CHAPTER II

LITERATURE REVIEW

Tuberculosis

TB is a disease of major public health concerns worldwide .It is estimated that nearly a third of the world population is infected with MTB. In human TB, 8-10 million individuals develop active disease and nearly 2 million people die of TB each year, mostly in developing or under developed countries. The incidence of TB has also increased in developed countries particularly involving HIV positive individuals such as immigrants, expatriated worker from countries with TB endemic area, the homeless and prison inmates. One of the most alarming trends in concerning TB is the steady increase of drug resistant MTB strains and several outbreaks caused by multi – drug resistant (MDR) strains of MTB (MDR-TB) from nearly all part of the world. The rifampin is a key first line drug and the presence of rifampin resistance increase MDR-TB.MTB strains with resistance to rifampin trends to be resistant to several other anti – TB drugs. Drug-resistant TB is widespread and found in all countries. It emerges as a result of treatment mismanagement, and is passed from person to person in the same way as drug-sensitive TB.

It can take two years to treat with drugs that are more toxic, and 100 times more expensive. If the drugs to treat MDR-TB are mismanaged, further resistance can be occured. Extensively drug-resistant TB (XDR-TB) is a form of TB caused by bacteria resistant to all of the most effective drugs (i.e. MDR-TB plus resistance to any fluoroquinolone and any of the second-line anti-TB injectable drugs: amikacin, kanamycin or capreomycin). Antituberculosis treatment has two main objectives (Onyebujoh, 2005). First, there is a need to rapidly kill those bacilli living extracellular in lung cavities, which are metabolically active and are dividing continuously; this is required in order to attain the negativization(sputum turn to completely Negative) and therefore to prevent further transmission of the disease. Second, it is necessary to achieve complete sterilization and elimination of those bacilli replicating less actively in acidic and anoxic closed lesions, and to kill semi dormant bacilli living intracellular

in other host tissues, otherwise these bacilli may persist and will be responsible for subsequent TB relapses. Isoniazid (INH) is the drug with the highest activity against rapidly dividing bacilli, whereas Rifampicin (RIF) and Pyrazinamide (PZA) have the greatest sterilizing activity against bacteria that are not dividing. These reasons, along with the prevention of drug resistance, support the use of a combination therapy for the treatment of TB. Drugs for treating TB are usually classified as first and second line drugs. Traditionally, there are five first line drugs: INH, RIF, PZA, Ethambutol (EMB), and Streptomycin (SM). Second line drugs are aminoglycosides kanamycin amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide prothionamide and several fluoroquinolones (such as moxifloxacin, levofloxacin and gatifloxacin). New drugs such as the rifamycin derivatives rifapentine and rifabutin can be considered among the first line drugs, and in the near future, it is quite likely that some fluoroquinolones could be incorporated into the standard antituberculosis treatment, thus being considered as first line drugs. The current short course treatment for the complete elimination of active and dormant bacilli involves two phases:

1. **initial phase**: three or more drugs (usually isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin) are used for two months, and allow a rapid killing of actively dividing bacteria, resulting in the negativization of sputum.

2. **continuation phase**: fewer drugs (usually isoniazid and rifampicin) are used for 4 to 7 months, aimed at killing any remaining or dormant bacilli and preventing recurrence.

Strengthening TB control through the Stop TB Strategy prevents MDR-TB and XDR-TB. The Global Plan to Stop TB 2006-2015 outlines the actions needed to prevent drug resistant TB and treat all diagnosed MDR-TB patients by 2015. Principal actions proposed by the WHO Global Task Force on XDR-TB: (World health organization, 2008)

1. Strengthen TB control through the Stop TB Strategy.
2. Scale-up MDR-TB and XDR-TB programmes.
3. Expand and improve laboratory services.
4. Expand MDR-TB and XDR-TB surveillance.
5. Prevent transmission through infection control.
6. Increase awareness and information.

7. Pursue funding and resources.
8. Promote research and development.

Mycobacteria are a family of small, rod-shaped bacilli that can be classified into 3 main groups for the purpose of diagnosis and treatment: (www.en.wikipedia.org/wiki/Nontuberculous mycobacteria,2008)

1. *Mycobacterium tuberculosis* complex which can cause tuberculosis: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti*.
2. *M. leprae* which causes Hansen's disease or leprosy.
3. Nontuberculous mycobacteria (NTM) are all the other mycobacteria which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease.

M. tuberculosis is obligate aerobe growing most successfully in tissues with a high oxygen content, such as the lungs, non-motile, slow-growing with a generation time of 12 to 18 hours. Hydrophobic with a high lipid content in the cell wall. Because the cells are hydrophobic and tend to clump together. They are impermeable to the usual stains, e.g. Gram's stain. It's acid-fastness bacilli because of their lipid-rich cell walls, which are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, the cells resistant to decolorization with acidified organic solvents and are therefore called "acid-fast".

Tuberculosis diagnosis

1. clinical diagnosis

The infectious agent of tuberculosis, TB is carried on airborne droplet nuclei. Droplet nuclei are produced when persons with pulmonary tuberculosis cough, sneeze, speak, or sing. After patient inhalation of a droplet nucleus, it passes down the bronchial tree without settling on the respiratory epithelium and implants in a respiratory bronchiole or alveolus beyond the mucociliary system. Here the bacilli may multiply with no initial resistance from the host. The organisms are slowly engulfed by macrophages, but they may remain viable and even multiply within the cells.

The tubercle bacilli are spread through the lymphatic channels to regional lymph nodes and through the bloodstream to more distant sites. Certain organs and

tissues are notably resistant to subsequent multiplication of these bacilli. The bone marrow, liver, and spleen are almost always seeded with mycobacteria, but uncontrolled multiplication of the bacteria in these sites is exceptional. Organisms deposited in the upper lung zones, kidneys, bones, and brain may find environments that favor their growth, and numerous bacterial divisions may occur before specific immunity develops and limits multiplication.

TB disease should be suspected in persons who have the following symptoms:

1. Unexplained weight loss.
2. Loss of appetite.
3. Night sweats.
4. Fever
5. Fatigue

If TB disease is in the lungs (pulmonary), symptoms may include: Coughing for > 3 weeks, Hemoptysis (coughing up blood), Chest pain.

