CHAPTER I

INTRODUCTION

Tuberculosis is a curable infectious disease of global importance. It is the most prevalent of all bacterial infections, existing actively or in latent form in a third of the world's population. In the developing world, almost everyone is infected and the tubercle bacillus might be almost regarded as part of the normal human condition. It is not surprising that the disease has played such an important role in the political, social, industrial, artistic and scientific history of mankind (Madkour et al., 2004).

There were 9.27 million estimated new cases of TB in 2007 (139 per 100000), including 4.1 million (62 per 100000) new smear positive cases and 1.7 million deaths due to TB in 2007 (WHO, 2009). These statistics show that TB remains a major global health problem.

In the last two decades, TB has re-emerged as one of the leading causes of death by a single infectious pathogen worldwide. The TB crisis is likely to escalate since the human immune deficiency virus (HIV) epidemic has triggered an even greater increase in the number of tuberculosis cases. Among the 9.27 million new cases of TB in 2007, 1.37 million (14.8%) were HIV positive (WHO, 2009).

Tuberculosis is a major public health problem in Nepal and recognized by the government as a priority number one (P_1) 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, however it is estimated 5,000-7,000 people still die per year from TB (NTC, 2008).

Tuberculosis remains the largest infectious cause of human mortality even decades after the introduction of effective chemotherapy. TB treatment is long, involving multidrug

therapy over 6 to 8 months. Multi drug resistance tuberculosis (MDR-TB), defined as resistance to at least isoniazid and rifampicin strains of *Mycobacterium tuberculosis*, has emerged due to non-adherence to treatment. Before the 1990s MDR TB was rare but over the last 10 years epidemic of MDR *Mycobacterium tuberculosis* strains have been observed around the world, with high mortality rates, particularly among HIV positive patients (Angarano et al., 1998). MDR TB is now also emerging in HIV negative populations and high risk populations.

According to a recent WHO report, drug resistance tuberculosis (DR-TB) and particularly MDR-TB is a ubiquitous problem worldwide and in some areas it is a serious and emerging threat to TB control (WHO/HTM/TB/2004). Globally there were about 4, 89,000 cases of MDR-TB in 2007 (WHO, 2009).

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 part because of the difficulty and expense associated with its treatment (STAC, 2008).

The rapid and accurate drug susceptibility testing of *Mycobacterium tuberculosis* is essential for effective patient treatment and to prevent transmission of the disease (CDC, 1995). Isolation identification and susceptibility testing are essential procedures that should be performed as quickly as possible, so that adequate treatment can be prescribed. The BACTEC 460 has been used as a method to obtain results within 5 to 10 days in developing countries, but it requires the use of radio-isotopes and a proprietary instrument, and thus is costly to perform. For developing countries, like Nepal it would be helpful to have a simple and inexpensive test that can rapidly detect MDR *Mycobacterium tuberculosis* strains.

One rapid drug susceptibility testing method that could potentially satisfy these criteria is a Nitrate Reductase Assay (NRA). It is a colorimetric assay also known as Griess method (Golyshevskaia et al., 1996). This assay was initially developed at the Central Tuberculosis Research Institute in Moscow, Russia, where it was called the Griess method, after J.P. Griess, who discovered the chemistry of the detection method used (Griess and Benerkungen, 1879). It is a low-cost DST method that can be employed in areas of limited resources and low technical capacity (Canetti et al., 1969). It is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is routinely used for biochemical identification of mycobacterial species. The presence of nitrite can easily be detected with specific reagents, which produce a color change (Kent and Kubica, 1985).

This method has the advantage of being based exclusively on conventional method and materials accessible to any laboratory without reliance on proprietary materials, reagents, and equipment (Baylan et al., 2004).

This study was carried out at National TB centre and SAARC TB and HIV/AIDS centre, Thimi, Bhaktapur, Nepal, to evaluate a Direct Nitrate Reductase Assay for drug susceptibility testing of *M. tuberculosis* directly from clinical sputum samples with positive microscopy results for acid-fast bacilli. The method has saved valuable time by omitting the pre isolation step. The performance of this technique was compared to that of the 1% proportion method in determining the susceptibilities to isoniazid (INH), rifampicin (RFP), streptomycin (SM), and ethambutol (EMB).

This study shall help to diagnose TB rapidly and also provide the drug susceptibility pattern of positive specimen in short period of times. It is mandatory that the new test be evaluated for their performance characters in terms of sensitivity, specificity and predictive values, before they are used routinely. Thus, the Direct Nitrate Reductase Assay method was evaluated with respect to the gold-standard method recommended by WHO for culture and susceptibility testing of *Mycobacterium tuberculosis*.

CHAPTER II

2. OBJECTIVES

2.1. General objective

To evaluate the performance of a direct Nitrate Reductase Assay (NRA) in determining the susceptibilities to four primary anti-tubercular drugs in smear-positive samples from patients with pulmonary tuberculosis.

2.2 Specific objective

i. To determine the sensitivity, specificity and predictive values of direct Nitrate Reductase Assay.
ii. To evaluate its feasibility and performance in the screening of MDR-TB

CHAPTER III

3. LITERATURE REVIEW

3.1 Mycobacteria

Mycobacteria were among the first bacteria to be ascribed to specific disease (Koch, 1882). The genus Mycobacteria belongs in:

Family : Mycobacteriaceae
Order : Actinomycetales, and
Class : Actinomycetes.

Currently, there are more than 100 recognized or proposed species in the genus Mycobacterium, including the causative agents of TB. Of these 14 are known to cause disease in human (Forbes et al., 2007).

Mycobacteria can be divided into two major groups based on fundamental differences in epidemiology and association with disease: Mycobacterium tuberculosis complex (MTC) and non-tuberculous bacteria also known as MOTT bacteria (Forbes et al., 2007).

Mycobacterium tuberculosis complex

M. tuberculosis, M. bovis, M. africanum and *M. microti* are grouped into the MTC on the basis of DNA homology (Baess, 1979; Imaeda, 1985). Among these *M. microti* is rarely encountered and is not considered an important pathogen for animals or humans (Thoen et al., 1984). The other 3 species in the MTC can cause tuberculosis.

Mycobacteria other than tuberculosis (MOTT)

A variety of terms have been applied to these nontuberculous mycobacteria, including anonymous, unknown, environmental, opportunist, nyrocine, tuberculoid and MOTT

(Collins et al., 1984). These organisms are widespread in nature and have been frequently found in environment habitants that may colonize and occasionally cause infection in humans and animals. Infection caused by these organisms is called mycobacteriosis. 1-3% of MOTT have been identified as cause of pulmonary infection (Woodering et al., 1990). The most commonly encountered MOTT that is isolated in clinical specimen includes *M. kansasii*, *M. avium-intercellular complex*, *M. fortuitum*, and *M. gordonae.* MOTT infections have a predilection for individuals with pre-existing chronic obstructive pulmonary disease, underlying debilitating disease, a previous tuberculous infection and patients who are immunocompromised (Shahed et al., 2004).

3.1.1. Morphological characteristics

In smears stained with carbol fuchsin or auramine and examined under light microscope, the tubercle bacilli typically appear as straight or slightly curved rods. According to growth conditions and age of the culture, bacilli may vary in size and shape from short coccobacilli to long rods. A typical curved shape has been described for *M. microti* (Vansoolingen, 1998). The dimensions of the bacilli have been reported to be 1-10 µm in length (usually 3-5 µm), and 0.2-0.6 µm in width (Barrera, 2007). *M. tuberculosis* is rarely pleomorphic, it does not elongate into filaments, and does not branch in chains when observed in clinical specimens or culture. In the experimental macrophage infection intracellular bacilli were described as being significantly elongated compared to broth-grown bacilli and, remarkably, to display bud-like structures (Chauhan et al., 2006). They are non-motile, non-sporing, and non-encapsulated (Wayne and Kubica, 1986).

3.1.2 Cell wall structure

The mycobacterial cell wall is unique among prokaryotes. The wall is constituted by an inner peptidoglycan which contains repeating disaccharide units of N-acetylglucosamine-(β1-4)-N-glycolylmuramic acid. The chains are cross-linked via L-

alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine tetrapeptides (Draper, 1986). The degree of peptidoglycan cross linking in the cell wall of *M. tuberculosis* is 70-80 % whereas that in *E. coli* is 20-30 % (Barrera, 2007). Covalently bound to the peptidoglycan is a branched polysaccharide, the arabinogalactan, whose outer ends are esterified with high molecular weight fatty acids, called mycolic acids. The mycolic acids are α-branched, β -hydroxy fatty acids with 60-90 carbon atoms in the primary chain (Good and Shinnick, 1998).

3.1.3 Genomic structure

The genomes of mycobacteria display a typical bacterial structure (a single, large circular DNA molecule), contain about 3×10^6 base pairs of DNA and have a G+C content of 60 to 70 mol% (Good and Shinnick, 1998). The genomes of most species of *Mycobacterium* contain repetitive DNA elements. The repeated DNA elements range from short stretches of sequences rich in G+C to insertion elements and transposons. The individual repeated DNA elements IS6110 is found only in members of the *M. tuberculosis* complex. Most *M. tuberculosis* strains contain 1 to 20 copies of IS6110 and the precise locations of the copies in the genome vary dramatically among individual isolates (Crawford, 1996).

3.1.4 Cultural characteristics

Mycobacteria are prototrophic (i.e. it can build all its components from basic carbon and nitrogen sources) and heterotrophic (i.e. it uses already synthesized organic compounds as a source of carbon and energy). They require oxygen as a final electron acceptor in aerobic respiration. Carbon dioxide is essential and may be taken from the atmosphere and also from carbonates or bicarbonates. *M. tuberculosis* is mesophile and neutrophile as its multiplication is restricted to 37ºC and a neutral pH. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow. High saline concentration such as that found in media containing 5 % sodium chloride inhibits the growth of the microorganism (Barrera, 2007).

M. tuberculosis is able to metabolize glycerol into pyruvate, whereas *M. bovis* is not. *M. bovis* grows much better in the presence of a pyruvate salt as a source of carbon. Albumin, which is normally provided by adding eggs or bovine serum albumin to the culture media, promotes the growth of these microorganisms (Barrera, 2007).

M. tuberculosis are slow-growing mycobacteria and may take 3-6 weeks to produce visible colonies, which are buff-coloured, rough and friable. In primary isolation, 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 (Osoba, 2004).

3.1.5. Culture media for isolation of mycobacteria

Different culture media are in use for the isolation of mycobacteria. The most common are based on egg and also contain high concentrations of malachite green to overcome contamination with other bacteria.

1. Solid media

Two types of solid media have been used for the isolation of Mycobacteria and each has its advantages: Egg-based media and Agar-based media

Egg-based media

The most widely known solid media is the Lowenstein-Jensen (LJ) medium, which is an egg-based non-selective medium. LJ medium has a long shelf life and is very cheap to prepare; it is used widely in developing countries. Its disadvantages are that it may be difficult to distinguish artifacts from true colonies on this medium and the fact that heat is required for solidification, which along with the presence of albumin inactivates some anti-tuberculous drugs. On the whole it is good for the recovery of *M. tuberculosis* but not so much for the non-tuberculous mycobacteria. It is generally regarded as the ‘gold

standard' as it results in a very high recovery rate of mycobacteria (Garcia et al., 1998).

The other types of egg-based media are the American Thoracic Society medium and a modified LJ medium by Petragnani, which contains a different concentration of malachite green used in the medium.

Agar-based media

The agar-based selective media are the Middlebrook 7H10 and 7H11 media, which contain defined salts, vitamins, cofactors, oleic acid, glycerol, glucose as enrichment, catalase and biotin, to stimulate the re-growth of damaged bacilli, and albumin to promote growth by combining with the toxic products in the media (Osoba, 2004). Their basic ingredients are commercially available: powder base, agar and Middlebrook OADC enrichment. Middlebrook media have been shown to achieve slightly higher isolation yields than egg-based media, but are considerably more expensive (WHO, 1998).

2. Liquid media

Liquid media are not generally employed for routine cultivation but are used for sensitivity test, biochemical tests and preparation of antigens and vaccines. Mycobacteria grow as a surface pellicle in liquid medium unless a surfactant, such as the non-ionic detergent Tween 80, is added to lower the surface tension and permit diffuse growth. In general, the use of liquid media system reduces the turnaround time for isolation of acid-fast bacilli to approximately 10 days, compared with 17 days or longer for conventional solid media (Forbes et al., 2007).

Different types of liquid media are available for the isolation of mycobacteria like Herman Kirchner liquid medium, Dubos oleic acid-albumin liquid medium etc. Herman Kirchner liquid medium is most useful and least expensive of the liquid media for culture *M. tuberculosis*. It has the additional advantage that it can support a large

inoculum. Dubos oleic acid-albumin liquid medium is recommended for the cultivation of tubercle bacilli from cerebrospinal, pleural and peritoneal fluid. It may be prepared from basic ingredients or may be obtained commercially as a ready-to use base to which sterile albumin or serum is added. Middlebrook 7H9 is a commercial liquid medium and may be prepared from commercially available powder base supplemented with Middlebrook ADC enrichment after sterilization (WHO, 1998).

