CHAPTER I

INTRODUCTION

Tuberculosis (TB) is chronic infectious disease that has afflicted humanity for over 35000 years (Hughes *et al.*, 2002), and, its etiological agent, *M. tuberculosis* has accounted for more human deaths than any other pathogen (Cole, 1998; Daniel, 2006). Despite the world wise use of a live attenuated vaccine and several antibiotics, it is still one of the biggest killers among the infectious diseases (Smith, 2003) besides the HIV with which it is intimately linked (WHO, 2008).

Although antibiotic treatment for TB was discovered more than half a century ago, an estimated one third of the world's population (2 billions) is currently infected with *M. tuberculosis* (Keshavjee *et al.,* 2008). In 2008, 9.4 million new cases of TB are reported to occur around the world and 1.8 million people died from TB in 2008 (WHO, 2009). A combination of HIV and TB has proven to be especially deadly. At least one third of the 33.2 million people living with HIV worldwide are infected with TB. HIV is the main reason for failure to meet TB control targets in high HIV settings (WHO, 2009). TB control is high on the international public health agenda, not just because of the enormous burden of disease, but also because of short course chemotherapy is recognized to be among the most cost effective of all health interventions (Dye *et al.,* 2010).

Tuberculosis is a major public health problem in Nepal and recognized by the government as a priority number one program. About 45% of the total population is infected with TB, out of which 60% are adult. Every year about 40,000 people develop TB, of whom 20,000 have infectious pulmonary disease. Introduction of treatment by DOTs has already reduced the number of death; it is estimated 5,000-7,000 people still dies per year from TB (NTC, 2008).

Multi dug resistant TB (MDRTB)-strains of TB that are resistant to the two principal first line TB drugs (isoniazid and rifampicin)- is a major and growing global problem

(Giffin *et al.,* 2009), since these bacteria often cause incurable disease, even when expensive second- and third line drugs are available. As a consequence, this would read to higher mortality and treatment failure rates and increased periods at disease transmission within a community (Gonzalez *et al.,* 1999). WHO estimated 440,000 MDR-TB cases and 150,000 deaths in 2008, but only 7% of all estimated MDR-TB patients diagnosed and notified, is a major concern (WHO, 2010). Furthermore MDR-RB patients co-infected to with HIV are more likely and are often the first to show active drug-resistant tuberculosis after transmission in an outbreak (Gandhi *et al.,* 2010). Recent Global anti TB Drug resistance surveillance studies have shown that drug resistance was ubiquitous and MDR-TB Strains have been described worldwide (WHO, 2008).

In Nepal, a national prevalence of MDR-TB is 2.9% among new TB cases and 11.7% among previously treated TB cases. Although treatment with standardized directly observed therapy with short-course chemotherapy (DOT-SCC) has been successfully implemented in Nepal, MDR-TB continues to be a major public health problem in past because of the difficulty and expense associated with its treatment (STAC, 2008).

In most resource poor countries of the world, detection and management of drug resistant TB is very expensive. Thus, the appearance and spread of MDR-TB is these countries would lead to higher case fatality rate (Kochi *et al.,* 1993). Earlier, the link between drug resistance and TB control programme has been noted. So, the importance to monitor drug resistance patterns in improving performance of TB control and in reducing the disease burden has also been advocated (Kim & Hong, 1992; WHO/IUATLD, 1998). For this reason, the rapid diagnosis of TB drug resistance is a priority in developing country (Musa *et al.,* 2005; Palomino, 2005).

Current standard methods for the detection of MDR *M. tuberculosis* include the proportion method (PM) performed on Lowenstein-Jensen(LJ) medium or agar, absolute concentration and resistance ratio methods (Canetti *et al.,* 1969; Kent & Kubica, 1985) are used globally but are time consuming. Other methods, such as the

BACTEC 460 TB System (Roberts *et al.*, 1983), and oxidation-reduction dyes, e.g. tetrazolium (Caviedes *et al.*, 2002), and microplate Alamar Blue assay (MABA), (Franzblau *et al.*, 1998) are faster but have the drawback of requiring either radioactive or expensive substrates, and are consequently not feasible in most resource-poor settings. A cost effective and rapid drug susceptibility method is required to guide TB treatment.

Angeby *et al.* have described a new nitrate reductase assay (NRA) for rapid and inexpensive DST of *M. tuberculosis* (Angeby *et al.*, 2002). The method depends on the ability of *M. tuberculosis* to reduce nitrate to nitrate which can be detected using specific reagents producing a change in colour (Kent & Kubica, 1985). It is a colourimetric method also known as Griess method (Golyshevskaia *et al.*, 1996), after J.P. Griess, who discovered the chemistry of the detection method. NRA has the advantage of being performed on the classical LJ media and requires neither fancy equipment nor expensive substrates or reagents (Angeby *et al.*, 2002).

This study was carried out at National TB Centre and SAARC TB and HIV/AIDS centre, Thimi, Bhaktapur, Nepal, to evaluate Indirect Nitrate Reductase Assay for drug susceptibility testing of *M. tuberculosis* clinical isolates. The performance of this technique was compared to that of the 1% proportion method in determining the susceptibilities to isoniazid (INH), rifampicin (RMP), Streptomycin (SM) and ethambutol (EMB).

There are different methods for detection of TB drug resistance. The proportion method (PM), based on the measurement of growth in culture media containing antibiotics, require several weeks to give results. For developing countries, it would be useful to have a simple and inexpensive test that could rapidly detect drug-resistant *M. tuberculosis* strains. An alternative method is the nitrate reductase assay (NRA), previously reported as a useful tool for rapid and accurate detection of resistance to first-line anti-tuberculous drugs. This study shall help to provide the drug susceptibility pattern of clinical isolates in short period of times.

CHAPTER II

2. OBJECTIVES

2.1 General Objective

To evaluate the performance of Indirect Nitrate Reductase Assay for routine detection of resistance to first-line anti-tubercular drugs.

2.2 Specific Objectives

1. To determine the sensitivity, specificity and predictive values of Indirect Nitrate Reductase Assay.
2. To determine the mean time of resistance detection.

CHAPTER III

3. LITERATURE REVIEW

3.1 HISTORICAL PERSPECTIVE OF TUBERCULOSIS

One may conclude from different historical sources (Webb, 1936; Keers, 1978), that the disease caused by a microbe, presumably identical or very similar to the currently recognized pathogen, *M. tuberculosis* has been extant in diverse human populations for many thousand years. Indeed, by using a variety of sophisticated molecular biological indicators estimated that the tubercle bacilli is at least 35,000 years old (Hughes *et al.*, 2002). Today TB is reappearing in many countries as a public health crisis. Thus, if not an emerging disease, it is an important reemerging disease, and though ancient, it is not a disease of past. A staggering yet tuberculosis still kills about two million people every year. Only today, it is a disease of poverty.

3.2 The Genus Mycobacteria

The genus Mycobacteria belongs to the class actinomycetes, order actinomycetales and family Mycobacteriaceae, and includes obligate parasites, saprophytes (Shinnick & Good, 1994) and opportunistic commensals that can be isolated from clinical samples, soil, water or other environmental sources (Kubica,1984). Currently, there are more than 100 recognized or proposed species in the genus Mycobacterium, including the causative agent of TB. Of these thirty are known to cause disease in human (Forbes *et al.*, 2007).

Together with other highly related bacteria, *M. tuberculosis* forms a complex, the M*ycobacterium tuberculosis* complex (MTC), a single species as defined by DNA/DNA hybridization on studies (Imaeda, 1985). The MTC comprises seven members which include *M. tuberculosis*, the primary causative agent of human tuberculosis, *M. bovis*, which is responsible for bovine TB and includes the vaccine strain *M. bovis* BCG; *M. africanum*, the main causative agent of TB in west Africa

(Kallenius *et al.,* 1999); *M. microti*, which is a pathogen of volves and rarely infects humans (Rastogi, 2001); *M. canneti*, a rare MTC strain so far isolated from individuals having been to the horn of Africa (Pfyffer, 1998); *M. pinnipeddi*, also known as the seal bacillus (Cousins, 2003); *M. caprae*, primarily isolated from goats (Aranaz, 2003).

All other mycobacterial species that do not belong to *M. tuberculosis* complex are called non tuberculous mycobacteria (NTM) or MOTT and present everywhere in the environment (Forbes *et al.,* 2007). They can also colonise man and cause clinical infection more commonly in the presence of predisposing factors/underlying disease (immunocompromised). They are notably resistant to commonly used antitubercular drugs. Clinically important NTM are *M. kansasii, M. genavense, M.marinum, M. simiae, M. scrofulaceum, M. szulgai, M. avium, M. haemophilum, M. intracellulare, M. malmoense, M. ulerans, M. xenopi, M abscessus, M. chelonae, M. fortuitum and rarely M. smegmatis* (Horsburgh, 1996). Their isolation from clinical samples has to be interpreted with caution, isolation from a single sample on a single occasion is unlikely to be significant with some exceptions (Rayner, 2006; Sungkanuparh *et al.,* 2003).

3.3 The Major TB organism: *M. tuberculosis*

M. tuberculosis is typically slender, straight or slightly curved and rod in shape. It occurs as a single cell or in a thread like form. The size of the bacillus ranges from 0.3µm in width and from 0.5µm to 4µm in length. It is slow growing, non encapsulated, non sporing, non motile and lipid rich organism (Salyers & Whitt, 1994). The organism exhibits extreme pleomorphism in certain circumstances and can exist even as a cell wall deficient or L-forms (Domingue & Woody, 1995). In animal tissues, they are generally longer and thinner than in culture.

M. tuberculosis has a lipid rich cell wall that consists of a typical bilayered plasma membrane followed by a layer of peptidoglycan-arabinoglactan covalently linked to mycolic acids (Hoffmann, 2008). The unique cell wall is responsible for its resistance to the lethal effects of acids, alkalis and detergents, a characteristic fully exploited for isolation of the organism from other bacteria for culturing (Evans, 1998). Lipids

contributes to the several biological features including the hydrophobicity of mycobacterium, the tendency of mycobacterium to form clumps or cords, the resistance of mycobacterium to common lysis procedure, the ability to survive intracellularly (Brennan *et al.*, 1995; Besra & Chaterjee, 1994), and present difficulty in staining (Laidlaw, 1989). Liproarabinomannan (LAM), a lipopolysaccharide from cell wall is considered to direct macrophage function, inhibit the processing of mycobacterial peptides by antigen presenting cells, and to induce tumor necrosis factor production (Barens *et al.*, 1990).

