EVALUATION OF RAPID SLIDE CULTURE TECHNIQUE FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS.

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Mymensingh, July, 2009
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<tr>
<td>AFB</td>
<td>Acid Fast Bacilli</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
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<tr>
<td>BCG</td>
<td>Bacilli Calmette Guerin</td>
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<tr>
<td>CDC</td>
<td>Centre for disease control</td>
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<tr>
<td>CD4+ T cell</td>
<td>Helper T cell</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
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<tr>
<td>DOTS</td>
<td>Directly Observed Treatment, Short course</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxid</td>
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<tr>
<td>DST</td>
<td>Drug susceptibility test</td>
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<tr>
<td>et. al</td>
<td>et alia (and others)</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
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<tr>
<td>gm</td>
<td>Gram</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>ICT</td>
<td>Immunochromatographic test</td>
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<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IUATLD</td>
<td>International Union Against Tuberculosis and Lung Disease</td>
</tr>
<tr>
<td>IFN - γ</td>
<td>Interferon – γ</td>
</tr>
<tr>
<td>LJ</td>
<td>Lowenstein- Jensen</td>
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<tr>
<td>MDR</td>
<td>Multi Drug Resistance</td>
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<td>MDR TB</td>
<td>Multi Drug Resistant Tuberculosis</td>
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<td>Abbreviation</td>
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<tr>
<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
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<td>P</td>
<td>Probability</td>
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<td>PANTA</td>
<td>Polymyxin, B Azlocillin, Nalidixic acid, Trimethoprim, Amphotericin B</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain Reaction</td>
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<td>PTB</td>
<td>Pulmonary Tuberculosis</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TST</td>
<td>Tuberculin skin test</td>
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<tr>
<td>TLCP</td>
<td>Tuberculosis – Leprosy control programme</td>
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<tr>
<td>WHO</td>
<td>World health organization</td>
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<tr>
<td>X - DR</td>
<td>Extensively drug resistant</td>
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<tr>
<td>Z – N</td>
<td>Ziehl-Neelsen</td>
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<tr>
<td>&gt;</td>
<td>Greater than</td>
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<tr>
<td>&lt;</td>
<td>Less than</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>(+Ve)</td>
<td>Positive</td>
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<tr>
<td>(-Ve)</td>
<td>Negative</td>
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<tr>
<td>µg</td>
<td>Micro gram</td>
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<tr>
<td>µl</td>
<td>Micro liter</td>
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SUMMARY

BACKGROUND

Tuberculosis (TB) is one of the most prevalent human infections and causes more death than any other infectious disease. Of all types of tuberculosis pulmonary tuberculosis is most common form of TB and accounts 80% of cases in Bangladesh. Moreover 65% of all the pulmonary tuberculosis are a smear positive cases. Tuberculosis is a major public health problem. Its diagnosis is based on clinical examination, microscopic examination, x-ray findings, culture, immunological tests and nucleic acid based technique. Microscopy with Ziehl – Neelsen (Z-N) staining has low sensitivity and x-ray is nonspecific. Other methods (like Ogawa culture, PCR) are expensive, time consuming and not available in all laboratory settings. Microcolony detection of M. tuberculosis using slide culture technique is being developed as a rapid, less expensive and sensitive technique for diagnosis of pulmonary tuberculosis.

OBJECTIVES

To diagnose pulmonary tuberculosis by microscopy, rapid slide culture in sula medium and standard culture on Ogawa medium and to compare these three methods to explore a rapid, less expensive and most effective technique.

METHODOLOGY

A cross sectional study was conducted among the suspected cases of pulmonary tuberculosis patients who fulfilled the inclusion criteria of the study at the DOT’S corner of Mymensingh Medical College Hospital and Netrakona during the period from July 2007 to Jun 2008. Sputum samples were taken from 214 suspected cases for microscopy and culture. Culture
was done on two different methods. One was standard culture on acidified egg medium and 
other was slide culture in sula medium.

RESULTS

Out of 214 samples, 42 (19.62%) showed growth on slide culture technique, where as 
microscopy and acidified culture were positive in 46 (21.49%) and 50(23.36%) respectively.
In slide culture method 8 were false negative but none were false positive. On the other hand 
Z – N stain showed 9 false negative and 5 false positive. The sensitivity and specificity of 
slide culture were 84% and 100% which was higher than that of microscopy (82% and 
96.95% respectively).

CONCLUSION

Analyzing the findings of this study it may be concluded that slide culture technique may be a 
suitable method for rapid diagnosis of pulmonary tuberculosis in settings requiring minimal 
equipments and it can be a routine diagnostic procedure for diagnosis of pulmonary 
tuberculosis. The sensitivity and specificity of slide culture were higher than microscopy 
when compared with gold standard Ogawa culture.
CHAPTER 1
INTRODUCTION & OBJECTIVES
INTRODUCTION

Tuberculosis (TB) is one of the most prevalent human infections and cause more death worldwide than any other infectious diseases. About one third of the global population is infected with *Mycobacterium tuberculosis* and eight million of new cases of TB occur each year, leading to nearly 3 million deaths annually (Guda *et al.*, 2004). World health organization (WHO) estimates that largest number of new TB cases in 2005 occurred in South East Asian region which accounts 36% of incidence. However the estimated incidence rate in sub Saharan Africa is nearly twice than that of South East Asian region. Every second a new one is infected with TB and affects 350 people/100,000, in Sub Saharan Africa (WHO, 2007). Tuberculosis is a global threat. Every year more than eight million people develop active tuberculosis and about two million die (WHO, 2006). Nearly two billion people (one third of the world’s population) harbouring latent infection (WHO, 2008). Furthermore 200,000 people died from HIV associated TB every year. The WHO declared TB is a global emergency in 1993. Multidrug-resistant tuberculosis (MDR-TB), extensively drug-resistant tuberculosis (XDR-TB), HIV-associated TB and weak health systems are major challenges in many countries for controlling TB (WHO, 2008). The WHO has been working to dramatically reduce the burden of TB, half TB deaths and prevalence by 2015, through the “Stop TB” strategy and supporting the Global Plan is to “Stop TB”. Among all the regions, the South East Asia Region carries 36% of the global TB cases. It is 26% in the Africa Region, 25% in the Western Pacific Region, 5% in the Eastern Mediterranean Region and 4% each in the American Region and the European Region (WHO, 2008).

Countries in the South-East Asia Region have continued to make steady progress with TB control. During the year 2006, more than two million TB patients were initiated on treatment
in the Region. Six countries in the Region have achieved the global targets for case detection and treatment success. As a result, the overall regional case detection rate was 70% in 2006 and the treatment success rate for the 2005 cohort of new smear-positive patients was 87% (WHO, 2007).

In 2006, Bangladesh ranked sixth as the highest burden TB countries among 22 countries in the world. On the basis of recent informations WHO estimated for 2007, the absolute numbers of prevalent cases was 559,000 (all forms), 321,675 new cases (all forms), 144,397 new smear-positive cases and 64,335 people died from TB. The MDR-TB rate is 3.6% among new cases and 19% among re-treatment cases. Although the HIV prevalence is still low in Bangladesh but it poses a threat to TB control. Three limited surveys conducted in 1999, 2001 and 2006 – 07 the HIV prevalence in adult TB patient was about 0.1% (WHO, 2007).

Early case detection and treatment remains the cornerstone for effective control of TB because, these two factors not only enable to cure but also reduce the transmission of infection to others in the community (Sarin, 1995).

Currently, the only confirmatory criterion for definite diagnosis of TB is the demonstration of the presence of tubercle bacilli in clinical specimens. This is based on traditional methods, microscopy with Ziehl-Neelsen’s acid fast stain and culture of *M. tuberculosis* on Lowenstein –Jensen (L-J) medium (Marei *et al*, 2003). Z-N staining lacks sensitivity, only 40 – 60% of truly active pulmonary tuberculosis cases being confirmed even with optimal staining and satisfactory microscopic examination (Diagbouga *et al*, 1997). Microscopic examination is time honored and economical but this technique requires a large number of bacilli between 5,000 to 10,000 per ml of sputum (Iqbal *et al*, 2003).
Culture techniques are complex and time consuming, require 4 - 8 weeks to yield growth of *M. tuberculosis* and also lack sensitivity, particularly in smear negative cases. Lowenstein - Jensen medium though widely used in tuberculosis laboratories but it presents certain difficulties because, it requires a well- equipped laboratory and must prepare the media at frequent intervals because of its short life (Olga Salazar Alegre, 1967). For this difficulties culture of *M. tuberculosis* not done in any of the medical colleges in Bangladesh.

Serological tests have not significant role for the diagnosis of pulmonary tuberculosis due to the unpredictable antibody detection. The delayed type of hypersensitivity (DTH) reaction following injection of tuberculin (PPD) is a well- established and widely used test for determining infection with tubercle bacilli (Bloom, 1994). Its sensitivity is ablated in subjects previously sensitized by BCG vaccination or by intensive exposure to environmental mycobacteria. It gives false negative results in severely ill TB patients and in subjects with associated viral infection which causes immunosuppression and other immunosuppressive condition or a state of anergy (Joklik *et al*, 1992).

Chest x-ray can be useful in the diagnosis of tuberculosis. But chest lesion identification by radiograph is not specific and can not identify the causal agent (Jenkins, 1994). Other chest conditions may be accompanied by radiographic abnormalities misread as tuberculosis and may be improperly treated for tuberculosis (Faulds, 1998). On the other hand, in one study, experts missed approximately 25% of tuberculosis cases in a series of films (Garland, 1959).

Although new, more rapid diagnostic methods have been developed, that are based ether on liquid culture techniques such as BACTEC460, MGIT960, or on molecular techniques like PCR for detecting *M. tuberculosis* DNA sequence, but they are expensive and require specialist personnel, sophisticated equipments and a separate, clean and preferably an aseptic
environment, the conditions lacking in many routine diagnostic laboratories, specially in developing countries (Marei et al, 2000). Though PCR is highly sensitive (88% to 100%) and has the capability of distinguishing between different species of mycobacteria. The technique gives false positive results (specificity of the PCR is 80%) due to contamination with DNA fragments from previous PCR debris from nonviable bacilli (Noordhock et al, 1994).

Consequently, there is a need for a rapid, sensitive and low-cost technique that will be suitable for routine use in the developing world. \( M.\) \( tuberculosis \) grows more rapidly on liquid sula medium than on solid medium. This medium can be prepared in an ordinary laboratory and its has long shelf life. It can be kept for six months at room temperature and one year in refrigerator. Based on these observations, a new, efficient, reliable and less expensive method, known as slide culture technique has been developed at Anantapur in Netrakona by modification of liquid culture method. By this culture technique \( M.\) \( tuberculosis \) can be detected by observing micro colonies within 7 – 10 days.

Having the described background, the present study was carried out to evaluate the slide culture technique, in the context of Bangladesh by comparing with the traditional culture method.
OBJECTIVES

General objectives:

To evaluate rapid slide culture technique for the diagnosis of pulmonary tuberculosis.

Specific objectives:

- To diagnose pulmonary tuberculosis by Ziehl-Neelsen staining.
- To diagnose pulmonary tuberculosis by standard culture on Ogawa medium.
- To diagnose pulmonary tuberculosis by rapid slide culture in sula medium.
- To compare the slide culture method with Ziehl-Neelsen staining method and standard culture method.
Chapter 2

REVIEW OF LITERATURE
2.1 HISTORY OF HUMAN TUBERCULOSIS

Tuberculosis (TB) has been present in humans since antiquity. The earliest unambiguous detection of *Mycobacterium tuberculosis* is in the remains of bison dated 17,000 years before the present (Rothschild B *et al.*, 2001). However, whether tuberculosis originated in cattle and then transferred to humans, or diverged from a common ancestor, is currently unclear (Pearce-Duvet J, 2006). Skeletal remains show prehistoric humans (4000 BC) had TB, and tubercular decay has been found in the spines of mummies from 3000-2400 BC (Zink A *et al.*, 2003).

*Phthisis* is a Greek term for tuberculosis; around 460 BC, Hippocrates identified phthisis as the most widespread disease of the times involving coughing up blood and fever, which was almost always fatal. Genetic studies suggest that TB was present in South America for about 2,000 years (Konomi N *et al.*, 2002). In South America, the earliest evidence of tuberculosis is associated with the Paracas-Caverna culture (South America: Prehistoric Findings, 2003).

In the 17th century the exact pathology and infectious nature of the disease began to appear in medical literature. In 1720, Benjamin Marten, an English physician theorized that TB could be caused by “wonderfully minute living creatures” which are contagious over a prolonged period of contract. In 1865, a French army physician Jean-Atoine Villemin demonstrated that consumption could be passed from humans to cows to rabbits. With this evidence he postulated that the disease was caused by a specific microorganism and this organism was first viewed in 1882 when Robert Koch discovered a special staining technique that allowed him to see the *Mycobacterium tuberculosis*. (Smith, 2003)
2.2 EPIDEMIOLOGY OF TUBERCULOSIS

2.2.1 GLOBAL INCIDENCE

With 1.7 million deaths, 9.2 million new active cases per year and nearly two billion people (one third of the world’s population) harbouring latent infection, TB is a global threat. In addition, another 200 000 people with HIV died from HIV associated TB. The WHO declared TB a global emergency in 1993. If TB disease is detected early and fully treated, people with the disease quickly become non-infectious and eventually cured. Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), HIV-associated TB and weak health systems are major challenges in many countries. The WHO is working to dramatically reduce the burden of TB and halve TB deaths and prevalence by 2015, through the Stop TB strategy and supporting the Global Plan to Stop TB. Among all the regions, the South East Asia Region carries 36% of the global TB cases. It is 26% in the Africa Region, 25% in the Western Pacific Region, 5% in the Eastern Mediterranean Region and 4% each in the American Region and the European Region (WHO, 2008).

Globally, the case detection rate for new smearpositive cases reached 61% in 2006 and the treatment success rate improved to 84.7% in 2005, marginally below the target of 85%. Major progress occurred in the implementation and planning of other parts of the strategy ranges from major such as provision of TB/HIV interventions for TB patients in the African Region while less progress was reported in other areas. There is a need for improved guidance on advocacy, communication and social mobilization (ACSM) activities, and more ambitious planning for treatment of patients with MDR-TB, in the European, South-East Asia and Western Pacific Regions.

2.2.2 SOUTH-EAST ASIA REGIONAL SCENARIO
Countries in the South-East Asia Region have continued to make steady progress with TB control. During the year 2006, more than two million TB patients were initiated on treatment in the Region. Six countries in the Region have achieved the global targets for case detection and treatment success. As a result, the overall Regional case detection rate was 70% in 2006 and the treatment success rate for the 2005 cohort of new smear-positive patients was 87%. All countries are implementing the Stop TB strategy launched in 2006. “TB control: Progress and Plans for implementing the new Stop TB strategy” was an agenda item which resulted in a resolution at the 60th Regional Committee in 2007. The resolution called on Member countries to ensure that necessary steps are taken to fully implement national plans for TB control, incorporating all elements of the Stop TB strategy, in order to achieve the TB targets set under the Millennium Development Goals by 2015 (WHO, 2007).

2.2.3 TUBERCULOSIS AND BANGLADESH

In 2006, Bangladesh ranked sixth on the list of 22 highest burden TB countries in the world. The WHO estimated that in 2006 there were approximately 391 TB cases (all forms) per 100 000 population. It is estimated that, per 100 000 people, 225 new cases occur each year. Of these, approximately 101 per 100 000 were infectious cases, i.e. able to transmit TB in the community. It is further estimated that about 45 per 100 000 people die of TB every year. Applying these most recent WHO estimates for 2007, this translates to the following absolute numbers: 559 000 prevalent cases (all forms), 321 675 new cases (all forms), 144 397 new smear-positive cases and 64 335 people dying from TB. Although the HIV prevalence is still low, HIV poses a threat to TB control. The HIV prevalence in adult TB patient was about 0.1% as revealed in three limited surveys conducted in 1999, 2001 and 2006-07. The MDR-
TB rate among new cases of TB was estimated to be 3.6% among new cases and 19% among re-treatment cases.