Liquid culture media has been proven to be significantly more sensitive than egg-based solid media for the isolation of mycobacteria from clinical specimens (Hines et al., 2006). However, one disadvantage is the much higher price, which is often too high for cost-effective TB diagnosis in resource-limited countries.

3. Biphasic media

The media which contain both agar and broth are referred as the biphasic media. The biphasic media used for the culture of Mycobacteria in some laboratories is the BBL-Septic-Chek AFB system. It uses the Middlebrook 7H9 broth in its lower chamber and agar slope of Middlebrook 7H11 in the upper part. Mycobacteria grow readily in this medium and at 2 or 3 days intervals during incubation the broth is made to run over the agar. Colonies are formed on the agar if they contain mycobacteria (Osoba, 2004).

3.1.6 Susceptibility to physical and chemical agents

Mycobacteria are as susceptible as other non-spore-forming bacteria to heat and to some other physical and chemical agents, although some early work on the heat susceptibility of mycobacteria may have suggested otherwise (Corper and Cohn, 1937). On the other hand, the bacilli are very sensitive to heat, sunlight and ultraviolet (UV) irradiation. In sputum or in aqueous suspension, they progressively lose viability between 30°C and 37°C within one week. Exposed to direct UV irradiation, moderate loads of tubercle bacilli die in a few minutes.

Mycobacteria are generally resistant to acids and alkalis, and this feature is used to advantage in isolation procedures. As much as 2% sodium hydroxide, 2% sulphuric acid or 2.5% oxalic acid can be used to kill contaminants in specimen prior to culture. However, killing activity of acids and alkalis increases with increasing temperature of exposure, and resistance varies greatly among different species. Tubercle bacilli are also resistant to quaternary ammonium compound and indeed, cetylpyridinium chloride has been used for decontamination of clinical specimens prior to culture (Kent and Kubica, 1985). With respect to chemical disinfectants, mycobacteria are susceptible to a variety of chemical agents including: alcohols (ethyl and isopropyl although the later is not as active as the former), chlorine, glutaraldehyde, iodophores, phenolic compounds, ethylene oxide, formaldehyde and hydrogen peroxide. Derivatives of phenol, in which a functional group replaces one of the hydrogen atoms on the aromatic ring, are effective and safe to use (Marsik and Denys, 1995).

3.1.7 Pathogenesis

3.1.7.1 Mode of infection

Mycobacterium tuberculosis is transmitted by inhalation of air-borne bacilli present in the cough particles from the sputum of a smear-positive patient. It is spreaded by air borne droplet nuclei, which are 1-5 µm particles containing 1 to 400 bacilli each. Inhaled droplet nuclei avoid the mucocilliary defenses of the bronchi and lodge in the terminal alveoli of the lungs. (Park, 2005).

3.1.7.2 Predisposing factors

Predisposing factors for progression of disease are old age, alcoholism, diabetes neoplastic disease, malnutrition, immunosuppressive drugs, stress and drug induced, congenital or acquired immunodeficiency including HIV infection. Also the vulnerable groups or the individuals more susceptible to the disease include persons under conditions like, chronic lung disease (bronchitis) and silicosis, cancer, advance kidney disease, heavy smokers, elderly, low income groups, intravenous drug users, living and

attending drug treatment centers, hospital, nursing homes etc (WHO, 1993; WHO, 1997).

3.1.7.3 Virulence factors

The virulence of the tubercle bacilli is due to its resistance to tissue cells and fluids rather than to the production of toxic substances (Grange, 1994). The virulence of tubercle bacilli appears to be related to their ability to survive within macrophages and the organisms exhibit long periods of metabolic inactivity. Mycobacterial virulence factors can have both direct and indirect effects on host cells and can ultimately lead to tissue destruction and disease. Direct cell-cell interactions, including attachment, invasion, and intracellular multiplication and indirect interactions, through secreted bacterial factors such as hemolysin and cytotoxin, can cause lysis of the host cells. In addition, lipoarabinomannans (LAMs), heat shock proteins, and mycobacterial products can stimulate host cell to produce inflammatory products or cytokines that can amplify tissue damage in the host (Quinn et al., 1997).

Mechanisms hypothesized for how tubercle bacilli avoid being killed by macrophages include:

i. Prevention of an oxidative burst in phagocytosing cells.
ii. Inhibition of phagosome-lysosome fusion.
iii. Resistance to lysosomal enzymes such as lysozyme.
iv. Secretion of inhibitors or in inactivators of bactericidal agents such as peroxide or oxygen radicals.
v. Exudation of lipids or capsules to block the assess of bactericidal agents to the targets and
vi. Escape from the phagosome into the cytoplasm

Several bacterial components have been implicated in these various hypotheses e.g. phenolic glycolipids can act as scavengers of free radicals and the mycolic acid layer

can act as a hydrophobic barrier to bactericidal agents. Similarly, a recently described cytolytic activity might be involved in the ability of mycobacteria to enter and exit host cells or perhaps escape from the phagosome. Phagocytized tubercle bacilli appear to inhibit acidification of the phagosome and the subsequent phagosome-lysosome fusion. Potential fusion-inhibiting substances made by the mycobacteria include ammonia, polyglutamic acid and sulpholipids, although the key molecule has not been identified. Some electron microscopic evidence suggests that the tubercle bacilli escape from the phagosome and replicate in the cytoplasm whereas other data suggest that *M. tuberculosis* ends up in another membrane bound vacuole which contains proteins characteristic of phagosomes and, early and late endosomes (Good and Shinnick, 1998)

3.1.7.4 Mechanism of infection

The inhaled bacilli usually deposited in the middle or lower lung field. The exact mechanism of the protective immunity response against the development of the disease in humans have not been totally clarified (Ellner, 1997). *M. tuberculosis* lipoproteins and glycolipids induce activation of macrophage, T-cell, and cytokine expression (Wallis and Johnson, 2001). Alveolar macrophase phagocytose the bacilli, and activation of the cell-mediated immune response (CMI) plays an essential role in combating infection. The bacilli may survive, especially within nonactivated monocyt /macrophages that enter the alveoli from the bloodstream (Dannenberg, 2001).

Early in the primary infection (within a few hours) the bacilli are transported by macrophages to the hilar and/or paratracheal lymph nodes (Milburn, 2001). Tissue-damaging delayed hypersensitivity (DTH) develops, and the bacilli-laden macrophages are killed. Key factors in this process include monocyte-derived interleukin (IL)-12 and tumor necrosis factor (TNF)-α as well as T-cell derived IL-2 and interferon (IFN)-γ. These cytokines play a crucial role in the induction of macrophage-mediated elimination of mycobacteria (Vanham et al., 1997; Garcia et al., 2002).

The balance of cytokines produced by lymphocytes in response to infection is believed to have a profound effect on the clinical outcome. As the infection progress, local areas of inflammation with granuloma formation and cellular infiltrates occur in the middle or lower zones of the lung. This is the pulmonary component of the primary Ghon focus and, in combination with lymphadenitis, forms the primary complex.

The outcome of the infection depends on the interaction between the host immune system response and the bacilli. In patients with strong and competent cellular immunity, the Ghon focus and regional lymphadenitis heal with fibrosis and calcifications within weeks or months. However, the primary complex lesion may progress into lung parenchymal or lymph node infection or both in what is known as progressive tuberculosis (Madkour, 2004). Within the first year of primary pulmonary infection, the incidence of clinically significant disease is approximately 1.5%, and the cumulative risk during the first five years is 5% -10% (Chiba and Kurihara, 1979).

3.2 Tuberculosis

Tuberculosis is a chronic granulomatous disease affecting human and many other mammals. It is caused by four very closely related species of Mycobacteria : *M. tuberculosis* (the human tubercle bacilli), *M. bovis* (the bovine tubercule bacilli), *M. microti* (the vole tubercle bacilli), and *M. africanum* . It is the disease, which most frequently affects the lung but it may involve virtually any organ or system of the body. Tuberculosis may therefore mimic many other diseases (Grange, 1998).

3.3 Epidemiology of Tuberculosis

3.3.1 Global situation of TB

There were an estimated 9.27 million new cases of TB in 2007, (139 per 100000 populations) including 4.1 million (44.5% of the total) new smear positive cases. Asia (South-East and Western pacific regions) accounts for 55% of global cases, and Africa

accounts for 31%; the other regions account for relatively small fractions of global cases. A total of 1.7 million people died of TB in 2007. There were an estimated 13.7 million prevalent cases and 0.5 million cases of 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 (WHO, 2009).

3.3.2 Situation of TB in Asia

The South East Asia Region (SEAR), with 4.97 million TB cases, carries over one third of the global burden of TB. It has the highest burden of TB cases among all the WHO regions. Every year 1.4 million new cases of TB appear and over 50 0000 deaths occur due to this disease. MDR-TB in the South East Asian Region is estimated at 2.8% among new cases, and 18.8% among previously treated cases (STAC, 2008).

3.3.3 Situation of TB in SAARC Region

About 50% of the adult population of this region has already been infected with *M. tuberculosis* and is at risk of developing disease. A total of 1.9 million all type of TB cases were notified in the year 2007. This represents 73.7% of the 2.6 million estimated incident cases; the 0.8 million new smear positive cases notified account for 69.5% of the 1.1 million estimates. India, Bangladesh, Pakistan and Afganistan account for 27% of the global absolute burden of TB (STAC, 2008).

3.3.4 Situation of TB in Nepal

Tuberculosis is a major public health problem in Nepal and recognized by the government as a priority number one (P_1) 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; however it is estimated 5,000-7,000 people still die per year from TB (NTC, 2008). The latest survey conducted during

2006/07 shows MDR-TB at 2.9% among new TB cases and 11.7% among previously treated cases (STAC, 2008).

3.4 Types of Tuberculosis

TB has been broadly divided into two types; pulmonary TB and extra-pulmonary TB.

3.4.1 Pulmonary tuberculosis (PTB)

Pulmonary TB refers to disease involving the lung parenchyma as the lung is the usual site of primary lesion and the principal organ involved. 80% of TB occurs in the lung and is called pulmonary tuberculosis (NTC, 1997).

Pulmonary tuberculosis is classically divided into primary and post primary disease.

Primary pulmonary tuberculosis

Primary pulmonary tuberculosis is a term used when the infected individual has not been previously exposed to *M. tuberculosis* and lacks hypersensitivity to tuberculo-protein (Shahed et al., 2004). Radiologically, primary pulmonary tuberculosis typically manifests in four major ways: Parenchymal disease, Lymphadenopathy, Pleural effusion and Miliary disease

Post primary tuberculosis

Post primary TB occurs after a latent period of months or years after primary infections. It may occur by reactivation of dormant bacilli i.e. dormant bacilli persisting in tissues for months or years after primary infection start to multiply or by re-infection. Reactivation may be in response to a trigger, such as weakening of the immune system by HIV infection. Post primary TB usually affects the lungs but can involve any part of the body. The characteristics features of post primary PTB are: extensive lung

destruction with cavitations, positive sputum smear, upper lobe involvement, usually no intrathoracic lymphadenopathy (Crofton et al., 1999; Grange, 1998).

3.4.2 Extra pulmonary tuberculosis

When TB affects organs other than lungs, it is referred to as Extra-pulmonary TB. Most, if not all, extra pulmonary lesions results by haematogenous spread of the organism from a primary focus which is not always detected (Chakraborty, 2003). The higher rate of infection of extra PTB cases in immune-compromised states. There are many types of extra pulmonary TB. These include: endobronchial TB, pleural TB, spinal TB, musculoskeletal TB, renal TB, skin TB, abdominal TB, and genitourinary TB. (Madkour et al., 2004).

3.5 Clinical diagnosis of pulmonary tuberculosis

The most important symptoms in the selection of TB suspects in adults (over 15 years of age) include: productive cough of more than 3 weeks, or haemoptysis, and significant weight loss. Patients with TB may also have other symptoms (which are more common, but less suggestive) such as: chest pain, breathlessness, fever/night sweats, tiredness, and loss of appetite) (WHO, 1997).

3.6 Radiological diagnosis of pulmonary tuberculosis

Chest radiology is often used for suspected TB patients, where and when available, as evidence of TB disease. Conventional chest radiography is the mainstay in the detection and follow up examination of patients with pulmonary TB. Good quality chest radiographs are essential and remain the first line of investigation (Shahed et al., 2004). But, normal radiographs do not exclude TB and diagnosis of TB should not be made on a single abnormal X-ray (except for miliary TB) (NTC, 1997).

Recently, conventional Computerized Tomography (CT) has been used in selected

cases. It is required in many circumstances such as in the detection of cavitations and in the evaluation of the route of spread (Kuhlman et al., 1990). CT is also used in the evaluation of complications that might occur in the process of the disease.