The *M. tuberculosis* H37Rv genome consists of 4.4×10^6 bp, 65.6% G+C content and contains approximately 4,000 genes (Cole *et al.*, 1998). Over 200 genes are annotated as encoding enzymes for the metabolism of fatty acids, comprising 6% of the total (Smith *et al.*, 2003). Fully 48% of the genes encode unknown or conserved hypothetical proteins, whereas 3-4% of the genome is composed of insertion sequences (Brennan, 2002) and prophages (Cole, 2002). IS elements have been implicated in genome plasticity of mycobacteria. The IS are mostly present in the *M. tuberculosis* complex species and their insertion position in the genome is variable between different strains which serve as the basis for differentiating between strains (Burgos, 2004).

M. tuberculosis is a strict aerobe equipped with catalase, peroxidase, and superoxide dismutase, the growth rate of which is highly dependent upon the oxygen concentration. It does not grow on ordinary culture media, but only on enriched media. However, the absence of growth on ordinary culture media is not related to any particular requirement for a growth factor or vitamin, even though various compounds potentiate the in vitro growth. Increased CO_2 tension enhances growth (Grange, 1998).

M. tuberculosis is mesophile and neutrophile as its multiplication is restricted to 37˚C and a neutral pH. *M. tuberculosis* is able to metabolise glycerol into pyruvate, where as *M. bovis* is not. *M. tuberculosis* is slow growing mycobacteria and may take 3-6 weeks to produce visible colonies, which are buff coloured, rough and friable. In primary culture, they hardly show any visible growth during the first week of culture. On egg

based media they produce characteristic non-pigmented colonies, with a general rough and dry appearance simulating breadcrumbs. On agar based media, the colonies appear flat, dry and rough with irregular edges (Osaba, 2004).

3.4 Transmission and Pathogenesis

M*ycobacterium tuberculosis* is transmitted from person to person mainly through air-borne droplet nuclei (i.e. bacilli-bearing particles less than 100μm in diameter) released from the lungs via the nose and mouth when a patient with active TB coughs, sneezes or speaks. Droplet nuclei can remain suspended in small air currents ready to be inhaled, until removed by ventilation. Risk of infection of a susceptible individual is especially increases in crowded and poor ventilated area (Basnet, 1998). Infection begins when the bacilli reach the alveoli and phagocytic alveolar macrophages are the first cells infected by *M .tuberculosis* (Bloom & Murry, 1992). Some of the bacilli may be killed immediately; others may multiply within the macrophages. Infrequently, but especially in immune-suppressed persons and in children, the bacilli spread to other sites in the body. This dissemination sometimes results in life threatening meningitis.

One of the key steps in the pathogenesis of *M. tuberculosis* infection is the ability of the tubercle bacillus to enter and replicate within the macrophages of its human host (Des Jardin & Schlesinger, 2000). It is well established that single gene encoded virulence factors are important for the pathogenesis of *M. tuberculosis*. These include: catalase peroxidase, katG gene encoded enzyme, which protects the bacillus against reactive oxygen species such as hydroxyl radical, superoxide and hydrogen peroxide produced by the phagocyte; macrophage colonizing factor (possibly encoding invasin), encoded by mce gene, which is responsible for the ability of the bacillus to bind and invade macrophages; a sigma factor gene (sigA), which is involved in the transcription specificity of RNA polymerase or to promote the transcription of a subset of genes involved in virulence (Cole *et al*., 1998); and isocitrate lyase (ICL), icl gene encoded enzyme that promotes persistence of the bacillus within inflammatory macrophages (McKinney *et al.,* 2000).

Furthermore, bacillary enzymes like phospholipases C, lipases, and esterases, which might attack cellular, or vacuolar membranes, and several proteases could act as virulence factors. For instance, phospholipases act as contact dependent haemolysin. These proteins may well be secreted or surface exposed to play a role in invasion of host cells (Cole *et al*., 1998).

In addition, the mycobacterial cell wall is also essential in pathogenesis by allowing intracellular survival of the bacillus and it also renders the bacillus impermeable to common drugs (Brennan & Draper, 1994; Kolattukudy *et al*., 1997). The mycobacterial cell wall components such as mycocerosic acid, phenolthiocerol, lipoarabinomannan and arabinogalactan may contribute to mycobacterial longevity, trigger inflammatory host reaction and act in pathogenesis (Cole *et al*., 1998).

Recently, it has been demonstrated that the bacteria may persist intracellularly not only in macrophages but also in non-professional phagocytic cells (Hernandez-Pando *et al*., 2000). These include fibroblasts, endothelial- and epithelial-cells in lung tissue in the absence of tuberculous lesions. This is actuality in spite of the dominant view that latent organisms exist in old classic tuberculous lesions (Hernandez-Pando *et al*., 2000).

Usually, within 2 to 8 weeks after initial infection, most individuals are able to mount an effective immune response. In people within intact immune systems, infected phagocytic alveolar macrophages present pieces of the bacilli, displayed on their cell surfaces, to immune lymphocyte called the T-cell. When stimulated, T-cells release an elaborate array of chemical signals. Some of the T-cell signals produce inflammatory reactions; other signals recruit macrophage reactivation and pro-inflammatory cytokine production (Enarson & Rouillon, 1998). As a result, they kill *M. tuberculosis* and wall-off infected macrophages in tinny, hard grayish nodules (tubercles) and thereby prevents dissemination of bacilli (Parish *et al.*, 1998). Hence, the host's immune response limits the proliferation of the bacilli and produces a long lasting partial immunity, both to new infection and to the reactivation of old infection (Sutherland *et*

al., 1976). However, in primary TB infection, even when successfully controlled by the immune system, not all bacteria are eliminated. Some remain dormant but viable (persistent) bacilli (Bloom & Murry, 1992).

If, however, as acquired immunity wanes through aging or immune suppression, progression or breakdown of infection leads to active disease in about 10 % of infected cases. Active disease (TB) usually results from the spread of the bacilli from the alveoli through the blood stream or lymphatic system to other sites of the body, usually in the lungs or local lymph nodes. Notably, the clinical manifestations of active TB depend on the site of *M. tuberculosis* spread and accordingly, TB can be grouped into two major forms: pulmonary form (PTB)-TB developed in the lungs and extra-pulmonary form (EPTB)-TB developed outside the lungs. Pulmonary tuberculosis (PTB) is the most common type of TB comprising about 80-85 % of all TB cases, while EPTB is found in about 15-20 % of cases (WHO, 1996a). In general, *Mycobacterium tuberculosis* accounts for 98% cases of PTB spread via air-borne droplet nuclei and 70% of EPTB form (Selwyne *et al*., 1989).

Although many bacilli are killed, a large proportion of infiltrating phagocytes and local tissue e.g. lung parenchyma cells, die as well producing characteristic solid caseous (cheese-like) necrosis/granuloma in which bacilli may survive but not flourish. If the necrotic reaction expands, breaking in to a bronchus, a cavity is produced in the lungs allowing a large numbers of bacilli to spread with coughing to the outside. As the disease progresses, the granulomas may liquefy, perhaps a result of released hydrolases from inflammatory cells, which creates a rich medium for the proliferation and spread of the bacilli. The pathologic and inflammatory processes produce the characteristic weakness, fever, chest pain, and, when a blood vessel is eroded, bloody sputum produced (Bloom & Murry, 1992).

3.5 Epidemiology of Tuberculosis

3.5.1 Global Scenario

WHO estimated 9.4 million new cases (including1.4 million cases among people living with HIV) in 2008 compared with 9.27 million new cases in 2007. 1.8 million people died from TB in 2008, including 500,000 people with HIV, equal to 4500 deaths a day (WHO, 2009). Among 9.27 million new cases in 2007, an estimated 44% (4.1 million) were new smear positive cases. Asia (South-East Asia and Western Pacific regions) accounts for 55% of global cases, and Africa account for 31%. There are an estimated 13.7 million prevalent cases and 0.5 million (511000) cases of multi-drug resistant TB (MDR-TB), of which 0.3 million were among people not previously treated for TB and 0.2 million were among previously treated TB cases. Of the 511000 incident cases of MDR-TB in 2007, 68% (349000) were smear positive (WHO, 2009).

3.5.2 Situation of TB in SAARC Region

The SAARC region, with an estimated 4.4 million prevalent cases and an annual incidence of 2.7 million TB cases, carries 29.3% of the global burden of TB. Four of the eight member countries in the region are among the 22 high burden countries, with India accounting for 21% of the world's cases. Among estimated 2.7 million TB cases, 1.2 million are sputum smear positive cases which are infectious for the community. Though there was progress in TB control after introduction of DOTS strategy in the region, the disease still claims more than 400000 lives each year. MDR-TB prevalence is estimated as 2.8% among new cases and 18.8% among previously treated cases (STAC Update, 2009).

3.5.3Tuberculosis in Nepal

Tuberculosis remains one of the major public health problems in Nepal. About 50% population is infected with TB, of which 60% are adult. Every year, 21,827 smear positive infectious TB incidence cases are expected to arise in the country. Although introduction of DOTS has already reduced the numbers of deaths, however 5000 to 7000 people still continue to die each year from TB. The latest survey conducted during

2006/07 shows MDR-TB as 2.9% among new TB cases and 11.7% among previously treated cases (STAC, 2009). During 2009, surveillance shows a prevalence of 5% of XDR-TB cases among MDR-TB cases registered (NTC, 2008/09).

3.6 Drug resistance tuberculosis

3.6.1 Definition

Drug resistance is defined as a decrease in susceptibility of sufficient degree from a wild strain that has never been exposed to the drug (WHO, 1997). With increasing use and misuse of antibiotics resistance has emerged in many microorganisms including *Mycobacterium tuberculosis* (WHO, 2008). Generally, when one percent or more of organisms in an isolate are found resistant to an anti-tubercular drug, therapeutic success is less likely to occur. It is then that the strain is considered resistant to the drug (Rom & Garay, 1996).