2.2.4 HIV AND TB

HIV and TB form a lethal combination, each speeding the other’s progress. HIV weakens the immune system. Someone who is HIV-positive and infected with TB bacilli is many times more likely to become sick with TB than someone infected with TB bacilli who is HIV-negative. TB is a leading cause of death among people who are HIV-positive. In Africa, HIV is the single most important factor contributing to the increase in incidence of TB since 1990. (WHO, 2007)

WHO and its international partners have formed the TB/HIV Working Group, which develops global policy on the control of HIV-related TB and advises on how those fighting against TB and HIV can work together to tackle this lethal combination. The interim policy on collaborative TB/HIV activities describes steps to create mechanisms of collaboration between TB and HIV/AIDS programmes, to reduce the burden of TB among people and reducing the burden of HIV among TB patients. (WHO, Fact sheet, 2007).
2.2.5 TB WITH HIV COINFECTION IN BANGLADESH

Bangladesh, although still considered as a low HIV/AIDS prevalent country, is at a critical moments in the course of its AIDS epidemic. It is estimated that there are 13,000 HIV positive people in the country and that HIV prevalence in the adult population is less than 0.01%. Among the adults tuberculosis cases HIV positivity rate is 0.1% (WHO, 2004b). In 1998 all six reported AIDS death were due to TB (WHO, 1998).

2.3 TRANSMISSION

When people suffering from active pulmonary TB cough, sneeze, speak, or spit, they expel infectious aerosol droplets 0.5 to 5 µm in diameter. A single sneeze can release up to 40,000 droplets. (Cole & Cook, 1998) Each one of these droplets may transmit the disease, since the infectious dose of tuberculosis is very low and the inhalation of just a single bacterium can cause a new infection. (Nicas M, et al, 2005)

People with prolonged, frequent, or intense contact are at particularly high risk of becoming infected, with an estimated 22% infection rate. A person with active but untreated tuberculosis can infect 10–15 other people per year. (WHO 2006) Others at risk include people in areas where TB is common, people who inject drugs using unsanitary needles, residents and employees of high-risk congregate settings, medically under-served and low-income populations, high-risk racial or ethnic minority populations, children exposed to adults in high-risk categories, patients immunocompromised by conditions such as HIV/AIDS, people who take immunosuppressant drugs, and health care workers serving these high-risk clients. (Griffith D & Kerr C 1996)
Transmission can only occur from people with active — not latent — TB (Abbas et al. 2007). The probability of transmission from one person to another depends upon the number of infectious droplets expelled by a carrier, the effectiveness of ventilation, the duration of exposure, and the virulence of the *M. tuberculosis* strain. (CDS 2003) The chain of transmission can, therefore, be broken by isolating patients with active disease and starting effective anti-tuberculous therapy. After two weeks of such treatment, people with non-resistant active TB generally cease to be contagious. If someone does become infected, then it will take at least 21 days, or three to four weeks, before the newly infected person can transmit the disease to others. TB can also be transmitted by eating meat infected with TB. *Mycobacterium bovis* causes TB in cattle (Wikipedia, 2009).

### 2.4 Reservoirs

Comprised within the *M. tuberculosis* complex and generically called the tubercle bacillus, the various etiologic agents of tuberculosis (TB) has distinct hosts and reservoirs. *M. tuberculosis*, and the regional variants or subtypes *Mycobacterium africanum* and *M. canettii* are primarily pathogenic in humans. *M. bovis* and *M. microti* are the causative agents of TB in animals, and can be transmitted to humans. Some particular strains isolated from goats and seals have been named *Mycobacterium caprae* and *Mycobacterium pinnipedi*, although sometimes they are identified as *M. bovis* subspecies or variants (Wayne et al., 1982).
2.5 HABITAT

Only a few *Mycobacteria* became successful pathogens of higher vertebrates, preferentially inhabiting the intracellular environment of mononuclear phagocytes. The host-dependent *Mycobacteria* that cannot replicate in the environment are *Mycobacterium leprae*, *Mycobacterium lepraemurium*, *Mycobacterium avium* subsp. *paratuberculosis*, and the members of the *Mycobacterium tuberculosis* complex. Bacteria within the *M. tuberculosis* complex are able to reproduce *in vitro*, in contrast to *M. leprae* and *M. lepraemurium*, which are uncultivable and require the intracellular milieu for survival and propagation. The major pathogens *M. tuberculosis* and *M. leprae* are incapable of replicating on inanimate subject whereas (Non tubercular mycobacterium) NTM are free living mycobacteria and are usually found in association with watery habitats such as lake, rivers and wet soil (Willett, 1992).
### 2.6 MICROBIOLOGY OF MYCOBACTERIUM TUBERCULOSIS

#### 2.6.1 TAXONOMY AND DESCRIPTION OF THE GENUS (Tuberculosis, 2007)

<table>
<thead>
<tr>
<th>Kingdom</th>
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<td><em>M. caprae</em></td>
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<td><em>M. pinnipedii</em></td>
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2.6.2. GENERAL PROPERTIES OF MYCOBACTERIUM

The genus *Mycobacterium* is the only genus in the family Mycobacteriaceae and is related to other mycolic acid containing genera. The high G+C contents of the DNA is a unique property of Mycobacterium species (61 to 71 mol%, except that of *M. lepra* (55 mol %) along with other mycolic acid-containing genera, Gordonia (63 to 69 mol %) Tsukamurella (68 to 74 mol %), Nocardia (64 to 72 mol %), and Rhodococcus (63 to 73 mol %). Mycobacteria are slender bacilli and sometimes exhibits filamentous forms resembling fungal mycelium (from greek, myces means fungus) hence they are so named (Chakroborty, 1999). Mycobacteria are aerobic (though some species are able to grow under a reduced-O$_2$ atmosphere), non-spore-forming, nonmotile, slightly curved or straight rods, 0.2 to 0.6 um by 1.0 to 10 um which may be branched. Colony morphology varies among the species, ranging from smooth to rough and from non pigmented (nonphotochromogens) to pigmented. Some species require light to form pigment (photochromogens); other species form pigment in either the light or the dark (Scotochromogens). Aerial filaments are very rarely formed and never visible without magnification. Filamentous or mycelium-like growth many sometimes occur but, on slight disturbance, easily fragments into rods or coccoid elements (Pfyffer, 2007).

The genus mycobacterium includes obligate pathogens, opportunistic pathogens, and saprophytes. With the advent of the molecular techniques for the appropriate identification, close to 200 *Mycobacterial* species have now been described. They could be classified grossly into Rapid grower and slow grower. Slow grower contains (1) *M. tuberculosis complex* (2) *M. lepra* and (3) NTM or non tubercular mycobacterium. Some human pathogenic NTM are *M.ulcerans, M. haemophelium, M. asiaticum*. A natural division exists between slowly rapidly
growing species of *Mycobacteria*. Slow growers require more than 7 days producing colonies on solid media under ideal culture conditions (Raviglione *et al*, 2005)

The rapid grower mycobacterium is the opportunistic pathogens that grow within 7 days on laboratory media that produce diseases in various clinical setting. The three major clinically important species are *M. fortuitum, M. chelonae, M. abscesssus*.

2.6.2. 1 MYCOBACTERIUM TUBERCULOSIS COMPLEX

The *M. tuberculosis* complex is the cause of TB and is comprised of *M. tuberculosis, M. bovis, M. africanum, M. canettii* and *M. microti*. The mycobacteria grouped in the complex are characterized by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences but differ widely in terms of their host tropisms, phenotypes, and pathogenicity (Boddinghaus *et al*, 1990).

2.6.2. 2 CELL WALL STRUCTURE

As the most distinctive anatomical feature of the bacillus, the cell envelope is composed of the (1) plasma membrane (2) a cell wall, and (3) an outer capsule like layer (Mahapatra *et al*, 2005). The mycobacterial cell wall, however, is unique among prokaryotes. The wall is constituted by an inner peptidoglycan layer, which seems to be responsible for the shape-forming property and the structural integrity of the bacterium. The structure of this stratum differs that of common bacteria as it presents some particular chemical residues and an unusual high number of cross-links. The arrangements of these mycolic acids are species-specific, a property that allows the identification of many species of mycobacteria by gas-liquid, high-performance liquid or thin-layer chromatography. The mycolic acids specific to *M. tuberculosis* are alpha, keto and methoxymycolates containing 76 to 82, 84 to 89, and 83 to 90 carbons respectively. The outer layer of the cell wall presents an array of free lipids.
Some glycolipids such as lipomannan (LM) and lipoarabinomannan (LAM), are anchored to the plasma membrane and extend to the exterior of the cell wall and LAMs are species-specific. Lipids constitute more than half of the dry weight of the Mycobacteria and act as carbon and energy reserves. The waxy coat confers the idiosyncratic characteristics of the genus: acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics, and distinctive immunological properties and probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients. The capsule like layer contains proteins, polysaccharides and minor amounts of inner lipids, might be protective and bioactive (Riley et al, 2006). The envelope of the tubercle bacillus seems to be a dynamic structure that can be remodeled as the microorganism is either growing or persisting in different environments (Kremer et al, 2005).

2.6.2. 3 ACID FASTNESS

The expression “acid-fastness” describes the resistance of certain microorganisms to decolorization with acid-alcohol solutions after staining with carbol fuchsin. This feature is of utmost practical importance in identifying the tubercle bacillus, particularly in pathological specimens. Evidence was provided sustaining the role of lipids in trapping the dyes. Indeed, there is a parallelism between the increasing degree of acid fastness displayed by microorganisms in the genera Corynebacterium, Nocardia, and Mycobacterium, and the increasing length of mycolic acid chains in their walls. However, the property is absolutely dependent on the integrity of the bacillus (Goren et al, 1978).

2.6.2. 4 CULTURAL CHARACTER

They grow only in special enriched medium containing egg, asparagine, potatoes, serum and meat extracts. M. tuberculosis more luxuriantly in culture (eugonic) than M. bovis (dysgonic).
Virulent strain often grows as twisted rope like colonies which are called serpentine cords (Chakroborty, 1999). In contrast, non-virulent *Mycobacteria* and tubercle bacilli attenuated by prolonged cultures usually develop smooth colonies on solid media. The recognition of that peculiarity of cord formation provides a reliable clue to the experienced microbiologist for the presumptive distinction of *M. tuberculosis*. These distinctive characteristics of the virulent bacilli have been attributed to the trehalose 6, 6′-dimycolate also known as cord factor (Chesnutt *et al*, 2007).

### 2.6.2. 5 PERMEABILITY BARRIERS

The tightly packed mycolic acids provide the bacillus with an efficient protection and an exceptional impermeability along with a thicker layer of carbohydrate and protein outside the lipid layer. The impermeability is at least one of the determinants for two *M. tuberculosis* characteristics: its slow growth and its intrinsic drug resistance (Chambers *et al*, 1995).

### 2.6.2.6 NUTRITIONAL AND ENVIRONMENTAL REQUIREMENTS FOR GROWTH

The microorganism macromolecular structure and metabolic capabilities result in high adaptation to the specific environment. During the course of infection in mice, *M. tuberculosis* metabolism may shift from an aerobic, carbohydrate-metabolizing mode to one that is more microaerophilic and utilizes lipids (Raviglione *et al*, 2005). Trace elements as Magnesium and iron are essential for life. In nature, the bacillus grows most successfully in tissues with high oxygen partial tension, such as the lungs, particularly the well-aerated upper lobes. In the laboratory, an atmosphere of 5 to 10 % carbon dioxide favors culture growth, at least during the early stage of incubation.

### 2.6.2. 7 GENERATION TIME
Under favorable laboratory conditions, *M. tuberculosis* divides every 12 to 24 hours. The slow growth rate might be partially determined by the cell wall impermeability that limits nutrient uptake. Ribonucleic acid (RNA) synthesis to be a major factor associated with the long generation time of the tubercle bacillus (Harshey *et al*, 1977). The low multiplication rate explains the typically sub-acute to chronic evolution of the disease and the long time required to attain visible growth in vitro.

### 2.6.2. 8 METABOLIC AND BIOCHEMICAL MARKERS

In addition to some susceptibility tests, the investigation of niacin accumulation, nitrate reductase and urease activity allows the distinction of *M. tuberculosis* complex and species differentiation within the complex. (Kasarov *et al*, 1972).

The main *M. tuberculosis* antioxidant enzyme is a heat labile catalase-peroxidase with both catalase and peroxidase activities. The catalase is not only self protective but can also be self-destructive as it activates the anti-tuberculous prodrug INH. The nitrate reductase activity is observed in both the *M. tuberculosis* (Sohaskey *et al*, 2003).

### 2.6.2. 9 RESISTANCE TO PHYSICAL AND CHEMICAL CHALLENGES

Although the tubercle bacillus is not a spore-forming bacterium, it has a remarkable capacity to endure unfavorable conditions. The bacillus is able to limit the access to the bacterial targets of hydrophilic antiseptics and antibiotics. The microorganism also withstands very low temperatures. Its viability may be increasingly preserved for a long term between 2-4°C to -70°C. When ultrafrozen, the viability of the bacilli remains almost intact as well as the taxonomic, serologic, immunologic, and pathogenic properties. On the other hand, the bacilli are very sensitive to heat, sunlight and ultraviolet (UV) irradiation (Pfyffer, 2007).

### 2.6.2. 10 VIRULENCE MECHANISMS AND VIRULENCE FACTORS
*Mycobacterium tuberculosis* does not possess the classic bacterial virulence factors such as toxins, capsules and fimbriae. However, a number of structural and physiological properties of the bacterium are beginning to be recognized for their contribution to bacterial virulence and the pathology of tuberculosis.

MTB has special mechanisms for cell entry. The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell wall-associated mannosylated glycolipid, LAM, or indirectly via certain complement receptors or Fc receptors.

The ability of MTB to grow intracellularly is an effective means of evading the immune system and as a result antibodies and complement are ineffective. Once MTB is phagocytosed, it can inhibit phagosome-lysosome fusion (Robbin *et al.*, 2005).

MTB interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by two mechanisms: 1. Compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism. 2. Macrophage uptake via complement receptors may bypass the activation of a respiratory burst.

Antigen 85 complex may aid in walling off the bacteria from the immune system and may facilitate tubercle formation (Todar, 2008).

Because of MTB's slow generation time, the immune system may not readily recognize the bacteria or may not be triggered sufficiently to eliminate them.

High lipid concentration in cell wall accounts for impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the intracellular and extracellular environment, and resistance to osmotic lysis via complement deposition and attack by lysozyme.

Cord factor is primarily associated with virulent strains of MTB. It is known to be toxic to mammalian cells and to be an inhibitor of PMN migration (Willett, 1992).
2.7 PATHOGENESIS OF TUBERCULOSIS

About 90% of those infected with *Mycobacterium tuberculosis* have asymptomatic, latent TB infection (sometimes called LTBI), with only a 10% lifetime chance that a latent infection will progress to TB disease (Abbas *et al.*, 2007). However, if untreated, the death rate for these active TB cases is more than 50% (Onyebujoh *et al.*, 2006).

TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within the endosomes of alveolar macrophages (Houben E *et al* 2006) The primary site of infection in the lungs is called the Ghon focus, and is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe (Abbas *et al.*, 2007). Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymph nodes. Further spread is through the bloodstream to other tissues and organs where secondary TB lesions can develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone (Herarrmann J & Lagrang P 2005). All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid (Agarwal R *et al.*, 2005).

Tuberculosis is classified as one of the granulomatous inflammatory conditions. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma, with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the mycobacteria, but also provides a local environment for communication of cells of the immune system. Within the granuloma, T lymphocytes (CD4+) secrete cytokines such as interferon gamma, which activates
macrophages to destroy the bacteria with which they are infected (Kaufmann et al, 2002). T lymphocytes (CD8+) can also directly kill infected cells (Houben E et al, 2006).