3.7 Serological diagnosis of tuberculosis

A number of serological tests developed for the diagnosis of TB have now become commercially available and these tests can be divided into 2 groups, they are: Immunochromatographic tests, and Enzyme-linked immunosorbent assay.

1. Immunochromatographic tests

"ICT TB test" is an immunochromatographic test based on the detection of IgG antibodies directed against five *M. tuberculosis* secreted antigens, using an anti-human IgG labeled with colloidal gold (Rasolofo et al., 2000). 5 highly purified antigens secreted by *M. tuberculosis* during active infection are immobilized in 4 lines on the test strip. The test detects the presence of IgG antibodies to these antigens. Rapid TB test is a one step colored immunochromatographic test which detects antibodies to the recombinant 38 kDa antigen from *M. tuberculosis* expressed and purified in *E. coli.*

2. Enzyme linked immunosorbent assay (ELISA)

TB IgA EIA test detects the IgA antibodies to a mycobacterial kp90 immuno-cross-reactive antigenic compound (ImCRAC). The pathozyme TB complex test detects serum IgG antibodies to a recombinant 38 kDa antigen from *M. tuberculosis* expressed and purified from *E. coli*, permitting the isolation of significant quantities of protein. This antigen has been reported as the single most important antigen for the serological diagnosis of TB as it is a unique disease-associated protein that appears to be completely specific to the MTC. PATHOZYME-MYCO IgG, IgA and IgM test is also an ELISA based test that measures the levels in serum of IgG, IgA and IgM, respectively to 2 antigens LAM and recombinant and recombinant 38 kDa antigens.

These kits detect infections due to *Mycobacteria species* (Osoba, 2004).

3.8 Tuberculin skin testing

The tuberculin skin test is still widely used to identify infection with *M. tuberculosis*. But, it is the only means of estimating the prevalence of infection in a population (Park, 2005). Tuberculin is a purified protein derivative from tubercle bacilli which when injected into the skin of an infected person, produces a delayed-type hypersensitivity reaction after 24-48 hours. There are three types of tuberculin tests: the Mantoux intradermal test, Heaf test and the Tine multiple puncture tests (Crofton et al., 1999).

Mantoux test

Intradermal injection of 0.1mg/0.1ml of the standard 5-tuberculin unit dose (TU) is done into the volar or dorsal surface of the forearm. The diameter of skin indurations at the site of the injection should be measured between 48 and 72 hour after injection. Three cut-off points have been recommended for defining a positive test: A cut-off point of ≥5mm of induration using the ballpoint pen method of Sokal is considered positive in a person with recent contact or in the presence of abnormal chest radiographs consistent with TB. A cut-off point of ≥10mm is suggested for individuals who have normal or mildly impaired immunity and a high likelihood of being infected with the disease but without other risk factors. A cut-off point of ≥15mm for individuals with no risk factors for TB is considered positive (American Thoracic Society 2000).

A “positive” tuberculin test does not mean the patient has active TB, and a “negative” tuberculin test does not exclude TB. A positive test may be due to infection with mycobacteria other than *M. tuberculosis* or *M. bovis.* The conditions which may suppress the tuberculin skin test include HIV infection, malnutrition, severe bacterial infection (including TB itself, Miliary TB), viral infection, cancer and use of immunosuppressive drugs (WHO, 1997).

3.9 Laboratory diagnosis of pulmonary tuberculosis

The confirmation of the clinical diagnosis of TB depends on the identification of the mycobacteria in smears, isolation on laboratory media, identification of the species and susceptibility testing of the isolate (Osoba, 2004).

3.9.1 Specimen collection and transport

Sputum is the specimen of choice in the investigation of TB and should always be collected (WHO, 1998). Swabs are not recommended for isolation of mycobacteria (Osoba, 2004).

Specimens should be collected in sterile, leak proof, disposable and appropriately labeled containers and placed into bags to contain leakage should it occur (Forbes et al., 2007). Pulmonary secretions may be obtained by any of the following methods: spontaneously produced or induced sputum, gastric lavage, transtracheal aspirations, brochoscopy, and laryngeal swabbing. Sputum samples should not be saliva but should be coughed up from the lungs.

Because of the high yield among multi-bacillary cases, the IUATLD (1998) has recommended the “on-the-spot-early morning-on-the-spot” collection of sputum. Three or more consecutive samples should be examined, collected first thing in the morning if possible (Collee et al., 1996). If sputum specimens can be kept refrigerated they could be sent to the laboratory once a week and if they have to be transported at ambient temperature, chemical preservation may be used (WHO, 1998). Three methods provide reasonable results, *viz*:

i. Mixing the fresh specimen with an equal volume of 1% cetyl pyridinium chloride in 2% sodium chloride. Tubercle bacilli will survive for up to a week, while the growth of unwanted organisms will be restricted.
ii. Mixing the fresh specimen with anhydrous sodium carbonate in the

proportion of 50 mg reagent to 2 ml specimen.

iii. If the delay before the cultural examination is to be less than 24 hours the specimen may be mixed with an equal volume of 23% trisodium phosphate.

However, none of the above mentioned preservation methods is optimal and speedy transportation is essential for good results (WHO, 1998).

3.9.2 Macroscopic examination

Macroscopic examinations include the observation of the color and appearance of the sputum sample- mucoid, salivary, purulent, mucopurulent, bloody sputum specimen.

Table 3.1: Macroscopic examination of sputum sample (Cheesbrough, 2002)

S. No.	Type	Observation
1	Purulent	Green looking, mostly pus
2	Mucopurulent	Green looking with pus and mucus
3	Mucoid	Mostly mucus
4	Mucosalivary	Mucus with a small amount of saliva
5	Blood stained	Presence of blood

3.9.3 Microscopic examination

The cornerstone of the diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. Microscopy provides a simple, sensitive and rapid means of detecting open infectious cases of pulmonary TB (WHO, 1998). Between 5,000 and 10,000 bacilli per millitre of sputum are required for direct microscopy to be positive (Rocillon et al., 1976; WHO, 1998). Mycobacteria retain the primary stain even after exposure to decolorizing acid-alcohol, hence the term “acid-fast”. A counter-stain is employed to highlight the stained organisms for easier recognition. There are several methods of determining the acid-fast nature of

mycobacteria. Two most widely used staining techniques to observe AFB are ZN staining and Fluorescence method (WHO, 1998).

ZN is the only method providing consistently good results without need for special equipment, and the required binocular microscope can be used for other purposes as well. In the carbol fuchsin (ZN) procedure, AFB appears red against a blue background. Fluorescence microscopy is cost effective and rapid if more samples are to be proceeded each day. Fluorescence staining utilizes basically the same approach as ZN staining, but carbol fuchsin is replaced by the fluorescent dye, the acid for discoloration is gentler, and the counter-stain is not essential although useful to quench background fluorescence. In Fluorochrome method, the acid-fast organisms appear as fluorescent rods, yellow to orange (the color may vary with the filter system used) against a paler yellow or orange background (IUATLD, 1998; WHO, 1998).

Table 3.2: Reporting of sputum smear by ZN staining method (WHO, 1998)

S NO.	Reading	Report
1	No AFB per 100 immersion fields	Negative
2	1-9 AFB per 100 immersion fields	Positive (Scanty)
3	10-99 AFB per 100 1mmersion fields	Positive (1+)
4	1-10 AFB per field	Positive (2+)
5	More than 10 AFB per field	Positive (3+)

Other methods for light microscopy include cold staining techniques (such as with Kinyoun's or Gabett's solution, or Tam Tham Hok's method). For fluorescence microscopy, other methods include staining with Auramine/rhodamine, rhodamine, acridine orange, and others. These methods are reliable but are more expensive so not used in the routine examination (Forbes et al., 2007).

AFB-microscopy can be used for accurate diagnosis, which is rapid with high

specificity (99%). The main disadvantage is low sensitivity (25-75%). Similarly, microscopy can not distinguish between live and dead AFB, so that some patients excreting non-viable bacilli at the end of the treatment may be roughly considered as failure-cases (WHO, 1998).

3.9.4 Processing of sputum specimen

The selection of a liquefaction and decontamination process that maintains the viability of the mycobacteria, eliminates the present bacterial flora and liquefies the mucin in the sample is desirable. The three most widely used digestion methods are the NaOH method, the Zephiran-trisodium phosphate (Z-TSP) method and the NALC-NaOH method (Roberts et al., 1991).

1. NaOH method:
This method utilises sodium hydroxide at concentrations ranging between 2 % and 4 % to digest and, at the same time, decontaminate the specimen. Each laboratory should determine the lowest concentration for optimal digestion and decontamination (Della Latta, 2004). Modified Petroff NaOH method is used widely in developing countries. The method uses 4% NaOH (WHO, 1998).

2. Zephiran-trisodium phosphate (Z-TSP) method:

This method utilizes trisodium phosphate and Zephiran (benzalkonium) to liquefies and decontaminate specimens. The methods results in the killing of approximately 30% of tubercle bacilli (WHO, 1998).

3. NALC-NaOH method:

The method utilizes a mucolytic agent, N-Acetyl-L-Cysteine (NALC) for digestion and NaOH for decontamination. The mucolytic agent enables the decontaminating agent (NaOH) to be used at a lower final concentration of 1%. Sodium citrate is included in the digestant mixture to bind the heavy metal ions which may be present in the

specimen and could inactivate the acetyl-cysteine (WHO, 1998).

It is the most commonly used method in clinical laboratories. The advantage of this method is that a large number of specimens can be processed in a short time; concentrated smears can be ready for staining immediately and it has been shown to give a high recovery of mycobacteria (Roberts et al., 1991).

3.9.5 Culture of sputum specimen

Culture is the "gold standard" for diagnosis of TB which is most sensitive than AFB staining method and can reliably find mycobacteria when they are present in a concentration of about 10-100 organisms/ml of specimen (IUATLD, 2007). Various steps have to be followed before the inoculation of sample for culture. They are as given below:

1. Bacterial flora present in the specimens must be removed or reduced in numbers before inoculation in special media, since these are faster growing than mycobacteria.
2. Mycobacteria trapped in mucin must be released by a liquefaction process without killing the mycobaceria, for easy identification and culture.
3. Specimens with low bacterial load must be concentrated to enable detection by Microscopy and culture.
4. Suitable media (solid and/or liquid media) and appropriate incubation environment must be chosen to ensure optimum recovery of mycobaceria.

Table 3.3 : Reporting of culture on LJ medium (WHO, 1998)

S No.	Reading	Report
1	No growth	Negative
2	1-19 colonies	Positive (Scanty)
3	20-100 colonies	Positive (1+)

4	100-200 colonies	Positive (2+)
5	200-500 colonies (almost confluent growth)	Positive (3+)
6	Contaminated	Contaminated

3.9.6 Identification of Isolates

Identification of mycobacteria can be rather complex and needs a multitude tests ascertain to which species a mycobacterium belongs. Conventional methods for the identification of mycobacteria are: growth rate at different temperatures, colonial morphology, pigmentation in dark or light, and biochemical tests.

The biochemical tests commonly used to differentiate mycobacteria are: Growth on medium containing p-nitrobenzoic acid (PNB), Niacin test, Nitrate reduction test, Catalase test, Urease test, Arylsulphatase test, Pyrazinamidase test and Thiophen-2-Carboxylic acid Hydrazide (TCH) susceptibility test.

3.9.6.1 Growth on medium containing p-nitrobenzoic acid (PNB)

Para-nitrobenzoic acid inhibits the growth of several species in the *M. tuberculosis* complex (Tsukamura, 1984; Leao, 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 (Fujiki, 2001).

3.9.6.2 Niacin test

Although, Nicotinic acid or niacin is produced by all mycobacteria, some species, such as *M. tuberculosis*, *M. 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. The test may be done either with chemical

reagents or with commercially available paper strips (Kent and Kubica, 1985).

3.9.6.3 Nitrate reduction test

M. tuberculosis is one of the strongest reducers of nitrate among the mycobacteria, which allow this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from the other mycobacteria. This test is based on the principle that the enzyme nitrate reductase causes the reduction of nitrate in the presence of a suitable electron donor to nitrite. This test is particularly useful for differentiating *M. tuberculosis*, which gives a positive reaction, from *M. bovis*, which is negative (Tsukamura, 1984; Vincent, 2003)

3.9.6.4 Catalase test

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually, all mycobacteria passes catalase enzyme, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis*. Mycobacteria posses several kinds of catalase that vary in heat stability: Drop catalase test (indicates the presence of catalase); Semi quantitative catalase test (indicates level of catalase production); and 68°C heat labile catalase test (indicates loss of catalase activity due to heat). If niacin test is not available, 68°C labile catalase test can be used to differentiate *M. tuberculosis* from other mycobacteria (Fujiki, 2001).