Multidrug-resistant tuberculosis (MDR-TB) is tuberculosis due to organisms which show high-level resistance to both isoniazid and rifampicin, with or without resistance to other anti-TB drugs. This is a very serious problem and pose a significant threat to the control of the disease globally. The loss of response to both the main bactericidal drug and the main sterilizing drug means that patients remain infectious for much longer, both in the community and in hospital, that treatment is lengthy, and that less effective and more toxic second-line drugs have to be use (BTS, 1998).

Extensively drug-resistant tuberculosis (XDR TB) is a relatively rare type of MDR TB. XDR-TB is defined as *Mycobacterium tuberculosis* infection that is resistant to isoniazid, rifampin, any fluoroquinolone, and at least one of three injectable drugs (i.e., amikacin, kanamycin, or capreomycin (CDC, 2008). XDR TB is of special concern for persons with HIV infection or other conditions that can weaken the immune system. These persons are more likely to develop TB disease once they are infected, and also have a higher risk of death once they develop TB disease.

3.6.2 Epidemiology of drug resistance tuberculosis

WHO estimates that 440 000 cases of MDR tuberculosis occurred in 2008 (3·6% of the estimated total incident tuberculosis episodes). Of these, 360 000 were new and relapse cases (i.e. likely transmission of an MDR strain), and 94 000 were in individuals previously treated for the disease (i.e. likely acquired resistance during previous treatment). India and China together carry nearly 50% of the global burden, followed by Russia (9%). MDR tuberculosis caused an estimated 150 000 deaths in 2008 (WHO, 2010). For the South East Asia region, MDR-TB prevalence is estimated as 2.8% among new cases and 18.8% among previously treated cases (STAC, 2010).

The initial drug resistance rate in Nepal is not so high comparing with that of other countries. First surveillance of TB drug resistance survey showed initial MDR 3.7% and acquired 12% (Shrestha, 1996). Gharti Chettri, 2002, reported that 8.57% of the isolates had primary MDR-TB while 100% of the isolates had acquired MDR-TB. Bhattarai, 2003, reported 4.61 % and 5% of the total isolates had primary and acquired MDR-TB respectively. Similarly the study carried out by Subba, 2007 reported Multi drug resistance (MDR) in untreated TB patients were found in 22.22 % and in treated TB patients was 37.20%. In Nepal, the latest surveys shows 2.9% among new TB cases and 11.7% among previously treated cases (STAC, 2010).

3.6.3 Development and acquition of drug resistance TB

Drug-resistant TB is not a recent phenomenon. Resistance to antituberculosis drugs arises as a result of spontaneous mutations in the genome of *M tuberculosis* and not as a result of horizontal gene transfer. These resistance-conferring mutations occur at predictable rates for each antituberculosis drug (eg, isoniazid 10^{-6}, rifampicin10^{-8}) (David, 1970). Thus, in patients with active tuberculosis disease, subpopulations of resistant mycobacteria spontaneously arise, and can emerge as the dominant strain in

the presence of drug-selection pressure (Post, 2004). Thus, drug-resistant tuberculosis is regarded as a man-made amplification of a natural phenomenon (Rijal *et al.,* 2002). With selection, drug-resistant organisms multiply to become the dominant strain; for example, isoniazid monotherapy selects for isoniazid-resistant mutants and allows them to multiply (figure 1). Resistance to additional tuberculosis drugs can be added in a step-wise manner to create tuberculosis strains that are resistant to several drugs; for example, treatment of isoniazid monoresistant tuberculosis with isoniazid and rifampicin selects for spontaneous rifampicin-resistant mutants. This process is referred to as acquired resistance (figure 1) (Victor, 2007) and is the rationale for the adage "never add a single drug to a failing regimen". However, for patients not responding to first-line tuberculosis treatment in high-burden settings, in the absence of drug susceptibility test data, common practice is to replace standard first-line treatment with an extended regimen that adds streptomycin as the only additional drug (category II regimen) (WHO, 2003). This practice has predictably led to acquisition of further drug resistance (Cox, 2007). Once created, drug-resistant strains can be transmitted, giving rise to drug-resistant tuberculosis in individuals never previously exposed to antituberculosis drugs (primary resistance) (Zhao, 2009). Drug-resistance mutations were initially postulated to exert a fitness cost, rendering those strains too weak to be transmitted. However, results of many studies have now confirmed that transmission of drug-resistant tuberculosis does occur. The global epidemic of drug-resistant tuberculosis is due to a combination of acquired resistance and primary transmission (Cox, 2007; Zhao, 2009).

The emergence of drug resistant strains of *M. tuberculosis* in a population has been associated with a variety of management, health provider and patient related factors (Malla, 1996). The most frequent causes of resistance in the community and generation of MDR-TB under epidemic conditions are poorly organized or funded national tuberculosis programme; lack of guidelines and treatment monitoring; inadequate supply or poor quality of drugs; inadequqte drug intake and malaborption or side

effects; poor infection control strategy and HIV (Rijal, 2002). A crucial element in the emergence of drug resistance is the lack of a properly organized system to ensure prompt diagnosis and effective treatment (Reichman, 1997). For this reason, the level of anti-TB drug resistance in a population is an indicator of the effectiveness of a National TB Programme (WHO/IUATLD, 1997).

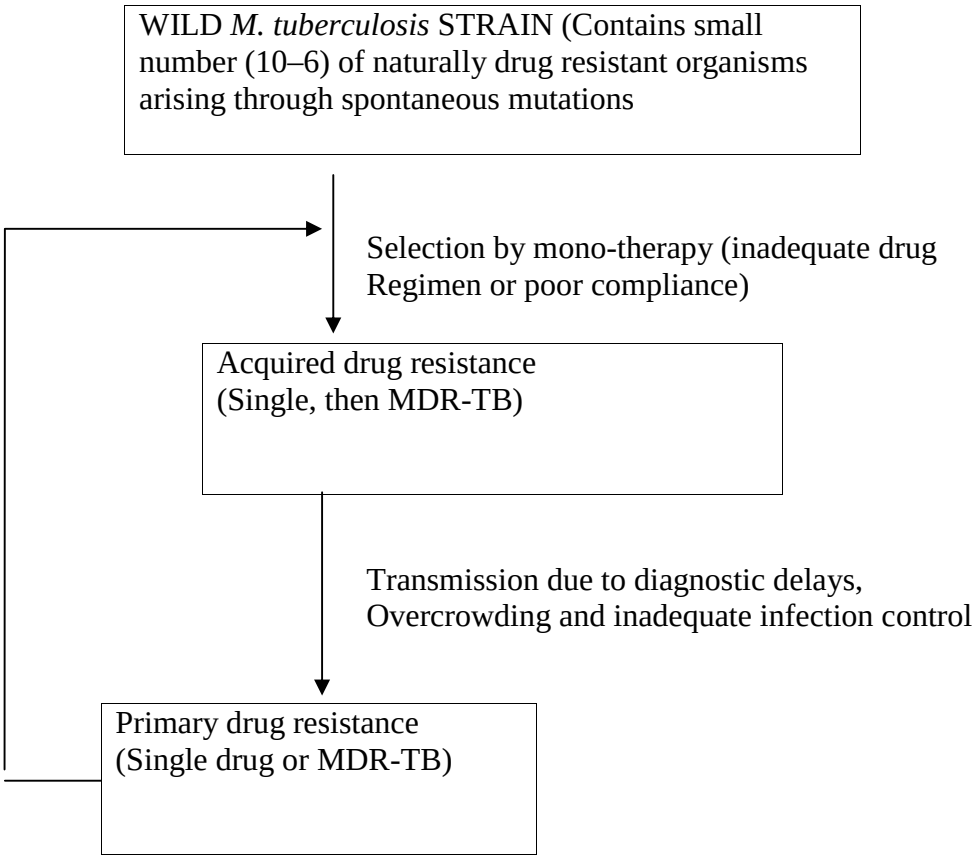

Figure 1. The development and spread of drug- and multi-drug resistant tuberculosis

3.6.4 Anti-tubercular drugs and their Molecular genetic basis of resistance

The genetic bases of resistance to most anti-TB drugs are established.

Isoniazid (INH)

INH is the most widely used first-line anti-tuberculosis drug in all effective regimens for the treatment of TB disease and latent infection. *M. tuberculosis* is highly susceptible to INH (minimum inhibitory concentration [MIC] 0.02–0.2 μg/ml). INH is only active against growing tubercle bacilli, and is not active against non-replicating bacilli or under anaerobic conditions. INH is a prodrug that is activated by the catalase peroxidase enzyme (KatG) encoded by the *kat*G gene to generate a range of highly reactive species such as superoxide, peroxide and hydroxyl radical, nitric oxide which then attack multiple targets in *M. tuberculosis*. The primary target of INH inhibition is thought to be the InhA enzyme (enoyl-acyl carrier protein reductase), involved in elongation of fatty acids in mycolic acid synthesis. The active species (isonicotinic-acyl radical or anion) derived from KatG-mediated INH activation reacts with NAD(H) (nicotinamide adenine dinucleotide) to form an INH-NAD adduct, and then attacks InhA (Zhang *et al.*, 1992). Resistance to INH occurs more frequently than for most anti-tuberculosis drugs, at a frequency of 1 in $10^{5–6}$ bacilli in vitro. INH-resistant clinical isolates of *M. tuberculosis* often lose catalase and peroxidase enzyme encoded by *kat*G. Mutation in *kat*G is the main mechanism of INH resistance. KatG S315T mutation is the most common mutation in INH-resistant strains, accounting for 50–95% of INH-resistant clinical isolates (Hazbon, 2006). Resistance to INH can also occur by mutations in the promoter region of *mab*A/*inh*A operon. Mutations in *inh*A not only cause INH resistance, they also confer cross-resistance to the structurally related drug, ethionamide (ETH) (Banerjee, 1994).

Rifampicin (RMP)

RMP is an important first-line drug for the treatment of TB. RMP is bactericidal for *M. tuberculosis*, with MICs ranging from 0.05 to 1 μg/ml on solid or liquid media, but the MIC is higher in egg media (MIC =2.5–10 μg/ml). RMP is active against both growing and stationary phase bacilli with low metabolic activity. The latter activity is related to

its high sterilizing activity in vivo, correlating with its ability to shorten TB treatment from 12–18 months to 9 months (Mitchison, 1985). RMP interferes with RNA synthesis by binding to the β subunit of the RNA polymerase. The RMP-binding site is located upstream of the catalytic center and physically blocks the elongation of the RNA chain. In *M. tuberculosis*, resistance to RMP occurs at a frequency of 10^{-7} to 10^{-8}. As in other bacteria, mutations in a defined region of the 81 base pair (bp) region of the *rpo*B are found in about 96% of RMP-resistant *M. tuberculosis* isolates. Mutations at positions 531, 526 and 516 are among the most frequent mutations in RMP-resistant strains (Telenti, 1993).

