresistant n = 5 (%)	MDR n = 28 (%)
katG MUT1	315 (S315T1)	0 (0)	0 (0)
katG MUT2	315 (S315T2)	0 (0)	0 (0)
katG WT	315	0 (0)	2 (7.1)
katG MUT1 , *katG* WT	315	5 (100)	24 (85.7)
katG MUT1 , *katG* MUT2, *katG* WT	315	0 (0)	1 (3.5)

Table 9: Pattern of *inhA* gene mutations in resistant *Mycobacterium tuberculosis* strains

Band Gene	Gene region of mutation	INH-monoresistant n = 5 (%)	MDR n = 28 (%)
*inhA*MUT1	-15 (C15T)	0 (0)	0 (0)
*inhA*MUT2A	-16 (A16G)	0 (0)	0 (0)
*inhA*MUT3A	-8 (T8C)	0 (0)	0 (0)
*inhA*MUT3B	-8 (T8A)	0 (0)	0 (0)
*inhA*WT1	-15 to -16	0 (0)	0 (0)
*inhA*WT2	-8	0 (0)	0 (0)
*inhA*MUT1, *inhA*WT1	-15 to -16	0 (0)	1 (3.5)

Abbreviations: INH = isoniazid; MDR = multidrug resistance; MUT= mutation probe; WT= failing wild type probe. T= Threonine; C= Cysteine; A= Alanine; G= Glycine. Values are numbers, with percentages in parentheses.

Among 34 rifampicin resistance strains only 6 strains were rifampicin monoresistant and among 33 isoniazid resistance strains only 5 strains were isoniazid monoresistant. Each of the six rifampicin monoresistant strains have unique mutation patterns and all were equally important for resistance development. Only two types of gene mutation pattern were observed in isoniazid monoresistant strains with disproportionate ratio. Majority of the isoniazid monoresistance strains were found to have mutation in *katG* gene with a very less number of isolates giving resistance due to mutation *inhA* gene. Ten types of gene mutation pattern were observed in multidrug resistant strains. Of those 28 multidrugresistance isolates, only one isolate have this resistance due to mutation in *rpoB* gene and *inhA* while all remaining isolates have developed multidrug resistance due to mutation in *rpoB* gene and *katG* gene.

Table 10: Pattern of gene mutations in rifampicin monoresistant *Mycobacterium tuberculosis* strains

Pattern of gene mutations in RIF-monoresistant strains (6 strains)	Percent (No.of mutation strains/ No. of resistant strains)
*rpoB*MUT1	16.6 (1/6)
rpoB WT7	16.6 (1/6)
rpoB WT1, *rpoB* WT6, *rpoB* WT7, *rpoB* WT8	16.6 (1/6)
rpoB WT6, *rpoB* WT7, *rpoB* WT8	16.6 (1/6)
rpoB MUT1, *rpoB* WT8	16.6 (1/6)
rpoB MUT3, *rpoB* WT8	16.6 (1/6)

Table 11: Pattern of gene mutations in isoniazid monoresistant *Mycobacterium tuberculosis* strains

Pattern of gene mutations in INH-monoresistant strains (5 strains)	Percent (No.of mutation strains/No.of resistant strains)
katG MUT1 , *katG* WT	80 (4/5)
*inhA*MUT1, *inhA*WT1	20 (1/5)

Table 12: Pattern of gene mutations in multidrugresistant *Mycobacterium tuberculosis* strains

Pattern of gene mutations in MDR-TB strains (28 strains)	Percent(No.of mutation strains/ No.of resistant strains)
rpoB MUT3,*rpoB* WT8, *katG* MUT1, *katG* WT	50 (14/28)
rpoB MUT3,*rpoB* WT8, *katG* WT	3.5 (1/28)
rpoB WT8, *katG* MUT1, *katG* WT	7.1 (2/28)
rpoB MUT3, *inhA* MUT1, *inhA* WT1	3.5 (1/28)
rpoB WT7, *katG* WT	3.5 (1/28)
rpoB WT2, *rpoB* WT3, *katG* MUT1, *katG* WT	3.5 (1/28)
rpoB WT3, *rpoB* WT4, *katG* MUT1, *katG* WT	3.5 (1/28)
rpoB MUT1, *rpoB* WT3, *rpoB* WT4, *katG* MUT1, *katG* WT	17.8 (5/28)
rpoB MUT1,*katG* MUT1, *katG* MUT2, *katG* WT	3.5 (1/28)
rpoB WT7, *katG* MUT1, *katG* WT	3.5 (1/28)

Table 13: Pattern of gene mutations in all rifampicin resistant *Mycobacterium tuberculosis* strains

Pattern of gene mutations in all RIF-resistant strains (34 strains)	Percent (No.of mutation strains/ No.of resistant strains)
rpoB WT1	2.9 (1/34)
rpoB WT2	2.9 (1/34)
rpoB WT3	20.5 (7/34)
rpoB WT4	17.6 (6/34)
rpoB WT6	5.8 (2/34)
rpoB WT7	14.7 (5/34)
rpoB WT8	61.7 (21/34)
rpoB MUT1	23.5 (8/34)
rpoB MUT3	50 (17/34)