3.9.6.5 Urease test

The ability of a culture to hydrolyze urea releasing ammonia is useful in identifying both scotochromogens and non-photochromogens. *M. scrofulaceum, M. flavescens, M. bovis M. tuberculosis,* and *M. gastri* are positive, whereas *M. avium* complex, *M. xenopi, M. terrae* complex, and *M. gordonaei* are negative (Forbes et al., 2007).

3.9.6.6 Pyrazinamidase test

Pyrazinamidase is an enzyme that hydrolyzes pyrazinamide to ammonia and pyrazinoic acid. The test is useful to differentiate *M. tuberculosis* (positive) from the other species of the *M. tuberculosis* complex (negative), with the exception of "*M. canettii"*, which is also positive. Some strains of *M. tuberculosis* may acquire resistance to pyrazinamide due to selective pressure induced by treatment with this drug. These strains give a negative pyrazimidase test (unable to transform pyrazinamide to pyrazinoic acid, the active form of the drug) (Vincent, 2003).

3.9.6.7 Thiophen-2-Carboxylic acid Hydrazide (TCH) susceptibility test

This test is is useful to distinguish *M. bovis* from *M. tuberculosis* and other non-chromogenic slowly growing mycobacteria. Only *M. bovis* is susceptible to low concentrations of TCH, 1-5 μg/ml. Isoniazid-resistant strains of *M. bovis* may be resistant to TCH. *M. tuberculosis* and other mycobacteria are usually resistant to the inhibitory action of this compound (Vincent, 2003; Leao, 2004).

3.9.6.8 Molecular techniques for identification of mycobacteria

Molecular techniques have been developed in the last decade with the aim of achieving faster commencement of appropriate treatment, shortening time to detection of anti-mycobacterial drug resistance and instituting appropriate control measures. There are various types of molecular techniques so far developed. These are : Nucleic acid probe method, The Accuprobe system, The BD probe Tec ET, The Amplified *M. tuberculosis* Direct (MTD) test, Amplicor *M. tuberculosis* (PCR) test, Ligase chain reaction (LCR), (Forbes et al., 2007; Madkour et al., 2004).

A number of other molecular techniques have been described for the rapid identification of mycobacteria growing on solid media. These are: Thin layer chromatography, High Performance Liquid Chromatography (HPLC), Gas liquid chromatography (GLC), and

Analysis of DNA Probes. Although these procedures can produce rapid identification of mycobacteria growing on solid media, they are too insensitive for the identification of isolates in liquid media, due to the relatively low cell mass often produced, or they are only capable of identifying a limited number of species (Madkour et al., 2004).

3.9.7 Antimicrobial susceptibility testing of Mycobacteria (AST)

Antimicrobial susceptibility testing (AST) of isolated mycobacteria is very important not only for appropriate therapy but also for control of the disease and the identification of resistant strains The source of the inoculum for a susceptibility test may be either a smear-positive specimen (direct method) or growth from a primary culture or subculture (indirect method). The direct method is used when antimicrobial resistance is known or suspected. However, the indirect method is considered the standard method for inoculum preparation, and results of the direct method are usually confirmed by subsequent testing by the indirect method. With both the direct and indirect methods, the inoculums must be a pure culture and careful attention must be given to avoid or under-inoculation (Inderlied and Pfyffer, 2003).

For the direct method, the inoculums is either a digested, decontaminated clinical specimen or an untreated, normally sterile body fluid, in which acid fast bacilli are seen in stained smears. To ensure adequate but not excessive growth in the direct susceptibility test on solid medium, specimens are diluted according to the number of organisms observed in the stained smear of the clinical specimen. Theoretically, this type of inoculums is more representative of the population of the tubercle bacilli in a particular lesion in the host. It is prudent to include an undiluted inoculums, if the smear-positive specimen is from a patient who is receiving antimicrobial therapy, since a significant proportion of the bacilli seen on the smear may be nonviable (Inderlied and Pfyffer, 2003).

The direct susceptibility test has two major advantages. First, results can be reported

within 4 weeks from the time of specimen receipt in the laboratory for a majority of smear- positive specimens. Second, the proportion of resistant bacteria better represents the patient's bacterial population. However, it is not possible to accurately calibrate the inoculums, which may result in insufficient growth or excessive growth on drug free media. In addition, if contaminants grow, results are not interpretable. According to the literature, the total rate of failure for direct susceptibility testing can reach 15% or more, necessitating retesting by the indirect method. Use of the direct method may be warranted in situations where there is a high prevalence of drug resistance (Inderlied and Pfyffer, 2003).

For the indirect method, the source of the inoculums is a subculture, usually from the primary isolation media. Careful attention should be paid to the selection of colony types so that the final inoculum is representative of all types present to ensure the there is a balance of potentially resistant and susceptible bacilli (Inderlied and Pfyffer, 2003).

3.10 Methods of detection of drug resistance

Methods that are commonly used to test rapidly growers aerobic and facultative anaerobic bacteria are, for a variety of reasons, unsuitable for testing most mycobacterial species. For example, the conventional disk diffusion method is unsuitable for testing slowly growing mycobacterium because the drug diffuse throughout the medium before growth of the mycobacteria is significantly affected. The methods generally accepted for determining the antimicrobial susceptibility of mycobacteria are based on the growth of the microorganisms on solid or liquid medium containing a specified concentration of a single drug (Inderlied and Pfyffer, 2003)..

These include both phenotypic and genotypic methods. In many cases, the genotypic methods in particular have been directed towards detection of RFP resistance, since it is considered a good surrogate marker for MDR-TB, especially in settings with a high prevalence of MDR-TB. Genotypic methods have the advantage of a shorter turnaround

time, no need for growth of the organism, the possibility of direct application in clinical samples, lower biohazard risks, and the feasibility of automation; however, not all molecular mechanisms of drug resistance are known. Phenotypic methods, on the other hand, are in general simpler to perform and might be closer to implementation on a routine basis in clinical mycobacteriology laboratories (Martin and Portaels, 2007).

3.10.1 Conventional phenotypic methods

In general, phenotypic methods assess inhibition of *M. tuberculosis* growth in the presence of antibiotics to distinguish between susceptible and resistant strains. This is possible since *M. tuberculosis* isolates from patients never treated before are very uniform in their level of susceptibility, as shown by the narrow ranges of minimal inhibitory concentrations (MIC) of the main anti-tuberculosis drugs (Heifets, 1996). The classical definition for a drug resistant *M. tuberculosis* strain is that it displays a degree of susceptibility significantly lower than that of a wild strain that has never been in contact with the drug (Canetti et al., 1963; Canetti et al., 1969).

Phenotypic methods based on cultivation of *M. tuberculosis* in the presence of antibiotics have been most commonly performed on egg-based or agar-based solid media, and can also be performed as a direct or indirect method. For the direct method, antibiotic-containing and control media are inoculated with a decontaminated and concentrated clinical specimen, while for the indirect method the antibiotic-containing and control media are inoculated with a bacterial suspension of the isolated strain. There are three conventional phenotypic methods for drug susceptibility testing based on solid media: the proportion method, the resistance ratio method and the absolute concentration method (Canetti et al., 1963; Canetti et al., 1969; Kent and Kubica, 1985). More recent methods are based on liquid media including the BACTEC radiometric and the Mycobacterial Growth Indicator Tube methods.

3.10.1.1 Proportion method

The proportion method is the most commonly used method worldwide amongst the three methods mentioned above. It allows the precise determination of the proportion of resistant mutants to a certain drug. Briefly, several 100-fold serial bacilli dilutions are inoculated into drug-containing and drug-free (control) media. One of those dilutions should produce a number of colonies that is easy to be counted. The number of colonies obtained in the drug-containing and control media are enumerated and the proportion of resistant mutants is then calculated. When performed in Lowenstein-Jensen medium tubes, the test is first read after 28 days of incubation at 37°C. If the proportion of resistant bacteria is higher than 1 % for isoniazid, rifampicin and para-aminosalycilic acid, or 10 % for the other drugs, the strain is considered resistant and the results are final; otherwise, the test is read again at 42 days of incubation to assess if the strain is susceptible to a certain drug (Heifets, 2000). If the test is performed on agar, a Middlebrook 7H10/11 is used and the medium is incubated in a 10 % CO2 atmosphere. Results are interpreted after 21 days of incubation or even earlier if they show the strain to be resistant (Kent and Kubica, 1985). The critical concentrations of the main drugs used in the proportion method are shown in Table 3.4

Table 3.4: Critical concentration of main antibiotics in the proportion method (µg/ml)

Antibiotic	Lowenstein-Jensen	7H10 agar	7H11 agar
Isoniazid	0.2	0.2,1.0	0.2,1.0
Rifampicin	40.0	1.0	1.0
Streptomycin	4.0	2.0	2.0,10.0
Ethambutol	2.0	5.0	7.5
Pyrazinamide	100	-	-
PAS	0.5	2.0	8.0
Kanamycin	20.0	5.0	6.0
Ethionamide	20.0	5.0	10.0
Ofloxacin	2.0	2.0	2.0
Capreomycin	20.0	10.0	10.0
Cycloserine	40.0	-	-

Adapted from: Kent and Kubica, 1985; WHO/CDS/TB/2001.288; and NCCLS 2000

3.10.1.2 Resistance ratio method

This method is based on the resistance ratio, which corresponds to the MIC of a test strain divided by the MIC of the drug-susceptible reference strain H37Rv tested at the same time. Thus, it compares the resistance of an unknown strain with that of a standard laboratory strain. For the performance of the test, parallel sets of tubes containing two-fold dilutions of the tested drug are then inoculated with a standardized inoculum of both test and reference strain. Reading of the test is performed after 4 weeks of incubation at 37°C. Tubes containing 20 or more colonies are considered as positive for growth and the MIC is defined as the lowest concentration of drug in the presence of which the number of colonies is lower than 20. An isolate with a resistance ratio value of 2 or less is considered susceptible, while a resistance ratio of 8 or more defines the isolate as resistant (Kent and Kubica, 1985; Heifets, 2000).

3.10.1.3 Absolute concentration method

This method uses a standard inoculum of the test strain grown in a two-fold dilution drug-containing media and drug-free control. The resistance of a strain is expressed in terms of the lowest concentration of a certain drug that inhibits all or almost all the growth of the strain. The critical concentrations included in the medium are similar to the ones used in the proportion method but the drug concentration considered as ‘critical’ should be determined in each laboratory (Heifets, 2000). For the interpretation of the test, the reading is performed after 4 weeks of incubation at 37°C, or at 5-6 weeks if there is not enough growth. A strain is considered to be susceptible if the number of colonies on the drug-containing medium is less than 20 with 3+ or 4+ (confluent) growths on the drug-free control.

3.10.1.4 BACTEC radiometric method

The radiometric method is based on the commercial system BACTEC TB-460 (Becton Dickinson, Sparks, MD), which uses an enriched Middelbrook 7H9 liquid medium containing ^{14}C-labeled palmitic acid as the sole carbon source. Growth of the mycobacteria and consumption of the labeled fatty acid will produce $^{14}CO_2$ that is detected inside the 12B vial by the BACTEC apparatus and expressed as a growth index. In the presence of a certain drug, susceptibility can be measured by inhibition of the daily increases in the growth index. For the performance of the test, a test vial containing the drug under study and a drug-free control are inoculated with a standard inoculum and incubated at 37°C. The vials are then read in the BACTEC 460-TB apparatus on a daily basis. Since two control vials are inoculated with a 100-fold serial dilution of the inoculum, results can be interpreted as in the proportion method with the 1 % proportion of growth. More recently, critical concentrations for second-line drugs have also been proposed and tested successfully for most drugs in a multi center evaluation (Pfyffer et al., 1999). The major advantage of the BACTEC radiometric method is the capacity to detect drug resistance faster than with the solid media-based methods; the major disadvantage is the cost of the system and the need for disposal of the radioactive waste from used vials.

3.10.1.5 Mycobacterial Growth Indicator Tube Method

The Mycobacteria Growth Indicator Tube (MGIT) (Becton Dickinson, Sparks, MD) is part of the ‘new generation’ of TB diagnostic tools both in its manual version as well as in its more recently introduced automated format. It is based on fluorescence detection of mycobacterial growth in a tube containing a modified Middlebrook 7H9 medium together with a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. Consumption of oxygen in the medium produces fluorescence when illuminated by a UV lamp. In the manual system, for the performance of the test a drug-containing tube and a control tube are inoculated with the standardized mycobacterial

suspension and incubated at 37°C (day 0). Starting on the third day (day 2), the tubes are controlled daily with an UV lamp. The presence of an orange fluorescence in the drug-containing tube at the same time as in the control tube or within two days of positivity in the control is interpreted as resistance to the drug; otherwise, the strain is considered to be susceptible. The test is valid if the growth control gives a positive signal until the 14^{th} day of incubation (day 12) (Palomino and Portaels, 1999). The MGIT system in its manual version has also been successfully used as a direct method using de- contaminated clinical specimens. The MGIT has also been recently introduced as an automated system. The BACTEC MGIT960 (Becton Dickinson, Sparks, MD) is based on the same principle of oxygen consumption and a fluorescence signal, but the tubes are incubated and controlled inside the MGIT960 apparatus. For the performance of the test, drug-containing and drug-free control vials are inoculated with a standardized inoculum of the *M. tuberculosis* isolate and entered into the machine in a special rack-carrier with a printed barcode; this is read by the machine when entering the tubes to identify the test and apply the adequate algorithm for susceptibility or resistance interpretation. All readings are performed inside the machine and the results are printed as susceptible or resistant (Ardito et al., 2001).