Pyrazinamide (PZA)

PZA plays a unique role in shortening TB treatment from the previous 9–12 months to 6 months because it kills a population of persister bacilli in acidic pH environment in the lesions that is not killed by other drugs (Mitchison, 1985). PZA is an unconventional and paradoxical antituberculosis drug that has high sterilizing activity in vivo, but no activity against tubercle bacilli at normal culture conditions near neutral pH. PZA is only active against *M. tuberculosis* at acid pH (e.g., 5.5). PZA activity is enhanced under low oxygen or anaerobic conditions. PZA is a prodrug that requires conversion to its active form, pyrazinoic acid (POA), by the pyrazinamidase/nicotinamidase enzyme encoded by the *pnc*A gene of *M. tuberculosis.* The POA produced intracellularly reaches the cell surface and disrupts membrane potential in *M. tuberculosis* which affects membrane transport (Zhang, 2003). PZA-resistant *M. tuberculosis* strains lose pyrazinamidase/ nicotinamidase activity. Cloning of *M. tuberculosis pnc*A66 shown that defective pyrazinamidase activity due to *pnc*A mutations is the major cause of PZA resistance. The *pnc*A mutations are highly diverse and scattered along the gene, which is unique to PZA resistance. PZA is active only against *M. tuberculosis* complex organisms (*M. tuberculosis, M. africanum* and *M. microti*), but not *M. bovis,* due to a characteristic mutation in its *pnc*A gene (Scorpio, 1996). Strains of *M. bovis*, including

bacille Calmette-Guérin (BCG), are naturally resistant to PZA and lack pyrazinamidase; these features are commonly used to distinguish *M. bovis* from *M. tuberculosis*.

Ethambutol (EMB)

EMB is a first-line drug that is used in combination with INH, RMP and PZA to prevent the emergence of drug resistance. The MICs of EMB for *M. tuberculosis* are in the range of 0.5–2 μg/ml. EMB is a bacteriostatic agent that is active for growing bacilli and has no effect on non replicating bacilli. It inhibits the polymerization of cell-wall arabinan of arabinogalactan and of lipoarabinomannan, and induces the accumulation of D-arabinofuranosyl-P-decaprenol, an intermediate in arabinan biosynthesis (Mikusov, 1995). Arabinosyl transferase, encoded by *emb*B, an enzyme involved in the synthesis of arabinogalactan, has been proposed as the target of EMB in *M. tuberculosis* and *M. avium.* In *M. tuberculosis, emb*B is organized into an operon with *emb*C and *emb*A in the order *emb*CAB. Mutation to EMB resistance occurs at a frequency of 10^{-5}. Mutations in the *emb*CAB operon, in particular *emb*B, and occasionally *emb*C, are responsible for resistance to EMB (Telenti, 1997).

Streptomycin (SM)

SM is an aminoglycoside antibiotic which kills actively growing tubercle bacilli with MICs of 2–4 μg/ml but it is inactive against non-growing or intracellular bacilli (Mitchison, 1985). SM inhibits protein synthesis by binding to the 30S subunit of bacterial ribosome, causing misreading of the mRNA message during translation. The site of action of SM is the 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA. Resistance to SM is caused by mutations in the S12 protein encoded by *rps*L gene and 16S rRNA encoded by *rrs* gene. Mutations in *rps*L and *rrs* are the major mechanism of SM resistance, accounting for respectively about 50% and 20% of SM-resistant strains (Honore, 1994).

3.7 Laboratory diagnosis of tuberculosis

No single diagnostic test for TB exists at present that can be performed rapidly, simply, inexpensively, and accurately as a stand-alone test for active TB. Although many new (molecular) diagnostic methods have been developed, acid fast bacilli (AFB) smear microscopy and culture on Löwenstein-Jensen medium are still the “gold standards” for the diagnosis of active TB and, especially in low-resource countries (Waard, 2007).

3.7.1 Specimen collection and transport

For patients with a productive cough, collection of an early morning, freshly expectorated specimen is recommended (Christie, 1995). For patients unable to produce sputum, induction of sputum production via inhalation of nebulized hypertonic saline should be considered. WHO recently recommend that “at least two sputum samples” should be collected (WHO 1994). If it is not possible to collect a sputum sample, even with induction, lavage of gastric secretion, bronchoscopy with lavage, brushings or even a transbronchial biopsy, or needle aspiration may be considered. When disease is isolated to an extrapulmonary site, collection of excretions (urine or stool), aspiration of fluid (e.g., pleural fluid, ascites, cerebral spinal fluid), or tissue biopsy for AFB smear, culture, and histology may be necessary for diagnosis (Talavera, 1994).

Specimens should be collected in sterile, leak-proof containers and labeled with the patient’s name and/or identification number before anti-tuberculosis chemotherapy is started. Blood and other specimens prone to coagulate, including bone marrow, synovial, pleural, pericardial and peritoneal fluids, should be collected in tubes containing sulfated polysaccharides or heparin. Sulfated polysaccharides are the preferred anticoagulants as they enhance the growth of mycobacteria. Lymph nodes, skin lesion material, and tissue biopsy specimens should come without preservatives or fixatives and should not be immersed in saline or any fluid. Once in the laboratory, tissue specimens are homogenized in a sterile tissue grinder with a small amount of

sterile saline solution before AFB smear staining or culture. Abscess contents or aspirated fluids can be collected in a syringe. If renal TB is suspected, the specimen of choice is the first-morning urine, at least 50 ml, obtained by catheterization or from the midstream clean catch on three consecutive days (Lam *et al.*, 2010).

Specimens should be transported rapidly to the laboratory to avoid overgrowth by other microorganisms. This is particularly true for specimens from non-sterile sites, such as sputum. When the transport or the processing is delayed, specimens should be stored for not more than five days at 4°C until transported or presented for bacteriological processing. The cetylpyridinium chloride (CPC) method is widely used for the transport of sputum specimens (Smithwick, 1975). Sodium carbonate was also found to be a better preservative of sputum specimens for AFB smear microscopy as well as culture (Bobadilla, 2003).

3.7.2 Processing of specimen

Specimen processing for the recovery of AFB from clinical specimens involves complex steps. Specimen from sterile sites can be inoculated directly to media (small volume) or concentrated to reduce volume. Other specimens require decontamination and concentration (Forbes *et al.*, 2007). Before processing, sputum specimens must be classified at the laboratory with regard to their quality, i.e. bloody, purulent, mucopurulent, saliva. In general, AFB smear microscopy from body fluids is rarely positive and the whole sediment from concentrated specimens should rather be cultured (Waard, 2007).

Sputum samples are digested and decontaminated to liquefy the organic debris surrounding the microbes, so that the decontaminating agents can kill the contaminants and to allow surviving mycobacteria access to nutrients of primary isolation media. The digestion decontamination procedures include: N- acetyl-L-cystein alkaline (NALC-NaOH), sodium hydroxide; zephiran trisodiumphosphate; and sodium laurylsulfate

methods, oxalic acid of which Modified petroff NaOH (4%) is widely used in developing countries (Kubica, 1984).

3.7.3 Smear microscopy

Smear microscopy continues to be the most widely used test for active TB (Pai *et al.*, 2009). The two most widely used staining techniques to observe AFB are ZN staining and fluorescence method (WHO, 1998). Direct microscopic examination of sputum for AFB is inexpensive, rapid, and relatively easy to perform. The sensitivity of a single sputum AFB smear is 30% to 40%. Because direct microscopy cannot distinguish between *M. tuberculosis* and nontuberculous mycobacteria (NTM), specificity is a concern but studies have shown that the AFB smear to continues to have a high specificity (>90%). The sensitivity of AFB smear is such that it requires 6000 to 10,000 organism per ml of sample to register as positive. While this limits the sensitivity of microscopy, it also serves to identify patients who are highly infectious and therefore the highest priority for immediate anti-TB treatment (Lam *et al.*, 2010).

Methods to improve the sensitivity of AFB smear include fluorescence microscopy, which uses an acid-fast fluorochrome dye with an intense light source. Fluorescence microscopy has higher sensitivity and comparable specificity to conventional AFB smear. Fluorochrome staining is simpler than Ziehl-Neelsen methods, and fluorescence microscopy requires less time and effort from microscopists. In addition, the adaptation of fluorescence microscopes with light-emitting diodes makes fluorescence microscopy more reliable in resource-limited laboratories (Pai *et al.*, 2009).

3.7.4 Culture

Cultivation of *M. tuberculosis* from clinical samples is the gold standard for the diagnosis of active TB. It can detect 100 bacilli/ml of sputum in comparison with

6,000–10,000 bacilli/ml needed for microscopy. It also provides material for further identification, genotyping and drug susceptibility testing (Kent & Kubica, 1985).

The sputum is liquefied and decontaminated from other bacteria and subsequently inoculated. Conventional methods of culture have relied on egg-based and agar-based media, such as the Lowenstein-Jensen (LJ) medium and Middlebrook agar. Egg based L-J media is most widely used in developing country and has a long shelf life and very cheap to prepare. It is generally regarded as the gold standard, as it results in a very high recovery rate of mycobacteria However, it is laborious and time consuming requiring from 3–8 weeks to obtain the results (Gercia, 1998). More rapid growth is achieved by using liquid media such as Middlebrook or Dubos broth .The introduction of BACTEC 460 radiometric technique has reduced detection time to 10 days, and with MGIT 960 to 12.7 days compared with 20 days with the solid media. Primary isolation in liquid media is more expensive and more sensitive to contamination furthermore aerosols are more easily created as compared to solid media (Kent & Kubica, 1985). Nevertheless, the specificity of culture has been reported to be as high as 98% and sensitivity greater than 80% (Levy, 1989). Culture, although it offers improved sensitivity, is time consuming and requires high cost safety facilities. Despite these limitations, culture has been recommended as a cost effective method for assessing and monitoring the prevalence of drug resistant TB in any TB control Programme (IUATLD, 1998)

3.7.5 Species identificatiion

An initial identification as *M. tuberculosis* is defined traditionally on AFB bacilli from slowgrowing, non-pigmented colonies that are niacin positive, are inhibited by p-nitrobenzoic acid and display nitratase activity. Additional tests that confirm an isolate as *M. tuberculosis* are susceptibility to pyrazinamide, growth on thiophene carboxylic acid hydrazide, absence of catalase production at 68°C and absence of iron uptake (Waard *et al.*, 2007).