A complete medical evaluation for TB includes the following:

1. Medical History

Clinicians should ask about the patient's history of TB exposure, infection, or disease. It is also important to consider demographic factors (e.g., country of origin, age, ethnic or racial group, occupation) that may increase the patient's risk for exposure to TB or to drug-resistant TB. Also, clinicians should determine whether the patient has medical conditions, especially HIV infection, that increase the risk of latent TB infection progressing to TB disease.

2. Physical Examination

A physical exam can provide valuable information about the patient's overall condition and other factors that may affect how TB is treated, such as HIV infection or other illnesses.

3. Mantoux Tuberculin Skin Test and/or QuantiFERON®-TB Gold Test

The Mantoux tuberculin skin test (TST) and the QuantiFERON -TB Gold test (QFT-G) are used to test forM. tuberculosis infection. Additional tests are required to confirm TB disease. The Mantoux tuberculin skin test is performed by injecting a small amount of fluid called tuberculin into the skin in the lower part of the

arm. The test is read within 48 to 72 hours by a trained health care worker, who looks for a reaction (induration) on the arm. The QFT-G is a blood test. It measures the patient's immune system reaction toM. tuberculosis. Once the blood samples are taken, they must be processed within 12 hours. Interpretation of QFT-G results is influenced by the patient's estimated risk for TB infection.

4. Chest Radiograph

A posterior-anterior chest radiograph is used to detect chest abnormalities. Lesions may appear anywhere in the lungs and may differ in size, shape, density, and cavitation. These abnormalities may suggest TB, but cannot be used to definitively diagnose TB. However, a chest radiograph may be used to rule out the possibility of pulmonary TB in a person who has had a positive reaction to a TST or QFT-G and no symptoms of disease.(TB elimination, CDC,2006)

The examination of clinical specimens suspected of containing mycobacteria by conventional methods involves several diagnostic tools: microscopic examination, culture of samples, biochemical testing, drug susceptibility testing and identification of isolates.

2. Laboratory diagnosis

2.1 Microscopic examination

The light microscope examination cannot resolve the internalstructures of the tubercle bacillus with the exception of some intracellular lipid vacuoles appearing as unstained spherules at regular intervals inside the bacilli (Draper, 1982) and de- posits of lipophilic material that might have a storage function (Garton, 2002). Despite considerable efforts, a more subtle resolution of the ultrastructure of the bacillus has not been achieved. This is probably due to technical problems arising from bio safety, from the minute size of the bacilli, and from the large amounts of complex lipids existing in their wall. With electron microscopy, some inner dense granules can be identified. They are believed to consist of polyphosphate and might be an energy store in the cell and also the site of oxidation-reduction reactions. In sections of the cell, the plasma membrane is seen to proliferate into vesicular or laminated internal bodies that might supply metabolic activities. Ribosomes, DNA filaments and radial bands, the latter postulated to be remaining scars of cell divi- sion, have also been described (Draper, 1982; Brennan, 1994).

Recently, the initiation of septum formation prior to division was clearly evidenced by tagging the mid-cell rings with green fluorescent protein (Chauhan, 2006). Also, impressive images of the surface of M. bovis BGG were obtained by atomic force microscopy (Verbelen, 2006).

The use of microscopy to reach a rapid preliminary diagnosis of TB is of great value, especially in the detection of actively infectious cases. The turnaround time for smear microscopy is one working day from receipt of specimen to reporting of results. Because the examination of direct smears can detect only concentrations at least 10^5 acid-fast bacilli per milliliter of specimen. This method has limited sensitivity. The presence of acid-fast bacilli in a specimen does not automatically imply the presence of *M. tuberculosis*. In fact, more often than not. The acid-fast bacilli are mycobacteria other than tuberculosis, and confirmatory testing is therefore unavoidable. Fluorescence acid-fast staining is more expensive than conventional Ziehl–Neelsen staining but is associated with a higher rate of detection because the slides can be examined faster at lower magnifications. Fluorescence-positive slides are confirmed by Ziehl–Neelsen staining.

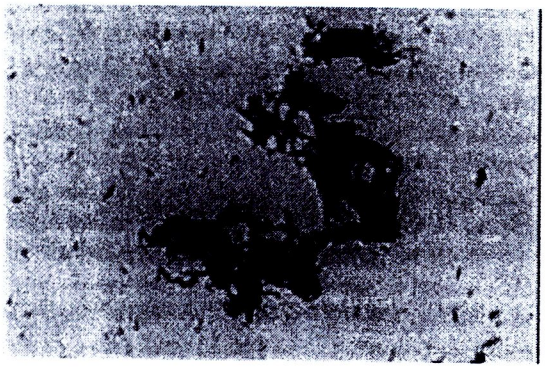


Figure 1 Ziehl-Neelsen staining of *Mycobacterium tuberculosis* growing in culture at 1000x magnification.

Source: Lucia barrera, 2007

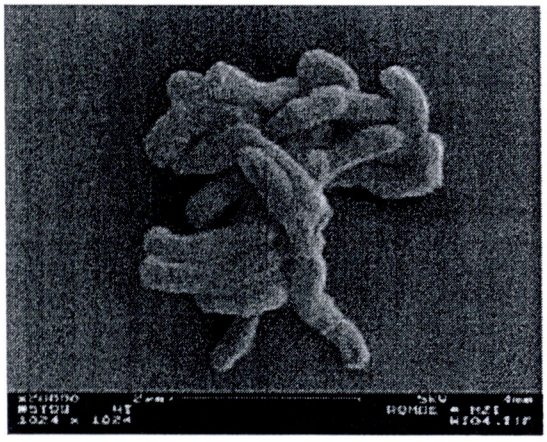


Figure 2 Electron microscopy of *Mycobacterium tuberculosis* growing in culture (Courtesy of M. Rohde -M. Singh).