Table 14: Pattern of gene mutations in all isoniazid resistant *Mycobacterium tuberculosis* strains

Pattern of gene mutations in all INH-resistant strains (33 strains)	Percent (No.of mutation strains/No.of resistant strains)
*katG*MUT1	90.9 (30/33)
*katG*MUT2	3.0 (1/33)
*katG*WT	96.9 (32/33)
*inhA*MUT1	3.0 (1/33)
*inhA*WT1	3.0 (1/33)

CHAPTER-V
DISCUSSION

Among those 62 culture positive samples, 88% of the sample were verified as MTBC by Genotype MTBDRplus molecular assay with majority comprising drug resistant isolates and remaining isolates were atypical mycobacteria since the latter showed no TUB (probe for *Mycobacterium tuberculosis* complex) band on the strip and hence cannot be evaluated further by this test system. Incidence of multiple drug resistance in new TB cases has remained stable at a level of 1.3% but it has not declined in Nepal (Ministry of Health and Population, 2011b). This shows interrupted treatment therapy and low level or no drug administration by the patient is still common in Nepal. This is because patients may feel better and halt their antibiotic course, they forget to take their medication from time to time, drug supplies may run out or or become scarce, or patient is medicated without any drug sensitivity testing or it is done late. Lack of rapid identification of drug resistant isolates has also heoped to transmit drug-resistant TB from one person to other in the community giving rise to MDR.

TB (both drug susceptible and drug resistant) affected female were more than double the number of male patients in this study. Moreover, drug resistance tuberculosis was found almost thrice in female than in male. Even though male/female ratio among newly detected cases is 2:1 in South East Asia (WHO, 2009c) and male tuberculosis patient are higher in Nepal as found in many past studies done in Nepal (Acharya, 2008; Koirala, 2008; Marahatta *et al*., 2011; Sharma, 2010) this might not be true at present time. This might be because of active participation of female population during diagnosis time, more approach to health facilities, improved socioeconomic conditions and declining issues relating health stigma and gender discrimination.

According to the age group, patients with their age between 20 and 45 were most affected with tuberculosis which indicates most of the adult population and the patients in their productive age group in Nepal were the major victim

of the disease. According to WHO report, most cases of tuberculosis occur in the age group of 15-54 years (WHO, 2009c). Age for majority of tuberculosis patients in Nepal were reported between 15 and 49 (Acharya, 2008; Pande 2002; Koirala, 2008; Sharma, 2010) which also coincides with our study. This is because people of this age group are more likely to be exposed to infected people and surrounding as they are the active members of the society. Secondly, TB is common among AIDS patients and people aged 25 to 44 are most affected by AIDS and hence the the largest increase in TB cases has occurred among people of this age group.

This study had covered samples from all five development regions of Nepal and majority of the patients with tuberculosis were from central region of Nepal which accounts for almost half of the total study population. Highest number of multidrugresistance isolates, rifampicin monoresistant isolates and drug susceptible isolates were also reported from this same region in this study except for isoniazid resistance from mid western region. This may be due to higher sample size from this region and also because GENETUP also runs its own diagnostic centre for tuberculosis patients who visit there besides being referral centre for drug sensitivity and culture tests.

Of those all CAT I failure samples only 86.6% were confirmed as Mycobacterum tuberculosis complex by Genotype MTBDRplus and all of these were found to be drug resistant isolates except for 20% of the isolates. Similar finding was obtained in Bangladesh in a study done by Rahman *et al.,* where he finds 13% cases were MDR-TB in category I (CAT – I) failure while there was no MDR TB in new cases (Rahman *et al.,* 2009). Drug resistant isolates is more common in CAT I failure patients because these isolates are already challenged by drugs and have evolved alternative pathway or altered target site as to evade drug effect. Drug resistance is most commonly seen in patient with recurrent TB who have previously received antitubercular drugs (Inderlied *et al*., 2003). This shows great agreement with our study as all CAT I failure patients have received antitubercular drugs in the past. Non compliance of patient is the main reason for failure of CAT I treatment and development of drug resistances in them which may be due to

irregular intake of anti-TB drugs, incorrect drug administration and absence of periodic DST to look drug efficacy during treatment period.

Various nucleic acid position analyzed by this method was from 505 to 533 for *rpoB* gene,315 for *katG* and from -8 to -16 for *inhA* gene. Mutation in *rpoB*, *katG* and *inhA* was detected in this assay either by observing band development in various mutation probe or absence of these bands in various wild type gene probes (such probes are called as failing wild type probes or WT) or combinely due to these both kind of probe. Different pattern of gene mutations in resistant *Mycobacterium tuberculosis* strain were noted. Among total of 4 different mutation gene probe of *rpoB* gene (MUT1, MUT2A, MUT 2B and MUT3) , 2 different mutation gene probe of *katG* gene (MUT1 and MUT2) and 4 diffferent mutation gene probe of *inhA* gene (MUT1, MUT2A, MUT3A and MUT3B) targeted only two mutation probe of *rpoB* gene i.e. *rpoB* MUT1 and *rpoB* MUT3 gives mutation independently. However, except for failing wild type probe WT5 from *rpoB* gene and failing wild type probe WT2 and mutation probes MUT2A, MUT3A and MUT3B from *inhA* gene all remaining probe (both failing wild type probes and mutation probes) give resistance for a particular drug monoresistance or multidrugresistnace or both for any of the total isolates of the study.