3.7.5.1 Conventional methods of identification

Niacin accumulation test:

Nicotinic acid or niacin is produced by all mycobacteria, but some species, such as *M. tuberculosis*, *Mycobacterium simiae* and *M. bovis* BCG, excrete it due to a blockade in their scavenging pathway. The excreted niacin accumulates in the culture medium and is evidenced in the presence of cyanogen halide with a primary amine. Niacin-negative *M. tuberculosis* strains are extremely rare (Waard *et al.*, 2007).

Growth in the presence of p-nitrobenzoic acid:

This compound inhibits the growth of several species in the *M. tuberculosis* complex: *M. tuberculosis, M. bovis, M. africanum* and *M. microti* (Leão, 2004). It has been used for the selective screening of *M. tuberculosis. M. tuberculosis* does not grow on PNB at 4 weeks of incubation and even if few colonies are observed on the medium, it should be regarded as negative. All other mycobacteria are resistant to PNB (Fujuki, 2001).

Nitrate Reduction Test:

Mycobacteria differ quantitatively in their abilities to reduce nitrate and the test measures the ability of a strain to produce the enzyme nitrate reductase. It is valuable for the identification of some mycobacteria that possess similar characteristics of colony morphology, growth rate and pigmentation. *M. tuberculosis, M. kansasii, M. szulgai* and most rapid growers are nitrate reductase positive. This test is particularly useful for differentiating *M. tuberculosis*, which gives a positive reaction, from *M. bovis*, which is negative (Vincent, 2003).

Thiophene 2 carboxylic acid hydrazide (TCH) test:

TCH test is used to distinguish niacin positive *M. bovis* from *M. tuberculosis* and other non-chromogenic slowly growing mycobacteria. *M. bovis* is susceptible to low

concentrations (1 to 5µg/ml) of TCH where as *M. tuberculosis* and other mycobacteria are resistant to the inhibitory action of this compound (Kent & Kubica, 1967).

Pyrazinamidase Test:

The enzyme pyrazinamidase hydrolyses pyrazinamide to pyrazinoic acid. This acid is detected by the addition of ferrous ammonium sulfate to the culture medium. The formation of a pink ferrous- pyrazinoic acid complex indicates a positive test. This test is most useful in separating *M. tuberculosis which is positive from M. bovis* (Wayne, 1974).

Catalase test:

Catalase is an intracellular enzyme that transforms hydrogen peroxide to oxygen and water. The 68°C catalase is a heat-tolerance test measuring the catalase activity at high temperature. Characteristically, *M. tuberculosis* gives negative results, as do other species in the *M. tuberculosis* complex (Vincent, 2003).

3.7.5.2 Non conventional methods for identification

These methods are advanced and nucleic acid based techniques, which show promise as more rapid, sensitive and specific for identification of mycobacteria and detection of *M. tuberculosis* directly from processed specimens or indirectly from primary culture, but are rather expensive. Moreover, they are helpful for strain characterization (van Embden *et al.,* 1993). Molecular methods include amplification of species-specific nucleic acid sequences, PCR amplification and restriction enzyme analysis, hybridization with species-specific oligonucleotide probes with or without prior DNA amplification, nucleic acid sequence determination, and restriction fragment length polymorphism (RFLP) analysis (Pai *et al.,* 2009). Mycolic acid profiles of mycobacteria were also proposed as a rapid alternative for identification.

Molecular methods offer several advantages over conventional techniques for the rapid detection and identification of *M. tuberculosis*, such as the turnaround time for results, reliability, reproducibility and the possibility to improve patient management (Palomino, 2005).

3.8 Diagnosis of drug resistance tuberculosis

Diagnosis of drug resistance TB is one of the essential steps in the management of tuberculosis. DST usually requires an additional 2-4 weeks after the primary isolation. To meet this requirement several methods have been developed: conventional (absolute concentration, resistance ratio and proportion) BACTEC, molecular methods etc. However, these methods have a number of limitations.

3.8.1 Conventional Phenotypic drug susceptibility testing

This method is essential to measure the ability of *M. tuberculosis* proliferation in a drug containing solid or liquid media. It can be performed either directly on clinical specimens confirmed AFB smear positive or indirectly on a culture isolate of *M. tuberculosis* (Canetti *et al.*, 1969). The following four standard methods are available for indirect drug susceptibility testing:

3.8.1.1 The Resistance Ratio (RR) method.

A ratio is obtained by dividing the minimum inhibitory concentration (MIC) of the test strain with the MIC of standard susceptible strain. RR of 8 defines drug resistance, while RR of 2-drug susceptibility. RR between 2 and 8 is considered intermediate and, on repeated test, it can be considered as resistant (Canetti *et al.*, 1969).

3.8.1.2 The Proportion Method.

This method consists in calculating the proportion of resistant bacilli present in a strain. Resistance is defined as more growth on drug containing media as compared to the growth on a drug free control containing ten fold serially diluted tubercle bacilli suspension or a ratio obtained (i.e. above the critical proportion) by dividing the number

of colonies grown on drug containing medium with the number of colonies grown on drug free control. The critical proportion of resistant bacilli required to define a strain as resistant is 1% for all drugs tested (Canetti *et al.,* 1969; Kubica, 1984).The most commonly used proportion method on LJ-medium or Middlebrook agar requires a minimum of 3–4 weeks to produce results (Heifets, 1991).

3.8.1.3 The Absolute Concentration Method.

Drug resistance is defined as the growth of 20 colonies on media containing a defined concentration of the test drug (Canetti *et al.,* 1969).

3.8.1.4 The BACTEC460 Radiometric Method.

It is a variant of the proportion method. The growth of mycobacteria in drug free control and drug containing vials is quantified as growth index based on the amount of 14C-labeled CO_2 produced. A strain is resistant if the difference in growth index (GI) of control is less than that of the vial with drugs (Roberts *et al.,* 1983). The BACTEC 460 system has the advantage of having shorter turnaround time requiring one week instead of three as compared to solid media DST. BACTEC uses 14-C labeled palmitic acid for the detection of bacterial growth, resulting in free $^{14}CO_2$, which is quantified daily (Siddiqi, 1981).

There are several other rapid methods in use: MGIT, ESP Myco, MB/BacT. In all these systems, a modified 7H9 broth is used. An indirect susceptibility test has a turnaround time of 7.1 days for MGIT 960, 18-22 days for BACTEC 460 and about 24 days for both ESP and MB/BacT systems (Bemer *et al.*, 2004; El-Sayed Zaki, 2007).

3.8.2 Genotypic methods of drug resistance detection

Genotypic approaches detect the genetic determinants of resistance involving nucleic acid amplification to detect gene mutations known to be associated with drug resistance. Genetic testing is often referred to as “rapid molecular testing” since results can be available with less than one day turnaround time if done directly on sputum.

Unfortunately, direct molecular testing generally requires a high bacterial load in the sputum, which essentially excludes smear-negative TB patients from this strategy. These tests are becoming cheaper and easier and have been shown to detect both isoniazid and rifampicin resistance with a high degree of accuracy (Bernard, 2008).

This method involve two basic steps: nucleic acid amplification such as polymerase chain reaction (PCR), to amplify the sections of the *M. tuberculosis* genome known to be altered in resistant strains; and a second step of assessing the amplified products for specific mutations correlating with drug resistance (Tortoli, 2007).

DNA sequencing of PCR-amplified products has been the most widely used method, becoming the gold standard. It has been performed by both manual and automated procedures although the latter has been the most commonly used. It has been thoroughly used for characterising mutations in the rpoB gene in rifampicin-resistant strains and to detect mutations responsible for resistance to other anti-TB drugs (Telenti, 1993).

Several other genotypic methods have been proposed to detect resistance to antibiotics, such as PCR-single strand conformation polymorphism, PCR-heteroduplex formation, and solid-phase hybridisation assays. Moreover, the Line Probe Assay (LiPA-Rif) based on the hybridisation of amplified DNA from cultured strains or clinical samples to ten probes encompassing the core region of the rpoB gene of *M. tuberculosis*, immobilised on a nitrocellulose strip (Rossau, 1997), and the GenoType *Mycobacterium tuberculosis* test (MTB-DR) (Hain, Germany), a commercial system for the detection of the *M. tuberculosis* complex and its resistance to rifampicin and isoniazid from culture samples based on the detection of the most common mutations in the rpoB and katG plus InhA genes, respectively. The LiPA-Rif assay has been evaluated in different settings with encouraging results (Rossau, 1997). The second generation GenoType® MTBDR*plus assay shows* 100% concordance with phenotypic resistance testing for RMP resistance, and 96.5% sensitivity and 95.8% specificity for INH resistance overall (Causse, 2008).

DNA microarrays and real-time PCR techniques have also been proposed as alternative methods for drug resistance detection; the former still beyond the reach of clinical diagnostic laboratories, and the latter being increasingly evaluated with promising results (Palomino, 2005).

3.8.3 New Phenotypic methods

New phenotypic methods assess inhibition of M. tuberculosis in the presence of antibiotics by detecting earlier signs of growth using various technologies, for example, the measurement of metabolism with the aid of colour indicators, or oxygen consumption, by early visualisation of micro-colonies, and by the use of phages.

3.8.3.1 The microscopic observation broth-drug susceptibility assay (MODS)

A MODS is based on the observation of the characteristic cord formation of *M. tuberculosis* when growing in a liquid medium (Caviedes *et al.*, 2000). Reading of the plates is performed with an inverted microscope at 40× magnification to identify the typical cord formation of *M. tuberculosis*. Growth in the drug-containing wells and in the growth control is interpreted as resistance. Although fairly accurate to detect resistance, especially to RMP, the requirement of an inverted microscope to read the plates and biosafety concerns related to the use of liquid medium may hamper its application (Moore, 2006).