3.10.2 Genotypic methods

Genotypic methods for drug resistance in TB look for the genetic determinants of resistance rather than the resistance phenotype, and 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 (García, 2003; Palomino, 2005).

3.10.2.1 Desoxyribonucleic acid (DNA) sequencing

Sequencing DNA of PCR-amplified products has become the most widely used genotypic method for detecting drug resistance in *M. tuberculosis*, DNA sequencing has

been widely for characterizing mutations in the rpoB gene in RFP-resistant strains and to detect mutations responsible for resistance to other anti-tuberculosis drugs (Telenti et al., 1993; García, 2003). Drug resistance detection in *M. tuberculosis* has also been described by pyrosequencing technology. This technology is a short-read (30–50 bp) sequencing technique, which is based on the quantitative detection of pyrophosphate released following nucleotide incorporation into a growing DNA chain.

3.10.2.2 Solid-phase hybridization techniques

There are currently two commercially available solid-phase hybridization techniques for the rapid detection of drug resistance in TB: the Line Probe Assay (INNO-LiPA Rif TB Assay, Innogenetics, Ghent, Belgium) for the detection of resistance to RFP and the GenoType MTBDR assay (Hain Lifesciences, Nehren, Germany) for the simultaneous detection of resistance to INH and RFP.

The LiPA assay was introduced several years ago and is based on reverse hybridization of amplified DNA from cultured strains or clinical samples to ten probes covering the core region of the rpoB gene of *M. tuberculosis*, immobilized on a nitrocellulose strip (De Beenhouwer et al., 1995). From the pattern of hybridization obtained, the presence or absence of mutated or wild regions is visualized by a colorimetric reaction and the strain can be considered as resistant or susceptible to RFP (Rossau et al., 1997).

The GenoType MTBDR, on the other hand, detects resistance to INH and RFP in culture samples based on the detection of the most common mutations in the katG and rpoB genes respectively (Makinen et al., 2006). It also utilizes PCR and reverse hybridization to probes immobilized on a DNA strip. Both solid-phase hybridization methods have proven relatively simple to perform; however, basic expertise in molecular biology and PCR techniques is required.

Another solid-phase reverse hybridization test for rapid detection of RFP resistance is rifoligotyping. This is an in house low-cost assay for the detection of RFP resistance-

associated mutations in the rpoB gene of *M. tuberculosis*. The test was developed at the National Institute of Public Health and the Environment in the Netherlands and initially evaluated at the Cetrangolo Hospital in Argentina. It also involves a combination of DNA amplification and reverse-line blot hybridization. DNA of the rpoB gene of *M. tuberculosis* is amplified by PCR with specific primers and the PCR products are hybridized to oligonucleotides on a DNA membrane, encoding the wild type rpoB sequence, and the most frequent mutations in RFP-resistant strains. Amplified products from RFP-resistant strains will fail to hybridize to one or more of the wild type oligonucleotides, and in most cases, will hybridize to one of the mutant oligonucleotides bound to the membrane. RFP resistant strains can be detected within a few hours with an enhanced luminescent reaction (Morcillo et al., 2002).

3.10.2.3 Real-time PCR techniques

Real-time PCR techniques have also been introduced recently for the rapid detection of drug resistance in TB. Different probes have been used for detection, such as the Taq Man probe, Fluorescence Resonance Energy Transfer probes, molecular beacons and biprobes. The main advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination. The main disadvantages would be the requirement for expensive equipment and reagents, and the need for skilled technical personnel. Real-time PCR techniques have been applied to *M. tuberculosis* strains and, more recently, directly to clinical samples. Results are generally obtained in an average of 1.5-2.0 hours after DNA extraction. Real-time PCR could eventually be implemented in reference laboratories with the required capacity to properly set up the technique and in settings where it can contribute to the management of TB patients (Shamputa et al., 2004).

3.10.2.4 Microarrays

Microarrays, also known as biochips or DNA chips, have been proposed as genotypic methods for detecting drug resistance in *M. tuberculosis*. They are based on the

hybridization of DNA obtained from clinical samples to oligonucleotides immobilized on a solid support, such as miniaturized glass slides. They have been tested to detect resistance to INH and RFP. The recently described CombiChip Mycobacteria drug-resistance detection DNA chip is an oligonucleotide microchip coupled with PCR for the detection of resistance to INH and RFP. For the time being, and due to the high cost involved, the use of microarrays for detecting drug resistance in M. tuberculosis is still beyond the reach of most clinical mycobacteriology laboratories, especially in high-burden countries (Gryadunov et al., 2005).

The laboriousness and long time required by conventional methods to give results and, on the other hand, the requirement for expensive equipment and the need for skilled technical personnel for most molecular techniques, continue to stimulate the search for alternative and affordable methods for drug resistance detection in TB.

3.10.3 Colorimetric methods

Several colorimetric methods have been proposed in the last few years for the rapid detection of drug resistance in *M. tuberculosis*. They use redox indicators or tetrazolium salts to detect mycobacterial growth. The tests are based on the reduction the colored redox indicator added to the culture medium after *M. tuberculosis* has been exposed in vitro to different antibiotics. Resistance is detected by a change in color of the indicator, which is directly proportional to the number of viable mycobacteria in the medium (Palomino et al., 2004).

Alamar blue (Trek Diagnostics, Ohio, USA) is a proprietary reagent that was the first to be used to detect drug resistance in *M. tuberculosis*. The reagent is blue in the oxidized state but changes to pink when reduced. In a study that evaluated the activity of INH, RFP, EMB, and SM on clinical isolates of *M. tuberculosis*, MICs were obtained after 1-2 weeks of incubation with an overall accuracy of 97 %, compared to the agar proportion method (Yajko et al., 1995).

The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT is a yellow compound that, when reduced by metabolically active cells, it produces crystals of insoluble purple MTT formazan that can be measured with a spectrophotometer after solubilization (Mosmann, 1983). With the purpose of speeding up the detection of drug resistance in clinical samples, MTT has also been applied as a direct assay in sputum samples for RFP-resistance detection. The sensitivity and specificity of this direct MTT assay matched those of the standard indirect drug susceptibility testing on 7H10 agar with 98.5 % of the samples giving interpretable results within two weeks (Abate et al., 2004).

Resazurin was also introduced in a rapid test to detect drug resistance in *M. tuberculosis.* The resazurin microtiter assay (REMA) allowed rapid detection of multidrug resistance in *M. tuberculosis* isolates with an overall accuracy of 97 % as compared to the proportion method in Lowenstein-Jensen medium. The REMA also showed its usefulness for the detection of resistance to other anti-tuberculosis drugs, including common second-line drugs, quinolones and pyrazinamide (Martin et al., 2006).

3.10.4 Nitrate reductase assay

The Nitrate Reductase Assay (NRA) is a quite 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. Resistant strains will reduce the nitrate revealed by a pink-red colour in the medium, while susceptible strains will loose this capacity as they are inhibited by the antibiotic, leaving the medium colourless (Angeby et al., 2002).

M. tuberculosis is cultivated on Lowenstein-Jensen medium in the presence of an antibiotic and its ability to reduce nitrate is measured after 10 days of incubation in a positive culture (Indirect method) or in 21-28 days when applied to a smear-positive sputum sample (Direct method). Resistant strains will reduce the nitrate, which is revealed by a pink-red color in the medium, while susceptible strains will lose this

capacity as they are inhibited by the antibiotic. It has the added advantage of using the same format and culture medium as the standard proportion method (Angeby et al., 2002).

The assay has been evaluated in several studies for first-line drugs and ofloxacin with good results. It has the added advantage of using the same format and culture medium as the standard proportion method. In a multicenter study that evaluated the performance of the NRA for detecting resistance to the first-line drugs, the test performed very well for INH, RFP and EMB with an accuracy of 96.6 % to 98 %. Lower values, were obtained for SM (Martin et al., 2005). However, the NRA was easily implemented in settings with limited laboratory facilities. Two recent studies applied the NRA directly on sputum samples and produced variable results for sensitivity and specificity; the best results were obtained for INH and RFP resistance detection (Musa et al., 2005; Solís et al., 2005). These two studies have shown the feasibility for implementation of the NRA as a direct test on sputum samples that warrant further evaluations in target populations.

M. tuberculosis has an enzyme bound to the cell membrane that rapidly reduces nitrate and leads to the accumulation of nitrite. Unlike those of other mycobacteria, *M. tuberculosis* nitrate reductase is permanently very active *in vitro* regardless of the culture conditions. Under hypoxic conditions or on exposure to nitric oxide, its activity may even be enhanced by induction of the protein NarK2. This protein is a nitrate transporter that might be able to sense the redox state of the cell and adjust its own activity accordingly (Sohaskey, 2005). The reductase activity may be hindered by very high concentrations of INH. Furthermore, some isolates of the tubercle bacillus that are resistant to INH and para-aminosalicylic acid (PAS) were found to be unable to reduce nitrate when growing in minimal media (Hedgecock, 1962). The nitrate reductase activity seems to be encoded by the constitutive *narGHJI* operon (Weber, 2000), which is present in both *M. tuberculosis* and *M. bovis*. However, *M. bovis* does not reduce nitrate. It was demonstrated that a single nucleotide polymorphism at position 215 in

the promoter of this gene cluster determines different levels of enzyme activity in both species (Sohaskey, 2003). *M. canettii* and some isolates of *M. africanum* produce detectable amounts of nitrite from nitrate in vitro.

3.11 Treatment of Tuberculosis

Excellent statements of recommendation for the treatment of tuberculosis have been produced by the American Thoracic society-centers for Disease Control and Prevention (American Thoracic society et al., 2003), world Health Organization (1997), and other Organizations. These statements are written by committees of experts and set the standard for treatment.

3.11.1 Anti-tubercular drugs

Most common drugs used in the treatment of TB patients include: isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide.

Isoniazid (INH)

Isoniazid (isonicotinic acid hydrazide) is a synthetic drug, introduced in 1952 and has been the most used anti-tubercular drug. It has excellent bactericidal activity that is largely limited to *M. tuberculosis* and to a lesser extent to other members of the mycobacterial family. It appears to interfere with the mycolic acid synthesis. Some evidence indicates that INH inhibits a desaturation step in the production long-chain fatty acids and may also inhibit the elongation of fatty acids and hydroxy lipids (Winder and Collins, 1970).

Resistance to INH was associated with less virulence in vitro and decreased catalase activity. This has been attributed to mutations of the KatG gene, which encodes a catalase-peroxidase required to activate INH (Zhang et al., 1992). Mutations of the regulatory region of the ahpC gene cause overproduction of alkyl hydroperoxide reductase but do not confer virulence (Heym et al., 1993).

Rifampicin (RFP)

Rifampicinn is 3, 4-(methylpiperazinyl-imidene)-rifamycin SV; it was introduced in 1968 as anti-tuberculosis agent. It is the most important drug for the treatment of all forms of TB. Short course chemotherapy can not be accomplished without it. RFP inhibits a DNA-dependent RNA polymerase. This enzyme appears to be a complex polymer that is made up of different subunits encoded by the rpo genes. Most RMP resistance comes from modification in the rpoB genes (Telenti et al., 1993).

Pyrazinamide (PZA)

Pyrazinamide is a synthetic derivative (pyrazine analog) of nicotinamide, and in combination with INH, is rapidly bactericidal for replicating forms of *M. tuberculosis*. PZA is inactive against non-replicating tubercle bacilli and totally inactive against other Mycobacterium species. It is active only at acidic pH. It may be converted to pyrazinoic acid in the secondary lysosome and lowers the pH, which increases the killing of the mycobacteria harbored there. Most PZA resistance occurs with mutated pncA genes (Sreevastan et al., 1997).

Ethambutol (EMB)

Ethambutol is dextro-2, 2-(ethylenediimino)-di-1-butanol-dihydrochloride. It is a potent synthetic anti-tuberculosis agent that was introduced in 1961. It can be given for all forms of TB and may be synergistic with most medications. EMB inhibits mycobacterial arabinosyl transferase and the synthesis of arabinogalactan, a component of the polymer core of the mycobacterial cell wall (Takayama and Kilburn, 1989). Most EMB resistance results from alternation of embAB gene products that causes an overproduction of arabinosyl transferase (Sreevastan et al., 1997).