Altogether 1, 3 and 11 different sets of gene patterns were found to be responsible for all *inhA*, *katG* and *rpoB* gene related drug resistances respectively in the study which reflects higher chance of mutation to occur due to rifampicin than isoniazid. Most MDR strains had mutation in a clustered region of their genome which was found to be 530-533 nucleic acid position particularly S531L mutation (i.e. within MUT3 probe) in *rpoB* region for rifampicin resistance, C15T mutation (i.e. within MUT1 probe) in *inhA* region and S315T1 mutation (i.e. within MUT1 probe) in *katG* region for isoniazid resistance. S531L mutation is the the most common mutation for detection of rifampicin resistance (Marahatta *et al*., 2011; Poudel *et al*., 2012; Rienthong *et al.,* 2009) which was true in this study where nearly all of the isoniazid resistance isolates (97%) examined had mutation in the *katG* gene with only a single isolate having mutation in the *inhA* gene. Studies from other countries

have confirmed this variability in the contribution of different mutations to INH resistance (Barnus *et al.,* 2008; Baker *et al.,* 2005). This study showed rifampicin resistance is most common (mostly 530-533 region especially S531 mutation) and prevalence of mutation is common in the high level isoniazid resistance of the *katG* gene in Nepal. INH resistance with *kat*GS315Thr mutation may be more likely to develop resistance to other first line drugs including rifampicin (Poudel *et al*., 2012; Marahatta *et al*., 2011; Hu *et al.,* 2010) which is also true in our study as almost all of the INH resistant strain (17/18 i.e. 94.4%) were found to have S531 mutation in *katG* gene and this mutation has nearly completely (15/16 i.e. 93.7%) also given rise to MDR.

A high prevalence of *katG* mutations has been reported to account for a high proportion of INH resistance in high TB prevalence countries and for a much lower proportion in lower TB prevalence setting (Poudel *et al*., 2012 ; Baker *et al.,* 2005). This presumably tells about the ongoing transmission of these strains in particular pocket of Nepalese society. Nepal, being geographically situated between China and India which carry almost 50% of worlds MDR-TB burden (Wright *et al*., 2009) which is further fostered by open border with India there is higher probabilitry of transmission of MDR cases inside country unless effective control program is implemented. This study also reveals MDR strains in Nepalese patient are varied and control strategy and drug resistance surveillance must be immediately adopted before these isolates further mutate themselves producing large number of MDR variants affecting more people and hindering control programs which could be very devastating for resource limited countries like Nepal.

CHAPTER-VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Mutation in different genes responsible for isoniazid and/or rifampicin resistance were identified in tuberculosis patients. S315T1 (87.8%) and S531L (50%) were the most common mutation for rifampicin and isoniazid respectively. All of the suspected tuberculosis patients were confirmed to be infected either due to *Mycobacterium tuberculosis* complex (87.1%) or atypical mycobacteria (12.9%). Among the drug resistant *Mycobacterium tuberculosis* complex isolates (72.2%) multidrug resistant isolates were predominant (71.7%) followed by rifampicin monoresitant (15.3%) and isoniazid monoresistant isolates (12.8%). More female (72.2%) were affected by tuberculosis than male (27.7%) and most patient (66.6%) with age between 20 and 45 had suffered from the disease. Majority (61.5%) of CAT I failure tuberculosis cases were found to have multidrug resistant isolates. Tuberculosis was reported from all five regional treatment centre of Nepal with highest proportion (51.8%) of multidrug resistance among all MTC isolates which was also true for each region. Different patterns of gene mutation were reported for a single drug (either rifampicin or isoniazid) even within each of these geographic regions. This suggests wider distribution and diversed population of *Mycobacterium tuberculosis* complex isolates in Nepal with increasing trend of multidrug resistance to develop in them.

6.2 Recommendations

1. Molecular line probe assay Genotype MTBDRplus must be used for rapid screening of resistant tuberculosis before prescribing any treatment.

2. Choice of drug for treatment must be considered according to drug susceptibility pattern of clinical isolate since this study revealed higher proportion of antitubercular drug resistances.

3. CAT I failure patients should be closely monitored for MDR development as this study finds most of these patients having higher MDR TB. DOTS need to be effectively implemented to prevent CAT I failure.

4. Rifampicin resistance isolates should be considered for MDR development as the study shows majority of them were MDR isolates.

6.3 Limitations of the study

1. Resistances of *M. tuberculosis* complex that have their origin outside *rpoB, katg and inhA* regions are not examined by by this study.

REFERENCES

Acharya S (2008). Comparison of the resistance ratio and proportion methods for drug susceptibility testing of *Mycobacteriuum tuberculosis* isolated from patients visiting national tuberculosis centre. M.Sc. Dissertation submitted to Central Department of Microbiology, Tribhuvan University, pp. 57-59.