3.8.3.2 Phage-based methods

Two formats of phage-based methods have been proposed as rapid techniques for drug-resistance detection in *M. tuberculosis*, the phage amplification method and the luciferase reporter method. Phage amplification methods are based on the replication of the phage D29 inside viable mycobacteria while the luciferase reporter method relies on the production of light by an engineered phage containing the luciferase gene. Results

are available, on average, in 2 days with a high sensitivity, but variable and slightly lower specificity (Pai, 2009).

3.8.4 Colorimetric methods

Colorimetric methods are based on the reduction of a coloured indicator added to the culture medium after *M. tuberculosis* has been exposed in vitro to different antibiotics. Resistance is detected by a change in the colour of the indicator, which is directly proportional to the number of viable mycobacteria in the medium (Solis, 2005). These assays can be performed in microtiter plates or in culture tubes with results available in 7–8 days. Various redox indicators have been proposed, with Alamar blue, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and resazurin giving the most reliable and accurate results for DST of INH and RMP. Colorimetric methods seem more appropriate for reference laboratories with the facilities and biosafety conditions to manipulate small volumes of liquid cultures in a microplate format (Palomino, 2007). A recent meta-analysis of colorimetric methods using redox indicators for DST of *M. tuberculosis* showed that there is evidence of high sensitivity and specificity of these methods (Martin *et al.*, 2007).

3.8.5 Nitrate Reductase Assay

The nitrate reduction assay is a very simple technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding a chemical reagent to the culture medium. *M. tuberculosis* is cultivated in the presence of the antibiotic and its ability to reduce nitrate is measured after 10 days of incubation. Resistant strains will reduce the nitrate revealed by a pink-red colour in the medium, while susceptible strains will lose the capacity to reduce nitrate, thus producing a non-coloured reaction, as they are inhibited by the antibiotic. It has the added advantage of using the same format and culture medium as used in the standard proportion method (Angeby *et al.*, 2002).

In a recent multicenter multi center study that evaluated the performance of the NRA for detecting resistance to the first-line drugs, the test performed very well for INH, RMP and EMB with an accuracy of 96.6 % to 98 %. Lower values, were obtained for SM (Martin, 2005a). However, the NRA was easily implemented in settings with limited laboratory facilities. Moreover the assay also applied directly on sputum samples and produced variable results for sensitivity and specificity; the best results were obtained for INH and RMP resistance detection (Musa, 2005; Solís, 2005). The studies have shown the feasibility for implementation of the NRA as a direct test on sputum samples that warrant further evaluations in target populations.

The programmatic implementation of rapid DST testing using the Griess method, on modified LJ medium in two populous districts of Lima, Peru, used as a rapid screen to test for MDR-TB, the Griess method was successfully incorporated into their diagnostic algorithms for only US$4.80 per sample (not including labour or capital costs), resulting in a shortened time to result of 31 days compared to 99 days using conventional DST (Asencios, 2008).

Members of *M. tuberculosis* complex contain two homologues of prokaryotic dissimilatory nitrate reductases (NRs) encoded by narGHJI and narX. The constitutive NarGHJI encodes membrane bound NR (Weber, 2000).This enzyme catalyses reduction of nitrate to nitrite.

M. tuberculosis is one of the most proficient reducers of nitrate amongst the mycobacteria. The inability of M. bovis and BCG to efficiently reduce nitrate under both aerobic and hypoxic conditions was ascribed to inactive narGHJI and narK2X gene/gene products (Stermann *et al.*, 2004; Sohaskey & Modesti, 2009).

The NR activity of *M tuberculosis* observed in culture is attributable exclusively to NarGHJI, with no discernable contribution from NarX. Unlike other organisms NarGHJI enzyme levels are not induced in *M. tuberculosis* in response to hypoxia or

stationary phase. Instead, the increase in NR activity observed in hypoxic culture is due elevated levels of the nitrate and nitrite transporter, NarK2 (Sohaskey & Wayne 2003). NarK2 is able to sense the redox state of the cell so that nitrate is transported into the cell under reducing but not oxidizing conditions (Sohaskey, 2005). However, NR doesnot appear to support the anaerobic growth of M. tuberculosis.

3.9 Treatment of Tuberculosis

Recently, WHO and Tuberculosis Coalition for Technical Assistance, has published the new guidelines (4^{th} edition) for the treatment and care of tuberculosis. New guidelines have abandoned Categories I–IV, which were used to prioritize patients for treatment. To replace Categories I–IV, this fourth edition groups patients (and standard regimens recommended for each group) according to the likelihood of their having drug resistance. This edition uses the same patient registration groups as those used for recording and reporting, which differentiate new patients from those who have had prior treatment. Registration groups for previously treated patients are based on the outcome of their prior treatment course: failure, relapse, and default (WHO, 2009).

According to WHO, National TB control programmes will need three standard regimens:

i. New patients presumed or known to have drug-susceptible TB

The 2-month rifampicin regimen is associated with more relapses and deaths than the 6-month rifampicin regimen. WHO therefore recommends the following for new patients presumed or known to have drug-susceptible TB.

New patients with pulmonary TB should receive a regimen containing 6 month rifampicin: 2HRZE/4HR. Wherever feasible, the optimal dosing frequency for new patients with pulmonary TB is daily throughout the course of therapy. However alternative to this are: New patients with pulmonary TB may receive a daily intensive

phase followed by three times weekly continuation phase [2HRZE/4$(HR)_3$] provided that each dose is directly observed or three times weekly dosing throughout therapy [2$(HRZE)_3$/4$(HR)_3$] is another alternative, provided that every dose is directly observed and the patient is NOT living with HIV or living in an HIV-prevalent setting.

In populations with known or suspected high levels of isoniazid resistance, new TB patients may receive HRE as therapy in the continuation phase as an acceptable alternative to HR.

ii. Standard regimens for previously treated patients

The approach to the initiation of retreatment depends on the country's laboratory capacity, specifically when (or if) DST results are routinely available for the individual patient. The WHO guideline on retreatment patient is described in table 3.9.

iii. MDR regimen

Prompt identification of MDR and initiation of MDR treatment with second-line drugs gives a better chance of cure and prevents the development and spread of further resistance. If their MDR is not detected and treated with second-line drugs, these patients will suffer poor outcomes and spread MDR in their communities.

FDCs are thought to prevent acquisition of drug resistance due to monotherapy, which may occur with separate ("loose") drugs. With FDCs, patients cannot be selective in the choice of drugs to ingest. Prescription errors are likely to be less frequent because dosage recommendations are more straightforward, and adjustment of dosage according to patient weight is easier. The number of tablets to ingest is smaller and may thus encourage patient adherence.

Table 3.9: Standard regimens for previously treated patients depending on the availability of routine DST to guide the therapy of individual retreatment patients

DST	Likelihood of MDR(patient registration group)	
Routinely available for previously treated patients	High (failure)	Medium or low (relapse, default)
Rapid molecular-based method	DST results available in 1–2 days confirm or exclude MDR to guide the choice of regimen	
Conventional method	While awaiting DST results: Empirical MDR regimen 2HRZES/1HRZE/5HRE Regimen should be modified once DST results are available.	Regimen should be modified once DST results are available.
None (interim)	Empirical MDR regimen Regimen should be modified once DST results or DRS data are available.	2HRZES/1HRZE/5HRE for full course of treatment. Regimen should be modified once DST results or DRS data are available.

8. REFERENCES

Angeby KA, Klintz L, Hoffner SE (2002) Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. J Clin Microbiol 40: 553–5

Aranaz A, Cousins D, Mateos A and Dominguez L (2003) Elevation of *M. tuberculosis* subsp. *caprae* Aranaz *et al.*, 1999 to species rank as *M. caprae*. Int **J** Syst Evol Microbiol 53: 1785-9

Banerjee A, Dubnau E, Quemard A *et al*. (1994) *inh*A a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 263: 227–30

Barnard M, Albert H, Coetzee G *et al*. (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: 787–92

Barnes P, Fong S, Brennan P, Twomey P, Mazumdar A, Modlin R (1990) Local production of tumor necrosis factor and IFN-gamma in tuberculosis pleuritis. J Immunol 145: 149-54

Basnet RB (1998) Cytodiagnosis of clinically suspected tuberculosis cervical lymphadenitis and its correlative study with tuberculin test, AFB stain and culture of Mycobacteria. MD thesis submitted to the TU, Kathmandu, Nepal

Bemer P, Bodmer T, Munzinger J, Perrin M, Vincent V, Drugeon H (2004) Multicenter evaluation of the MB/BacT system for susceptibility testing of *Mycobacterium tuberculosis.* J Clin Microbiol 42: 1030–4

Besra GS, Chatterjee D (1994) Mycobacterium In: Collier L, Balows A and Sussman M (eds.) Topley & Wilson's Microbiology and Microbial Infections, Systematic Bacteriology. 9th edition, Arnold, a member of the Hodder Headline Group, London 549-76

Bhattarai NR (2003) Anti-tuberculosis treatment resistance in pulmonary tuberculosis patients visiting German Nepal Tuberculosis Project (GENETUP), Kalimati, Kathmandu, Nepal. MSc Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kirtipur, Katmandu, Nepal

Bloom BR, Murry CJL (1992) Tuberculosis: Commentary on a re-emerging killer. Science 257: 1055-64

Bobadilla-del-Valle M, Ponce-de-Leon A, Kato-Maeda M *et al.* (2003) Comparison of sodium carbonate, cetyl-pyridinium chloride, and sodium borate for preservation of sputa for culture of *Mycobacterium tuberculosis*. J Clin Microbiol 41: 4487-8

Brennan MJ, Delogu G (2002) The PE multigene family: a 'molecular mantra' for mycobacteria. Trends Microbiol 10: 246-9

Brennan PJ (2003) Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis.* Tubercle 83: 91–7

Brennan PJ, Draper P (1994) The cell envelope of *M. tuberculosis*. In: Bloom, R.L. (eds.). Tuberculosis: Pathogenesis, protection and control. ASM Press. Washington, DC, USA 271-84

Burgos MV, Méndez JC, Ribon W (2004) Molecular epidemiology of tuberculosis: methodology and applications. Biomedica 24 (supl): 188-201

Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, Rist N, Smelev NA (1969) Advances in techniques of testing mycobacterial drug sensitivity and the use of sensitivity tests in tuberculosis control programmes. Bull World Health Org 41: 21-43

Canetti G, Froman S, Grossett J, Handiroy P Langerov'a M, Mahler HT *et al*. (1963) Mycobacteria: laboratory methods for testing drug susceptibility and resistance. Bull World Health Organ 29: 565-78.

