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Role of Histopathology, Cytopathology and Bleach Sedimentation Technique in the 'Diagnosis of Tuberculosis

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University of Rajshahi

ROLE OF HISTOPATHOLOGY, CYTOPATHOLOGY AND BLEACH SEDIMENTATION TECHNIQUE IN THE DIAGNOSIS OF TUBERCULOSIS

THESIS SUBMITTED FOR THE DEGREE
OF
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DECLARATION

I, hereby, declare that, the research work submitted as a dissertation entitled "ROLE OF HISTOPATHOLOGY, CYTOPATHOLOGY AND BLEACH SEDIMENTATION TECHNIQUE IN THE DIAGNOSIS OF TUBERCULOSIS" submitted to the Institute of Biological Sciences, University of Rajshahi, Rajshahi, Bangladesh for the degree of Master of Philosophy (M. Phil) in Pathology has been carried out by me under the supervision of Dr. Parvez Hassan, Professor, Institute of Biological Sciences, University of Rajshahi, Rajshahi, Bangladesh and Dr. Md. Shamim Farooq, Assistant Professor, Department of Pathology, Khwaja Yunus Ali Medical College & Hospital, Sirajgonj, Bangladesh.

I further declare that this dissertation has not been the basis for the award of any degree or diploma of any other similar title.

.....

April 2012

(Md. Mahbur Rashid Sarker)
Signature of the candidate

CERTIFICATE

This is to certify that Md. Mahbur Rashid Sarker is the sole author of the dissertation entitled "ROLE OF HISTOPATHOLOGY, CYTOPATHOLOGY AND BLEACH SEDIMENTATION TECHNIQUE IN THE DIAGNOSIS OF TUBERCULOSIS". This dissertation has not been previously submitted for the award of any degree or diploma of any other similar title.

We are forwarding this dissertation to be examined for the degree of Master of Philosophy (M. Phil) in Pathology to the Institute of Biological Sciences, University of Rajshahi, Bangladesh. Md. Mahbur Rashid Sarker has fulfilled all the requirements according to the rules of the University for Submission of a dissertation for the M. Phil (Pathology) degree.

His work is authentic and up to our full satisfaction.

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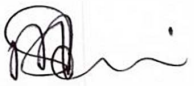

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TO

MY PARENTS & TEACHERS

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The Author

ABSTRACT

Background:

Tuberculosis remains a major threat to world health. About one third of the world's populations are suffering from this dreadful disease. Approximately 8 to 10 million new cases of tuberculosis occur and about 3 million people die annually from this disease. The incidence of TB continues to rise especially in developing countries including Bangladesh where more than 90% of the global TB cases and deaths occur. After HIV; tuberculosis is the leading infectious causes of death in the world.

Laboratory procedure is essential for diagnosis as well as for treatment of tuberculosis. Microscopy of smears made directly from sputum for detection of AFB is the most commonly used method for diagnosis of tuberculosis. However, microscopy of smears made directly from sputum has a low sensitivity and requires the presence of 5000-10000 organisms per ml. Thus, reliance on smear microscopy may cause missed or delayed tuberculosis diagnosis, potentially increasing morbidity, mortality and tuberculosis transmission. Increasing the sensitivity of tuberculosis diagnostic testing is a public health priority and there is an urgent need for improved methods.

We have compared microscopy of smear made directly from sputum with microscopy of sputum made after liquefaction and concentration of sputum with household bleach.

Diagnosis of tuberculosis is often difficult to diagnose specially when no acid-fast bacilli (AFB) seen in sputum and no pulmonary lesion seen radiologically. In such situations, Histopathological or cytopathological examination is helpful for diagnosis of tuberculosis. These methods are reliable and reasonably safe and have increased value in terms of diagnostic accuracy, sensitivity and specificity.

Objectives:

To evaluate the performance and feasibility of tuberculosis diagnosis by sputum microscopy after bleach sedimentation compared with by conventional direct smear microscopy and to evaluate/verify the value of Histopathological and Cytopathological examination in the diagnosis of tuberculosis among the study patients /cases.

Study population:

A total of one hundred fifteen (115) clinically suspected tuberculosis patients aged between 7-85 years attending the indoor and outdoor departments of Khwaja Yunus Ali Medical College Hospital (KYAMCH), Sirajgonj were included in the study. Among them 64 were male and 51 were female.

Materials and Methods:

A total 115 clinically suspected tuberculosis patients were selected for taking history and examination. After taking verbal and written consent, sputum and specimens for Histopathological / Cytopathological examination were collected. These studies were done in the laboratory of Khwaja Yunus Ali Medical College Hospital (KYAMCH), Sirajgonj, during the period July 2009 to June 2011.