American Thoracic Society (ATS) and CDC (2000). Diagnostic standards and classification of tuberculosis in adults and children. *Am J Respir Crit Care Med* **61**: 1376-1395.

Arnvig KB and Young DB (2009). Identification of small RNAs in *Mycobacterium tuberculosis*. *Mol Microbiol* **73** (3): 397-408.

Baker LV, Brown JT, Maxwell O, Gibson AL, Fang Z, Yates MD and Drobniewski FA (2005). Molecular analysis of isoniazid-resistant-isolates from England and Wales reveals the phylogenetic significance of the ahpC-46A polymorphism. *Antimicrob Agents Chemother* **49**: 1455-1464.

Barnard M, Albert H, Coetzee G, O'Brien R and Bosman ME (2008). Rapid molecular screening for drug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *Am J Respir Crit Care Med* **177** (7): 787-92.

Barnus M, Alber H, Coetzee G, O'Brien R and Bosman ME (2008). Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume Public Health laboratory in South Africa. *Am J Respir Crit Care Med* **177**: 787-792.

Bartfai Z, Somoskovi A, Kodmon C, Szabo N, Puskas E, Kosztolanyi L, Farago E, Mester J, Parsons LM and Salfinger M (2001). Molecular characterisation of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Hungary by DNA sequencing and the line probe assay. *J Clin Microbiol* **39** (10): 3736-3739.

Bell E (2005). Vaccines: A souped-up version of BCG. *Nat Rev Immunol* **5** (10): 746.

Brimacombe M, Hazbon M, Motiwala AS and Alland D (2007). Antibiotic resistance and single nucleotide polymorphism cluster grouping type in a mutational sample of resistant *M. tuberculosis* isolates. *Antimicrob Agents Chemother* **51** (11): 4157-4159.

Brossier F, Veziris N, Truffot-Pernot C, Jarlier V and Sougakoff W (2006). Performance of the Genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. *J Clin Microbiol* **44** (10): 3659-3664.

Cavusoglu C, Hilmiioglu S and Bilgic A (2002). Characterization of *rpoB* mutations in rifampicin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay. *J Clin Microbiol*. **40**: 4435-4438.

CDC (2005). National plan for reliable tuberculosis laboratory services using a systems approach: recommendations from CDC and the Association of Public Health Laboratories Task Force on Tuberculosis Laboratory Services. **54** (6): 1-12.

CDC (2000). Update: nucleic acid amplification tests for tuberculosis. **49**: 593-594.

CDC (2003). Treatment of tuberculosis. American Thoracic Society, CDC, and Infectious Diseases Society of America.

Chakraborty P (2003). *A Textbook of Microbiology*, 2nd edn. New Central Book Agency (P) Ltd., India, pp. 396-414.

Cheesbrough M (1998). *Medical laboratory manual for tropical countries: Microbiology*. ELBS, pp.**2**: 207-208.

Cirillo DM, Piana F, Frisicale L, Quaranta M, Riccabone A and Penati V (2004). Direct rapid diagnosis of rifampicin-resistant *Mycobacterium tuberculosis* infection in clinical samples by line probe assay (INNO LiPA Rif.TB). *New Microbiol* **27**: 221-227.

Daniel WW (1999). *Biostatistics: A Foundation for Analysis in the Health Sciences*, 7th edn. New York: John Wiley & Sons.

de Siqueira R.H, de Freitas FAD, de oliveira ND, Barreto AMW, Dalcolmo MP and Albano RM (2009). Isoniazid-resistant *Mycobacterium tuberculosis* strains arising from mutations in two different regions of the *kat*G gene. *J Bras Pneumol* **35** (8): 773-779.

Dickinson JM and Mitchison DA (1981). Experimental models to explain the high sterilizing activity of rifampin in the chemotherapy of tuberculosis. *Am Rev Respir Dis* **123**: 367-371.

Dooley SW and Simone PM (1994). The extent and management of drug-resistant tuberculosis: the American experience. In *Clinical Tuberculosis*. London: Chapman & Hall, pp. 171-189.

Driscoll J, Lentz A, Sikes D, Hartline D and Metchock B (2010). The first month of a new diagnostic service for molecular detection of MDR and XDR tuberculosis. Am J Crit Care Med **181**: A259.

Drobniewski FA, Watterson SA, Wilson SM and Harris GS (2000). A clinical, microbiological and economic analysis of a nationalservice for the rapid molecular diagnosis of tuberculosis and rifampicin resistance in *Mycobacterium tuberculosis*. *J Med Microbiol* **49**: 271-278.

Drobniewski FA and Wilson SM (1998). The rapid diagnosis of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis* - a molecular story. *J Med Microbiol* **47**: 189-196.

Enarson DA, Rieder HL, Arnadottir T and Trebucq A (2000). *Management of tuberculosis: A guide for low income countries*, 5th edn. International Union against Tuberculosis and Lung Disease (IUATLD) Publisher, Paris, France, pp. 52.

Feklistov A, Mekler V, Jiang Q, Westblade LF, Irschik H, Jansen R, Mustaev A, Darst SA and Ebright RH (2008). Rifamycins do not function by allosteric modulation of binding of Mg2+ to the RNA polymerase active center. *Proc Natl Acad Sci* USA **105** (39): 14820-14825.