Causse M, Ruiz P, Gutierrez J B, Zerolo J, Casal M (2008) Evaluation of new GenoType® MTBDRplus for detection of resistance in cultures and direct specimens of *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 12: 1456–60

Caviedes L, Delgado J, Gilman RH (2002) Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. J Clin Microbiol 40: 1873–4

Caviedes L, Lee TS, Gilman RH, *et. al.*, (2000) Rapid, efficient detection and drug susceptibility testing of *Mycobacterium tuberculosis* in sputum by microscopic

observation of broth cultures. The Tuberculosis Working Group in Peru. J Clin Microbiol 38: 1203-8

Center for Disease Control and Prevention (2008) TB Elimination: Extensively Drug-Resistant Tuberculosis (XDR TB). US Department of Health and Human Services, Atlanta, Georgia

Christie JD, Callihan DR (1995) The laboratory diagnosis of mycobacterial diseases. Challenges and common sense. Clin Lab Med 15(2): 279–306

Cole ST (2002) Comparative and functional genomics of the *Mycobacterium tuberculosis* complex. Microbiol 148: 2919-28

Cole ST, Brosch R, Parkhill J, Garnier T, Churchur C, Harris D, Gordon SV, *et. al*., (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537-44

Cousins DV, Bastida R, Cataldi A, Murray A, Butler NR, Romano MI, Ahmed N, Collins DM (2003) Tuberculosis in seals caused by a novel member of the *M. tuberculosis* complex*: M. pinnipedi* sp. nov. Int J Syst Evol Microbiol 53: 1305-14

Cox HS, Niemann S, Ismailov G, *et. al*., (2007) Risk of acquired drug resistance during short-course directly observed treatment of tuberculosis in an area with high levels of drug resistance. Clin Infect Dis 44: 1421–7

Daniel TM (2006) The history of tuberculosis. Respir Med 100: 1862-70

David HL (1970) Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. Appl Microbiol 20: 810–14

De Waard JH, Robledo J (2007) Conventional diagnostic methods In: Polomino JC, Leao SC, Ritacco V (eds.) Tuberculosis 2007 from basic science to patient care, 1st edition, Bourcillier kamps 18: 401-24

Des Jardin LE, Schlesinger LS (2000) Identifying *Mycobacterium tuberculosis* virulence determinants: new technologies for a difficult problem. Trends Microbiol, 8(3): 97-9

Domingue GJ, Woody HB (1995) Bacterial persistence and expression of disease. Clin Microbiol Rev 10: 320-44

Dye *et. al.* (2010) The Global Tuberculosis Epidemic: Scale, Dynamics, and Prospects for Control In: Mario C (eds) Tuberculosis: the essentials.4th ed. Informa Healthcare USA, Inc. New York: 1-23

El-Sayed Zaki M, Goda T (2007) Rapid phenotypic assay of antimycobacterial susceptibility pattern by direct mycobacteria growth indicator tube and phage amplified biological assay compared to BACTEC 460 TB. Tubercle (Edinb) 87: 102–8

Enarson DA, Rouillon A (1998) The epidemiological basis of tuberculosis control. In: Davis PDO (eds.). Clinical tuberculosis. London: Chapman and Hall Medical, England: 35-52

Evans CC (1998) Historical background: Origins of Mycobacteria. In: Clinical Tuberculosis. (Ed) Davies P.D.O. Chapman and Hall, London: 21-43.

Forbes BA, Sahm DF, Weissfeld AS (2007) Bailey & Scott's diagnostic microbiology. 12th edition, Mosby Inc, USA; 45: 478-503

Franzblau S, Witzig R, McLaughlin J, *et al*., (1998) Rapid, lowtechnology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 36: 362–6

Fujuki A (2001) TB Bacteriology examination to stop TB. The Reasaearch Institute of Tuberculosis. JICA, Japan; 20-6

Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, van Soolingen D, Jensen P, Bayona J (2010) Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet 375: 1830–43

Gercia FG (1998) Evaluation of the MB/BACT automated mycobacteria culture system versusculture on LJ medium. J Clin Microbiol 4: 339-43

Gharti Chhetri G (2001) Prevalence of Tuberculosis among the suspected patients visiting Tribhuvan University Teaching Hospital and their Antimicrobial Resistance Pattern. MSc Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kirtipur, Katmandu, Nepal

Giffin R, Robinson S (2009) Addressing the threat of drug-resistant tuberculosis: A realistic assessment of the challenge: Workshop Summary; The National Academies Press. Washington, DC: 19-35

Golyshevskaia VI, Korneev AA, Chernousova LN, Selina LG, Kazarova TA, Grishina TD, Safonova SG, Puzanov VA, Nikolaeva GM, and Fadeeva NI (1996) New microbiological techniques in diagnosis of tuberculosis. Probl Tuberk 6: 22–5

Gonzalez N, Torres MJ, Aznar J (1999) Molecular analysis of rifampicin and Grange JM (1998) Mycobacteria and human disease, 2nd edition, Arnold publication, Oxford university press Inc.

Hazbon MH, Brimacombe M, Bobadilla del Valle M, *et. al*. (2006) Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 50: 2640–9

Heifets LB, Good RC (1994) Current laboratory methods for the diagnosis of tuberculosis. In: Bloom, R.L. (eds.). Tuberculosis: pathogenesis, protection and control. ASM Press. Washington, DC, USA; 85-110

Hernandez-Pando R, Jeyanathan M, Mengistu G, Aguilar D, Orozco H, Harboe M, Rook GAW, Bjune G (2000) Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. Lancet 356: 2133-8

Hoffmann CA, Niederweis LM, Plitzko JM, Engelhardt H (2008). Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc. Natl. Acad. Sci. USA; 105: 3963-7

Honore N, Cole ST (1994) Streptomycin resistance in mycobacteria. Antimicrob Agents Chemother 38: 238–42

Horsburgh CR Jr (1996) Epidemiology of disease caused by non tuberculous mycobacteria. Semin Respir Infect 11(4): 244-51

Hughes AL, Friedman R, Murray M (2002) Genome-wide pattern of synonymous nucleotide substitution in two complete genomes of *Mycobacterium tuberculosis*. Emerg Infect Dis 8: 1342-6

Imaeda T (1985) Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium bovis BCG, Mycobacterium microti* and *Mycobacterium africanum*. Int J Syst Bacteriol 35: 147-50

Joint Tuberculosis Committee of the British Thoracic Society (1998) Chemotherapy and management of tuberculosis in the United Kingdom: recommendations 1998. Thorax 53: 536–48

Kallenius G, Koivula T, Ghebremichael S, Hoffner SE, Norberg R,Sevensson E, Dias F, Marklund BI and Svenson SB (1999) Evolution and clonal traits of *Mycobacterium tuberculosis* complex in Guinea-Bissau. J Clin Microbiol 37: 3872-8

Keers RY (1978) Pulmonary tuberculosis: A journey down the centuries. Cassell Ltd, London; 6-28

Kent PT, Kubica GP (1985) Pubic health mycobacteriology: A guide for the level III laboratory. U.S. Department of Health and Human Services. Centers for Disease Control and Prevention. Atlanta, Georgia, USA; 159-84

Keshavjee S, Seung K (2008) Stemming the tide of multidrug-resistant tuberculosis: Major barriers to addressing the growing epidemic. A White Paper for the Institute of Medicine of the National Academies;14-16

Kim SJ, Hong YP (1992) Drug resistance of *Mycobacterium tuberculosis* in Korea. Tuberc Lung Dis 73: 219-24

Kochi A, Vareldzis B, Styblo K (1993) Multi-drug resistant tuberculosis and its control. Res Microbiol 144: 104-10

Kolattukudy PE, Fernandez ND, Azad AK, Fitzmaurice AM, Siraoa TD (1997) Biochemistry and molecular genetics of cell wall lipid biosynthesis in Mycobacteria. Mol Microbiol 24: 263-70

Kubica GP (1984) Clinical Microbiology. In: Kubica GP, and Wayne LG (eds.). The Mycobacteria: a source book. New York and Basel; Marcel Dekker, Inc., USA;.133-75

Laidlaw M (1989) Mycobacterium: Tubercle bacilli. In: Practical medical microbiology, 13th edition. Collee J.G (eds) et al. Churchill Livingston. London; 122-89

Lam PK, Catanzaro A, Perry S (2010) Diagnosis of Pulmonary and Extrapulmonary Tuberculosis In: Tuberculosis the essentials 4th edition (Edited by Mario C. Raviglione) Informa Healthcare USA, Inc; (5): 81-112

Leão SC, Martin A, Mejia GI, Palomino JC, Robledo J, Telles MAS, Portaels F (2004) Practical handbook for the phenotypic and genotypic identification of mycobacteria. Brugges, Vanden Broelle: 164-87

Levy H, Feldman C, Sacho H, *et. al.*, (1989) A reevaluation of sputum microscopy and culture in the diagnosis of pulmonary tuberculosis. Chest 95(6): 1193–97

Malla P (1996) Factors associated with treatment failure in TB patients at National TB centre. Journal of Nepal Medical Association 34: 41-46

Mandal PK (2009) Evaluation of direct nitrate reductase assay for culture and susceptibility testing of *M. tuberculosis* to four primary anti-tubercular drugs. A MSc Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal

Martin A, Montoro E, Lemus D *et al.* (2005a) Multicenter evaluation of the nitrate reductase assay for drug resistance detection of *Mycobacterium tuberculosis*. J Microbiol Methods 63: 145-50

Martin A, Portaels F, Palomino JC (2007) Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. J Antimicrob Chemother 59: 175-83

McKinney JD, Bentrup KH, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, Swenson D, Sacchettini JC, Joabs WR, Russel DG (2000) Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406: 735-8

Mikusov K, Slayden R, Besra G *et al*. (1995) Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob Agents Chemother 39: 2484–9

Minion J, Zwerling A, Pai M (2009) Diagnostics for tuberculosis: what new knowledge did we gain through The International Journal of Tuberculosis and Lung Disease in 2008? Year In Review Int J Tuberc Lung Dis 13(6): 691–7