Importantly, bacteria are not always eliminated within the granuloma, but can become dormant, resulting in a latent infection (Abbas et al, 2007). Another feature of the granulomas of human tuberculosis is the development of cell death, also called necrosis, in the center of tubercles. To the naked eye this has the texture of soft white cheese and was termed caseous necrosis (Parrish et al, 1998).

If TB bacteria gain entry to the bloodstream from an area of damaged tissue they spread through the body and set up many foci of infection, all appearing as tiny white tubercles in the tissues. This severe form of TB disease is most common in infants and the elderly and is called miliary tuberculosis. Patients with this disseminated TB have a fatality rate of approximately 20%, even with intensive treatment (Kim J et al, 2003).

In many patients the infection waxes and wanes. Tissue destruction and necrosis are balanced by healing and fibrosis. (Grosset J 2003) Affected tissue is replaced by scarring and cavities filled with cheese-like white necrotic material. During active disease, some of these cavities are joined to the air passages bronchi and this material can be coughed up. It contains living bacteria and can therefore pass on infection. Treatment with appropriate antibiotics kills bacteria and allows healing to take place. Upon cure, affected areas are eventually replaced by scar tissue. (Grosset J 2003)

2.7.1 FORMATION OF GRANULOMA
The granulomatous response is the hallmark of chronic *M. tuberculosis* infection, which is a desperate attempt by the host immune system to contain multiplication and further dissemination of bacteria to other organs. It is postulated that stimulated alveolar macrophages in the airways invade the lung epithelium following internalization of inhaled bacteria (Ulrichs *et al.*, 2006). Along with other inflammatory cascade regulated and superceded by a specific, cellular immune response is linked to formation of the 'stable' granuloma responsible for immune containment during latent or subclinical infection becomes recognizable and stratification of the structure emerge (Tully *et al.*, 2005). The granuloma subsequently develops central areas of necrosis (called caseum, from the word ‘cheese’), resulting in the death of the majority of bacteria and destruction of the surrounding host tissue. The surviving bacilli exist in a latent state and can become reactivated leading to development of active disease. The granuloma serves (Robins *et al.*, 2005) major purposes as a local environment in which immune cells can interact to kill bacteria, a focus of inflammatory cells that prevent inflammation from occurring throughout the lungs, and a barrier to dissemination of bacteria throughout the lungs and other organs (Algood *et al.*, 2005) Disruption of the granuloma structure or function appears to be detrimental to the control of bacterial replication and the control of immunopathology in the lung.

### 2.8 IMMUNOLOGY OF TUBERCULOSIS

#### 2.8.1 CELLS INVOLVED IN IMMUNE RESPONSE

##### 2.8.1.1 MACROPHAGES

Macrophages play a central effector role in the immune response to *M. tuberculosis* infection. Once infected by the bacterium, macrophages presents antigens on both class I and II major histocompatibility complex (MHC) to T cells, which in turn secrete IFN-γ resulting in
activation of the macrophages to kill the bacteria (Huygen et al, 1994). Tumor necrosis factor (TNF) is an important proinflammatory cytokine secreted by activated monocytes/macrophages (Bekker et al, 2001). Furthermore, the pro inflammatory cytokines IL-1 and IL-6 secreted during inflammation play an important role in recruitment of cells to the site of infection. A major effector mechanism is the induction of nitric oxide (NO) and related reactive nitrogen intermediates (RNIs) by macrophages (Giacomini et al, 2001) and phagolysosome fusion (Robins et al, 2005).

2.8.1.1 PHAGOSOME - LYSOSOME FUSION

*M. tuberculosis* has the ability to produce ammonia in abundance and is thought to be responsible for inhibitory effect of virulent mycobacteria on phagolysosome fusion. In addition, ammonium chloride (NH$_4$Cl) has been shown to effect the sultatory movement of lysosome and to alkalinize the intralysosomal compartment (D. Acry Hart et. al., 1983). Thus by virtue of its ability to produce a significant amount of ammonia, the tubercle bacillus can potentially evade the toxic environment within the lysosomal vacuole by (i) inhibiting phagosome-lysosome fusion (ii) diminishing the potency of the intralysosomal enzymes via alkalinization. Another mycobacterial product through to have the ability to inhibit phagolysosomal fusion is the sulphatides (Goren et.al, 1976).

2.8.1.1 2. THE RESPIRATORY BURST

It has been shown that lipoarabinomannan (LAM) can incapacitate the oxygen radical-dependent antimicrobial mechanisms at two levels: (i) LAM is an effective reactive oxygen intermediates (ROI) scavenger, and (ii) LAM can down regulate the oxidative burst by
inhibiting protein kinase C (Chan et al., 1991). Other mycobacterial components that interfere with the oxygen radical dependent antimicrobial-mechanism of macrophage are phenolicglycolipid -1 (PGL-1) and the sulphatides. Another mechanism by which *M. tuberculosis* could evade the toxicity of ROI is to avoid binding to macrophage cell surface, component, such as Fc receptors, that would provoke oxidative burst. Instead, the tubercle bacillus MP via compliment receptors CRI and CR3, whose interaction with a ligand does not trigger ROI production in resting macrophage (Wright and Silverstein, 1983).

**2.8.1.1. 3 THE ROLE OF MONONUCLEAR PHAGOCYTES**

When *M. tuberculosis* organisms are inhaled into the lung, they are engulfed by alveolar macrophages, which perform three important functions. First, the macrophages produce proteolytic enzymes and other metabolites that exhibit mycobactericidal effects. Second, macrophage produce characteristic patterns of soluble mediators (cytokines), including IL – 1, IL – 6, IL – 10, tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGB-β). These cytokines have the potential to exert potent immunoregulatory effects and to mediate may of the clinical manifestations of tuberculosis. The third critical function of macrophage is to process and present mycobacterial antigens to T-Lymphocytes. Expression of mycobacterial antigen in association with major histocompatibility complex (MHC) molecules induces expansion of specific CD4+ lymphocytes, the cell population that is central to acquired resistance to *M. tuberculosis* (Toossi et al., 1991).
2.8.1.2 DENDRITIC CELLS

It is now established that DCs are also involved in an effector role against *M. tuberculosis* infection and are central to the generation of acquired immunity after carriage of antigens to draining lymph nodes, where recognition by T cells can be maximized (Flynn *et al.*, 2004). The efficient antigen-capturing causes immature DCs are transformed into mature T cell stimulating DCs, which migrate into draining lymph nodes. In these compartments, the stimulatory capacity of mature DCs ultimately leads to effector T cell differentiation and memory T cell expansion, which in turn, confer protection against *M. tuberculosis* in the lungs (Kaufmann *et al.*, 2001).

2.8.1.3 NATURAL KILLER CELLS

Natural killer cells play a very important role in the development of the innate immune response associated with the development of cytotoxicity to target cells and the first cell populations to produce IFN-γ during the immune response (Junqueira-Kipnis *et al.*, 2003).

2.8.1.4 NEUTROPHIL LEUKOCYTES

Even though macrophages are considered the main targets for infection by *Mycobacterium tuberculosis* Neutrophil leukocytes are among the earliest cells recruited into infection sites and inflammatory signals are triggered along with the formation of neutrophil extracellular traps (Urban *et al.*, 2006).
2.8.1.5 CD4+ T CELLS

CD4+ cells play a dominant but not exclusive role in immune defense against tuberculosis. CD4+ cells are selectively expand at the site of disease in patient with a resistant immune response, such as those with tuberculosis pleuritis. Depletion of CD4+ cells by HIV infection markedly increases susceptibility to primary and reactivation tuberculosis. Frequency of mycobacteremia rises from 4% in patient with more than 200 CD4 cells/µl to 94% in those with 100 or fewer CD4+ cells/µl (Jonson et al., 1993). CD4+ cells mediate their antimycobacterial effects through cytokine production and activation of macrophages. Cytolytic CD4+ cells that specifically recognized mycobacterial antigens can lyse these macrophages releasing bacilli to be engulfed and killed by macrophages with greater antimycobacterial activity. Alternatively, cytokine T cell may play a scavenger role by lysing dead macrophages containing large number of dead bacilli so they can be catabilized by the surrounding mononuclear cells (Orme et al., 1992).

2.8.1.6 CD8 T CELLS

CD8 T Cells constitute the major cytolytic T cell population in defense against many intracellular pathogens but their role in human antimicrobial defense remains uncertain. CD8+ dependent effector mechanisms contribute to anti *M. tuberculosis* immune response. CD8+ depended cytotoxicity (perforin, granzyme) is associated with killing of *M. tuberculosis* infected macrophages (Ellner et al., 2000). γδ T cells: γδ T cells play a role in the initial immune response to *M. tuberculosis* infection. Human γδ T cells have an innate capacity to recognized mycobacterial antigen. γδ T cells contribute to the control of primary
infection before the αβ T cell response has become established by producing cytokine similar to αβ T cell and causing lysis of the target cells infected with *M. tuberculosis* (Munk *et al*, 1990).

### 2.8.2 CYTOKINES INVOLVED IN IMMUNE RESPONSE PROTECTION AND IMMUNOPATHOLOGY

The human immune response eliminates microbial pathogens through an inflammatory response that may also be harmful to host tissue. In tuberculosis, tissue necrosis and fibrosis are characteristic manifestations that are through to result in part from cytokines produced during inflammatory response. Inhaled *M. tuberculosis* organisms are initially engulfed by alveolar macrophages that can produce a plethora of cytokines, including IL-1, IL-8, IL-10, TNFα, TGF-β and GM-CSF. Of them IL-1, IL-8, TNF-α and GM-CSF are proinflammatory molecules that facilities recruitment of lymphocytes and monocytes. TNFα and GM-CSF also activate macrophages and may augment mycobactericidal activity. T lymphocytes recruited to the site of infection can produce cytokines that include IFN-γ and IL-2, IL-5, and IL-10 and recruited macrophages can produce TNF-α and 1,25- dyhydroxy vitamin D (Burnes *et al*, 1989). IFN-γ and 1,25- dyhydroxy vitamin D further enhance macrophage activation and monokine production. The protective immune response may also cause significant tissue necrosis as well as fever and wasting from release of cytokine such as TNF-α into circulation. To reduce excessive inflammation and tissue damage, immunosuppressive cytokines such as IL-10 and TGF-β, produced by macrophages at the site of disease in tuberculosis. (Toosi *et al*, 1991) may down regulate the immune response and limit the extent of tissue injury.

### 2.8.3 IMMUNE RESPONSE TO *M. TUBERCULOSIS*
2.8.3.1 INNATE IMMUNE RESPONSE

After the *M. tuberculosis* invades the activation of signaling pathways occurs upon recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). Although T cells provide the crucial element of specificity, the immune response is sensed primarily by innate immune mechanisms. TLRs are expressed on many cells, including phagocytes, and mediate the activation of cells of the innate immune system, resulting in destruction of the invading microorganism through activation of several signaling cascades. TLR signalling also triggers differentiation of monocytes into macrophages and DCs, generating the cellular populations necessary for a potent innate and adaptive immune response (Hill, 1998). The pro-inflammatory cytokines IL-1 and IL-6 secreted by macrophage during inflammation play an important role in recruitment of cells to the site of infection. A major effectors mechanism is related to reactive nitrogen intermediates (RNIs) by macrophages. Other antimycobacterial mechanisms of macrophages are; phagolysosome fusion, a process which exposes ingested bacteria in the phagosome to lytic enzymes in the lysosome. Neutrophil leukocytes are the earliest cells recruited into infection sites and inflammatory signals are triggered along with the formation of neutrophil extracellular traps (Urban *et al.*, 2006). Natural killer cells are associated with the development of cytotoxicity to target cells (Junqueira-Kipnis *et al.*, 2003).

2.8.3.2 SPECIFIC IMMUNE RESPONSE

Specific immune responses can be divided into cell-mediated mechanisms, which include T-cell activation and effector mechanisms, and the humoral immune response, consisting of B-cell maturation and antibody production. Both mechanisms are not mutually exclusive, and T
helper cells are required for antibody maturation, isotype switching and memory. B cells also function as antigen presenting cells by activating T cells in a specifically driven manner.

The protective immunity against TB is mediated exclusively by T cells rather than antibodies as antibodies cannot reach the bacilli within the phagosomes of infected macrophages (Reljic, 2006).

2.8. 3.3 CELLULAR IMMUNE RESPONSE

Since the tubercle bacilli reside inside a compartment within the macrophage, their antigens are presented by MHC class II molecules to CD4+ T lymphocytes. These cells play an important role in the protective response against M. tuberculosis (Caruso et al., 1999). The main function of CD4+ Th1 cells is the production of cytokines including IFN-γ, which activates macrophages and promotes bacilli destruction and it also helps to develop the CD8+ T cell mediated response (Serbina et al, 2001). In the same way, CD4+ cells may participate in the induction of apoptosis of infected cells and the subsequent reduction of bacterial viability through the CD95 Fas ligand system (Oddo et al, 1998). In addition, CD8+ T cells proved to be efficient in lysing infected cells and in reducing the number of intracellular bacteria (Keane, et al, 2001).
2.8.3.4 THE IMMUNE RESPONSE RELATED TO PROGRESSIVE DISEASE

There is substantial evidence that demonstrates a Th$_2$ response in human TB (Seah et al, 2000). Indeed, TB patients have several IL-4-dependent manifestations, including high IgE antimycobacterial antibodies (Yong 1989). CD8+ cells are another source of IL-4, and this correlates with cavitation (Crevel et al, 2000). The presence of IL-4 at late stages of the disease has a direct pathogenic role because it downregulates the protective Th$_1$ responses (Biedermann et al, 2001). The phase of progressive disease starts after one month of infection characterized by a drop in the number of cells expressing INF-γ, IL-2, TNF-α, and iNOS, high bacillary counts and very high levels of IL-4 and TGF-β, produced foamy macrophages. TNF-α plays an essential role in protection but may also be a significant factor in its pathology.

2.8.3.5 FACTORS THAT DEREGRULATE THE PROTECTIVE TYPE 1 RESPONSE

An increase in antigen load is clearly a participating factor of the Th1/Th2 imbalance and deregulates the protective immune response to *M. tuberculosis*. Low antigen loads, prime the Th1 response in contrast high antigen loads efficiently induce the Th2 response (Robbins et al, 2005). In the early stage of infection (21 days after infection), while the Th1 cytokine response predominated and controlled the growth of bacilli, the DTH response was the highest. In contrast, during the progressive phase of the disease (50 days after infection) extensive tissue damage and high IL-4 production are manifested, the DTH response was very low (Hernandez-Pando et al, 1998).

2.8.4 IMMUNE EVASION

Although the immune response against *M. tuberculosis* is usually sufficient to prevent progression to active disease, the microorganism persists in the host. Thus, the strong immune response can control, but not eliminate the infection, indicating that *M. tuberculosis* has
evolved mechanisms to modulate or avoid detection by the host. *M. tuberculosis* persist within macrophages through a variety of immune evasion strategies

(1) ENTRY INTO MACROPHAGES VIA MULTIPLE RECEPTORS

Entry of Mycobacteria into phagocytic cells can occur through binding to multiple receptors, all leading to the delivery of the bacilli into macrophage phagosomes.

(2) MANIPULATION OF THE PHAGOSOME

To persist in the host, *M. tuberculosis* arrests the maturation of bacilli-containing phagosomes into phagolysosomes (Russell *et al*, 2002). It could interfere with phagolysosomal fusion by retention of host protein on the phagosome behaving as self antigens.

(3) AVOIDANCE OF THE TOXIC EFFECTS OF REACTIVE NITROGEN INTERMEDIATES

*M. tuberculosis* expresses genes that counteract the bactericidal or bacteriostatic effects of RNI and NO.