Source: Lucia barrera, 2007

Table 1 AFB grading recommend by World Health Organization and the International Union Against Tuberculosis and Lung Disease

Count on Ziehl-Neelsen /Kinyoun stain (1000x)	Report
0	Negative
1-9/100 fields	Exact count
10-99/100 fields	1+
1-10/field	2+
> 10/field	3+

Source: Palomino, 2007

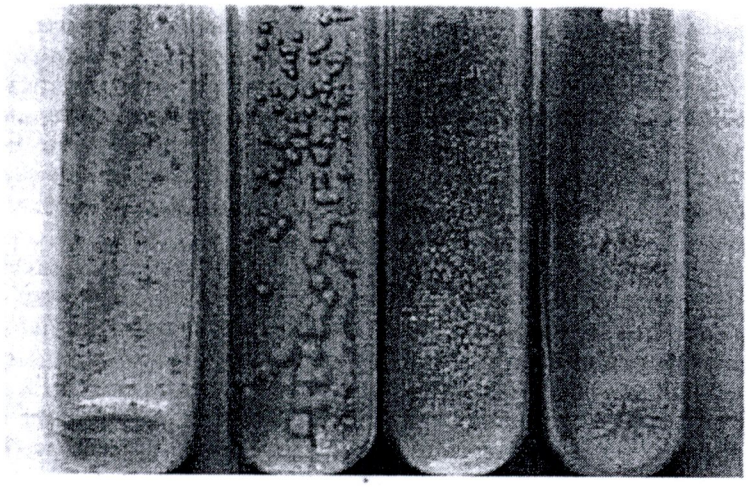
Table 2 AFB grading for concentrate smear.

Number of AFB seen (1000x)	Report
0	Negative
1-2 AFB/ Whole smear	Doubtful positive. Confirm by observing another smear from the same specimen or from another specimen from the same patient.
1-9/100 fields	1+
1-9/10field	2+
1-9/field	3+
>9/field	4+

Source: Salman H. Siddiqi, Ph.D., 2006

2.2 Culture

A definitive diagnosis of TB can be obtained only by culturing clinical specimens and testing the isolates further after preliminary identification. The specimens should be sent to the laboratory as soon as possible. Because of mycobacteria in sputum die within a few days. Culture of clinical specimens is associated with higher case detection rates, because the sensitivity of culture is much higher than that of smear microscopy; with this technique, concentrations of 10 to 100 bacilli/mL can be detected. Culture methods based on a combination of liquid or biphasic (solid and liquid) media, together with solid media, are used to ensure maximum sensitivity of detection and are considered the current "gold standard" for culture. The mean time for detecting the *M. tuberculosis* complex is about 2 days for the solid media currently in use. Rapid methods can be used to complement conventional methods for the recovery of mycobacteria. The only well established rapid method for detecting mycobacteria in clinical specimens is the BACTEC 460TB system (Becton-Dickinson Diagnostic Instruments Systems, Maryland). This system is based on the detection of radioactive carbon Laszlo.



a b c d

Figure 3 Mycobacteria growing on Löwenstein-Jensen slants. a. *Mycobacterium gordonae*; b. *Mycobacterium fortuitum*; c. *Mycobacterium avium*; d. *Mycobacterium tuberculosis.*

Source: Lucia barrera, 2007

2.3 *Mycobacterium tuberculosis* identification

Biochemical tests

Niacin test : Niacin (nicotinic acid) plays a vital role in the oxidation mycobacteria produce niacin, comparative studies have shown that, because of a blocked metabolic pathway *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis. Niacin negative *M. tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests.

The steps involved in testing Niacin begin with the addition of 1 ml of distilled water or normal saline in the culture medium and slant test tube (scrape medium for extract niacin substance). Next, wait 15 minutes and add water 0.5 ml to the test tube. Third, add aniline or benzidine 0.5 ml and cyanogens bromide 0.5 ml and check the color of the liquid to determine results.

Results are indicated by a positive yellow color in aniline or pink in benzidine. No color change indicates a negative result.

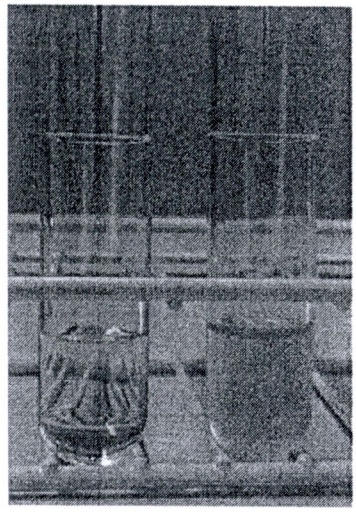


Figure 4 Niacin test (Courtesy of A. Martin)

Source: Palomino, 2007

Nitrate reduction test : *M. tuberculosis* is one of the strongest reducers of nitrate among the mycobacteria, which allows for this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from the other mycobacteria. Cultures to be tested for nitrate reduction should have abundant growth on Löwenstein Jensen egg medium about 4 weeks.

In order to test for nitrate reduction, begin by preparing 1.01 M. sodium nitate in 0.022 M phosphate buffer , pH 7.0 sterilization. Next, prepare 50 ml HCl in 50 ml distill water and 0.2 g. sulphanilamide in 100 ml distilled water. Prepare 0.1 g. N naphtylethylenediamine dihydro chloride in 100 ml distilled water and add 0.01 M sodium nitrate in 0.02 M phosphate buffer ,pH 7.0 20 ml in test tube and scrape colony 1 loop put in test tube. Mix all of the above ingredients until no separation of liquids is observed. Warm mixture in a water bath 37 c for 2 hours and add HCl 1 drop , 0.2 g sulphanilamide in distilled water 100 ml 2 drops and 0.1 g N–naphtylethylenediamine dihydro chloride in distill water 2 drops in test tube.

Positive results are indicated by a pink-violet color (1+ - 5+ , positive 3+ up). A negative outcome results in no color (add ZN dust to confirm true negative color must not change).

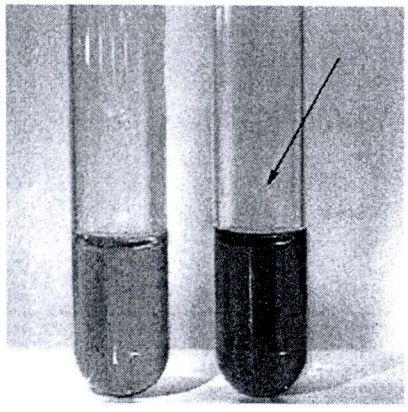


Figure 5 Nitrate reduction test result

Source: Palomino, 2007

Catalase test : Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen; eg. $2H_2O_2$ 6 $2H_2O + O_2$. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually all mycobacteria passes catalase enzymes, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis*.