Mitchison DA (1985) The action of anti-tuberculosis drugs in short course chemotherapy. Tubercle 66: 219–25

Moore DA, Evans CA, Gilman RH, *et. al.,* (2006) Microscopic-observation drug-susceptibility assay for the diagnosis of TB. N Eng J Med 355: 1539-50

Musa HR, Ambroggi M, Souto A & Angeby KA (2005) Drug susceptibility testing of Mycobacterium tuberculosis by a nitrate reductase assay applied directly on microscopy-positive sputum samples. J Clin Microbiol 43: 3159–61

Musa HR, Ambroggi M, Souto A, Angeby KA (2005) Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy-positive sputum samples. J Clin Microbiol 43: 3159–61

National Tuberculosis Centre (2009) Annual report (2008/2009) National Tuberculosis Control Programme Nepal, NTC, Thimi, Bhaktapur: 5-12

Osaba AU (2004) Microbiology of TB In: Madkour MM, Saif AA, Shahed MA, Moutaery KA, Kudwah AA (eds.) (2004) Tuberculosis. Springer-verlag Berlin Heidelberg, Germany 8: 115-30

Palomino JC (2005) Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. Eur Respir J 26: 339–50

Parrish NM, Dick JD, Bishai WR (1998) Mechanisms of latency in *Mycobacterium tuberculosis*. Trends Microbiol 6: 107-12

Pfyffer GE, Auckenthaler R, Van Embden JD and Van Solingen D (1998) *M. canetti*, the smooth variant of *M. tuberculosis*, isolated from a swiss Patient exposed in Africa. Emerg Infect Dis 4: 631-4

Post FA, Willcox PA, Mathema B, *et. al*. (2004) Genetic polymorphism in *Mycobacterium tuberculosis* isolates from patients with chronic multidrug-resistant tuberculosis. J Infect Dis 190: 99–106

Ramachandran R, Paramasivan CN (2003) What is new in the diagnosis of tuberculosis? Part II: techniques for drug susceptibility testing. Indian J Tuberc 50: 197–202

Rastogi N, Legrand E and Sola C. (2001) The mycobacteria: an introduction to nomenclature and pathogenesis. Rev Sci Tech 20: 21-54

Rayner A, Watt B, Harris G (2006) Mycobacteria In: Collee JG, Fraser AG, Mamion BP, Simmons A Mc Cartney practical medical microbiology. 14th edition, Churchill Livingstone, London; 329-41

Reichman LB (1997) Tuberculosis elimination-what's to stop us? Int J Tuberc Lung Dis 1: 3-11

Rijal B, Rehman MM, Bam DS (2002) Multidrug resistance tubarculosis: an overview of the SAARC region. STC newsletter 12(1): 13-14

Roberts GD, Goodman NL, Heifets LB, Larsh HW, Linder TH, McClatchy JK, Mc Ginnis MR, Siddiqi SH, Wright P (1983) Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear positive specimens. J Clin Microbiol 18: 689-96

Rom NW, Garay SM (1996) Tuberculosis. Little Brown and company USA.; 92- 3

Rossau R, Traore H, De Beenhouwer H, Mijs W, Jannes G, De Rijk P *et al*. (1997) Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob Agents Chemother 41: 2093-8

SAARC Tuberculosis and HIV/AIDS Centre (2010) Tuberculosis Control SAARC Region Update 2009. Thimi Bhaktapur, Nepal: 4-14

Salyers AA, Whitt DD (1994) Bacterial pathogenesis, A molecular approach, ASM Press, Washington DC; 67-103

Selwyne PA, Hartel D, Lewis VA, Schoembaum EE, Vermund SH, Klein RS, Walker AT, Friedland GH (1989) A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. New Eng J Med 320: 545-50

Shinnick TM, Good RC (1994) Mycobacterial taxonomy. Eur J Clin Microbial Infect Dis 11: 884-901

Shrestha, B Nehar A and Breyer U (1996) The pattern of anti-tuberculosis drug resistance in patients treated at an urban TB clinic in Kathmandu Valley. J Nep Med Assoc TB special 1996, Vol 117: 36-40

Siddiqi SH, Libonati JP, Middlebrook G (1981) Evaluation of rapid radiometric method for drug susceptibility testing of *M. tuberculosis*. J Clin Microbiol 13: 908-12

Smith I (2003) *Mycobacterium tuberculosis* Pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 16.3: 463–96

Smithwick RW, Stratigos CB, David HL (1985) Use of cetylpyridinium chloride and sodium chloride for the decontamination of sputum specimens that are transported to the laboratory for the isolation of *Mycobacterium tuberculosis*. J Clin Microbiol 1: 411-3

Sohaskey CD (2005) Regulation of nitrate reductase activity in *Mycobacterium tuberculosis* by oxygen and nitric oxide. Microbiology 151: 3803–10

Sohaskey CD, Modesti L (2009) Differences in nitrate reduction between *Mycobacterium tuberculosis* and *Mycobacterium bovis* are due to differential expression of both narGHJI and narK2. FEMS Microbiol Lett 290: 129–34

Sohaskey CD, Wayne LG (2003) Role of narK2X and narGHJI in hypoxic upregulation of nitrate reduction by *Mycobacterium tuberculosis*. J Bacteriol 185: 7247–56

Solis LA, Shin SS, Han LL, Lanos F, Stowell M, Sloutsky A (2005) Validation of a rapid method for detection of *Mycobacterium tuberculosis* resistance to isoniazid and rifampin in Lima, Peru. Int J Tuberc Lung Dis 9: 760–4

Stermann M, Sedlacek L, Maass S, Bange FC (2004) A promoter mutation causes differential nitrate reductase activity of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. J Bacteriol 186: 2856–61

Subba S (2007) Study of antibiotic susceptibility pattern of *M tuberculosis* in pulmonary tuberculosis patients visiting National Tuberculosis Center, Thimi, Bhaktapur, Nepal. MSc Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal

Sungkanuparh S, Sathapatayavongs B, Pracharktam R (2003) Rapidly growing mycobacterial infections: spectrum of disease, antimicrobial susceptibility, pathology and treatment outcome. J Med Assoc Thai 86(8): 772-80

Sutherland I, Savandova E, Radhakrishna S (1976) Alternative models for the development of tuberculosis disease following infection with tubercle bacilli. Bull Int Union Tuberc 51: 171-9

Talavera W, Lessnau KK, Handwerger S (1994) Extrapulmonary tuberculosis. In: Friedman LN, ed. Tuberculosis: Current Concepts and Treatment. Boca Raton, FL: CRC Press, 1994: 113–51

Telenti A, Imboden P, Marchesi F *et al*. (1993) Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet 341: 647–50

Telenti A, Philipp WJ, Sreevatsan S, *et. al*., (1997) The *emb* operon, a unique gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nature Med 3: 567–70

Tortoli E, Palomino JC (2007) New Diagnostic Methods In: In: Polomino JC, Leao SC, Ritacco V (eds.) Tuberculosis 2007 from basic science to patient care, 1st edition, Bourcillier kamps; 14: 441-87

Van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick TM, Small PM (1993) Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standard methodology. J Clin Microbiol 31: 406-9

Victor TC, Streicher EM, Kewley C *et al*. (2007) Spread of an emerging *Mycobacterium tuberculosis* drug-resistant strain in the western Cape of South Africa. Int J Tuberc Lung Dis 11: 195–201

Vincent V, Brown-Elliot B, Jost KC, Wallace RJ (2003) Mycobacterium: Phenotypic and genotypic identification. In: Manual of Clinical Microbiology 8th edition. Murray PR, JoBaron E, Jorgensen JH, Pfaller MA, Yolken RH, editors. ASM Press, Washington, DC; 560-84

Virtanen S **(**1960) A study of nitrate reduction by mycobacteria. Acta Tuberc. Scand. Suppl 48: 78–82

Webb GB (1936) Tuberculosis In (ed), Clio Series. Hoeber, New York

Weber I, Fritz C, Ruttkowski S, Kreft A, Bange FC (2000) Anaerobic nitrate reductase (narGHJI) activity of *Mycobacterium bovis* BCG in vitro and its contribution to virulence in immunodeficient mice. J Mol Microbiol 35: 1017–25

WHO (1994) Framework for effective tuberculosis control. World Health Organization Document; WHO/TB/94.179:1-7.

WHO (1996a) Treatment of tuberculosis: Guidelines for national programmes.WHO, Geneva, Switzerland. WHO/TB/96.99; 1-15

WHO (1997) Anti-tuberculosis drug resistance in the world. The WHO/IUATLD global project on anti-tuberculosis drug resistance surveillance 1994-1997. WHO/TB/97.229.

WHO (1998a) Laboratory Services in Tuberculosis Control. Part I: Organization and management. Geneva, Switzerland. WHO/TB/98.259: 11-34

WHO (1998b) Laboratory services in tuberculosis control: Culture (Part III). WHO/TB/98.258 Geneva

WHO (2003) Treatment of tuberculosis: guidelines for national programmes. WHO, Geneva, Switzerland

WHO (2008) Anti-tuberculosis drug resistance in the world: fourth global report. WHO/HTM/TB/2008.394 WHO Geneva

WHO (2008) The Global Burden Of Disease: 2008 Update. Geneva, Switzerland

WHO (2009) TB/HIV Facts 2009 Update, World Health Organisation, Geneva

WHO (2009) Tuberculosis Facts 2009 Update, World Health Organisation, Geneva

WHO (2010) Multidrug and extensively drug-resistant tuberculosis: 2010 global report on surveillance and response. Geneva, Switzerland: World Health Organization; 6-25

WHO (2010) Treatment of tuberculosis Guidelines Fourth edition,WHO, Geneva; WHO/HTM/TB/2009.420: 5-18

WHO/IUATLD (1998). Guidelines for surveillance of drug resistance in tuberculosis. Int J Tuberc Lung Dis 2: 72-89

Zhang Y, Heym B, Allen B *et al*. (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 358: 591–3
Zhang Y, Wade MM, Scorpio A *et al*. (2003) Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid. J Antimicrob Chemother 52: 790–5
Zhao M, Li X, Xu P *et al*. (2009) Transmission of MDR and XDR tuberculosis in Shanghai, China. PLoS One; 4: 4370