Streptomycin (SM)

Streptomycin was the first effective drug to be used against TB. It is still considered to

the fifth drug for TB and is supplemented by the quinolones. It is effective but must be given intramuscularly. It penetrates the inner and outer membrane of mycobacteria probably through porin channels and exert their influence by binding to bacterial ribosomes. This reduces bacterial protein synthesis and induces misreading of mRNA. There appears to be several mechanism of SM resistance, but most arise from altered genes, e.g. rpsL, which encodes the ribosomal protein S12, and rrs, a 16S rRNA gene (Finken et al., 1993; Nair et al., 1993).

Other drugs that are given to TB patient according to the level of treatment and requirement are: rifabutin, ethionamide, fluoroquinolones, capreomycin, cycloserine, para-aminosalicylic acid (PAS), linezolid, kanamycin etc.

3.11.2 Notification of cases

New case: Smear-positive persons who have never previously been treated or have only received treatment for less than one month.

Relapse: a patient who has been declared cured of any form of TB in the past by a physician, after one full course of chemotherapy, and has become sputum smear-positive.

Treatment failure: a patient who, while on treatment, remained or became again smear-positive 5 months or later after commencing treatment; and

Treatment after interruption (after default): a patient who interrupts treatment for months or more, and returns to the health service with smear-positive sputum.

Chronic case: a patient who remained or became again smear-positive after completing a fully supervised re-treatment regimen (WHO, 1997)

3.11.3 Treatment regimens

The chemotherapeutic regimens are based on standardized combinations of 5 essential anti-TB drugs viz: INH, RFP, SM, EMB and PZA/Z. Each standardized chemotherapeutic regimens consists of 2 phases:

1 Initial (intensive) - 2 to 3 months, with 3-5 drugs given daily under direct observation, to maximally reduce the number of TB organism. The number of drugs used relates to the risk of failure of treatment due to bacterial resistance; &
2 Continuation – 4 to 6 months, with 2-3 drugs given 3 times a week under direct observation, or in some cases (e.g. during repatriation of refugee) 2 drugs for 6 months given daily unsupervised, but in fixed-dose combination form.

3.11.4 Treatment Categories

Treatment categories are essential for prioritization of TB treatment. For each TB patient, the recommended regimen depends on the treatment category determined by the case definition.

Category I

These patients are: New cases and severely ill patients with other forms of TB (new smear-negative PTB with extensive parenchymal involvement, and new cases of severe forms of TB).

Recommended regimens

Intensive phase: 2HRZE (S) i.e. isoniazid, rifampicin, pyrazinamide and either ethambutol or streptomycin, daily for 2 months.

Continuation phase: 4HR or $4H_3R_3$ i.e. isoniazid and rifampicin given daily or three times a week for 4 months. For patients with TB meningitides, disseminated TB or Spinal disease with neurological complications, isoniazid and rifampicin should be given daily for 6-7 months (i.e. a total of 8 months of therapy).

Category II

Patients who were previously treated and now sputum smear-positive, include: Treatment after interruption (after default), treatment failure, and relapse after treatment

Recommended regimens

Intensive phase: 2HRZES/ 1HRZE i.e. rifampicin combined with isoniazid, pyrazinamide and ethambutol, given daily for 3 months and supplemented with streptomycin for the first 2 months.

Continuation phase: $5H_3R_3E_3$ i.e. isoniazid, rifampicin and ethambutol three times per week for 5 months.

Category III

These patients are: Smear-negative pulmonary patients (with limited parenchymal involvement) and non-serious extra pulmonary disease in adults and children (including symptomatic primary disease)

Recommended regimens

Intensive phase: 2HRZ/ $2H_3R_3Z_3$ i.e. isoniazid, rifampicin and pyrazinamide given daily or three times a week for 2 months.

Continuation phase: 6HE (T)/ $4H_3R_3$ i.e. isoniazid and ethambutol or thioacetazone, given daily for 6 months, or isoniazid and rifampicin three times a week for 4 months.

Table 3.5: Recommended treatment regimens for each treatment category (WHO, 1997)

Treatment Category	Patients	Initial (Intensive) Phase	Continuation Phase
I	New smear-positive PTB; new smear –negative PTB with extensive parenchymal involvement; new cases of severe forms of extra-pulmonary TB.	2 EHRZ (2 SHRZ) Or 2 $E_3H_3R_3Z_3$ (2 $S_3H_3R_3Z_3$)	4 H_3R_3 (6 HE)
II	Sputum-smear positive; relapse; treatment failure; treatment after interruption.	2 HRZE/ 1 HRZE	5 $H_3R_3E_3$
III	New smear-negative PTB (other than in Category I); new less severe forms of extra-pulmonary TB.	2 HRZ Or 2 $H_3R_3Z_3$	4 H_3R_3 (6 HE)

3.11.5 Adverse effects of anti-TB drugs

Adverse effects are classified as minor or major. In general, a patient who develops minor adverse effects should continue the anti-TB treatment, usually at the same dose but sometimes at the reduced dose. The patient also receives symptomatic treatment. If a patient develops a major side effect, the treatment or the offending drug is stopped. Patients with major adverse reactions should be managed in a hospital (WHO, 1993; WHO, 1997; WHO, 2004).

3.12 Drug resistant tuberculosis

For *M. tuberculosis* complex drug resistance is defined in terms of the critical concentration of drug. The critical concentration of drug is the amount required to prevent growth above the 1% threshold of the test population of tubercle bacilli. Drug resistant TB and, particularly, multi drug-resistant TB (MDR-TB), have recently received heightened attention. Drug resistant TB is a case of TB (usually pulmonary) excreting bacilli resistant to one or more anti-tuberculosis drugs. Resistance of *M. tuberculosis* to anti-TB drugs is the result of a spontaneous genetic event and, worse "a

man-made amplification of the natural phenomenon" (Pfyffer, 2000).

3.12.1 Types of drug resistance

"Acquired or Secondary" resistance occurs in patients with some record of previous treatment (WHO, 2002). This is mostly because of single drug-due to irregular drug supply, inappropriate prescriptions or poor adherence to treatment-suppresses the growth of susceptible bacilli to that drug but permitted the multiplication of drug resistance organisms.

"Primary or Pretreatment" resistance occurs in patients who have not had prior treatment with anti-tuberculosis drugs. This type of resistance is observed when a patient develops TB after being infected by another patient who has resistant TB organisms.

3.12.2 Factors associated with anti-tuberculosis drug resistance:

According to Rijal *et al.* (2002), the factors associated with anti-tuberculosis drug resistance are:

Programmatic factors: These include lack of standardized therapeutic regimen, poor implementation compounded by frequent or prolonged shortage of drug supply in areas with inadequate resources, political instability,and Use of anti-tuberculosis drugs of unproven quality

Health provider related factors: These include departure from the correct management of individual cases, selection of inappropriate regimen due to lack of recognition of prior treatment, ignorance of importance of standardized regimens, addition of a single drug to a failing regimen, and lack of proper monitoring and supervising patients while on therapy.

Patients related factors: These include Non-adherence to prescribed treatment, HIV infection

3.12.3 Mechanism of resistance

Resistance to anti-tuberculosis medications is due to unlinked chromosomal mutations of the tubercle bacilli. In *M. tuberculosis* spontaneous mutations occur at a frequency of approximately 10^{-5} to 10^{-8} (Pfyffer, 2000; Iseman, 1993). Since resistance to various drugs arises independently, the likelihood of spontaneous mutation to Isoniazid and Rifampicin, for instance, is 1 in 10^{14} ($10^{6} \times 10^{8}$). At first sight, the probability of dual mutation seems minimal. However, since PTB is always associated with enormous bacterial masses (cavities contain as many as 10^{7}-10^{9} organisms), dual mutations will be seen with a certain frequency. This threat of multi-drug resistance is one reason why combination regimens must always used for TB.

Resistance to RFP arises due to mutations in the beta subunit of RNA polymerase encoded by rpo B, inhibiting RNA synthesis. Missense mutation in RNA polymerase and alteration in cell wall permeability is responsible for RFP resistance. Similarly, resistance to INH could occur either through a missense mutation that blocks INH from binding to the gene or through formation of large amounts of enzyme which could nullify the effect of INH. Kat G gene product has also been shown to be responsible for INH resistance. Resistance to streptomycin emerges through mutations in rrs and rpsl gene that produce alteration in the streptomycin binding site. Most pyrazinamide resistant organisms have mutations in the pyrazinamidase gene, although the gene may also be inactivated through the insertion of IS *6110* (Gillespie, 2002).

3.12.4 MDR-TB: Multi-drug resistant TB

MDR TB refers to *M. tuberculosis* isolates that are resistant to at least both INH and RFP, the two most powerful anti-TB drugs. This is a very serious problem. People with MDR TB disease can only be treated with reserve or second-line drugs. These drugs are

not as effective as the first-line drugs and cause more side effects.

People who have spent time with someone sick with MDR-TB disease can become infected with TB bacteria that are resistant to several drugs. Historically, MDR TB has spread gradually with alarming rates seen in 2001 in India, China, UK, Russia, Peru, Spain and Puerto Rico (WHO, 2004; Swaminathan *et al.*, 2005). The magnitude of anti-TB drug resistance is not well documented in the SEAR; however, the mean prevalence of MDR TB among new smear positive cases in the SEAR is believed to be low, at an overall 2%. In 1996/97, an MDR TB case was 1.20%, with 3.60% in 1998/99 which later decreased to 1.30% in 2001/2002 (NTC, 2007). Isolated reports of higher levels of MDR TB are reported mainly from hospital settings. Levels as high as 60% are reported among previously treated cases in tertiary care facilities.

3.13.5 XDR-TB: Extensive drug-resistant TB

XDR-TB was defined as MDR-TB with further resistance to second-line drugs . In addition to being MDR, XDR TB was initially defined as having resistance to at least three of the six main classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and paraaminosalicylicacid) (CDC 2006). More recently, at a consultation meeting of the World Health Organization (WHO) Global Task Force on XDR-TB, held in Geneva, a revised laboratory case definition was agreed. According to this "XDR-TB is TB showing resistance to at least rifampicin and isoniazid, which is the definition of MDR-TB, in addition to any fluoroquinolone, and to at least 1 of the 3 following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin." XDR-TB now constitutes an emerging threat for the control of the disease and the further spread of drug resistance, especially in HIV-infected patients, as was recently reported (Gandhi, 2006).

CHAPTER IV

4. MATERIALS AND METHODS

4.1 Materials

All the materials used in the study are listed in Appendix II.

4.2 Study setting

This study was the collaborative study between the Central Department of Microbiology, Tribhuvan University, Kathmandu, National Tuberculosis Centre and SAARC TB and HIV AIDS Centre, Thimi, Bhaktapur.

4.3 Study design

The study is a prospective study comparing the sensitivity and specificity of the Direct Nitrate Reductase Assay with the LJ-proportion method in determining Drug Susceptibility pattern to four primary anti-tubercular drugs among clinical isolates.

4.4 Study period

The study was carried out during June 2008 to March 2009.

4.5 Study population

A total of 302 previously treated pulmonary tuberculosis patients visiting National Tuberculosis Centre, Thimi, Bhaktapur were included in this study.

4.6 Data collection

Standard questionnaires were prepared and used to collect the data on personal information (Age, Sex, Case type etc. as given in Appendix I).

4.7 Laboratory methodology

4.7.1 Collection of sputum sample

The sputum sample was collected in wide mouth, transparent, plastic, sterile, leak proof, screw capped 50 ml falcon tube. About 3-5 ml, sputum specimen was collected. The container was labeled and filled in a request form available in the hospital. The specimen was kept at 4^0C (WHO, 1998).

When a sputum specimen was being collected, adequate safety precautions were taken to prevent the spread of infectious organisms. Aerosols containing tuberculosis bacteria may be formed when the patient coughs to produce a sputum specimen, therefore the patients were instructed to produce specimen either outside in the open air or away from other people and not in confined spaces such as toilets.

4.7.2 Evaluation of sputum

Collected sputum samples were evaluated using standard protocols (WHO, 1998).

4.7.3 Macroscopic examination of sputum sample

Macroscopic evaluation of sputum samples was done using standard methodology (Cheesbrough, 2002).
When the sputum was mostly saliva, the specimen was reported as “unsuitable” for microbiological investigation and requested another specimen.

4.7.4 Processing of Sputum spcimen

Sodium hydroxide (Modified Petroff) method was used for the homogenization and decontamination of sputum samples. The composition and preparation of digestion and decontamination reagent is given in Appendix III. Detailed procedure of NaOH (Modified Petroff) method is mentioned in Appendix V.

4.7.5 Microscopic examination of processed sputum

Microscopic examination of sputum was done using standard methodology (WHO, 1998). Detailed of ZN-staining procedure is mentioned in Appendix V.