Mycobacteria posses several kinds of catalase that vary in heat stability. Quantitative differences in catalase activity can be demonstrated by one or more of the following tests:

1. Room temperature or drop method (indicates the presence of catalyse)
2. Semiquantitative test (indicates level of catalyse production)
3. 68 °C test at pH7 (indicates loss of catalyse activity due to heat)

Drug susceptible strains of *M. tuberculosis* do form catalyse as indicated by the drop method, produce less than 45 mm of bubbles in the semiquantitative test and lose catalase activity when heated to 68 °C for 20 minutes. For these tests 14 day-old cultures on LJ butts should be used, ie. the media tubes should be inspissated in an upright position to provide a butt and should not be slanted. The tubes must have stoppers which permit exchange of air, eg. Cap-o-Test stoppers. The cultures should be incubated in a well-humidified incubator at 35-37 °C, with loose caps, for 14 days.

To test cultures, add phosphate buffer, pH 7.0 0.5 ml and scrape 1 loopful colony in a test tube. Warm the test tube to 68° C for 20 minutes. Prepare Tween 80 solution and hydrogen peroxide 1:1 immediately. Wait for the test tube to cool (heat helps hydrogen peroxide collapse) and add 0.5 ml solution slowly, avoiding air bubbles. If the solution will not mix after 20 minutes, assume air bubbles are present.

Positive results are indicated by the appearance of air bubbles while a lack of air bubbles indicates a negative result.

Table 3 Colony morphological and biochemical characteristics of species in the *M. tuberculosis* complex

Test	M. Tubercubsis	M. Bovis	M.bovis BCG	M. Africanum	M. Microti	"M. canettii"
Morphology	Rough	Rough	Rough	Rough	Rough	Smooth
Pyruvate rather than glycerol as carbon source	-	+	+	-	-	-
Pyrazimidase	+	-	-	+	+	+
Niacin	+	-	-	+/-	+	-
Nitratase	+	-	-	+/-	-	+
Urease	+/-	-	+	+/-	+/-	+
Susceptibility to TCH	R	S	S	S	S	R
O_2 requirement	aerobic	Micro-aerophilic	aerobic	Micro-aerophilic	Micro-aero-philic	Unknown

Note: R = resistant, S = susceptible, TCH = Thiophene-2-carboxylic acid hydrazide

Source: Palomino, 2007

2.4 Drug susceptibility test

Isolates of the *M. tuberculosis* complex are first tested against first-line antituberculosis agents, specifically isoniazid, streptomycin, rifampin, ethambutol and pyrazinamide. Drug-resistant *M. tuberculosis* isolates are then tested for susceptibility to second-line drugs, including amikacin, capreomycin, clofazimine, cycloserine, ethionamide, kanamycin, ofloxacine and rifabutin. Testing of isolates of the *M. avium* complex for susceptibility to amikacin, clarithromycin, clofazimine, ethambutol, rifampin, rifabutin and streptomycin is also performed. Testing for susceptibility to other agents is performed at the National Reference Centre for Tuberculosis upon request and on an experimental basis. As a rule, testing of susceptibility to isoniazid and rifampin is more reliable than testing of susceptibility to streptomycin, ethambutol and pyrazinamide. Furthermore, testing of susceptibility to secondline drugs is less reliable than testing of susceptibility to first-line drugs. To generate drug susceptibility results within a week, the testing is performed with the BACTEC 460 rapid radiometric method. The average turnaround time is less than 30 days from the time of specimen receipt (2 to 3 weeks for primary isolation and 1 week for drug susceptibility testing).

2.4.1 Direct drug susceptibility test

The direct drug susceptibility test is a procedure based on inoculation of drug-containing media with processed (concentrated after digestion and decontamination) sputum specimens that are smear-positive for acid fast bacilli (AFB) to determine the proportion or percentage of resistant MTBC in the patient's bacterial population. The test should be performed only on smear-positive specimens, and only by the agar proportion method or by a commercial method that has been approved and validated by FDA for direct susceptibility testing.

The advantages of the direct test are:

Results can be reported within three weeks (from the time of specimen receipt in the laboratory) for a majority of smear-positive specimens. The proportion of resistant bacteria recovered better represents the patient's bacterial population. It is cost-efficient.

The disadvantages of the direct test are:

The inability to accurately calibrate the inoculum, may result in insufficient or excessive growth on drug-free control quadrants. The Possible growths of contaminants make the results uninterruptable. The results of the test are valid only if the isolate is MTBC or *M. kansasii* (RIF only). The total rate of failure for the direct method can reach 10 to 15% or more, which results in frequent re testing by one of the indirect methods.

2.4.2 Indirect drug susceptibility test

In Agar Proportion Method, susceptibility testing usually is performed using cultures already isolated in or on a growth medium. The preparation of a standard inoculum is critical, because variations in the number of bacilli in the inoculum can alter the interpretation of the test.

2.4.3 The 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 Löwenstein-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, 1985).