(4) MODULATION OF ANTIGEN PRESENTATION

*M. tuberculosis* avoids elimination by the immune system through the inhibition of antigen processing or presentation by macrophages.
2.9 HOST GENETICS AND SUSCEPTIBILITY

Several aspects of TB epidemiology are not explained by the germ theory, and suggest that there are individual differences in susceptibility as not everyone exposed to \( M \) \( \text{tuberculosis} \) becomes infected. TB was more common in those who additionally had a family history of TB, suggesting the greater importance of familial susceptibility. Asians and especially Africans and African Americans had less innate resistance than Whites (Ottenhoff \textit{et al}, 2005).

Mutations causing Mendelian susceptibility to Mycobacterial diseases (MSMD) have been found due to different genes. The polymorphic human leukocyte antigens (HLA) were the first proteins to be examined for associations with TB susceptibility. HLA B13 has a protective effect while and lower while individuals with DR8 have a greater probability of developing TB (Bolhamley \textit{et al} 1989).

2.10 LABORATORY DIAGNOSIS OF PULMONARY TUBERCULOSIS

The presence of mycobacteria in a pulmonary sample may be confirmed either by demonstrating acid first bacilli by microscopy examination of stained smear or by cultivation of organism on primary isolation medium. The diagnostic value of serological test by demonstrating of antibodies to mycobacteria in serum and those of demonstrating cell mediated immunity by the tuberculin test is less reliable (Laid law, 1989).

2.10.1 SAMPLE COLLECTION

2.10.1.1 CONTAINER: Sputum should be collected in a robust, leak-proof and clean container. Container must be rigid to avoid crushing in transit and must possess a water tight wide mouthed screw top to prevent leakage and contamination (Kantor \textit{et al}, 1998)

2.10.1.2 TRANSPORT OF SPECIMEN
When the transport or the processing is delayed, specimens should be stored for not more than five days at 4°C. The cetylpyridinium chloride (CPC) method is widely used for the transport of sputum specimens (Smithwick et al, 1975). Good laboratory practice is required for the protection of laboratory staff from infectious airborne bacilli, i.e. good ventilation, use of laboratory coats, surgical gloves and face masks, hand washing and regular disinfection of the laboratory floor and surfaces, especially benches, with a disinfectant that is active against mycobacteria. This disinfectant may be 70% ethanol or sodium hypochlorite (house bleach) at a concentration of 0.2-0.5%. Ultraviolet light, emitting rays of wavelength 254 nm, is very effective in killing the tubercle bacillus and other mycobacteria (Riley et al, 1989).

2.10.2 ACID – FAST MICROSCOPIC EXAMINATION

Mycobacteria process cell walls that contain mycolic acids, which are long chain, cross-linked fatty acids. These long chain mycolic acids and lipids in the mycobacterial cell wall probably account for the unusual resistance of the organisms to the effects of drying and harsh decontaminating agents as well as characteristic acid fastness that distinguishes them from most of the other bacteria. Microscopy is the easiest and most rapid diagnostic procedure that detect the acid fast bacilli in clinical specimen. It is less sensitive than culture and requires high bacillary load 5,000 to 10,000/ml (Davie, 1976) and its sensitivity varies from 30% to 70% (Nair et al, 1976). Its sensitivity can be increased up to 74 – 78% when centrifuged concentrated deposited are used (Peterson et al, 1999). Microscopy is helpful in the clinical laboratory because it (i) provides presumptive diagnosis of mycobacterium tuberculosis, (ii) Enables rapid identification of the most infectious person i.e. smear positive case (iii) Confirm the acid fastness of organisms of culture growth (iv) May be use to follow the progress of patients treatment (v) Is also useful in determining the appropriate dilution of smear positive
sputum to be used as inocula for direct drug susceptibility testing (Kent and Kubica, 1985). Three types of staining procedures are used in the laboratory for rapid detection and confirmation of acid fast bacilli. (i) Fluorochrome stain (ii) Ziehl-Neelsen stain and (iii) Kinyoun stain. A 2×2cm smear is prepared from an uncentrifuged specimen or centrifuged deposit. Visualization of acid-fast bacilli is considered as presumptive evidence of tuberculosis because stained does not identify M. tuberculosis specifically. M. gordonae, a nonpathogenic schotochromogen found in tap water may create problem and M. smegmatis may also contaminate urine sample. However incidence of false positivity is very low and the specificity is 98% (Lipsky et al, 1984).

2.10.3 CULTURE

Examination by bacteriological culture provides the definitive diagnosis of tuberculosis depending on the decontamination method and the type of culture medium used, as few as 10 to 100 viable tubercle bacilli/ml of sample can be detected. Compared to other bacteria which typically reproduce within minutes, M. tuberculosis proliferate extremely slowly (generation time 18 – 24 hours). Furthermore, growth requirements are such that it will not grow on primary solution on simple chemically defined media. The only media which allow abundant growth of M. tuberculosis are egg-enriched media containing glycerol and asperagine, and agar or lipid medium supplemented with serum or bovine albumin. Culture increases the number of tuberculosis cases found, often by 30 – 50%, and detects cases earlier, often before they become infectious. Since culture technique can detects few bacilli, the efficiency of diagnosing failure at the end of treatment can be improved considerably. Culture is also needed to performed during susceptibility testing. Culture of
specimen is, however, much more costly than microscopy and requires facilities for media preparation.

Culture should be used selectively, in the following order of priority:

1. Diagnosis of cases with clinical and radiological signs of pulmonary tuberculosis where smears are repeatedly negative.
2. Diagnosis of extra pulmonary and childhood tuberculosis.
3. Follow-up of tuberculosis cases who fail a standardized course of treatment and who may be at risk of harboring drug resistance organisms.
4. Surveillance of tuberculosis drug resistance as an integral part of the evaluation of control programme performance.
5. Investigation of high risk individuals who are symptomatic, example laboratory workers, health care worker looking after multidrug resistant patient. (Kentor et al, 1998).

2.10.3.1 CULTURE MEDIA

Many different media have been devised for cultivating tubercle bacilli and three main groups can be identified, such as egg-based media, agar-based media and liquid media. The ideal medium for isolation of tubercle bacilli should (a) be economical and simple to prepare from readily available ingredients, (b) inhibit the growth contaminants, (c) support luxuriant growth small number of bacilli, (d) permit preliminary differentiation of isolates on the basic of colony morphology and (e) enable the performance of susceptibility test (Kentor et al, 1998). As no single medium meets all the requirements so if available it is better to use both an egg-based and an agar-based medium for isolation of mycobacterium. But if only one medium has
to be used for the culture of sputum specimens, egg-based media should be the first choice, since they meet all these requirements (Kent and Kubica, 1985).

2.10.3.1.1 EGG-BASED MEDIA

By the beginning of the century, Dorset (1903) introduced egg-media and during the ensuing years many different formulation were proposed. The well known egg-based media are Lowenstein-Jensin media (1930), Ogawa media (1949), American trudae society (ATS) (1962), Stone brink media (1958) etc. (Kentor et al, 1998).

2.10.3.1.1.1 ADVANTAGES OF EGG-BASED MEDIA

- It is easy to prepare.
- It is least expensive of all media available and support good growth of tubercle bacilli
- It may be stored in the refrigerator for several weeks provided it was made from fresh eggs and cultures bottles caps are tightly closed to minimise drying by evaporation.
- Contamination during preparation is limited because it is inespissated after being placed in bottles.
- In addition, the malachite green added to the media suppress the growth of non mycobacterial organisms (Kentor et al, 1998).

2.10.3.1.1.2 DISADVANTAGES OF EGG-BASED MEDIA

- It may take as long as eight weeks before culture becomes positive, specially if specimen contained few bacilli or if decontamination procedure have been overly harsh
- When contamination does occur, it often involves the total surface of the medium and the culture is usually lost.
• Drugs susceptibility test are more difficult to perform on egg-based media because the concentration of certain drugs must be adjusted to account for their loss by hating or by interaction with certain component of the egg (e.g. phospholipids).

2.10.3.1.2 AGAR BASED MEDIUM

Middlebrook 7H – 10 and 7H – 11 are commonly used agar based media for primary isolation and drug susceptibility testing of *Mycobacterium tuberculosis* (Kent and Kubica, 1985). Middle group 7H – 10 may be maid from basic ingredients or may be prepared from commercially available 7H – 10 agar powdered base and Middlebrook oleic acid – albumin – dextrose – catalase (OADC) enrichment. 7H – 11 is a 7H – 10 agar enriched by the addition of enzymatic digest of casein. It is best to prepare 7H – 10 and 7H – 11 medium in small quantities of 200 to 400ml to minimize the amount of heat needed to melt the agar. Boiling the basal medium before autoclaving (either to solubilise the agar or to provide stocks of prepared base that may be stored and boiled for later use) should be avoided because the repeat heating produces medium of inferior quality (Kantor *et al*, 1998).

When Middlebrook 7H – 10 or 7H – 11 medium is used for isolation cultures must be incubated in an atmosphere of 10% CO2. Exposure of Middlebrook 7H – 10 or 7H – 11 agar to either daylight or heat results in the release of formaldehyde in sufficient concentration to inhibit the growth of mycobacteria (Kantor *et al*, 1998).

2.10.3.1.3 INCUBATION OF CULTURES

All cultures should be incubated at 35\(^0\) to 37\(^0\)C until growth is observed or discarded as negative after 8 weeks.
Inoculated media should preferably be incubated in a slanted position for at least 24 hours to ensure even distribution of inoculum. Thereafter, if incubator space is needed, bottles could be placed upright. Tops should be lightened to minimize evaporation and drying of media. The various Middlebrook agars require and atmosphere of 10% CO₂ and 90% air to ensure growth. CO₂ is not essential to initiate growth on egg-based medium but does not stimulate earlier and more luxuriant growth. Agar plates can be placed in impermeable Mylar plastic bags and these charged three times a week with CO₂ (Kantor et al 1998).

2.10.3.1.4 EXAMINATION SCHEDULE

All culture should be examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants. Thereafter, cultures are examined weekly, or if this is not operationally feasible, on at least three occasions,

- After one week to detect rapidly growing mycobacteria which may be mistaken for M. tuberculosis
- After three or four weeks to detect positive culture of M. tuberculosis as well as others slow growing mycobacteria which may be either harmless saprophytes or potential pathogens
- After eight weeks to detect very slow-growing mycobacteria, including M. tuberculosis, before judging the culture to be negative

Should contaminated culture be found during the examination, those where the surface has been completely contaminated or where medium has been liquefied or discoloured should be sterilized and discarded. Certain contaminating organisms produce acid from constituents of the medium and the lowering of pH unbinds some of the malachite green from egg (indicated
by the medium changing to dark green). Tubercle bacilli will not grow under these conditions and cultures should be discarded. Cultures with partial contamination should be retained until the eight week. Late contamination does not exclude the presence of *M. tuberculosis*; it is therefore advisable to prepare a smear from the surface of the medium. Should microscopy indicate the presence of acid-fast bacilli, and attempt could me made to re-contaminate and re-inoculate the culture (Kantor *et al* 1998).
2.10.3.1.5 READING OF CULTURE ON SOLID MEDIUM

Culture procedures for tuberculosis bacteriology are notoriously time consuming, often taking weeks or months to complete. For this reason, interim reports should be issued. The following schedule is recommended.

- If the culture have been contaminated, a report should be sent out immediately and a repaid specimen requested.
- If cultures are positive and growth has been identified as *M. tuberculosis* a report should be sent out immediately.
- At four weeks an interim report optional could be sent out on all negative specimens, staining that another report will be issued in the event of the specimen becoming positive later on.
- At eight weeks a final report should be issued containing all the data previously reported so that earlier interim reports can be destroyed and only the final report retained in the patients file. (Kantor *et al*, 1998)

Culture report should be qualitative (i.e. positive or negative) as well as quantitative (i.e. number of colonies isolated). The average number of colonies on all the bottles/tubes per specimen should be reported. The following scheme is recommended:
### Interpretation of the growth on solid medium (WHO and IUATLD)

<table>
<thead>
<tr>
<th>Colonies</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>No growth</td>
<td>Negative</td>
</tr>
<tr>
<td>&lt; 50 colonies</td>
<td>Count the number of colonies</td>
</tr>
<tr>
<td>50 – 100 colonies</td>
<td>+</td>
</tr>
<tr>
<td>100 – 200 colonies</td>
<td>++</td>
</tr>
<tr>
<td>&gt;200 colonies</td>
<td>+++</td>
</tr>
<tr>
<td>Confluent growth</td>
<td>++++</td>
</tr>
</tbody>
</table>

#### 2.10.3.1.6 LIQUID MEDIA

In general, the use of liquid media system reduces the turn–around time for isolation for acid–fast bacilli to approximately 10 days, compared with 17 days or longer for conventional solid media (Forbes, Sham and Weissfeld, 2002). Numerous formulations have been proposed in the past and a few are still in use. Herman Kirchner liquid medium is the most useful and least expensive of the liquid media for culture tubercle bacilli. It has the additional advantage that it can support a large inoculum. Dubus Oleic Acid – Albumin liquid medium is recommended for the cultivation of tubercle bacilli from cerebrospinal, pleural and peritoneal fluid (Kantor *et al*, 1998). Middlebrook 7H9 and 7H12 are most widely used liquid media for subculturing and preparing inocula for drug susceptibility test. The addition of Tween 80 to the media allows for homogenous growth and dispersal of the clump of mycobacterial growth (Kent and Kubica, 1985). Sula media is routinely used by Damien Foundation in Bangladesh for slide culture and slide DST (Van Deun *et al*, 2008).

#### 2.10.3.1.7 RADIOMETRIC METHOD
BACKTEC 460 TB Method (Beckton Dickinson)

This technique is specific for mycobacterial growth, where $^{14}$C labeled palmitic acid in 7H12 medium is use. This system detect the presence of mycobacteria based on their metabolism rather than visible growth. When the $^{14}$C labeled substance present in the medium is metabolized, $^{14}$CO$_2$ is produced and measured by the BACTEC system instruments and reported in terms of growth index (GI) value (Ramehandran and Paramasivan, 2003). Growth index greater than or equal to 10 are considered positive (Forbes et al, 2002). Growth of mycobacteria may be detected within 5 – 7 days and tubercle bacilli can be differentiated from other mycobacteria within 5 days by using –nitro-a-acetyamino-b-priphenone (NAP) which inhibits the growth of mycobacterium tuberculosis and usually does not effect the growth of MOTT bacilli (Kantor et al, 1998).

Comparative tests have shown that the method is very successful and reliable and that confirmatory results for *M. tuberculosis* can be obtained within two weeks (Ramehandran and Parmasivan, 2003). However the high cost of both the apparatus and the radio-labeled medium and biohazard associated with the disposal of such medium prohibits its routine use in most high tuberculosis prevalence countries (Kantor et al, 1998).

2.10.3.1.8 SEPTI-CHECK AFB METHOD (BECTON DICKINSON)

It is a biphasic culture system made up of a modified middlebrook 7H9 broth with a three sided paddle containing chocolate, egg-based and modified 7H11 solid agars. The bottle is inverted regularly to inoculate the solid media. Growth is detected by observing the three sided paddle (Forbes et al, 2002).

MGIT (BD) and MB Redox (Heipha Diagnostica Biotest, Heidelberg, Germany) to semi automated systems (the BACTEC 460TB system; BD) and fully automated system (e.g., The
BACTEC 9000 MB and BACTEC MGIT 960 (BD); the ESP Culture system II (Trek Diagnostic Systems, Westlake, Ohio); and the MB/BacTALERT 3D system.

The optimum incubation temperature for most cultures is 35 to 37°C. 5 to 10% CO\textsubscript{2} in air stimulates the growth of \textit{Mycobacteria} in primary isolation culture using conventional media. Mycobacterial cultures on solid and in liquid media are generally held for 6 to 8 weeks before being discarded as negative. Specimens with position smears that are culture negative should be held for and additional 4 weeks. \textit{Mycobacteria} are relatively slowly growing organisms, and thus cultures can be examined less frequently than routine bacteriologic cultures. All solid media should be examined within 3 to 5 days after inoculation to permit early detection of rapidly growing mycobacteria and to enable prompt removal of contaminated cultures (Pfyffer, 2007).