Foulds J and O' Brien R (1998). New tools for the diagnosis of tuberculosis, the perspective of developing countries. *Int J Tuberc Lung Dis* **2** (10): 778-783.

Forbes BA, Sahm DF and Weissfeld AS (2002). *Bailey and Scott's diagnostic Microbiology*, 8th edn. Mosby Inc, USA, pp. 538-571.

Fox A (2010). *General aspects of bacterial pathogenesis*. University of South Carolina School of Medicine: Microbiology and Immunology On-line Textbook.

Frothingham R and Meeker-O'Connell WA (1998). Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology* **144** (5): 1189-96.

Gagneux S (2009). Strain variation and evolution. In *Mycobacterium: Genomics and Molecular Biology.* Parish T and Brown A (eds). Caister Academic Press, Norfolk, pp. 1-18.

Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K and Andrews J (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* **368**: 1575-1580.

Gillespie SH (2002). Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. *Antimicrob Agents Chemother* **46** (2): 267-274.

Glickman MS and Jacobs WR (2001). Microbial pathogenesis of *Mycobacterium tuberculosis*: dawn of a discipline. *Cell* **104** (4): 477-485.

Goble M, Iseman MD, Madsen LA, Waite D, Ackerson L and Horsburgh CR Jr (1993). Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. *N Engl J Med* **328** (8): 527-532.

Good RC and Shinnick TM (1998). Mycobacterium. In *Topley and Wilson's Microbiology and Microbial Infections,* 9th edn. Collier L, Balows A and Sussman M (eds). Edward Arnold, London, UK, **2**: 463-467.

Grange JM (1990). Tuberculosis. In *Topley and Wilson's Principle of Bacteriology, Virology and Immunology,* 8th edn. Parker MJ, Duerden BI (eds). Buttler and Tanner Ltd London **2**: 564-567.

Grange JM (1998). Tuberculosis. In *Topley and Wilson's Microbiology and Microbial Infections, Bacterial Infections*, 11th edn. Collier L, Balows A and Sussman M (eds). Arnold, a member of the Hodder Headline Group, London, pp. 391-442.

Groothius DG and Yates MD (1991). *Manual of Diagnostic and Public Health Mycobacteriology,* 2nd edn. Bureau of Hygiene and Tropical Diseases, London, UK, pp.150-153.

Guerra RL, Hooper NM and Baker JF (2007). Use of the Amplified *Mycobacterium tuberculosis* Direct Test in a public health laboratory: test performance and impact on clinical care. *Chest* **132**: 946-951.

Hafner R, Cohn JA, Wright DJ, Dunlap NE, Egorin MJ, Enama ME, Muth K, Peloquin CA, Mor N and Heifets LB (1997). Early bactericidal activity of isoniazid in pulmonary tuberculosis. *Am J Respir Crit Care Med* **156**: 918-923.

Hass DW (2000). Principle and Practice of Infectious Diseases, 5th edn. Churchill Livingstone, pp. 200-214.

Hawkey PM, Smith EG and Evans JT (2003). Mycobacterial interspersed repetitive unit typing of *Mycobacterium tuberculosis* compared to IS6110-based restriction fragment length polymorphism analysis for investigation of apparently clustered cases of tuberculosis. *J Clin Microbiol* **41** (8): 3514-3520.

Heym B, Stavropoulos E and Honore N (1997). Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. *Infect Immun* **65**: 1395-1401.

Hillemann D, Rusch-Gerdes S and Richter E (2007). Evaluation of the Genotype MTBDRplus Assay for Rifampin and Isoniazid Susceptibility Testing of *Mycobacterium tuberculosis* Strains and Clinical Specimens. *J Clin Microbiol* **45**: 2635-2640.

Hillemann D, Weizenegger M, Kubica T, Richter E and Niemann S (2005). Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* **43**: 3699-36703.

Hirano K, Abe C and Takahashi M (1999). Mutations in the *rpoB* gene of rifampicin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. *J Clin Microbiol* **37**: 2663-2666.

Hoechst (2000). Rifamate, 54th edn. Oradell, NJ: Medical Economics Data, pp. 1379-1382.

Hu Y, Hoffner S and Weili J (2010). Extensive transmission of isoniazid resistant *M.tuberculosis* and its association with increased multi-drug resistant TB in two rural countries of eastern China: A molecular epidemiological study. *BMC Infect Dis* **10** (43).

Inderlied C and Pfyffer GE (2003). Susceptibility test methods: Mycobacteria. In *Manual of Clinical Microbiology*, 8th edn. Murray PR, Baron EJ, Pfaller MA, Jorgensen JH and Yolken RH (eds). ASM Press, Washington D.C. **1**: 1149-1177.

Iseman MD (1993). Treatment of multidrug-resistant tuberculosis. *N Engl J Med* **329** (11): 784-791.

Ismael Kassim and Ray CG (2004). *Sherris Medical Microbiology*, 4th edn. McGraw Hill.

Jindani A, Aber VR, Edwards EA and Mitchison DA (1980). The early bactericidal activity of drugs in patients with pulmonary tuberculosis. *Am Rev Respir Dis* **121**: 939-949.