Table 4 Critical concentration of main antibiotics in the proportion method (μg/mL)

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

Source: Palomino, 2007

2.5 New diagnostic tools (www.cmaj.ca/cgi,2008)

Several new methods based on molecular biology techniques are increasingly being used in diagnostic TB bacteriology. The polymerase chain reaction (PCR) which is based on DNA amplification methods, has been proposed for rapid detection of mycobacterial DNA in clinical specimens as a replacement for culture and identification of the *M. tuberculosis* complex. This technique has been extensively used. It be done for diagnosis within 24 hours. This method gives the result in highly sensitive and specific more than conventional method. The nucleic acid probes with nonradioactive detection systems have gained increased acceptance in the clinical TB laboratory as a replacement for fastidious, time-consuming mycobacterial identification tests. These probes can be used to identify isolates growing on conventional or

radiometric media and are available for the detection of the *M. tuberculosis* complex. In this regard, they compare favourably with the BACTEC 460 NAP radiometric inhibition test. Turnaround time for test results once the sample has been cultured is 24 hours. Because conventional methods for identifying mycobacteria are cumbersome, rapid methods for identifying many species of *Mycobacterium*, such as nucleic acid sequencing, have been developed. One of these methods is based on the sequencing of 16S ribosomal RNA (rRNA), also referred to as ribotyping. This form of rRNA has minor variations in its base sequence, which appear to correspond closely with established mycobacterial species. Preliminary results12 have shown good correlation with conventional testing for some well-defined reference and test isolates, but the method fails to distinguish between the members of the *M. tuberculosis* complex, between *M. kansasii* and *M. gastri*, and between *M. marinum* and *M. ulcerans*. Attempts to identify problematic, intermediate-type mycobacterial isolates have so far yielded disappointing results. New genetic tests for drug resistance based on the sequencing of DNA have been developed following the recent identification of mutations leading to either the modification of the streptomycin, rifampin, isoniazid or ethambutol target molecules or to the loss of activation of pyrazinamide or isoniazid. Because the targets for each Loewenstein–Jensen + 7H10 + 7H11. Isolation rates were calculated by dividing the number of isolates detected with a particular media by the total number of isolates detected with all 4 media. With has different. No gene has been found that accounts for all resistance to any of the main antituberculosis drugs. Current genetic tests typically detect only 50% to 95% of mutations, depending on the drug. The technique of DNA fingerprinting, referred to a RFLP (restriction fragment length polymorphism) has in the investigation of TB outbreaks. This method can distinguish the difference between exogenous reinfection and reactivation. Moreover it can protect the laboratory cross-contamination. But the result analysis is difficult if the isolate has fewer than 5 bands.

2.5.1 Phenotypic methods (Martine Guillerm,Martine Usdin ,James Arkinstall,2006)

1) MTT assay

Traditionally, the determination of cell growth is done by counting viable cells after staining with a vital dye. Several approaches have been used in the past. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for highthroughput screening. Measuring the uptake of radioactive substances, usually tritium labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption max is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. The use of the MTT method does have limitations influenced by:

1. the physiological state of cells.
2. variance in mitochondrial dehydrogenase activity in different cell types.

Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli,

growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves. The MTT method of cell determination is most useful when cultures are prepared in multi well plates. For best results,cell numbers should be determined during log growth stage. Each test should include a blank containing complete culture medium without cells.

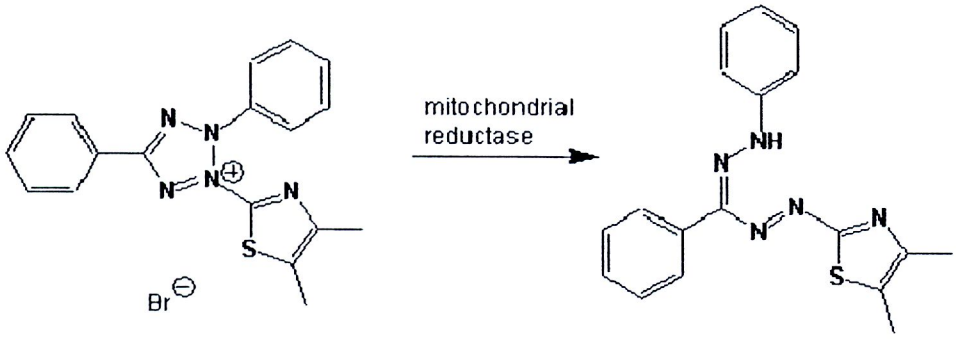


Figure 6 MTT structure

Source: Mosmann, 1983

MTT assay is development proportional method. Using for growth rate detection of MTB in liquid medium (M7H9) with drug and without drugs within 4-7 days.The test, performed on 92 clinical isolates of M. tuberculosis, matched the results obtained with the BACTEC radiometric method used as the gold standard. More recently, the MTT test has also been applied in the detection of resistance to other anti-tuberculosis drugs with good results (Foongladda ,2002; Caviedes, 2002; Morcillo, 2004). 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 RIF-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, 2004).

2) BACTEC 460

This system detects the presence of mycobacteria based on their metabolism rather than visible colonial growth. A radioactive marker is present in the tube that is detected by the machine when growth occurs.

Advantages : Faster than solid media.

Disadvantages : Costs: the machine is expensive, and tests for DST cost Euro 13 for two drugs tested per sample. These combine to make the technology more expensive than the conventional culture method.The machine requires appropriate laboratory infrastructure, including nuclear waste disposal. In addition, the need for radioisotopes, needles, and the cost of equipment limits its use to reference laboratories.

3) BACTEC MGIT 960® (automated)

Diagnosis through culture of samples (pulmonary and others). The MGIT system is based on a glass tube, recently replaced by a plastic tube, containing 7H9 broth together with a fluorescence quenching-based oxygen sensor. When inoculated with M.Tb, consumption of oxygen produces fluorescence when illuminated by a UV lamp. The fully automated version can incubate up to 960 samples for M.Tb diagnosis through culture of samples (pulmonary and others), and drug sensitivity testing for first-line drugs.

Advantages : High throughput capacity, Automated, standardized reading of samples Speed: diagnosis 7 days for sputum positive, up to 42 days for a negative result .DST 8 to 12 days (starting from culture)

Disadvantages : Machine is extremely expensive.Requires specific training of technical personnel. Liquid media are technically limited as they are prone to contamination. The machine must be maintained, this requires very frequent technical support from the company.

4) MB/Bact T® system

This technique for diagnosis and DST for first- and second-line drugs is an automated non-radiometric continuous monitoring system with computerized database management.The system is based on detection of CO_2 as an indicator of bacterial growth in cultures in a closed and a fully automated system.

Advantages : This system can be used for first- and second-line detection of drug resistance.System modular (machine and tubes can be bought separately), increasing flexibility of use.

Disadvantages : Relatively slow: diagnosis 17 days (range 7-40), DST 8 to 12 days. Requires an expensive and non-robust machine, complicated and cumbersome.