4.7.6. Direct Nitrate Reductase Assay Method

4.7.6.1. Inoculation and Incubation

The test was performed only on specimen with an AFB positive smear result. 0.1 ml of the processed sputum was inoculated on each of modified LJ media tube containing $NaN0_3$ (2 plane control tubes and 4 tubes containing critical concentration of drugs isoniazid, rifampin, streptomycin, and ethambutol respectively). Detailed procedure of preparation of media for test is mentioned in Appendix IV. The caps of inoculated media were kept loose and incubated at 37°C in a slanting position. When the surfaces of media were dry after few days, the caps were tighten and further incubated for up to 28 days.

4.7.6.2 Interpretation of Results

After 28 days of incubation at 37°C, 0.5 ml of freshly prepared Griess reagent is transferred into one of the plane growth control tube, and development of color is observed. (The composition and preparation of Griess reagent is given in Appendix III). If the color intensity is sufficient, the same amount of Griess reagent is transferred into the drug-containing tubes. The color intensity in the drug-containing tubes is then compared to the control tubes. The results were classified as negative if no color changes or a very pale pink color were observed. Positive results varied from pink to deep red or violet. The results were thus, interpreted as follows:

Resistant (R): An isolate was considered resistant to a certain drug if there was a positive color change in the drug tube in question and in the drug-free control tube.
Sensitive (S): In contrast, an isolate was considered sensitive to a drug if there was no

color change in the drug tube in question and positive color change in the drug-free control tube.

Invalid: If no color changes or pale pink color were observed in the control tube, the test was considered to be invalid.

4.7.7 Conventional Lowenstein-Jensen Method (Gold Standard Method)

4.7.7.1 Inoculation and Incubation

One loopfull of sediment from homogenized and decontaminated sample was inoculated on two culture tubes of LJ media. The caps of inoculated media were kept loose and incubated at 37°C. When the surfaces of media were dry after few days, the caps were tightened and further incubated for up to 8 weeks.

4.7.7.2 Culture examination

All the cultured tubes were examined after one week to detect any rapidly growing mycobacteria which might be mistaken for *M. tuberculosis*. The growth on the media was observed at every week after inoculation. The negative cultures were discarded only after 8 weeks.

4.7.7.3 Recording and reporting of culture results

Result of culture was reported as negative, positive (scanty, 1+, 2+ and 3+) and contamination according to WHO guidelines (WHO, 1998).

4.7.7.4 Microscopic examination by ZN-staining

The acid-fastness of the growth was confirmed by ZN-staining. A very small amount of growth was removed from the culture using a loop and gently rubbed into one drop of sterile saline on a clean slide. The smear was allowed to dry, fixed by heat and stained by ZN-method (Fujiki, 2001). The composition and preparation of staining reagents for

ZN-microscopy is mentioned in Appendix III. The ZN-staining procedure is described in detail in Appendix V.

4.7.8 Identification of Isolates

Although a presumptive diagnosis was made by colony morphology and serpentine cord formation, confirmatory test was carried out by growth on medium containing p-nitrobenzoic acid.

4.7.8.1. Inoculum preparation

One loopful of growth with a 3 mm diameter loop from a L-J medium was mixed with 5 ml of sterile distilled water to give 4 mg/ml suspension of growth (Fujiki, 2001).

4.7.8.2 Inoculation on medium containing p-nitrobenzoic acid (PNB)

One loopful of the suspension taken with a 3 mm diameter loop was inoculated onto two slants of PNB containing and PNB free media. The slants were incubated at 37°C up to 4 weeks. The slants were observed on 1st week and 4th week of incubation. The preparation of 0.5 mg/ml PNB containing L-J media is given in Appendix IV.

4.7.9 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test was performed by proportion method as recommended by IUATLD and WHO. Each strain was tested against four primary anti-tubercular drugs viz. INH, RFP, SM and EMB.

4.7.9.1 Preparation of drug containing media

The drug containing media were prepared with critical concentrations of primary anti-tubercular drugs as shown in Table 4.1

Table 4.1: Critical concentrations of primary anti-tubercular drugs used in the study

Drug	INH	RMP	SM	EMB
concetration µg/ml	0.2	40	4	2

The preparation of drug solutions and drug containing media for the Antimicrobial susceptibility testing by proportion method is mentioned in Appendix IV.

4.7.9.2 Preparation of bacillary suspension

1. 0.1 ml of sterile distilled water was taken in a screw-capped tube, containing six to seven 3 mm diameter glass beads.
2. One loopful of growth from sub-cultured L-J medium was transferred to the tube by using standard 3 mm diameter loop.
3. The tube was vortexed for few minutes and 1-2 ml sterile distilled water was added to give smooth suspension.
4. The turbidity of the suspension was adjusted with a Mac Farland No.1 by adding sterile distilled water (preparation of Mac Farland No.1 is in Appendix III).

4.7.9.3 Dilution of bacillary suspension for inoculation

1 mg/ml bacillary suspension was diluted by serial 100 fold dilutions with sterile distilled water. The dilutions were made as follows:

1. The dilution of 10^{-2} was produced by discharging One loopful i.e. 0.01 ml (3 mm diameter loop) of the bacillary suspension (standardized at 1 mg/ml) into a tube containing 1 ml of sterile distilled water and shaking.
2. Similarly, the dilution of 10^{-4} was produced by discharging One loopful of the dilution 10^{-2} into another tube containing 1 ml of sterile distilled water and shaking.

4.7.9.4 Inoculation and Incubation

1 From 10^{-2} dilution, one loopful of bacillary suspension was inoculated into two slopes of each of the four drug containing media of critical concentrations.
2 From 10^{-2} dilution, one loopful of bacillary suspension was inoculated into a slope of controls of plain L-J media and from 10^{-4} dilution, one loopful of bacillary suspension was inoculated into slope of controls of another plain L-J media i.e. 10^{-2} dilution in control II and 10^{-4} dilution in control IV.
3 All these tubes were incubated at 37°C for 6 weeks and observed first at 28^{th} day and then at 42^{nd} day.

4.7.9.5 Interpretation of results

10^{-4} dilution represents 1% of 10^{-2} dilution. Therefore, any colonies growing on drug containing medium inoculated with the 10^{-2} dilution that equal or more the number of colonies growing on the control medium inoculated with the 10^{-4} dilution (i.e. control IV) represents 1% or more of the test population. The results were thus, interpreted as follows:

Sensitive: No growth on the drug media with the critical concentration of the respective drugs or was less than growth on control IV was classified as "sensitive" (i.e. <1%).
Resistant: Growth on the drug media with the critical concentration of the respective drug was equal or more than on control IV was classified as "resistant" (i.e. ≥1%).

4.7.10 Quality control

Quality control ensures that the information generated by the laboratory is accurate, reliable and reproducible. During this study, quality control was applied in various fields (Fujiki, 2001; WHO, 1998; IUATLD, 1998).

1 Any leaking and broken specimen containers were discarded by autoclaving

and requested for a repeated specimen.

2 Smears were prepared only inside the Biological safety cabinet.

3 Fresh reagents were used for the staining procedure.

4 Slides were stained in batches with maximum of 12 slides per batch.

5 Temperature in all instruments (Inspissator, Water bath, Refrigerator and Incubators) were checked daily and recorded in the chart.

6 The inspissator was cleaned after each batch of culture media prepared. Its temperature was also checked during each period of media preparation.

7 Those media with faulty coagulation, discoloration or bubbles were discarded.

8 While using readymade dehydrated media, the manufacturer's instructions for preparation, sterilization and storage were followed to prevent the alteration of the nutritional, selective, inhibitory, and biochemical properties of the media.

9 The sputum specimens were processed in batches according to the capacity of centrifuge.

10 Each batch of prepared media was checked for sterility by incubating at 37°C for 24 hours.

11 All prepared media were kept in the dark in the refrigerator and unused media were discarded after 4 weeks.

12 Cross-contamination of culture was avoided by using individual pipettes or loops and strict aseptic techniques

13 When each newly batch of drug media was prepared, the quality of media was checked by inoculating standard strain *M. tuberculosis* H_{37}Rv suspension onto a series of drug containing media. The detailed procedure for quality check of drug containing media and interpretation table is given in Appendix VII.

4.8 Data analysis

All the tests results were recorded in Microsoft Excel 2007 spread sheets. Statistical analysis for the calculation of sensitivities, specificities, PPV and NPV was done. Chi Square test was used, wherever applicable.

CHAPTER V

RESULT

A total of 302 previously treated patients of pulmonary tuberculosis (PTB), attending National TB Centre, Thimi, Bhaktapur, Nepal was included in this study. All the sputum samples from the patient included in the study were processed and tested for acid fast bacilli (AFB) by ZN- microscopy and culture. Sputum samples those showing positive for AFB were tested by Direct Nitrate Reductase Assay method.

5.1 Case and gender-wise distribution of PTB patients enrolled in the study

The highest number of cases was Relapse 39.40% (119) followed by DOTS plus follow up 34.10% (103), Follow-up 16.23% (49), Chronic 9.94% (30) and defaulter 0.33% (1). Among all cases, 74.50% (225) were male patients and 25.50% (77) were female patients. The distribution is shown in Table-5.1.

Table 5.1: Case and gender-wise distribution of PTB patients enrolled in the study

S.N.	Case type	Male		Female		Total	
		No.	%	No.	%	No.	%
1.	Relapse	93	41.34	26	33.76	119	39.40
2.	Chronic	25	11.11	5	6.49	30	9.94
3.	Defaulter	1	0.44	0	0	1	0.33
4.	Follow up	34	15.11	15	19.50	49	16.23
5.	D0TS plus follow up	72	32.00	31	40.25	103	34.10
Total		225	74.50	77	25.50	302	100

5.2 Age and gender-wise distribution of the PTB patients included in the study

The highest number of cases was in the age group of 21-30 years 30.79% (93), followed by 41-50 years 20.20% (61) and 31- 40 years 16.89% (51). The distribution is shown in Table-5.2

Table 5.2: Age and gender-wise distribution of the PTB patients included in the study

Age	Male		Female		Total	
	No.	%	No.	%	No.	%
11-20	23	10.22	11	14.28	34	11.26
21-30	62	27.56	31	40.26	93	30.79
31-40	38	16.88	13	16.88	51	16.89
41-50	50	22.22	11	14.28	61	20.20
51-60	27	12.00	8	10.38	35	11.59
61-70	16	7.12	2	2.60	18	5.96
71-80	9	4.00	1	1.30	10	3.31
Total	225	74.50	77	25.50	302	100

5.3 AFB smear positivity among cases

On AFB staining, Out of 302 patients, 158 (52.32%) were AFB staining negative and 144 (47.68%) was positive. However, the positivity rate varied amongst different cases. Among 144 smear positive cases 82 (56.94%) was 3+, 31 (21.52%) was 2+, 25 (17.36%) was 1+ and 6 (4.16%) was scanty. The distribution is shown in Table-5.3

Table 5.3: Results of AFB smear by microscopy

Case type	Smear result					Total positive(%)	Total
	Negative	Scanty	1+	2+	3+		
Relapse	18	2	17	21	61	101(84.87)	119
Chronic	11	2	2	5	10	19(63.33)	30
Defaulter	-	-	-	1	-	1(100)	1
Follow up	35	2	3	3	6	14(28.57)	49
DOTS plus follow up	94	0	3	1	5	9(8.73)	103
All cases	158	6	25	31	82	144(47.68)	302

5.4 Culture positivity among cases

Out of 302 specimens 164 (54.30%) was culture negative and 127(42.05%) was culture positive, 11(3.64%) were contaminated. The positivity rate is varying amongst different cases. Among 127 culture positive cases 92 (72.44%) was 3+, 26 (20.47%) was 2+, 6 (4.72%) was 1+ and 3 (2.36%) was scanty. The distribution is shown in Table-5.4

Table 5.4: Results of sputum culture

Case type	Culture result						Total positive (%)	Total
	Negative	Scanty	1+	2+	3+	Conta-mination		
Relapse	18	1	3	19	69	9	92 (77.31)	119
Chronic	13	1	1	2	13	-	17 (56.66)	30
Defaulter	-	-	-	1	-	-	1 (100)	1
Follow up	37	1	1	3	6	1	11 (22.44)	49
DOTS plus follow up	96	-	1	1	4	1	6 (5.82)	103
All cases	164	3	6	26	92	11	127 (42)	302

5.5 Correlation between microscopy and culture of sputum specimen.

Of the 144 smear-positive specimens, 121 were culture-positive, 12 were culture-negative and 11 were contaminated. Of the 158 smear-negative specimens, 152 were culture-negative and 6 were culture-positive.

Table 5.5: Results of microscopy and culture of sputum specimen.