2.10.3.1.9 MB/BacT system (Organon Teknika, Belgium)

This is a non-radiometric continuous monitoring system with a computerized database management based on colorimetric detection of CO\textsubscript{2} (Ramehandran and Parmasivan, 2003).

ESP culture system H (Difco-AccuMed International)

This is a fully automated continuous monitoring system based on the detection pressure changes within the headspace above the broth culture medium in a sealed bottle, i.e. either gas production or gas consumption due to microbial growth. A special detection algorithm is present in this system for the detection of very slowly growing mycobacteria (Ramehandran and Parmasivan, 2003).

2.10.3.1.10 MICRO COLONY DETECTION ON SOLID MEDIA

In this method, plates poured with layer of middlebrook 7H11 agar medium are incubated and examined microscopically on alternate days for the first 2 days and less frequently thereafter.
In less than 7 days, microcolonies of slow growing microbacteria such as mycobacterium tuberculosis can be detected. Though this method is less expensive and requires about half the time needed for conventional culture, the recovery of mycobacteria is less efficient and it is labour intensive. (Ramehandran and Parmasivan, 2003).

2.10.4 BIOCHEMICAL TESTS

The final species identification of *M. tuberculosis* is based on characteristics such as slow growth, colony morphology, and biochemical tests. From a practical point of view, most isolates from human disease belong to the species *M. tuberculosis*. An initial identification as *M. tuberculosis* is defined on AFB bacilli from slow growing, non-pigmented colonies that are niacin positive, are inhibited by p-nitrobenzoic acid and display nitratase activity. Additional tests that confirm an isolate as *M. tuberculosis* are susceptibility to pyrazinamide, growth on thiophene carboxylic acid hydrazide, absence of catalase production at 68°C and absence of iron uptake.

**2.10.4.1 NIACIN ACCUMULATION TEST:** Nicotinic acid or niacin is produced by all mycobacteria, but niacin produced by *M. tuberculosis*, *M. simiae* and *M. bovis* BCG excreted and accumulates in the culture medium. Niacin-negative *M. tuberculosis* strains are extremely rare.

**2.10.4.2 GROWTH IN THE PRESENCE OF PARA-NITROBENZOIC ACID:** This compound inhibits the growth of several species in the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* (Leao et al, 2004).

**2.10.4.3 NITRATE REDUCTION TEST:** This test is useful for differentiating *M. tuberculosis*, which gives a positive reaction, from *M. bovis*, which is negative
2.10.4.4 **CATALASE TEST:** Catalase is an intracellular enzyme that transforms hydrogen peroxide to oxygen and water. Characteristically, *M. tuberculosis* gives negative results, as do other species in the *M. tuberculosis* complex.

2.10.4.5 **PYRAZINAMIDASE TEST:** Pyrazinamidase test is useful to differentiate *M. tuberculosis* (positive) from the other species of the *M. tuberculosis* complex (negative), with the exception of *M. canettii*.

2.10.4.6 **GROWTH IN THE PRESENCE OF THIOPHEN-2-CARBOXYLIC ACID HYDRAZIDE:** This test is useful to distinguish *M. tuberculosis*, which grows in the presence of this compound, from other members of the *M. tuberculosis* complex although *M. canettii* and most non-tuberculous mycobacterial species are also positive to this test.

2.10.5 **OTHER DIAGNOSTIC PROCEDURES**

2.10.5.1 **IMMUNODIAGNOSTIC METHODS**

The gold standard for tuberculosis (TB) diagnosis is the demonstration of Mycobacteria but which is often not possible. Though microscopic identification and culture of *Mycobacteria* in sputum are the most common methods for diagnosis of pulmonary disease but in the search for rapid and cost-effective diagnostic methods for TB, Immunodiagnosis is considered an attractive option. There is strong evidence both *in vivo* and *in vitro* of cellular immune reaction in those infected with *M. tuberculosis*. *In vivo*, this reaction can be measured by DTH response to PPD; and in vitro, by the proliferation of lymphocyte to different compounds of the bacteria (Pai *et al*., 2004).

Basically, it uses the specific humoral and cellular immune responses of the host to infer the presence of infection or disease. The tuberculin skin test (TST) or Mantoux test (Huebner 1993) and, more recently, the antigen-specific induction of interferon gamma production have
been used to detect infection with *Mycobacterium tuberculosis* (Pai *et al*, 2004). At the same time, a wide variety of serological tests for the detection of antibodies in individuals suspected to have TB have also been evaluated to detect active disease (Chan, 2000).

Historically speaking, serology for the diagnosis of TB has been explored since 1898, when crude cell preparations containing carbohydrates, lipids, and proteins from *M. tuberculosis* or *M. bovis* BCG were used as antigen preparations showing high sensitivity but low specificity. Modern developments in the purification of antigens, generation of monoclonal antibodies and chromatographic techniques, have led to a considerable improvement in specificity. During the last three decades, a large number of purified (native and recombinant) antigens have been assessed, showing substantial progress in the serodiagnosis of TB (Jackett *et al*, 1988). In order to produce a useful serological tool for TB diagnosis, several antigens must be combined as a cocktail.
2.10.5.2 SEROLOGICAL TESTS

2.10.5.2.1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

In TB patients, the serological response to *Mycobacterial* antigens has been primarily evaluated using standard ELISA with *in house* methodologies and protocols which certainly differ from laboratory to laboratory. Few commercial tests based on the detection of specific antigens, such as the 38 kDa protein, Antigen A-60, have been developed and have been in use, primarily in developing countries (Wilkinson *et al.*, 1997). There is still a need to improve the sensitivity or specificity of commercial serological tests.

2.10.5.2.2 IMMUNOCHROMATOGRAPHY (ICT)

Immunochromatographic assays, also called lateral-flow tests or simply strip tests are a logical extension of the technology used in latex agglutination tests. The benefits of immunochromatographic tests include: • User-friendly format • Very short time to test result • Long-term stability over a wide range of climates • Relatively inexpensive to make. These features make strip tests ideal for applications such as home testing, rapid point-of-care testing, and testing in the field. In addition, they provide reliable testing that might not otherwise be available to low-resource countries (Pfyffer, 2007).

T CELL BASED IMMUNOLOGICAL TESTS

Since *M. tuberculosis* is sometimes difficult to culture from patients with active TB, and impossible to culture from latently infected healthy people, it is therefore vital to have efficient tools for diagnosis of active TB and screening for latent *M. tuberculosis* infection. The only widely used test is the century-old MT, based on the intradermal injection of PPD, a crude mixture of *M. tuberculosis* proteins widely shared among *M. tuberculosis*, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), and most environmental mycobacteria
(Andersen et al, 2000). Hence, false-positive results are common in people exposed to environmental mycobacterial and/or previously vaccinated with BCG (Aagaard et al, 2004).

2.10.5.3 TUBERCULIN SKIN TEST

Tuberculin skin test can be done by two methods

2.10.5.3.1 MANTOUX TEST

Mantoux test has been used to identify patients actively infected with TB, to measure the prevalence of infection in a community, and to select susceptible or high-risk patients for BCG vaccination. The test has been in existence for more than 100 years and has remained more or less unchanged for the last 60 years (Huebner et al, 1993).

TST works by intradermally injecting 0.1 mL of 5 TU PPD on the forearm. On examination, after 48-72 hours, a positive reaction is indicated by erythema and in duration of > 10 mm in size. Erythema (redness) alone is not taken as a positive reaction. All persons with prior infection with tubercle bacilli will mount an immune response to bacilli proteins (Huebner et al, 1993). As the active ingredient used in the skin test contains a whole series of proteins that are shared with the BCG vaccine and other Mycobacteria common in the environment, the skin test is often falsely positive. It is currently estimated that almost one third of people positive to TST do not actually have TB infection. The sensitivity of the skin test is estimated to be around just 70 % in known active TB cases; so the test misses up to 30 % of people who are infected. This sensitivity decreases to as low as 30 % in immunocompromised people.

2.10.5.3.2 HEAF TEST

This test is done with multiple puncture apparatus. Although it is not widely used, it is used in epidemiological survey and as test for immunity before BCG vaccination (Laidlaw, et al 1989).
2.10.5.4 INTERFERON-GAMMA DETERMINATION

One of the most significant developments in the diagnosis of TB in the last hundred years seems to be the assays based on IFN-γ determination based on the principle that T cells of sensitized individuals produce IFN-γ when they re-encounter the antigens of *M. tuberculosis* (Tufariello *et al*, 2003). Recent evaluations showed that IFN-γ assays have advantages over tuberculin skin testing (Lalvani *et al*, 2001).

IFN-γ assays that are now commercially available are: the enzyme-linked immunospot (ELISPOT), T SPOT-TB assay (Oxford Immunotec, Oxford, United Kingdom), the original Quantiferon-TB, and its enhanced version Quantiferon-TB Gold assay.

Quantiferon-TB test measure the release of interferon-gamma in whole blood from human subjects infected with *M. tuberculosis* and *M. bovis* respectively, in response to stimulation by PPD. The IFN-γ secreted by T-cells into the plasma is measured by ELISA to indicate the likelihood of TB infection. Quantiferon-TB test was less affected by BCG vaccination, discriminated responses due to non-tuberculous mycobacteria (Pottumarthy *et al*, 1999).

2.9 TREATMENT

Drug treatment is fundamental for controlling TB, promoting the cure of the patients and breaking the chain of transmission. Anti tuberculosis drug treatment started in 1944 after streptomycin (SM) and para aminosalicylic acid (PAS) were discovered. Combined drug therapy is more effective at curing TB than monotherapy as treatment of active TB with a single drug results in the selection of drug resistant bacilli and different populations of tubercle bacilli shows distinct pattern of susceptibility for anti tuberculosis drugs (Levinson, 2006)
Drugs for treating TB are usually classified as first- and second-line drugs. Traditionally, there are five first-line drugs: Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), Ethambutol (EMB), and Streptomycin (SM). Second-line drugs include the Aminoglycosides, Kanamycin and Amikacin, Capreomycin, PAS, Cycloserine, the Thioamides, Ethionamide and Prothionamide and several Fluoroquinolones such as Moxifloxacin, Levofloxacin and Gatifloxacin.

Drugs used to treat tuberculosis are classified as first-line and second-line gents. First-line essential antituberculous agents are the most effective and are a necessary component of any short course therapeutic regimen. The three drugs in this category are rifampicin, isoniazid, and pyrazinamide. The first-line supplemental agents, which are highly effective and infrequently toxic, include ethambutol and streptomycin, second-line antituberculous druds are clinically much less effective than first-line agents and much more frequently elicit severe reactions. These drugs are rarely used in therapy and than only by caregivers experienced with there use (Wright and Wallance, 2001).

2.9.1 FIRST-LINE DRUGS

**RIFAMPICIN (RIF)**

RIF, first introduced in 1972, is extremely effective against MTB. It is bactericidal against replicating MTB. Because of its high bactericidal action, RIF, along with INH, forms the backbone of short course chemotherapy (Rattan, Kalia and Ahmad, 1998).

**ISONIAZID (INH)**

INH a synthetic isonicotinic acid hydrazide, is an analog of NADH, a critical cofactor in many biochemical reactions needed for mycolic acid cell wall synthesis. Its spectrum is limited and it is selective for MTB complex (Rattan et al, 1998).
ETHAMBUTOL (EMB)

EMB, a synthetic compound with profound antimycobacterial effects, is a first-line anti-MTB drug (Rattan et al, 1998).

PYRAZINAMIDE (PZA)

PZA, a structural analog of nicotinamide, was shown to have considerable anti MTB activity in 1952, but it became an important component of short-course chemotherapy only in the mid-1980s (Rattan et al, 1998).

STREPTOMYCIN (SM)

It was the first clinically effective drug to be available for the treatment of tuberculosis, streptomycin is bactericidal for the tubercle bacillus (Rattan et al, 1998).
2.9.2 SECOND LINE DRUGS

Table 6. Second line (reserve) antituberculosis drugs (WHO, 2003a).

<table>
<thead>
<tr>
<th>Name</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin (Am)</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Capreomycin (Cm)</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Ciprofloxacin (Cx)</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Cycloserine (Cs)</td>
<td>Bacterosatic</td>
</tr>
<tr>
<td>Ethionamide (Et)</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Kanamycin (Km)</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Ofloxacin (O)</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Para-aminosalycilic acid (PAS)</td>
<td>Bacterosatic</td>
</tr>
<tr>
<td>Prothionamide (Pt)</td>
<td>Bactericidal</td>
</tr>
</tbody>
</table>

The current short-course treatment for the complete elimination of active and dormant bacilli involves two phases:

**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.

**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.

2.9.3 TREATMENT REGIMENS
**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.

**DRUG SCHEDULE**

First two months (daily) $4 \times 4$FDC (Rifampicin 150 mg + Isoniazid 75 mg + Pyrazinamide 400 mg + ethambutol 275 mg) for the body wt. 55-70 kg & $4 \times 2$FDC (Rifampicin 150 mg + Isoniazid 75 mg) for next 4 months 3 times weekly.

**CATEGORY II** (relapse, treatment failure or default)

**DRUG SCHEDULE**

First three months (daily) $4 \times 4$FDC (Rifampicin 150 mg + Isoniazid 75 mg + Pyrazinamide 400 mg + ethambutol 275 mg) with injection streptomycin 1 gm (2 months) for the body wt. 55-70 kg & $4 \times 2$FDC (Rifampicin 150 mg + Isoniazid 75 mg) for next four months 3 times weekly.

**CATEGORY III** (new smear-negative pulmonary TB other than in Category I and less severe forms of extrapulmonary TB)

**DRUG SCHEDULE:**

First two month (daily) $4 \times 3$FDC (Rifampicin 150 mg + Isoniazid 75 mg + Pyrazinamide 400 mg) for the body wt. 55-70 kg & $4 \times 2$FDC (Rifampicin 150 mg + Isoniazid 75 mg) for next four months 3 times weekly (National guideline, 2005).
2.9.4 DRUG RESISTANCE

Drug resistance in tuberculosis (TB) is a matter of great concern for TB can be divided as Multi drug resistance TB (MDR-TB) and Extensively drug resistance TB (XDR-TB).

2.9.4.1 NATURAL DRUG RESISTANCE

The natural drug resistance of *M. tuberculosis* is an important obstacle for the treatment and control of TB. This resistance has traditionally been attributed to the unusual multi-layer cell envelope and active multidrug efflux pumps (Rossi et al, 2006).

2.9.4.2 ACQUIRED DRUG RESISTANCE

In other bacterial species, acquired drug resistance is mediated by plasmids or transposons, but in contrast, *M. tuberculosis* acquired drug resistance is caused by mutations in chromosomol genes. The MDR phenotype is caused by sequential accumulation of mutations in different genes involved in resistance to individual drugs, due to inappropriate treatment or poor adherence to treatment (Zhang et al, 2000).

ISONIAZID AND ETHIONAMIDE

Resistance to INH is mostly associated with mutations or deletions in katG; other mutations related with INH resistance occur in the coding region of inhA gene (or its promoter) and kasA (Ramaswamy et al, 2003).

RIFAMPICIN

All clinical isolates of *M. tuberculosis* resistant to RIF show mutations in rpoB, resulting in conformational changes that determine the low affinity of this subunit for RIF and consequently, resistance to the drug (Jin et al, 1988).

PYRAZINAMIDE
It has been postulated that PZA resistance could be due to mutations in an unknown $pncA$ regulatory gene (Cheng et al, 2000).

ETHAMBUTOL

Mutation in the $M. tuberculosis$ embB gene has been shown to be the most frequent and predictive for EMB resistance (Srivastava et al, 2006).

2.10 DRUG INTERACTIONS

In general, when two or more drugs are administered simultaneously to a patient, there is a possibility of drug interaction between them that may result in changes (increase or decrease) of the effective concentration of one or more of the drugs involved. Since the anti tuberculosis treatment itself consists of the administration of two or more drugs it is very important to consider those drug interactions affecting the TB drugs. Few drugs interact to alter the concentration of the antituberculosis drugs (Centers for Disease Control and Prevention, 2003). More frequently, antituberculosis drugs affect the other drugs. Most of the clinically relevant interactions involve the Rifamycin drugs (RIF, Rifapentine and rifabutin).