5) Phage-based tests

Diagnostic (FASTPlaque TB test) and rifampicin DST (FastPlaque TB-RIFTM).Phage tests are based on the ability of viable M. Tuberculosis to support the replication of an infecting mycobacteriophage (a virus that infects mycobacteria).Plaques of lysed cells in a lawn culture of mycobacteria are counted.

Advantages : Speed (2-3 days). For in-house systems: relatively cheap.

Disadvantages : Phage-based tests are technically complex to perform, requiring a well-functioning bacteriology laboratory, a strict incubation protocol and well-trained technicians.

They are very labour intensive and some studies also report a high rate of contamination, making the test and its results both difficult to perform and to interpret. FASTPlaque cannot be used for children or HIV-positive patients as it needs sputum.

6) Luciferase reporter phages

The technique is used for DST. This recombinant phage (phage which incorporated the gene for luciferase) can express the luciferase gene when infecting a mycobacterium. In the presence of luciferin substrate, infected bacteria emit light that can be detected with a luminometer or by photosensitive film in a Polaroid film box called the "Bronx Box".

Advantages : Rapid result (2 days post culture).

Disadvantages : To date, only limited reports of clinical application are available. The Bronx box is not easy to manipulate. Conditions of use not clearly defined; needs repeated testing.Results obtained 40 hours post-culture.

7) **MODS**

This method is performed in liquid medium (7H9) with or without drug Incorporated in the medium.

Advantages : Quicker than solid culture, may be quicker than MGIT as volumes are smaller. Fairly cheap, non-commercial, adaptable technique.

Disadvantages : MODS is a delicate method that requires very experienced personnel. As the test is performed in liquid medium, and needs to be handled often, it is more of a biosafety risk for laboratory staff. The test requires an inverted microscope, which is very seldom available or useful in field labs.

2.5.2 Genotypic methods

1) Techniques using antibody detection

In 2005 WHO/TDR performed an evaluation of commercially available rapid diagnostic tests (RDTs). All tests detect antibodies in serum.

2) Techniques using antigen detection

LAM urine test

The test detects lipoarabinomannan (LAM) in urine as a surrogate marker for TB infection. LAM is a component of the TB bacterial cell wall.The test exists in Elisa and simplified 'tube' format. Clinical trials to develop a dipstick format are ongoing. The simplified tube formatis apparently robust and does not need cold chain. The use of the test needs to be evaluated within a careful choice of algorithms, in order to determine whether it will help guide clinical decision making. The tube format requires at least three hours, several manipulations, a supply of distilled water and some amount of training. It also requires reading the result in a machine, but apparently a portable format has been developed. However, a dipstick format would be very welcome. Advantages: The test may be suitable for children, co-infected patients, and extra-pulmonary patients. A dipstick would be extremely useful as a high-throughput point-of-care test. It holds potential for monitoring of treatment as LAM is in theory quickly eliminated. A urine sample is a good non-invasive approach. Possibly, there will be no need to treat the urine sample prior to testing. This is currently a limiting step in many field tests. Disadvantages :In its current form as an Elisa test, it is not suitable for the peripheral laboratory setting, as it will require skilled staff, electricity supply, cold chain and specific equipment. The tube format

does not seem to completely address these problems,but a dipstick, if it were to become available, will be a significant improvement.Chemogen recommends boiling and centrifuging the urine, which is technically difficult in many peripheral settings.

Antigen-based detection test

Proteome Systems are using high throughput protein assays to identify antigens that are specific to M. Tuberculosis and that could potentially be used to develop a rapid test and diagnose active TB and infection, or to monitor treatment efficacy. They will examine all types of biological fluids such as sputum, saliva, plasma or whole blood.

Advantages : Potentially interesting because antigen detection avoids some of the problems associated with antibody detection. But there is no information about the preliminary results on characterized samples, nor on the technical background nor on performances of their format.

Disadvantages : Information on the test is currently severely lacking. It appears there may be a need for a reader or some kind of machine to perform the test.

Nucleic Acid Amplification (NAA)

NAA techniques require strong laboratory capacities, good quality Control procedures, and remain relatively expensive. In recent years, some improvements have been made, such as isothermal amplification steps, the inclusion of internal amplification controls to ensure that inhibitors (resulting in false negatives) are not present, the design of single-tube reactions to reduce contamination and the development of detection by emitted light or by dipstick. The use of NAA techniques remains technically challenging. Despite being usually highly specific, NAA tests have lower (and greatly variable) sensitivity. A positive NAA test is considered good evidence of infection but a negative result is not informative enough. Use of NAA tests has not beenrecommended for sputum negative patients.

Simplified NAT test, TB-LAMP test

LAMP (loop mediated isothermal amplification) is a method to amplify TB DNA directly from clinical samples. A positive result is signalled by a colour reaction visible to the naked eye.The format under development is an adaptation of an existing technology. The technique requires sample preparation

after the decontamination process and a special extraction device, then isothermal amplification and detection of a fluorescence signal, leading to a qualitative result. Apparently no cross-reaction with *M. avium*. A more direct method using sputum without decontamination of samples gives less specific results on negative samples, and a higher incidence of false positive results.

Advantages : This is a simplified NAT test, suitable in theory for the monitoring of treatment.Results are supposed to be obtained in two hours. Sample preparation apparently has been simplified.We have no recent information about the stage of development or whether the format is feasible.

Disadvantages : The feasibility of using the test at the district hospital level is of concern, and the test may be unsuitable for use at the periphery due to the complexity of the process. Certain specific equipment is needed. Reagents currently require cold storage. Heat stability studies are on going. Data on cross contamination and test performance are needed.

GenoType Assays (Hain test)

Two GenoTypes are commercialised. The first is for tuberculosis diagnosis (GenoType Mycobacteria Assay), the second for detection of rifampicin and isoniazid resistance (GenoType MTBDR Assay). Isolation is commonly done by PCR amplification of the 16S-23S ribosomal DNA spacer region followed by hybridization of the biotinylated amplified DNA products with 16 specific oligonucleotide probes. The specific probes are immobilized as parallel lines on a membrane strip. The GenoType MTBDR detects resistance to isoniazid and rifampicin in culture samples, based on the detection of the most common mutations in the katG and rpoB genes respectively .