Joanne LF and John C (2003). Immune evasion by *Mycobacterium tuberculosis*: living with the enemy. *Curr Opin Immunol* **15** (4): 450.

Johnson R, Streicher EM, Louw GE, Warren RM, van Helden PD and Victor TC (2006). Drug resistance in *Mycobacterium tuberculosis*. *Curr Issues Mol Biol* **8** (2): 97-111.

Koirala B (2008). Comparative study of polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) for direct detection of *Mycobacterium tuberculosis* in sputum. M.Sc. Dissertation submitted to Central Department of Microbiology, Tribhuvan University, pp. 58.

Kommareddi S, Abramowsky C, Swinehart G and Hrabak L (1984). Nontuberculous mycobacterial infections: comparison of the fluorescent auramine-O and Ziehl-Neelsen techniques in tissue diagnosis. *Hum Pathol* **15** (11): 1085-1089.

Konstantinos A (2010). Testing for tuberculosis. *Australian Prescriber* **33** (1): 12-18.

Kramnik I, Dietrich WF, Demant P and Bloom BR (2000). Genetic control ofresistance to experimental infection with virulent *Mycobacterium tuberculosis*. *PNAS* 2000: **97** (15): 8560-8565.

Kumar V, Abbas AK, Fausto N and Mitchell RN (2007). *Robbins Basic Pathology* , 8th ed. Saunders Elsevier, pp. 516-522.

Lawn SD and Zumla AI (2011). Tuberculosis. *Lancet* **378** (9785): 57-72.

Lin SYG, Lin SY, Probert W, Lo M and Desmond E (2004). Rapid detection of isoniazid and rifampin resistance mutations in *Mycobacterium tuberculosis* complex from cultures or smear-positive sputa by use of molecular beacons. *J Clin Microbiol* **42**: 4204-4208.

Ling DI, Zwerling AA and Pai M (2008). GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: a meta-analysis. *Eur Respir J* **32**: 1165-1174.

Livny J, Brencic A, Lory S and Waldor MK (2006). Identification of 17 Pseudomonas aeruginosa sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2. *Nucleic Acids Res* **34** (12): 3484-3493.

Maher D, Chaulet P, Spinaci S and Harries A (1997). Treatment of tuberculosis: guidelines for national programmes for the global tuberculosis programmes, 2nd edn. WHO, Geneva, Switzerland.

Makinen J, Marttila HJ, Marjamaki M, Viljanen MK and Soini H (2006). Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*. *J Clin Microbiol* **44**: 350-352.

Mani C, Selvakumar N, Narayanan S and Narayanan PR (2001). Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from India. *J Clin Microbiol* **39**: 2987-90.

Marahatta SB, Gautam S, Dhital S, Pote N, Jha AK, Mahato R, Mishra S, Poudel BH, Ramasoota P, Kaewkungwal J and Singhasivanon P (2011). katG (SER 315 THR) Gene Mutation in isoniazid resistant *Mycobacterium tuberculosis*. *Kathmandu Univ Med J* **33** (1): 19- 23.

Martin-Casabona N, Xairo MD, Gonzalez T, Rossello J and Arcalis L (1997). Rapid method for testing susceptibility of *Mycobacterium tuberculosis* by using DNA probes. *J Clin Microbiol* **35**: 2521-2525.

Mathema B, Kurepina NE, Bifani PJ and Kreiswirth BN (2006). Molecular Epidemiology of Tuberculosis: Current Insights. *Clin Microbiol Rev* **19** (4): 658-685.

Mazars E, Lesjean S and Banuls AL (2001). High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. *Proc Natl Acad Sci* USA. **98** (4): 1901-1906.

Ministry of Health and Population, Nepal (2011a). Annual Report 2010/2011. Department of Health Services, Kathmandu.

Ministry of Health and Population, Nepal (2011b). Drug Resistance Tuberculosis. Available at: http://nepalntp.gov.np/content.php?id=204. Accessed 18 October 2011.

Minnikin DE, Ridell M, Wallerstrom G, Besra GS, Parlett JH, Bolton RC and Magnusson M (1989). Comparative studies of antigenic glycolipids of mycobacteria related to the leprosy bacillus. *Acta leprol* **7** 1: 51-54.

Miotto P, Piana F, Penati V, Canducci F and Migliori GB (2008). Genotype MTBDRplus: a further step toward rapid identification of drug-resistant *Mycobacterium tuberculosis*. *J Clin Microbiol* **46**: 393-394.

Mitchison DA (1985). Mechanisms of the action of drugs in short-course chemotherapy. *Bull Int Union Tuberc* **60**: 36-40.

Mokrousov I, Otten T, Filipenko M, Vyazovaya A, Chrapov E, Limeschenko E, Steklova L, Vyshnevskiy B and Narvskaya O (2002). Detection of Isoniazid- Resistant *Mycobacterium tuberculosis* strains by multiple allele specific PCR assay targeting *kat*G codon 315 variation. *J Clin Microbiol* **40** (7): 2509-2512.