2.11 ANTITUBERCULOUS DRUGS SUSCEPTIBILITY TESTING

The most important requirement of drug susceptibility testing is the ability to make a clear distinction between susceptible and resistant $M. tuberculosis$ strains. There are a variety of methods, some based on traditional phenotypic strategy and others on newly developed genotypic techniques.

Basically there are two types of susceptibility test, direct method and indirect method.

2.11.1 DIRECT METHOD

In the direct test a set of drug-containing and drug-free media is inoculated directly with concentrated specimen. This test can be performed with smear positive specimens only. The
smear size is adjusted on the basic of the number of the bacilli observed in the smear. The advantage to the direct method over indirect method is that the results are available sooner (within three weeks on agar plats), and better represent the patient’s original bacterial population (Kent and Kubica, 1985).

2.11.2 INDIRECT METHOD

The indirect test, the inoculum is prepared from pure culture and is inoculated in drug containing and drug-free slopes either in egg-based Lowenstein-Jensen medium or agar based 7H11 medium. It takes a longer time (8 – 12 weeks) and the sensitivity pattern might not reflect the true situation of original bacterial population because the inoculum is prepared from selected colonies, not the entire culture. But still the indirect test is more suitable than direct test because the amount of bacilli in inoculum can easily be standardized (Kent and Kubica, 1985).

2.11.3 PHENOTYPIC METHODS

(A) ABSOLUTE CONCENTRATION METHOD

This method uses a standardized inoculum grown on drug free media and media containing graded concentrations of the drug (s) to be tested. Several concentrations of each drug are tested, and resistance is expressed in term of the lowest concentration of the drug that inhibits growth (less than 20 colonies): i.e. minimal inhibitory concentration (MIC). Drug concentrations, and particularly inoculum size, must be carefully standardized with reference to wild type cultures. Variations in oculum size are the major source of erroo in this method (Ramehandran and Paramasivan, 2003b).

(B) PROPORTION METHOD
This method enables a precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of inoculum are plated onto both control (drug-free) and drug-containing media; at least one dilution should yield isolated countable (50-100) colonies. When these numbers are adjusted by multiplying by the dilution of the inoculum used, the total number of viable colonies on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be estimated. The proportion of bacilli resistant to a given drug is then determined (Ramehandran and Paramasivan, 2003b). If growth at the critical concentration of a drug is more than 1%, that isolate is considered critically resistant (Kent and Kubica, 1985).

The proportion method is currently the method of choice for estimating drug resistance and this principle is being applied to the following rapid testing methods:

(i) ACTEC 460 (First and second line)
(ii) MGIT 960
(iii) MB/BacT system: and
(iv) VSP II system (Ramehandran and Paramasivan, 2003b).
2.11.4 RECENTLY DEVELOPED PHENOTYPIC METHODS

(A) SLIDE DRUG SUSCEPTIBILITY TEST (DST)
Smears are made from microscopically FDA positive sputum they are then left to dry, but no fixation is used. This is followed by incubation of smeared slides in liquid medium in strong universal glass bottles for ten days, with and without anti-TB drugs. After incubation, unopened containers are heated for sterilization. Subsequently slides are removed, fixed and stained. Microscopic reading looks for development of microcolonies, comparing drug-free controls and slides incubated with various drugs. Clear colony development in presence of a drug is interpreted as resistance to it.

(B) E – TEST (COMMERCIALLY AVAILABLE AS AB BLODISK)
The E-test is based on determination of drug susceptibility using strips containing gradients of impregnated antibiotics. There are reports about a high rate of false resistance by the method when compared with BACTEC or conventional LJ proportion methods.

(C) MYCOLIC ACID INDEX SUSCEPTIBILITY TESTING
Chromatographic method of drug susceptibility by detection of mycolic acid. Depending on the single and quantification of this procedure, drug susceptibility pattern can be carried out as a rapid method. (Ramehandran and Paramasivan, 2003b).

(D) MICROSCOPIC OBSERVATION DRUG SUSCEPTIBILITY ASSAY: This novel method of microscopic observation of both culture with drugs is used for drug sensitivity testing. It is a relatively inexpensive and fairly rapid drug susceptibility testing method with a high sensitivity and specificity and is suited for disease endemic developing countries (Ramehandran and Paramasivan, 2003b). Cavides et al (2000) reported levels of concordance were 99% for INH and 90% for RIF between MODS and microwell Alamar Blue Assay and
the MODS results were available at a median of 9.5 days. Park et al. (2002) found 100% agreement between MODS and 7H10 proportion method for INH and rifampicin and EMB and streptomycin the percentage of agreement were 70% and 77% respectively at day 11. (Moore et al, 2004) reported 92% agreement for INH and RIF both, that for EMB 85% and SM 87% between MODS and MABA – TEMA (Microplate Alamar Blue Assay – Tetrazolium Microplarte Assay) and the susceptibility detection time was only 10 days in their study.

(E) LUCIFIERASE ASSAY

The Luciferase assay, which can detect drug – resistant organisms in a few days, is a distinct improvement. Luciferase is an enzyme isolated from fireflies that produces flashes of light in the presence of adenosine triphosphate (ATP). If the organism is isolated from the patient is resistant, it will not be damaged by the drug; i.e., it will make a normal amount of ATP, and the Luciferase will produce the normal amount of light. If the organism is sensitive to the drug, less ATP will be made and less light produce (Warren Levinson, 10th ed, 2009).

(F) NITRATE REDUCTASE ASSAY (NRA)

Nitrate Reductase assay (NRA) is a rapid method for detection of antituberculous drug susceptibility develop at the central tuberculosis research institute in Moscow, Russia. 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 nitrate can be easily detected with a specific reagent which produces a colour change. Concordance for susceptibility results were 96.5% for isoniazid and 100% for rifampicin between NRA and BACTEC 460 method (Angeby et al, 2002). Sethi et al, (2004) reported 99% agreement beween NRA and proportion method on L-J media for isoniazid and ethambutol while
complete agreement 100% for rifampicin and streptomycin. The susceptibility result in NRA are available in 7 – 14 days. This method is simple rapid, reliable and highly reproducible. Furthermore it can be done in most laboratories having facility for culture and does not require any expensive reagents for chemicals (Paramasivan, 2004). NRA has all the features to become an important tool for rapid and accurate detection of MRD –TB strength in developing countries (Angeby et al, 2002).

2.12 PREVENTION

TB prevention and control takes two parallel approaches. In the first, people with TB and their contacts are identified and then treated. Identification of infections often involves testing high-risk groups for TB. In the second approach, children are vaccinated to protect them from TB. Unfortunately, no vaccine is available that provides reliable protection for adults. However, in tropical areas where the incidence of atypical mycobacteria is high, exposure to nontuberculous mycobacteria gives some protection against TB (Fine P et al, 2001).

2.12.1 VACCINES OF TUBERCULOSIS

Many countries use BCG vaccine as part of their TB control programs, especially for infants. This was the first vaccine for TB and developed at the Pasteur Institute in France between 1905 and 1921.(Bonah C,2005). However, mass vaccination with BCG did not start until after World War II.(Comstock G, 1994) The protective efficacy of BCG for preventing serious forms of TB (e.g. meningitis) in children is greater than 80%; its protective efficacy for preventing pulmonary TB in adolescents and adults is variable, ranging from 0 to 80% (Bannon M,1999).
In South Africa, the country with the highest prevalence of TB, BCG is given to all children under the age of three. However, the effectiveness of BCG is lower in areas where mycobacteria are less prevalent, therefore BCG is not given to the entire population in these countries. In the USA, for example, BCG vaccine is not recommended except for people who meet specific criteria (CDC, 2003)

- Infants or children with negative skin-test results who are continually exposed to untreated or ineffectively treated patients or will be continually exposed to multidrug-resistant TB.
- Healthcare workers considered on an individual basis in settings in which a high percentage of MDR-TB patients has been found, transmission of MDR-TB is likely, and TB control precautions have been implemented and were not successful.

Several new vaccines to prevent TB infection are being developed. The first recombinant tuberculosis vaccine entered clinical trials in the United States in 2004, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID, 2007). A 2005 study showed that a DNA TB vaccine given with conventional chemotherapy can accelerate the disappearance of bacteria as well as protect against re-infection in mice; it may take four to five years to be available in humans(Ha S, 2005). A very promising TB vaccine, MVA85A, is currently in phase II trials in South Africa by a group led by Oxford University, (Ibanga H et al, 2006) and is based on a genetically modified vaccinia virus. Because of the limitations of current vaccines, researchers and policymakers are promoting new economic models of vaccine development including prizes, tax incentives and advance market commitments. (Barder et al , 2005)
Chapter 3
Materials and Methods
A total of 214 sputum samples were collected from clinically suspected pulmonary tuberculosis cases during the period from July 2007 to June 2008 and examined by microscopy standard culture and slide culture in the department of Microbiology Mymensingh Medical College and reference laboratory of Damien foundation in Netrakona.

3.1 STUDY DESIGN – The study was a cross sectional study.

3.2 PLACE OF STUDY – The study was carried out in the department of Microbiology, Mymensingh Medical College & the reference laboratory of Damien foundation, Netrakona. All the cases were selected from DOT’S corner of Mymensingh Medical College Hospital (MMCH) and DOT’S corner of Netrakona. The protocol of this study was approved by protocol approval committee of the department of Microbiology, Ethical Review Committee of Mymensingh Medical College & also by the ethical review committee of Bangladesh Medical Research Council (BMRC).

3.3 STUDY POPULATION: A total of 214 sputum sample were collected purposively from patients attending at DOT’S corner of Mymensingh Medical College Hospital & DOT’S corner of Netrakona during the study period. In case of cross sectional study sample sizes was calculated by the following formula.

\[ n = \frac{Z^2 pq}{d^2} \times \text{design effect} \]

\[ n \quad = \quad \text{sample size} \]

\[ z \quad = \quad \text{is the normal variate at 5% level with 95% confidence interval} \]

\[ p \quad = \quad \text{expected proportion of event (prevalence or proportion of occurrence)} \]

\[ q \quad = \quad 1 - p \]

\[ d \quad = \quad \text{precision level or error level} \]
Here

\[ P = 0.10 \text{ (case positivity rate in Mymensingh region is 10\% i.e. 0.01)} \]

\[ q = 0.9 \]

\[ z = 1.96 \text{ at 5\% level} \]

\[ d = 0.05 \]

Design effect \( = 1.5 \)

\[ n = \frac{(1.96)^2 \times 0.10 \times 0.90}{(0.05)^2} \times 1.5 \]

\[ = 3.84 \times 0.10 \times 0.90 \times 1.5 \]

\[ = 207 \]

So, according to the proportion of occurrence or case positivity rates in Mymensingh region sample size should be 207. But in our study our sample size was 214 which was justifiable.

3.4 DATA COLLECTION & ANALYSIS: A structured pre-designed data sheet were used to collect relevant data (Appendix – I). Subsequently data were analyzed by computer programme software SPSS.

3.5 INCLUSION CRITERIA

- Cough for more than 3 weeks.
- Evening rise of temperature for at least one month.
- Weight loss and night sweating for one month.
- Patient not responding to conventional antibiotic for suspected infection.

3.6 EXCLUSION CRITERIA
Patient with similar sign & symptoms but diagnosed as having other disease e.g. carcinoma of lungs, atypical pneumonia, pleural effusion.

All diagnosed tuberculosis patients

3.7 LABORATORY PROCEDURE

3.7.1 SAMPLE COLLECTION

Sputum was collected in a robust, leak-proof and clean container by standard procedure. (Kantor et al, 1998). It was then transported to the laboratory. A wet film preparation of sputum were made and examined under microscope. When pus cell were more than 10/HPF then it was considered as sputum. It was then processed for microscopic examination and culture.

3.7.2 MICROSCOPIC EXAMINATION OF SPUTUM.

3.7.2.1 ZIEHL-NEELSEN STAINING

A smear was prepared on a clean and oil free slide. It was then dried in the air and fixed by alcohol and heating. The entire slides were flooded with carbol fuchsin and heated slowly by a Bunsen burner for 5 minutes after steaming. Then the slides were washed by clean water and were decolorized by 25% H₂SO₄ and allowed for 10 minutes. Then it was rinsed thoroughly with clean water and counter stained with Methylene Blue and kept for 1 minute. Slides were washed thoroughly with clean water, dried in air and examined under microscope by oil immersion lens.

3.7.2.2 INTERPRETATION
Microscopic examination were recorded according to WHO & National tuberculosis control programme (Appendix – IV)

3.8 CULTURE

3.8.1 STANDARD CULTURE ON OGAWA MEDIUM

Sputum was taken in a 50ml Falcon tube then equal volume of 4% Sodium hydroxide (NaOH) was added to it and kept for 20 minutes at room temperature. A homogenous mixture of sputum was prepared by vortexing. Everything was done within the safety cabinet. Then 4 drop of homogenous sputum was inoculated into the Ogawa medium. Then the surface of the medium was covered with the inoculums and kept at inclined position overnight at room temperature. It was then incubated at 37°C for eight weeks. The media were checked after two days for contaminations. If contamination occur the process were repeated by the remaining sputum kept in the refrigerator. Then the medium were checked every week for growth.

3.8.2 INTERPRETATION OF GROWTH

Results of the culture were recorded by a standard method (Appendix – V)

3.9 SLIDE CULTURE IN SULA MEDIA

Three Smears were prepared for every specimen on the non-frosted end of the half of a microscopic slide. Two of the prepared slides were used for plain sula medium and one for Para nitrobenzoicacid (PNB). All slides were dried on the sterile paper in the safety cabinet, but fixations were not done. With the help of a sterile forceps slides were placed in the respective sterile 28 ml universal bottle containing 7-8 ml of the appropriate medium. After 2-3 days of incubation all the bottles were checked for contamination, the procedure was repeated for the contamination specimen, using sputum kept in the refrigerator. After ten
days, bottles were removed from the incubator and moved to the safety cabinet. Bottles were opened and slides were removed with the help of a forceps or pincers, dipped them briefly in a universal container with absolute alcohol for fixation and let them dried on a brown paper in the safety cabinet. When the slides were dried they are arranged in a staining dish filled with sufficient 1% carbolfuchsin to immerse the slides completely and kept for 30 minutes. Staining dish was removed from the safety cabinet and Ziehl – Neelsen staining was done as usual. The stained slides were dried in the air and examined under bright field microscope.

3.9.1 READING AND RECORDING

Reading & Recording were done by observing for micro-colonies under microscope using 10X magnification according to standard operative procedure (SOP) (Appendix – VI)
Chapter 4

RESULTS
RESULTS

The present study was conducted with a total of 214 clinically suspected pulmonary tuberculosis respondents of which 201 were from DOT’S corner of Mymensingh Medical College Hospital and 13 were from DOT’S corner of Netrakona.

Age and sex distribution of the study population is shown in the Table 1 (Figure 1). Out of 214 respondents, 172 (80.37%) were male and 42 (19.62%) were female. Majority of the respondents 83 (38.78%) belonged to the age group of 21 – 40 years.

Table 2, shows the occupational distribution of respondents. Most of the respondents were labourer 92 (42.99%), followed by service holder 46 (21.49%), house wife 39 (18.22%), students 19 (8.87%) and garments worker18 (8.41%).

Table 3 (Figure 2), shows the number of positive cases by three different methods. Out of 214 respondents, 46 (21.49%) were Z – N stain positive, 42 (19.62%) were slide culture positive and 50 (23.36%) were standard culture positive.

Table 4, shows the clinical presentation of pulmonary tuberculosis patients. Fever and cough were found in all the 50 (100%) of the patients, followed by weight loss in 46 (92%), weakness in 44 (88%), anorexia in 42 (84%) and night sweating in 41(82%) of the pulmonary tuberculosis patients.
Table – 1. Distribution of suspected cases of tuberculosis according to age and sex (n = 214).