Advantages : It is possible to use Genotype MTBDR Assay to confirm TB infection and detect resistance to rifampicin and isoniazid at the same time.

Disadvantages : The tests are not validated yet on direct clinical samples and sensitivity is not known. Test is only for use on smear positive samples.Tests not easy to perform in the very large numbers likely be needed if routine resistance testing is adopted.

PCR sequencing

Specific M. Tuberculosis genetic material is amplified and sequenced, allowing the DNA to be "read". This is the gold standard and the most widely used method for defining genetic resistance for drug sensitivity testing. It has been commonly used for characterizing mutations in the rpoB gene in rifampicin-resistant strains and to detect mutations responsible for other anti-tuberculosis drugs. Most protocols include the repeat insertion sequence IS6110 as a target for amplification. This sequence is specific of *M. Tuberculosis* complex and is present in many copies in the *M. Tuberculosis* genome.

Advantages : PCR sequencing gives specific strain and mutation information.

Disadvantages : Using the technique requires sequencing capacity and sophisticated laboratory technology.PCR sequencing detects only some mutations, and only gives a "theoretical" result (as opposed to culture, which gives a "functional" result).

PCR directly from AFB slides

The basic technique consists of conducting PCR directly on samples washed off sputum slides. Different methods have been used.

Advantages : The technique may be more sensitive than microscopy alone. It may also be a way to ship samples to reference laboratories for testing.

Disadvantages : The technique is not well validated. There is a high risk of contamination, in addition to all the associated difficulties of PCR.

2.5.3 Other non-culture techniques

1) T-cell based tests

These were touted as a replacement to Mantoux test (tuberculin skin testing). Blood is taken from a patient, and placed in an incubator for 12 hours in sterile culture with the tuberculin antigen. The tests measure release of a protein that is associated with immune activation. If a patient has previously mounted an immune response to TB, they will have cells in their blood that respond to the presence of the tuberculin antigen. These "primed" cells then produce the immune response measured in the test.

Advantages : 24 hour result; requires blood therefore can be used on patients unable to produce sputum, and may detect extra-pulmonary form.

Disadvantages : Blood must be incubated within 12 hours of the draw. A result is known after 24 hours, which means that the patient needs to come back to the clinic to be treated, which is less desirable.The tests require complex and sensitive laboratory manipulations. The Elisa format tests require precise pipetting, reagents require storage at 4 - 8°C and an electricity supply. There are also individual variations in the immune response that can make the results difficult to interpret.

2) DNA Microarrays

This proposed new molecular method for detecting drug resistance in M.Tb is based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilised on a solid support, such as miniaturized glass slides.Microarrays have been mainly used to detect resistance to rifampicin .

Advantages : Rapid (~2 hrs); can be used to answer specific research questions

Disadvantages : Expensive; beyond reach of most clinical mycobacteriology laboratories anywhere in the world. Technology complex and not adapted to routine use.

3) Non-NAA based DNA detection

Various methods exist to detect DNA sequences without amplification. For example, a probe binds to the specific target, thereby uncovering the catalytic site buried in the probe molecule. The exposed catalyst acts to change the colour of the buffer. Stage of development still in early stages of development, although very little information available

Advantages : The techniques do not need NAA and therefore may be simpler to perform and more robust, as well as being less prone to contamination problems.

Disadvantages : Sample will nevertheless need preparation (DNA extraction and/or purification and concentration); sensitivity of detection may be difficult to achieve.

Related research

1. Guney, C. and Partal, M. (2005) Comparision of BactT/ALERT and Lowenstein- Jensen media and rapid differentiation of Mycobacterium tuberculosis complex from other mycobacteria using para-nitrobenzoic acid from clinical specimen at a chest disease hospital in Istanbl,Turkey. It has been showed that the performing of *p*-nitrobenzoic acid test in BacT/ALERT MP bottles is sensitive and rapid. Thus total testing time of mycobacteria at the system provides the ability to meet the 21 days target for detection and identification of *M. tuberculosis* complex.

2. Morcillo, N., et al. (2004) A microplate indicator-based method for determining the susceptibility of multidrug-resistant *Mycobacterium tuberculosis* to antimicrobial agent. Conclusion of this research is MICs by MTT were obtained at on average 8 days and correlated with those obtained using the proportion method. Then MTT could be used as a simple, rapid, low-cost technology to test the susceptibility of MDR-TB strains to several second-line and alternative drugs, with the objective of orienting future treatment regimens.

3. Pontino, MV., et al. (2006) Evaluation of a colorimetric micromethod for determining the minimal inhibitory concentration of antibiotics against *Mycobacterium tuberculosis*. A total of 603 clinical isolates, 507 from respiratory cases (84.1%) and 96 from non-respiratory cases (15.9%) were processed. The proportion method on a Löwenstein-Jensen medium (PM) with isoniazid (INH), 0.20 microg/ml; streptomycin (SM), 4.00 microg/ml; ethambutol (EMB), 2.00 microg/ml and rifampin (RMP), 40.00 microg/ml, was used as the gold standard. The drugs and the concentration range tested were: INH, 1.00-0.03 microg/ml; SM, 8.00-0.25 microg/ml; EMB, 32.00-1.00 microg/ml and RMP, 2.00-0.06 microg/ml. MIC results were obtained on an average of 8 days (range: 7-12). The cut-off values for each drug, calculated by the ROC curve method, were: INH, 0.25 microg/ml, RMP, 0.50 microg/ml, SM, 4.00 microg/ml and EMB, 4.00 microg/ml. Sensitivity and specificity for RMP were 100 %, while for INH, they were 97.8% and 99.5% respectively. The results obtained suggested that MTT is a low cost and easy to set up method that could be applied to MDR clinical diagnosis in developing countries.

4. Martin, A., et al. (2005) Multicenter study of MTT and resazurin assay for testing susceptibility to first-line andti- tuberculosis drugs. Conclusion of this research

is MTT and resazurin assays are promising, accessible new alternative methods for middle- and low-resource countries that need low-cost methods to perform rapid susceptibility testing of M. tuberculosis to key anti-tuberculosis drugs.