Microscopy	Culture			Total
	Positive	Negative	Contamination	
Positive	**121**	12	11	144
Negative	6	152	0	158
Total	127	164	11	302

5.6 Growth pattern of culture positive samples on LJ medium containing PNB

All the culture positive specimens showed growth on LJ-media on sub culture

Table 5.6: Results of culture positive isolates on LJ medium containing PNB

Culture positive samples	Sub- culture on plane L-J medium		Growth on L-J medium containing PNB	
	Positive	Negative	Positive	Negative
121	121	0	0	121

5.7 Age and Genderwise distribution of smear and culture-positive cases

Among 302 specimens, 121 (40.07%) specimens were found to be positive by both microscopy and culture. Among 225 male patients, 99 (44%) were positive and among 77 female patients 22 (28.58%) were positive. The distribution is shown in Table-5.7

Table 5.7: Age and Gender-wise distribution of smear and culture-positive cases

Age	Sample	Male			Female		
		Number	Positive	%	Number	Positive	%
11-20	34	23	10	43.44	11	3	27.27
21-30	93	62	26	41.94	31	7	22.58
31-40	51	38	17	44.73	13	2	15.38
41-50	61	50	24	48.00	11	5	45.45
51-60	35	27	12	44.44	8	4	50.00
61-70	18	16	6	37.50	2	1	50.00
71-80	10	9	4	44.44	1	-	0
Total	302	225	99	44.00	77	22	28.58

5.8 Drug Susceptibility Pattern of culture positive isolates

The pattern of susceptibilities to four anti-tuberculosis drugs determined by proportion method and direct NRA method is in Table-5.8

Table 5.8: Drug Susceptibility Pattern of culture positive isolates (n=121) determined by the proportion method and direct NRA method.

Drugs	Proportion Method		Direct NRA Method	
	Resistant	Sensitive	Resistant	Sensitive
INH	28	93	36	85
RFP	26	95	27	94
SM	25	96	32	89
EMB	21	100	23	98

There is no difference in results obtained by standard proportion method and direct NRA method. Statistical analysis is given in Appendix IX

Table 5.9: Comparison of Direct Nitrate Reductase Assay results with Conventional proportion method

Drugs	Conventional proportion method	Direct Nitrate Reductase Assay method					
		Resistant	Sensitive	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
INH	Resistant = 28	28	0	100	91	78	100
	Sensitive = 93	8	85				
RFP	Resistant = 26	26	0	100	98.95	96.30	100
	Sensitive = 95	1	94				
SM	Resistant = 25	24	1	96	91.66	75	98.88
	Sensitive = 96	8	88				
EMB	Resistant = 21	21	0	100	98	91.30	100
	Sensitive=100	2	98				

Calculations of sensitivity, specificity and predictive values are given in Appendix VIII.

Table 5.10: Resistance profiles of *M. tuberculosis* isolates (n = 121).

Pattern	Number of patients		
	Male (%)	Female (%)	Total (%)
Total patients tested	99 (81.81)	22 (18.19)	121 (100)
Sensitive to all drugs	72 (72.72)	16 (72.72)	88 (72.72)
Drug resistance	27 (28.28)	6 (28.28)	33 (28.28)
Any resistance			
INH	22 (22.22)	6 (27.27)	28 (23.14)
RFP	21 (21.21)	5 (22.72)	26 (21.49)
SM	20 (20.20)	5 (22.72)	25 (20.66)
EMB	18 (18.18)	3 (13.63)	21 (17.35)
Mono resistance	6 (6.06)	1 (4.54)	7 (5.78)
INH	4 (66.66)	1 (100)	5 (71.43)
RFP	0	0	0
SM	1 (16.66)	0	1 (14.28)
EMB	1 (16.66)	0	1 (14.28)
Resistance to 2 drugs	3 (3.03)	0	3 (2.48)
INH + RFP	1 (33.33)	0	1 (33.33)
RFP + SM	2 (66.66)	0	2 (66.66)
Resistance to 3 drugs	3 (3.03)	2 (9.09)	5 (4.13)
INH+RFP+SM	1 (33.33)	2 (100)	3 (60)
INH+RFP+EMB	1 (33.33)	0	1 (20)
RFP+SM+EMB	1 (33.33)	0	1 (20)
Resistance to all 4 drugs			
INH+RFP+SM+EMB	15 (15.15)	3 (13.63)	18 (14.88)
Multidrug resistance	18 (18.18)	5 (22.72)	23 (19)

CHAPTER VI

DISCUSSION AND CONCLUSIONS

6.1 DISCUSSION

During the study period, a total number of 302 sputum samples from previously treated patients of pulmonary tuberculosis (PTB) were processed and tested for acid fast bacilli (AFB) by ZN-microscopy and culture. Out of 302 sputum samples, 144 sputum samples positive for AFB were tested by Direct Nitrate Reductase Assay method.

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. Resistant strains will reduce the nitrate revealed by a pink-red colour in the medium, while susceptible strains will loose this capacity as they are inhibited by the antibiotic, leaving the medium colorless (Angeby et al., 2002).

In the present study, Direct Nitrate Reductase Assay method was tested on 144 AFB smear-positive sputum specimens. Out of these 144 smear-positive specimens, the method was completed on 121 specimens. On remaining 23 specimen the test was not completed as 12 were culture-negative by both gold standard and Nitrate reductase assay method and 11 were contaminated on culture by either one or both method.

Among smear positive samples processed for culture, 7.6% (11) were contaminated. According to WHO (1998), a contamination rate of 2 % to 3% is acceptable in laboratories that receive fresh specimens; but it may be as high as 5% to 10% if specimens (especially sputum) take several days to reach the laboratory. In this study, the contamination rate was slightly higher than the acceptable range which might be due to improper transportation of the sample to the laboratory, inefficient decontamination and other laboratory procedures.

Considering proportion method as the standard method, direct nitrate reductase assay was subjected for evaluation. Sensitivities (the ability to detect a true drug resistance in a strain) and specificities (the ability to detect a true drug susceptibility in a strain) for drugs as determined by the NRA method compared to those determined by the proportion method were 100% and 91% for isoniazid, 100% and 98.95% for rifampicin, 96% and 91.66% for streptomycin, and 100% and 98% for ethambutol respectively.

Direct Nitrate Reductase Assay has been successfully used by many researchers. In a similar study by Sloutsky et al., (2005) in Lima, Peru showed the sensitivity and specificity of the method for INH resistance was 99.1% and 100%, and for RMP resistance was 93.5% and 100% respectively. Study by Angeby et al., (2005) showed sensitivity and specificity of the direct NRA using the direct proportion method as reference for INH, RFP, SM, and EMB were 100 and 100% , 93 and 100% , 76 and 100% and 55 and 100% respectively. In another report by Angeby et al., (2002), sensitivities and specificities for drugs as determined by the NRA method compared to those determined by the BACTEC 460 method were 100%and 100% for rifampicin, 97% and 96% for isoniazid, 95% and 83% for streptomycin, and 75% and 98% for ethambutol, respectively. Other study of the performance of the indirect NRA method by Golyshevskaia et al., (1996) in Sweden, sensitivity and specificity for INH were 97% and 96% respectively, and 100% for RMP.

In this study sensitivity of all drugs gave full agreement concerning the results of the direct NRA and proportion methods. The method showed the best agreement in RFP as sensitivity of 100% and specificity of 98.95% with a PPV of 96.30% and NPV of 100%. This is essential, for the reason that RFP, jointly with INH, is the most valuable anti-tuberculosis agent. In addition, RFP resistance is mostly combined with INH resistance (Vareldzis et al., 1994) and might therefore be used as a marker of multidrug resistance if resources are inadequate.

The spread of multidrug-resistant (MDR) strains of *M. tuberculosis* is an increasing

public health concern in many parts of the world, especially in low-income countries, where most cases occur. The time lag to diagnose this is a significant threat to the patient, the community, and health care workers. So, earlier identification of MDR-TB cases is important as this would minimize the risk of disease progression and amplification of drug resistance due to optimal therapy. Conventional DST requires on average 20-40 days for initial culture growth, plus an additional 28-42 days for DST itself. In contrast, the turnaround time for the direct NRA in this study was uniformly 28 days, a time savings of 4-6 weeks. Further reductions in turnaround time are possible if the initial colorimetric readings are taken on the 21st day, as suggested in the original protocol (Safonova et al., 2001).

Other low-cost methods have been proposed, such as the MTT or resazurin assays (Palomino et al., 2004). They have been shown to be comparable to the NRA, at least for RFP when perform indirectly (Palomino et al., 2004). However, they make use of liquid medium in a micro-plate format and that makes the techniques more complex and might also constitute a biohazard. Instead, the NRA utilizes standard solid L-J medium, although with NaNO3 incorporated and (apart from being safer), it could therefore be easily adopted in any culture laboratory.

The ability to reduce nitrate is typical for *M. tuberculosis*, although some other mycobacterial species, like *M. kansasii*, and most rapid growers share this characteristic (Kent and Kubica, 1985). Nitrate reductase-negative strains of *M. tuberculosis* are rare (1%) (Kent and Kubica, 1985) and would create no false results since the control would be negative and the test would therefore be invalid. No such strains were encountered in this study. Strains of *M. bovis* do not reduce nitrate, for which reason the NRA technique is not applicable to DST of them. Another possible limitation is that nitrite might be further reduced to nitric oxide, which cannot be detected by the reagents used. When the nitrate reduction test is performed for the purpose of species identification, zinc powder is added to all negative tubes (Kent and Kubica, 1985). Zinc reduces nitrate rapidly, and a true negative test will directly turn red, while there will be no color

change in a tube where reduction has passed beyond nitrite. Since the result was always confirmed, this step was omitted in this study. However, further studies will be needed to clarify the role of zinc powder in the NRA.

This study demonstrates the potential usefulness of the direct NRA as a rapid, susceptible and specific screening tool for MDR-TB. Even though more studies are needed to further assess the accuracy and applicability of the method, the direct nitrate reductase assay has the potential to become an inexpensive alternative for DST where resources are scarce, especially for RFP, the most important anti-tuberculosis drugs. It might then be used either as a rapid screening tool alone or in combination with other methods. In addition to its rapidicity, the direct NRA has further obvious benefits that would facilitate its institution in resource poor setting. Furthermore, the test uses only simple reagents that are inexpensive and easily obtained, does not require maintenance of any specialized equipment, and requires minimal laboratory space and staffing.

This study would be helpful in identifying MDR-TB where the prevalence of MDR-TB is more. Finally, this work has also shown that direct NRA can be effectively used to detect DR and MDR-TB and the method is easy, cheaper and reliable.

6.2 Conclusion

In this study, direct NRA test for culture and susceptibility testing was evaluated in comparison to gold standard proportion method. The method was found to be having high sensitivity and specificity. This study revealed that the direct Nitrate Reductase Assay method has capability of accurate and rapid drug susceptibility testing for primary four anti-tuberculosis drugs viz, INH, RFP, SM, and EMB.

More importantly, the test will provide patients and clinicians with the benefits of greater access to fast and accurate drug susceptibility testing result for these first-line drugs

CHAPTER VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

This study was carried out at National TB reference laboratory and SAARC TB reference laboratory, Thimi, Bhaktapur, Nepal and the primary goal of the study was to evaluate the direct NRA method for culture and susceptibility testing of *M. tuberculosis*. The main objective of the study was to evaluate feasibility and performance of Direct Nitrate Reductase Assay in the screening of MDR-TB.

The aim was also to study the prevalence of the MDR-TB in previously treated patients of pulmonary tuberculosis (PTB) visiting NTC, Thimi Bhaktapur.

A total of 302 samples from previously treated patients of pulmonary tuberculosis (PTB) were taken in the study period. Out of total, 225 (74.5%) samples were from male and 77 (25.5%) samples from female.

The results of the study revealed following findings:

1 Among 302 sputum specimen screened against *M. tuberculosis*, 144 (47.68%) were found to be positive to AFB on Z-N microscopy.
2 All the smear-positive specimens were tested for culture and susceptibility testing by direct NRA and gold standard proportion method. 121 (84%) of the smear positive cases for AFB were positive for culture; and 12 (8.33%) were culture negative by the both methods and 11 (7.63%) were found to be contaminated.
3 There was no growth of the isolates when inoculated and incubated in LJ media containing PNB.

4 Considering proportion method as standard, the sensitivity and specificity of direct NRA was determined to be 100% and 91% for INH; 100% and 98.95% for RFP; 96% and 91.96% for SM; and 100% and 98% for EMB. Similarly, the PPV was found to be 78%, 96.30%, 75% and 91.30% with NPV being 100%, 100%, 98.88% and 100% respectively.

5 Of the total 121 samples tested for drug susceptibility, 27.28% (33) were resistant to one or more drugs whereas 72.72% (88) were sensitive to all four drugs.

6 The highest resistance was observed with INH i.e. 23.14% (28) followed by RFP 21.49% (26), SM 20.66% (25) and EMB 17.35% (21) and 19% (23) of the isolates were multi-drug resistant.

7.2 RECOMMENDATIONS

On the basis of the findings made by this study, following recommendations are put forward:

1 Direct Nitrate Reductase has the potential to become an alternative for conventional DST, especially for RFP and INH, the most important anti-tuberculosis drugs for the rapid detection of MDR-TB.
2 The method should be implemented in resource poor setting for rapid screening of MDR-TB.

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