<table>
<thead>
<tr>
<th>Age group</th>
<th>Sex</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>01 - 20 yrs</td>
<td>25 (86.20%)</td>
<td>04(13.79%)</td>
</tr>
<tr>
<td>21 – 40 yrs</td>
<td>65(78.31%)</td>
<td>18(21.68%)</td>
</tr>
<tr>
<td>41 – 60 yrs</td>
<td>52(75.36%)</td>
<td>17(24.63%)</td>
</tr>
<tr>
<td>61 – 80 yrs</td>
<td>30 (90.90%)</td>
<td>03(9.09%)</td>
</tr>
<tr>
<td>Total</td>
<td>172(80.37%)</td>
<td>42(19.82%)</td>
</tr>
<tr>
<td>Ratio</td>
<td>Male: Female = 4:1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Distribution of suspected cases of tuberculosis according to age and sex (n = 214).
Table – 2. Distribution of suspected cases of tuberculosis according to their occupation (n = 214).

<table>
<thead>
<tr>
<th>Occupation</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labourer</td>
<td>92</td>
<td>42.99</td>
</tr>
<tr>
<td>Service</td>
<td>46</td>
<td>21.49</td>
</tr>
<tr>
<td>House wife</td>
<td>39</td>
<td>18.22</td>
</tr>
<tr>
<td>Students</td>
<td>19</td>
<td>8.87</td>
</tr>
<tr>
<td>Working in Garments factory</td>
<td>18</td>
<td>8.4</td>
</tr>
</tbody>
</table>
Table – 3. Number of positive cases by three different methods (n = 214).

<table>
<thead>
<tr>
<th>Methods</th>
<th>Total positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z – N stain</td>
<td>46</td>
<td>21.49</td>
</tr>
<tr>
<td>Slide culture</td>
<td>42</td>
<td>19.62</td>
</tr>
<tr>
<td>Standard culture</td>
<td>50</td>
<td>23.36</td>
</tr>
</tbody>
</table>
Figure 2: Number of positive cases by three different methods

(n = 214).
Table – 4. Clinical presentation of pulmonary tuberculosis patients

(n = 50).

<table>
<thead>
<tr>
<th>Clinical symptoms</th>
<th>Present</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Fever</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Weight loss</td>
<td>46</td>
<td>92</td>
</tr>
<tr>
<td>Weakness</td>
<td>44</td>
<td>88</td>
</tr>
<tr>
<td>Anorexia</td>
<td>42</td>
<td>84</td>
</tr>
<tr>
<td>Night sweating</td>
<td>41</td>
<td>82</td>
</tr>
</tbody>
</table>
Sensitivity and specificity of slide culture is shown in Table 5, when compared with gold standard method. The sensitivity and specificity were 84% and 100% respectively.

Table 6, Shows the sensitivity and specificity of Ziehl – Neelsen (Z – N) stain when compared with gold standard Ogawa culture. The sensitivity and specificity were 82% and 96.95% respectively.

Comparison between standard culture and slide culture among smear positive cases is shown in the Table 7 (Figure 3). Among the 46 smear positive cases 41 (89.13%) were positive on Ogawa medium and 38(82.60%) were positive in sula medium. There is no statistical significant difference between the two methods. (‘p’ is >0.05 by chi-square test)

Table 8, Shows the comparative growth period of mycobacteria in positive cultures on Ogawa medium and sula medium. The mean growth time in sula medium were 8.5 days and in Ogawa medium were 24 days. This difference is statistically significant (‘p’ <0.001 by ‘t’ test).
Table – 5. Sensitivity & specificity of slide culture.

<table>
<thead>
<tr>
<th>Test results</th>
<th>Disease positive (standard culture positive)</th>
<th>Disease negative (standard culture negative)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slide culture positive</td>
<td>42(a)</td>
<td>00(b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide culture negative</td>
<td>08(c)</td>
<td>164(d)</td>
<td>84%</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td>50(a+c)</td>
<td>164(b+d)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Formula for Sensitivity**

\[
\text{Sensitivity} = \frac{\text{True positive (a)}}{\text{True positive (a) + False negative (c)}} \times 100
\]

**Formula for Specificity**

\[
\text{Specificity} = \frac{\text{True negative (d)}}{\text{True negative (d) + False}} \times 100
\]
positive (b)

<table>
<thead>
<tr>
<th>Test results</th>
<th>Disease positive (standard culture positive)</th>
<th>Disease negative (standard culture positive)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-N stain</td>
<td>41 (a)</td>
<td>05 (b)</td>
<td>82%</td>
<td>96.95%</td>
</tr>
<tr>
<td>positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-N stain</td>
<td>09 (c)</td>
<td>159 (d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50 (a+c)</td>
<td>164 (b+d)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table – 7. Comparison between standard culture and slide culture among smear positive cases

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard culture</td>
<td>41</td>
<td>05</td>
<td>46</td>
</tr>
<tr>
<td>Slide culture</td>
<td>38</td>
<td>08</td>
<td>46</td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>13</td>
<td>92</td>
</tr>
</tbody>
</table>

Calculated $\chi^2$ value is 0.76 which is much lower than the table value at 5% level in 1 d.f, i.e. 3.84

So, there is no significant difference between standard culture and slide culture technique.
Figure 3: Comparison between standard culture and slide culture among smear positive cases
Table – 8. Comparison of growth period of Mycobacterium tuberculosis on Ogawa and sula medium

<table>
<thead>
<tr>
<th>Method</th>
<th>Range of detection time in day</th>
<th>Mean detection time in day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ogawa medium</td>
<td>18 – 30</td>
<td>24</td>
</tr>
<tr>
<td>Sula medium</td>
<td>7 – 10</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Calculated value is 10.33 (by independent sample ‘t’ test) which is must greater than tabulated value at 15 d.f. and 5% level i.e. 4.07.

The growth of *M. tuberculosis* in sula media is significantly rapid than in Ogawa medium.
Chapter 5

DISCUSSION
DISCUSSION

Despite all the advances made in the treatment and management, tuberculosis still remains as one of the major public health problem, particularly in the developing countries. Though tuberculosis is a preventable and curable disease, the alarming news is that there has been no decline in the number of new tuberculosis cases (Sethi et al, 2004). Tuberculosis has been a grave health problem in Bangladesh with adverse social and economic consequences. The emergence of drug resistance strain, especially MDR –TB has made the situation even worse. Factors contributing to the recent outbreak and continued spread of MDR –TB include upsurge of HIV infection/ AIDS, insufficient tuberculosis control and laboratory delays in the identification of M. Tuberculosis (Paramasivan and Vankataraman, 2004).

Current recommendation for the control of tuberculosis case detection so as to allow treatment of patients and thereby to limit the transmission of the bacilli (Schluger, 1994). The mainstay for its control is the rapid and accurate identification of the infected individuals. The simplest rapid diagnostic method is the detection of acid-fast bacilli by microscopy. Conventional culture is the gold standard for diagnosis of pulmonary tuberculosis, but it takes several weeks to become positive (Wilkins, 1998). Therefore an alternative, rapid, less expensive and most effective method have been developed (Van Deun et al, 1993).

AFB (Acid fast bacilli) microscopy and Lowenstein-Jensen (L-J) culture remains the cornerstone for the diagnosis of tuberculosis (Vijayshekharan et al, 2006) and has remained the gold standard (Bhatia et al, 2003). Although the diagnosis is dependent on the clinical findings and the radiological evidences but is confirmed by microscopic examination by Z-N staining method, as over 65% of pulmonary tuberculosis (PTB) remains smear positive in Bangladesh (National guide line, 2005).
Cases found by direct microscopy relatively more important than the culture for the purpose of control (Harishur, 1999) which is important in middle income country where primarily identification of bacilli is highly specific for the diagnosis of tuberculosis (Steingert et al, 2006).

There are several methods for isolation of mycobacteria. Methods based on solid media are most commonly used in developing countries. Although less costly, these methods are labour intensive and time consuming. Sophisticated and automated broth based culture methods are rapid but too expensive to be used routinely in TB laboratories of developing countries. Slide culture technique is a liquid based rapid, less expensive, less time consuming and effective method for isolation of \textit{M. tuberculosis} (Salim et al, 2006). It can also be done in poor setting with minimal equipment and does not require reference laboratory. The present study was carried out to evaluate the rapid slide culture technique for the diagnosis of pulmonary tuberculosis.

In the present study, we found majority of the clinically suspected cases 83 (38.78\%) were in the age group of in between 21 – 40 years (Table 1 & Figure 1). A study by Adnan et al from Kwai in 2004 reported that most of the suspected cases were in the 20 – 59 years of age group having very close relationship with present study (Adnan et al, 2004). According to National Guidelines and Operational Manual for Tuberculosis Control, 75\% of cases are in the most economically productive age group (15 – 54) years in the world which was closed to our study.
Among the clinically suspected cases 172 (80.36%) were male and 42 (19.82%) were female and the male to female ratio was 4.09:1 (Table1 & Figure 1). Adnan et al, from Kuwait in 2004, reported (70.91%) were male and (29.08%) were female giving a male to female ration of 2.4:1. This finding differs from our study. Another study by Kame et al, from Alexandria, Egypt in 2003 showed that most of the suspected cases were male than female and the male to female ratio was 1.7:1 (Kame et al, 2003) which is not consistent with our study.

In the present study, fever and cough were present in all the 50 (100%) of tuberculosis patients diagnosed by standard culture method (Table 3 & Figure 2), was followed by weight loss 46 (92%), weakness 44 (88%), anorexia 42 (84%) and night sweating 41 (82%).

In another study by Kame et al, from Alexandria, Egypt in 2003 showed that cough were present (100%) tuberculosis patients, which was similar to our study. They showed weight loss in (83.1%) cases, which was also close to our study. Fevers were present only in (32.5%) cases which differs from our study. Less common symptom in there studies were anorexia, weakness and night sweating, which differs from our study (Kame et al, 2003).

In the present study, the sensitivity and specificity of slide culture were 84% and 100% respectively (Table 5). Slide culture technique was used in very few settings in home and abroad. Few studies by L. sula (1968), Olga Salazar Alegre (1967), Ives and McCormick (1955) and Shitaye et al, (2009) used sula media in slide culture technique but they did not see the sensitivity and specificity of this technique. So, the sensitivity and specificity of slide culture could not be compared with other studies.

Among the smear positive cases 41 (89.13%) were positive in Ogawa medium and 38 (82.60%) were positive in sula medium (Table 7 & Figure 3). In a study by Olga Salazar Alegre from, Lima, Peru, in 1966 showed that the conventional culture positive were
(66.23%) and (66.75%) culture positive were in sula medium and the contamination rates were (5.97%) in Ogawa medium and (5.45%) in sula medium respectively (Salazar Alegre, 1966).

In another study by L. Sula from Czechoslovakia in 1968 showed that the culture positive in sula medium were (30.55%) and in Ogawa medium were (33%) (L. Sula, 1968). These studies did not correlate with our study. These differences were due to the use of different types of digestive and decontaminating reagents. In our study, we used fresh sputum but in the study by L. sula used lyophilized sula medium. The composition of the sula medium used our study was modified.

In the present study, sensitivity and specificity of Ziehl-Neelsen staining were 82% and 96.95% respectively when compared with gold standard culture technique in Ogawa egg based solid medium (Table 6). In a study from India by Deepak Aggarwal et al, in 2008, showed sensitivity and specificity of Z – N staining were 80.51% and 78.26% respectively (Deepak Aggarwal et al, 2008).

In another study by Sajjad et al, from Pakistan in 2003, found 05 false positive cases by Z – N stain when compared with Lowenstein-Jensen medium (Sajjad et al, 2003). From our study it was observed that sensitivity of Z – N staining was almost similar to the present study but specificity was much high. This was due to 05 false positive cases, which was positive by Z – N staining but negative by standard culture. This false positive detection on microscopy may be due to many reasons, as has been reported in other studies (Boy and Marr, 1975). The reasons may probably the organisms (bacteria) were not able to grow on the culture medium because the environment were not ideal for the growth of the tubercle bacilli. It may also be due to the over digestion or decontamination by 4% NaOH. This destructive effect sometimes
involved 95% of all the mycobacteria in all pathological material and in this way, the results obtained with this method may be rather questionable, specially for specimens containing few mycobacteria (L. Sula, 1968).

In another study by Sujatha et al, from India 1991, found sensitivity of Z – N staining were 76.1%, which does not support our study. But our results were remarkably consistent with the findings of Sujaata et al, in 1991, showed the specificity by Z – N staining was 98.4% (Sujaata et al, 1991). In a study by Myma et al, in 1986 from Philippines found sensitivity and specificity of Z – N staining were 50.0% and 99.0% respectively (Myma et al, 1986). In another study by Yvonne et al, in 2009, from Uganda showed the sensitivity and specificity of Z – N staining were 93.1% and 100% respectively (Yvonne et al, 2009). In a study by Guy et al, in 2004, from United Kingdom found the sensitivity and specificity were 52% and 100% respectively (Guy et al, 2004). In another study by Mustafa et al, in 2000, from Turkey found he sensitivity and specificity of Z – N staining were 61% and 100% respectively (Mustafa et al, 2000).

From the above findings it was observed that, some findings were close to and some were remarkably distant from our present study. The differences of results were may be due to the studies conducted at different geographical areas as the disease prevalence differs from region to region. The differences may also be due to the quality of different laboratories.

In the present study, slide culture showed statistically significant faster culture detection time than Ogawa media - 8.5 days versus 20.75 days (p<0.05). Olga Salazar Alegre in 1966 found the growth detection time were 15 days in sula medium. Van Deun et al in 2006 from Paris, France reported that the growth detection time by slide culture on sula media were 10 days.
Analyzing the finding of the present study, we can easily understand that the slide culture technique is a rapid, simple, less expensive and alternative method of egg based solid medium. It can be kept at room temperature for six months and in lyophilized condition it can be preserved for up to two years. It can therefore be used even in the most modest laboratories, which means that in the less developed regions of the developing countries, where the tuberculosis problem is serious, bacteriological culture test could be made for the diagnosis of this disease. On the other hand conventional solid media have certain difficulties since requires a well equipped laboratory and must be prepared at frequent intervals because of its short life (Olga Salazar Alegre, 1966).

Lastly, one of the limitations of the study should be mentioned. Even after extensive and Vigorous search, sufficient numbers of literatures were not available regarding the study of slide culture technique. So, some of the variables of the present study could not be compared with others adequately.
CONCLUSION AND RECOMMENDATION

Analyzing the findings of present study it could be concluded that slide culture may be a suitable system for rapid diagnosis of *M. tuberculo* in resource poor settings requiring minimal equipments & it can be a routine diagnostic procedure. By applying this culture system slide drug sensitivity test (DST) can be done within 10 days. So rapid diagnosis, standard treatment & management of pulmonary TB & MDR TB will be very easier & will save more life.

Study of slide culture is going on routinely, only in Damien foundation of Bangladesh. But training had been given to the people from Benin, Burundi & Cameroon. Those countries have not yet used it in routine and are waiting for a follow-up visit to implement it.

In laboratories of tertiary level hospitals like BSMMU and other well funded laboratories both in government and private sectors, where facilities such as biosafety cabinet, laminar airflow and setup for *M. tuberculosis* culture are available and can afford sula media, slide culture can be an excellent alternative to L-J or acidified egg medium for isolation of *M. tuberculosis* and there susceptibility testing for antitubercular drugs. Two week training may be sufficient for the laboratory personnel to developed skill in detecting microcolonies in slide culture technique.

So it is strongly recommended to take necessary steps for setup and start slide culture and slide DST in the department of Microbiology, Mymensingh Medical College as well as other Medical colleges & also different chest disease hospitals of Bangladesh.