5. Foongladda, S., et al. (2006) Rapid and simple MTT method for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis*. Conclusion of this research is MTT assay results with 279 *M. tuberculosis* clinical isolates were compared with those of the conventional proportion method on Löwenstein-Jensen medium, high specificity and sensitivity values of 100% and 94.1%, respectively, for RMP susceptibility testing, and 99.5% and 89.2%, respectively, for INH susceptibility testing were obtained. The accuracy of the MTT method for RMP and INH was > 0.97 concordance with the proportion method. The MTT method is simple, inexpensive and rapid. The high level of agreement with the conventional proportion method suggests a potential to rapidly detect drug-resistant M. tuberculosis in developing countries, as only basic microbiological equipment is need.

Treatment regimens

There are many different anti-tuberculosis regimens described in the literature, mostly matching the premises, indications and doses indicated in the sections above (Centers for Disease Control and Prevention, 2003a, World Health Organization, 2003). Several drug regimens are recommended depending on many factors, such as disease localization and severity, result of sputum smear microscopy, human immunodeficiency virus (HIV) co-infection, prevalence of drug resistance in the setting, availability of drugs, cost of treatment and medical supervision, whether the patient has previously received any anti-tuberculosis drug, the country's budget, health coverage by public health services, and qualifications of health staff at the peripheral level. Then, the selection of a particular drug regimen must be done considering all these factors. The World Health Organization (WHO) has established four TB diagnostic categories, assuming from a public health perspective that the highest priority of national TB programs is to identify and cure those patients with sputum smear-positive pulmonary TB, i.e. infectious TB patients (World Health Organization, 2003).Category I comprises those patients with a high priority for treatment who are

new smear-positive patients, new smear-negative pulmonary TB patients with extensive parenchymal involvement, patients with concomitant HIV/acquired immunodeficiency syndrome (AIDS) disease or severe forms of extrapulmonary TB. Patients with a lower priority for treatment are classified as follows: Category II (relapse, treatment failure or default), Category III (new smear-negative pulmonary TB other than in Category I and less severe forms of extrapulmonary TB) and Category IV (chronic sputum-positive TB after re-treatment and proven or suspected MDR-TB).Preferred and optional treatment regimens for each category, as recommended by the WHO. In addition to these guidelines for TB treatment, there are other alternatives. For example, the Center for Disease Control and Prevention (CDC) of the United States (US) also suggests continuation phases consisting of INH and rifapentine once per week for four months for patients in Category I (Centers for Disease Control and Prevention 2003). This treatment can be used when sputum is negative for acid fast bacilli after the first two months of treatment but should be extended to nine months if the result of the culture at that time point is sill positive. These guidelines apply only to HIV-negative patients as regimens containing rifapentine should not be administered to HIV/AIDS patients.In general, the duration of the continuation phase must be estimated once the first two months of treatment (initial phase) have been completed. If the patient had cavitations on initial chest radiography and cultures are still positive after two months of treatment, the continuation phase should be extended to 31 weeks (seven months). When drug resistance develops, patients should be treated with a new combination containing at least three drugs that they had never received before (or that do not show cross-resistance with those to which resistance is suspected). In these conditions, the treatment is longer, more toxic, more expensive and less effective than regimens containing first-line drugs, and should be directly observed.

Since HIV/AIDS patients have a higher probability of acquiring TB (either pulmonary or extrapulmonary) or other mycobacterial opportunistic infections, particular drug regimens have been designed for treating active TB disease in them (Tuberculosis Coalition for Technical Assistance, 2006). Also, the severity of adverse effects of anti mycobacterial drugs (due to the interactions with anti-retroviral drugs) and mortality is higher among HIV positive patients. Although, in general, HIV

positive patients respond well to a standard short course treatment of TB, treatment failure due to malabsorption of antimycobacterial drugs has been re- ported. The WHO recommends not using SM or thiacetazone in HIV-positive patients in order to prevent the adverse effects of these drugs, often enhanced by anti- retroviral drugs; EMB can be used instead. Rifamycins (rifampicin, rifabutin, etc.) have clinically relevant interactions with some drugs used in the antiretroviral therapy, since they induce the metabolism of anti-retroviral agents such as zidovudine, non-nucleoside reverse transcriptase inhibitors,and HIV protease inhibitors,whose concentrations may fall to sub-therapeutic levels.Then, rifampicin free regimens have been suggested. They consist of INH, EMB, PZA, and SM, daily for two months, followed by INH, PZA, and SM two or three times weekly for seven months. However, it has also been described that the use of RIF throughout antituberculosis treatment improves outcome in HIV patients. Chemoprophylaxis of TB is indicated for asymptomatic patients having a positive tuberculin skin test (TST) but not showing active disease (latent TB infection), especially when they are at risk of developing the disease (for example, HIV positive patients) (Balcells, 2006; Centers for Disease Control and Prevention 2003(b); Stout, 2004). This is aimed at preventing the occurrence of TB. Prophylaxis is most frequently achieved by the administration of INH only, at doses of 300 mg daily for six to nine months (although there is a risk of developing INH resistance). When resistance to INH is suspected, other regimens include RIF, PZA or EMB, can be administered, although there is a greater chance of having adverse effects. In TB prophylaxis, RIF can be given concurrently with INH, reducing the prophylaxis treatment to three months. It is of prime importance to ensure the patient's adherence to the antituberculosis treatment in order to achieve complete elimination of the bacilli (and hence avoid disease relapse) and also to prevent the emergence of drug resistance. For this reason, the antituberculosis treatment has to be supervised. Both the patient's adherence and supervision are often difficult to manage when the antituberculosis treatment has to be administered on a daily basis. Alternative treatments based on an intermittent administration of drugs (three times, twice and even once per week) facilitate the patient's adherence and the supervision of treatment. Intermittent treatment is possible because antituberculosis drugs have a marked post-antibiotic effect. After the tuberculous bacillus has been exposed to

drugs, there is a lag period (up to several days) during which its growth is interrupted even after the drug concentration has fallen to sub-inhibitory levels. Thus, there is no need to maintain a continuous inhibitory drug concentration to kill the bacilli or prevent growth. (Anandi martin, 2007)