Moore D, Evans C, Gilman R, Caviedes L, Coronel J, Vivar A, Sanchez E, Piñedo Y, Saravia J, Salazar C, Oberhelman R, Hollm-Delgado M, LaChira D, Escombe A and Friedland J (2006). Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *N Engl J Med* **355** (15): 1539-50.

Morgan M, Kalantri S, Flores L and Pai M (2005). A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *BMC Infect Dis* **5**: 62.
Mycobacterium tuberculosis (2007). Sanger Institute. Available at:http://www.sanger.ac.uk/resources/downloads/bacteria/*Mycobacterium* .html. Accessed 05 November 2011.

National Committee for Clinical Laboratory Standards (NCCLS) (2000). Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes, 2nd edn. Tentative standard M24--T2. Wayne, PA: National Committee for Clinical Laboratory Standards. Available at http://www.nccls.org/microbiology.htm. Accessed 26 October 2011.

National Tuberculosis Control Programme, Annual report (2004/2005), NTC, Thimi, Bhaktapur, Nepal, pp. 10-12.

National Tuberculosis Programme of Nepal: A clinical manual (2009). National Tuberculosis Center, Nepal, pp. 24-32.

Noorden SK and Godal T (1988). Introduction. In *Tuberculosis and leprosy*. Rees RJW (eds). Br Med Bull **44** (3): 523-527.

Park K (2005). Park's Textbook of Preventable and Social Medicine, 18th edn. M/S Banarsidas Bhanot Publishers, India, pp. 146-161.

Poudel A, Nakajima C, Fukushima Y, Suzuki H, Pandey BD, Maharjan B and Suzuki Y (2012). Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated in Nepal. *Antimicrob Agents Chemother* **56** (6): 2831-2836.

Pande JN, Singh U.B, S*inhA* S, Agrawal RC and Singh SPN (2002). Evaluation of risk factors and prevalence of drug resistant tuberculosis in North India. *Chest* **128** (4): 404-408.

Pfyffer GE (2000). Antimicrobial susceptibility testing in management of multidrug-resistant tuberculosis. *J Int Assoc Phys AIDS Care* **6** (2): 52.

Rahman M, Kamal SMM, Mohammed FR, Alam MDB and Ahasan HN (2009). Anti-tuberculosis drug resistance pattern among different category of tuberculosis patients. *J medicine* **10**: 45-47.

Ramaswami S and Musser JM (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tubercle Lung Dis* **79**: 3-29.

Rastogi N, Goh KS and David HL (1989). Drug susceptibility testing in tuberculosis: a comparison of the proportion methods using Lowenstein-Jensen, Middlebrook 7H10 and 7H11 agar media and a radiometric method. *Res Microbiol* **140**: 405-417.

Rattan A, Kalia A and Ahmed N (1998). Multi-Drug resistant *Mycobacterium tuberculosis*: molecular perspectives. *Ind J Tub* **4** (2): 195-210.

Rienthong D, Rienthong S, Boonin C and Kasetjaroen Y (2009). Rapid detection of early rifampin and isoniazid resistance in *Mycobacterium tuberculosis*. *Siriraj Med J* **61**: 49-55.

Siddiqi SH, Hawkins JE and Laszlo A (1985). Interlaboratory drug susceptibility testing of *Mycobacterium tuberculosis* by a radiometric procedure and two conventional methods. *J Clin Microbiol* **22**: 919-923.

Snider DE Jr, Good RC, Kilburn JO, Laskowski LF Jr, Lusk RH, Marr JJ, Reggiardo Z and Middlebrook G (1981). Rapid drug-susceptibility testing of *Mycobacterium tuberculosis*. *Am Rev Respir Dis* **123**: 402-406.

Sola C, Filliol I, Legrand E, Mokrousov I, and Rastogi N (2001). *Mycobacterium tuberculosis* phylogeny reconstruction based on combined numerical analysis with IS1081, IS6110, VNTR, and DR-based spoligotyping suggests the existence of two new phylogeographical clades. *J Mol Evol* **53** (6): 680-689.

Sharma BK (2010). A case study of tuberculosis patients folllowing DOTS treatment in GENETUP DOTS clinic working together with NATA,Kathmandu. M.A. Dissertation submitted to Department of Sociology/ Anthropology,Trichandra multiple campus,Tribhuvan University. pp. 50-51.

Singh R, Manjunatha U and Boshoff HI (2008). PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. *Science* **322** (5906): 1392-1395.

Singh SP, Salamon H, Lahti CJ, Farid-Moyer M and Small PM (1999). Use of pulsed-field gel electrophoresis for molecular epidemiologic and population genetic studies of *Mycobacterium tuberculosis*. *J Clin Microbiol* **37** (6): 1927-1931.

Steingart K, Henry M and Ng V (2006). Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis* **6** (9): 570-581.

Suarez J, Ranguelova K and Jarzecki AA (2009). An oxyferrous heme/protein-based radical intermediate is catalytically competent in the catalase reaction of *Mycobacterium tuberculosis* catalase-peroxidase (KatG). *J Biol Chem* **284** (11): 7017-7029.

Supply P, Allix C and Lesjean S (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* **44** (12): 4498-4510.

Tarrand JJ and Groschel DH (1985). Evaluation of the BACTEC radiometric method for detection of 1% resistant populations of *Mycobacterium tuberculosis*. *J Clin Microbiol* **21**: 941-946.

Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S and Colston MJ (1993). Detection of rifampicinresistance mutations in *Mycobacterium tuberculosis*. *Lancet* **341**: 647-650.

Timmins GS, Master S, Rusnak F and Deretic V (2004). Nitric oxide generated from isoniazid activation by KatG: source of nitric oxide and activity against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **48** (8): 3006-3009.

Tracevska T, Jansone I, Broka L, Marga O and Baumanis V (2002). Mutations in the *rpo*B and *kat*G genes leading to Drug resistance in *Mycobacterium tuberculosis* in Lativa. *J Clin Microbiol* **40** (10): 3789-3792.

van Soolingen D, Hoogenboezem T, de Haas PE, Hermans PW, Koedam MA, Teppema KS, Brennan PJ, Besra GS, Portaels F, Top J, Schouls LM and van Embden JD. (1997). A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. *Int J Syst Bacteriol* **47** (4): 1236-1244.

Varma JK, Wiriyakitjar D and Nateniyom S (2007). Evaluating the potential impact of the new Global Plan to Stop TB: Thailand, 2004-2005. *Bull World Health Organ* **85**: 586-592.

Vasconcellos SE, Huard RC and Niemann S (2010). Distinct genotypic profiles of the two major clades of *Mycobacterium africanum*. *BMC Infect Dis* **10**: 80.

Vattanaviboon P, Sukchawalit R, Jearanaikoon P, Chuchottaworn C and Ponglikitmongkol M (1995). Analysis of RNA polymerase gene mutation in three isolates of rifampicin resistant *Mycobacterium tuberculosis*. *Southeast Asian J Trop Med Public Health* **26** (11): 333-336.

Victor TC, van Helden PD and Warren R (2002). Prediction of drug resistance in *M. tuberculosis*: molecular mechanisms, tools, and applications. *Life* **53**: 231-237.

Victor TC, Warren R, Butt JL, Jordaan AM, Felix JV, Venter A, Sirgel FA, Schaaf HS, Donald, PR, Richardson M, Cynamon MH and van Helden PD (1997). Genome and MIC stability in *Mycobacterium tuberculosis* and indications for continuation of use of isoniazid in multidrug-resistant tuberculosis. *J Med Microbiol* **46**: 847-857.

WHO (2001). Guidelines for drug susceptibility testing for second-line anti-tuberculosis drugs for DOTS-plus. Geneva, Switzerland, pp. 8-24.

WHO report (2003): Global Tuberculosis Control. Geneva, Switzerland, pp. 9-12.

WHO (2006). Guidelines for the programmatic management of drugresistant tuberculosis. Geneva, Switzerland, pp. 18-21.

WHO (2008b). Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB): policy statement. Geneva, Switzerland, pp. 28-32.

WHO (2009a). Country profiles: Nepal. Available at: http://www.searo.who.int/en/section10/ section2097/section2100_14801.htm. Accessed 25 Feb, 2012.

WHO (2009b). Epidemiology. Global tuberculosis control: epidemiology, strategy, financing. pp. 6-33.

WHO (2009c). TB in South-East Asia: Epidemiology of TB in the Region. Available at: http://www.searo.who.int/en/Section10/Section2097/Section210o_10639.htm. Accessed 27 Feb, 2012.

WHO (2010). Global tuberculosis control 2011. Available at: http://www.who.int/tb/publications/global_report/en/. Accessed 5 January 2012.

WHO (2011). The sixteenth global report on tuberculosis. Geneva, Switzerland.

WHO (2012). Health topics:tuberculosis. Available at: http://www.who.int/ topics/tuberculosis /en/. Accessed 29 Feb, 2012.

Woods GL (2000). Susceptibility testing for Mycobacteria. *Clin Infect Dis* **31**: 1209-1215.

Wright A, Zignol M, Deun AV, Falzon D, Gerdes SR, Feldman K, Hoffner S, Drobniewski F, Barrera L, van Sollingen D, Boulabhal F, Paramasivan CN, Kam KM, Mitarai S, Nunn P and Raviglione M (2009). Epidemiology of anti-tuberculosis drug resistance 2002-07: an updated analysis of the global project on anti-tuberculosis drug resistance surveillance. *Lancet* **373** (9678): 1861-72.

Yagui M, Perales MT, Asencios L, Vergara L, Suarez C, Yale G, SalazarC, Saavedra M, Shin S and Ferrousier O (2006). Timely diagnosis of MDRTB under program conditions: is rapid drug susceptibility testing sufficient? *Int J Tuberc Lung Dis* **10**: 838-843.

Zhang Y, Mazurek GH and Cave MD (1992). DNA polymorphisms in strains of *Mycobacterium tuberculosis* analyzed by pulsed-field gel electrophoresis: a tool for epidemiology. *J Clin Microbiol* **30** (6): 1551-1556.

Zhang Y and Telenti A (2000). Genetics of drug resistance in *Mycobacterium tuberculosis*. In *Molecular genetics of Mycobacteria*. Hatfull GF and Jacobs WR Jr (eds.). Washington, DC: ASM Press, pp. 235-254.