Shitaye, E.J., Beran, V., Svobodova, J., Moravkova, M., Babak, V. and Pavlik, I. 2009, ‘Comparison of the conventional culture, the manual fluorescent MGIT system and the automated fluorescent MGIT 960 culture system for the detection of mycobacterium avium ssp. avium in tissues of naturally infected hens’, Folia Microbiologica, 54 (2), 137 – 141.


Todar, K. 2008, ‘Mycobacterium tuberculosis and Tuberculosis’ in Todars online Textbook of Bacteriology, published online, Available at: www.textbookofbacteriology.net.


WHO, 2004b. Tuberculosis and health sector reforms in Bangladesh. World Health Organization, Regional office for South East Asia, NewDelhi (SEA –TB – 262)

WHO, 2005a. WHO, Bangladesh, TB. (online) Last update 23rf March 2005. available at: 
http://www.whoban.org/communicabledista.html

http://www.who.int/mediacentre/facsheet/fs104/en/


Appendices
APPENDIX – I

DATA SHEET

Data sheet for the suspected cases of pulmonary tuberculosis at DOT’S corners of

Mymensingh Medical College Hospital & Netrakona

A. S.L No: __________ Identification number __________ __________

B. Particulars:

1. Name: __________ 2. Age: __________ 3. Sex: Male/Female

4. Father/Mother/Husbands name: __________

5. Address:

Village: __________ P.O: __________ Union: __________

Thana: __________ Dist: __________

6. Occupation:

Working in

Jute mil __________ Tobacco factory __________ Asbestos factory __________

Rice mill __________ Textile mill __________ Garments factory __________

7. Education:

No education __________ Primary __________ Secondary __________

College __________ University __________

C. Clinical Symptoms/Signs:

1. Cough:

Yes __________ No __________
<table>
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<td>Yes</td>
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<tr>
<td>Colour</td>
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<tr>
<td>2. Fever:</td>
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<tr>
<td>Yes</td>
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<tr>
<td>Nature:</td>
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<tr>
<td>Continuous</td>
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<tr>
<td>Irregular</td>
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<td>3. Weight loss:</td>
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<td>Previous weight</td>
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<td>4. Night sweating:</td>
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<td>Yes</td>
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<td>5. Anorexia:</td>
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<td>Yes</td>
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<td>6. Fatigue:</td>
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<td>Yes</td>
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<td>7. Malaise:</td>
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<td>Yes</td>
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<td>8. Lassitude</td>
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<td>Yes</td>
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</tbody>
</table>
9. Mental status

   Sound   Irritated   Anxious   Relaxed

10. Lymphadenopathy

   Regions of enlarged lymphnode   Duration of enlargement
D. Risk factors:

1. Monthly income of the family
   - [ ] Yes
   - [ ] No

2. Number of people living in the family
   - [ ] Yes
   - [ ] No

3. Working in TB clinic
   - [ ] Yes
   - [ ] No

4. Suspected case of TB in the family
   - [ ] Yes
   - [ ] No

5. Family History of TB
   - [ ] Yes
   - [ ] No

6. Over crowding
   - [ ] Yes
   - [ ] No

7. Episodes of deadly infection in the family
   - [ ] Yes
   - [ ] No

8. Housing condition
   - Katcha
     - [ ] Yes
     - [ ] No
   - Pacca
     - [ ] Yes
     - [ ] No
   - Semi pacca
     - [ ] Yes
     - [ ] No

9. Habit
   - Sound sleep
     - [ ] Yes
     - [ ] No
   - Disturbance of sleep
     - [ ] Yes
     - [ ] No
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<tr>
<th>Condition</th>
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<tr>
<td>Hubble bubble</td>
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<td>Gull</td>
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<td>Alcohol</td>
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<td>10. Travelled to different places</td>
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<td>11. Immigration</td>
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<td>12. Immunosuppression</td>
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<td>Previous history of measles</td>
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<td>History of chronic infection</td>
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<td>Diabetes mellitus</td>
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<td>Malnutrition</td>
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</tbody>
</table>
AIDS

Yes  No

Malignancy

Yes  No

Use of Steroid

Yes  No

Use of anti cancer drugs

Yes  No

Intravenous drugs users

Yes  No

E. Others

1. Marital status

Married

Unmarried

2. Spending on food

Taka per meal:  
APPENDIX – II

POSITIVE CASES BY THREE DIFFERENT METHODS

<table>
<thead>
<tr>
<th>Case No</th>
<th>Growth on standard Culture</th>
<th>Growth on slide Culture</th>
<th>Result of AFB staining</th>
<th>Comments</th>
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These 36 cases were positive in all three methods.
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<th>Case No</th>
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<th>Result of AFB staining</th>
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<td>125</td>
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These 6 cases were positive by both standard culture & slide culture but negative in AFB staining.

These 5 cases were positive by both standard culture & AFB staining but negative in slide culture.

These 3 cases were positive in only standard culture but negative in slide culture and AFB staining.

These 5 cases were positive by only AFB staining but negative by both standard and slide culture.
APPENDIX – III

SAMPLE COLLECTION:

CONTAINER

Sputum was collected in a robust, leak-proof and clean container. Container must be rigid to avoid crushing in transit and must possess a water tight wide mouthed screw top to prevent leakage and contamination (Kantor et al, 1998).

SPUTUM SPECIMENS

Although M. tuberculosis is capable of causing disease in almost any organ of the body, more than 85% of tuberculosis disease in high prevalence countries is pulmonary. Sputum is the specimen choice in the diagnosis of pulmonary tuberculosis. A good sputum specimen consist of recently discharged material from the bronchial tree, with minimum amount of oral or nasal material. Satisfactory quality implies the presence of mucoid or mucopurulent material and is of greater significance than volume. Ideally, a sputum specimen have a volume of 3 – 5 ml, although smaller quantities are acceptable if the quality is satisfactory.

It is best to obtained a sputum specimen early in the morning before the patient has eaten since food or taken medication (which may interfere with the growth of tubercle bacilli or particles in smears make them difficult to examine). If sputum specimens are collected for diagnostic purposes, specimens were collected before starting tuberculosis chemotherapy (Kantor et al, 1998).

Because tuberculosis lesions in the lungs may drain intermittently, it is possible for a specimen to be negative on one day and positive the next. For this region three specimens were collected for diagnosis as follows.

- One spot specimen when the patient first present to the health service
- One early morning specimen (preferably the next day)
- One spot specimen when the early morning specimen is submitted for examination.

These were sent a laboratory as single specimen. For follow up of treatment at regular intervals and to determine outcome at the end of treatment, one specimen should be collected.

**SPUTUM COLLECTION PROCEDURE**

The patients were given confidence by explaining to him/her the reason for sputum collection. The patients were instructed to rinse his/her mouth with water before producing the specimen. This will help to remove food and any contaminating bacteria in the mouth. The patients were take two deep breaths, holding the breath for a few seconds after each inhalation and then exhaling slowly. Patients were breath in a third time and then forcefully blow the air out. Patients were breath in again and then cough. This process produces a specimen from deep in the lungs. The patients were asked to hold the sputum container close to the lips and to spit in to it gently after a productive cough. Specimens were transported o the laboratory as soon as possible after collection. If a delay is unavoidable the specimens were refrigerated or kept in as cool a place as to inhibit the growth of unwanted microorganism.
APPENDIX – IV

ZIEHL – NEELSEN METHOD

REAGENTS

ZIEHL-NEELSEN CARBOL FUCHSIN STAIN (1% fuchsin, 5% phenol)

Basic fuchsin                                      5 gm
Phenol (Carbolic acid)                      25 gm
Alcohol (95% or absolute)       50 ml
Distilled water                   425 ml

Dissolve the fuchsin in phenol by placing them in a litre of flask over a boiling water bath for about 5 minutes, shaking the contents from time to time. When there is complete solution alcohol is added and mixed thoroughly.

DECOLOURISING AGENT (25% sulphuric acid)

Water                          750ml
Concentrated sulphuric acid  250 ml

The acid should be poured slowly down the side of the flask into the water about 50ml at a time.

COUNTER STAIN (Methylene blue, 0.1%)

Methylene blue chloride - 100mg
Distilled water - 100ml
Methylene blue were mixed in distilled water and store in a amber coloured bottle after labeling with name of the reagent and dates of preparation and expirary. It can be stored at room temperature for 6 – 12 months.

**INTERPRETATION OF ZIEHL – NEELSEN STAIN**

Microscopic examination were recorded according to WHO & National tuberculosis control programme (WHO and IUATLD)

<table>
<thead>
<tr>
<th>AFB</th>
<th>RESULT</th>
<th>FIELDS TO BE EXAMINED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/100Field</td>
<td>Negative</td>
<td>100 fields</td>
</tr>
<tr>
<td>1-9/100 Field</td>
<td>Scanty</td>
<td>200 fields</td>
</tr>
<tr>
<td>10-99/100 Field</td>
<td>1+</td>
<td>100 fields</td>
</tr>
<tr>
<td>1-10/ Field</td>
<td>2+</td>
<td>20 fields</td>
</tr>
<tr>
<td>&gt;10/ Field</td>
<td>3+</td>
<td>20 fields</td>
</tr>
</tbody>
</table>
APPENDIX -V

PREPERATION OF OGAWA MEDIA

INGREDIENTS FOR 2400ML

Potassium di-hydrogen phosphate (KH$_2$PO$_4$) = 16gm
Magnesium citrate = 0.8gm
Sodium glutamate = 04gm
2% Malachite green =32ml
Glycerin (86 – 88%) =32ml
Distilled water = 800ml

Then all above mixture were autoclaved and cooled it at room temperature. Now 1600ml of homogenized eggs were added with this mixture.

HOMOGENIZATION OF WHOLE EGG

Fresh hen’s eggs not more than 7 days old are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. The eggs were soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soap them in absolute alcohol for 30 minutes. The eggs were cracked with a sterile probe in to a sterile scott bottles containing glass bids and homogenize by hand shaking.

PREPARATION OF COMPLETE MEDIUM

The mixture of homogenized eggs and autoclaved ingredients were taken in several scott bottles. With the help of a burret 7-8ml of solution were taken in the universal bottles and the tops were securely closed.
COAGULATION OF COMPLETE MEDIUM

Before loading inspissator were heated to 85°C to quicken the build-up of temperature bottles were placed in a slanted position in the inspissator and coagulate the medium for 1 hour. The quality if the egg media deteriorates when coagulation is done at 2 high a temperature or for too long. Discoloration of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures. Poor quality media should be discarded (Van Deun, et al, 1999).

INTERPRETATION OF GROWTH ON OGAWA MEDIUM (Van Deun, et al, 1999)

<table>
<thead>
<tr>
<th>No growth</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 50 colonies</td>
<td>Counting the number of colonies</td>
</tr>
<tr>
<td>50 – 100 colonies</td>
<td>+</td>
</tr>
<tr>
<td>100 – 200 colonies</td>
<td>++</td>
</tr>
<tr>
<td>200 – 500 colonies</td>
<td>+++</td>
</tr>
<tr>
<td>Confluent growth</td>
<td>++++</td>
</tr>
</tbody>
</table>
APPENDIX – VI

PREPARATION OF SULA MEDIA

INGREDIENTS FOR 400ML

Potassium di-hydrogen phosphate (KH$_2$PO$_4$) = 0.54gm
Sodium citrate = 0.54gm
Sodium hydrogen phosphate (Na$_2$PO$_4$) = 0.9gm
Magnesium sulphate (MgSO$_4$) = 0.225gm
Triptone soya = 1.8gm
Ferric (F) Ammonium citrate = 0.02gm

Then all the above mixtures were dissolved in 360ml of distilled water and 9ml of glycerol and 2ml of malachite green (0.2%) were added to it. Then all the mixture were autoclaved and 40ml of the goat serum added to it.

READING AND RECORDING OF SLIDE CULTURE

Reading & Recording were done by looking for micro-colonies under microscope on 10X magnification according to standard operative procedure (SOP, WHO, 2008).

- No colonies or single AFB only – negative
- Small colonies only, like clumps of few AFB – 1+
- Larger colonies, can be elongated but no serpentine cording is seen – 2+
- Larger colonies with some serpentine cording – 3+
- Large micro colonies with clear cording – 4+
HOMOGENIZATION AND DECONTAMINATION OF SPUTUM

The majority of clinical specimens submitted to the tuberculosis culture laboratory are contaminated to varying degrees by more rapidly growing normal flora organisms. These would rapidly overgrow the entire surface of the medium and digest it before the tubercle bacilli start to grow. Most specimens must, therefore, be subjected to a harsh digestion and decontamination procedure that liquefies the organic debris to free the bacilli from the mucus, cell or tissue in which they may be embedded and eliminates the unwanted normal flora. All currently available digesting/ decontamination agent are to some extent toxic to tubercle bacilli; therefore, to ensure the survival of the maximum number of bacilli in the specimen, the digestion/ decontamination procedure must be precisely followed. In order for enough tubercle bacilli to survive to give a confirmatory diagnosis, it is inevitable that a proportion of cultures will be contaminated by other organisms. As a general role, a contamination rate of 2% to 3% is acceptable in laboratories that receive fresh specimens; if specimens specially sputum takes several days to reach the laboratory then losses due to contamination may be as high as 5% to 10%. It is also important to note that a laboratory which experiences no contamination is probably using a method that kills too many of the tubercle bacilli.

Many different methods of homogenization and decontamination of sputum specimens for culturing have been described but there is no universally recognized best technique. Since sputum specimen are the most common clinical specimens submitted for tuberculosis culture, homogenization and decontamination procedures have been largely targeted towards there processing. While processing sputum specimens should not be polluted because of the risk of
cross-contamination and the whole specimens should be digested/decontaminated rather than attempt to select portion of the specimen as is done for direct microscopy. Specimens other than sputum demand even more care during processing because of the low numbers of tubercle bacilli present in positive specimens. (Kantor et al, 1998)

DIGESTION AND DECONTAMINATION PROCEDURES OF SPUTUM

PETROFF’S SODIUM HYDROXIDE (NAOH) METHOD

This method is used widely in developing countries because of its relative simplicity and the fact that the reagents are easy to obtain. NaOH is both a digestant and decontaminant and its most effective mucolytic activity occurs at a concentration of 4%. However this concentration is toxic not only for contaminants but also for tubercle bacilli; therefore, strict adherence to the indicated timing is crucial for the effective recovery of mycobacteria. Any way, this method is quite harsh, killing 60% to 70% Mycobacteria in this specimen (Kemnt and Kubica, 1985).
APPENDIX – VIII

PREPARATION OF PARA NITROBENZOIC (PNB) SOLUTION

(Concentration used in Sula plain medium: 500 µg/mL)

1. 0.5gm of PNB were dissolved in 10mL of Dimethylsulfoxid (DMSO)
2. Then mixing of the solution were done
3. This is a 50,000 µg/mL solution and will be store for later purpose.
4. Then 100 mL of the Sula plain medium were added to 1000 µl of the 50,000 µg/mL solution
STATISTICAL FORMULA

FORMULA FOR MEAN

\[ \bar{x} = \frac{\sum x}{n} \]

\( \bar{x} \) = mean of observations
\( x \) = individual observations
\( n \) = number of observation

Formula for Standard Deviation

\[ SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \]

\( SD \) = Standard deviation
\( x \) = Individual observation of a series
\( \bar{x} \) = mean of observations
\( n \) = number of observation

(n-1) = applicable for Sample
FORMULA FOR SENSITIVITY

\[
\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100
\]

FORMULA FOR SPECIFICITY

\[
\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \times 100
\]

STUDENTS T – TEST

\[
\text{STUDENTS T – TEST} = \frac{m^1 - m^2}{\sqrt{SE_1^2 + SE_2^2}}
\]

\[
\text{SE} = \sqrt{\sum d^2 \over n (n - 1)}
\]

FORMULA FOR CHI-SQUARE TEST (\(\chi^2\))

\[
\chi^2 = \sum \frac{(O - E)^2}{E}
\]

Here,

O = Observed frequencies

E = Expected frequencies

df = Degrees of freedom \((c - 1)(r - 1)\)

\(\Sigma\) = Summation