Results:

Sputum specimens were collected from 115 clinically suspected tuberculosis patients and processed for detection of AFB by direct smear microscopy and bleach sedimentation microscopy. Among them 35 (30.4%) patients were found to be TB positive by routine (Direct microscopy) method; where as, by bleach concentration method 42 (36.5%) were found positive; diagnosing 07 additional patients. The rise of 16.7% in sputum positivity by bleach sedimentation microscopy over the direct smear microscopy was found to be statistically significant ($p = <0.005$).

On the other hand, by Histopathological & cytopathological examination 73 (63.5%) cases were found tuberculosis positive indicating detection of 31 additional positive cases which were missed by direct and bleach method. These were performed by simple fine needle aspiration (e.g. from lymph node) or excision followed by examination of the stained slide and so we can diagnose tuberculosis patients by these methods without any special laboratory settings.

Majority of the patients of the study group presented with the features of cough, low-grade evening rise of temperature, lymph node swelling, weight loss and occasional bloody cough. The highest prevalence of tuberculosis was noticed among patients belonging to the age group of 20- 40 years and the prevalence of tuberculosis was higher among males than the females. The results of socio-economic demography of the present study revealed that the incidence rate of tuberculosis was higher among the poor and middle class.

Paired samples *t* test analysis of sputum between Direct and Bleach method indicated for the 115 subjects showed that the mean score on the bleach method (M==1.273) was significantly greater at the $p < 0.001$ level than mean score on the direct method. These results also indicates that a significant correlation exists between this two methods ($r=0.896$, $p < 0.001$).

Conclusion:

The results of the present study show that after processing of sputum by bleach sedimentation technique followed by staining with Ziehl-Neelsen (ZN) stain, 07 additional cases were found to be positive which were missed by direct microscopic method. This study suggests that digestion and liquefaction of sputum with bleach and concentration by centrifugation increases the sensitivity of direct microscopy with improved laboratory safety.

Sodium hypochlorite (bleach) is easily available, cheaper and it kills *Mycobacterium tuberculosis*, making the handling of specimens safer for the laboratory staff. This method is simple and can be used in all peripheral laboratories across the country.

So this undertaken study will be helpful for diagnosis of both pulmonary and extra-pulmonary tuberculosis either sputum positive or negative.

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AIDS	Acquired immunodeficiency syndrome
BCG	Bacilli calmette Guerin
DGHS	Directorate General of Health Services
DOTS	Directly observed therapy
FNA	Fine needle aspiration
HIV	Human Immunodeficiency Virus
M	Mycobacterium
MDR	Multidrug resistant
MoHFW	Ministry of Health and Family Welfare
MT	Mantoux test
NaOCl	Sodium hypochlorite
NIDCH	National Institute of Diseases of Chest and Hospital
NTP	National Tuberculosis Control Programme
PCR	Polymerase chain reaction
TB	Tuberculosis
WHO	World Health Organization
ZN	Ziehl-Neelsen

CHAPTER-1

INTRODUCTION

1. INTRODUCTION

Mycobacterium tuberculosis causes Tuberculosis, which is a worldwide problem especially in developing countries (Vinay *et al* 2010). Tuberculosis remains as a major cause of morbidity and mortality globally (Warren Levinson 2007). Franco *et al* (2001) mentioned *Mycobacterium tuberculosis* as the leading cause of death than any other single microbial agent. Approximately one-third of the world's population infected with this organism (Vinay *et al* 2010)). World Health Organization (WHO) declared TB as a Global Health Emergency in 1993.

In spite of discovery of the causative agent of Tuberculosis in 1982 by Robert Koch and adoption of National tuberculosis control programs, the incidence of TB cases is not only increasing but is appearing with new ferocity with Drug Resistant (DR) strain even with Multi Drug Resistant as well as Extreme Drug Resistant (EDR) strain which is virtually incurable. The problem is aggravated by high density of population, rapid uncontrolled urbanization, poverty, malnutrition, illiteracy and HIV infection. WHO (2005) reported that all of these factors are prevailing in Bangladesh except low incidence of HIV. Each year, nearly one percent of the world's population is newly infected with TB and 5 to 10% of them become sick or infectious at some time during their life (Hossain *et al* 2007). Currently about 3 million people die of tuberculosis and 8 to 10 million new cases occur annually (Vinay *et al* 2010). More than 90% of global TB cases and death occur in the developing countries. After HIV, tuberculosis is the leading infectious causes of death in the world (WHO 2007).

In South-East Asian region total number of tuberculosis cases notified to the WHO was 1,380,341 (42 % of global notified cases) in 1995 of which 42,610 cases (about 3 %) in Bangladesh; 1,214,876 cases (88 %) in India, 19,804 cases in Nepal and 45,428 cases in Thailand.

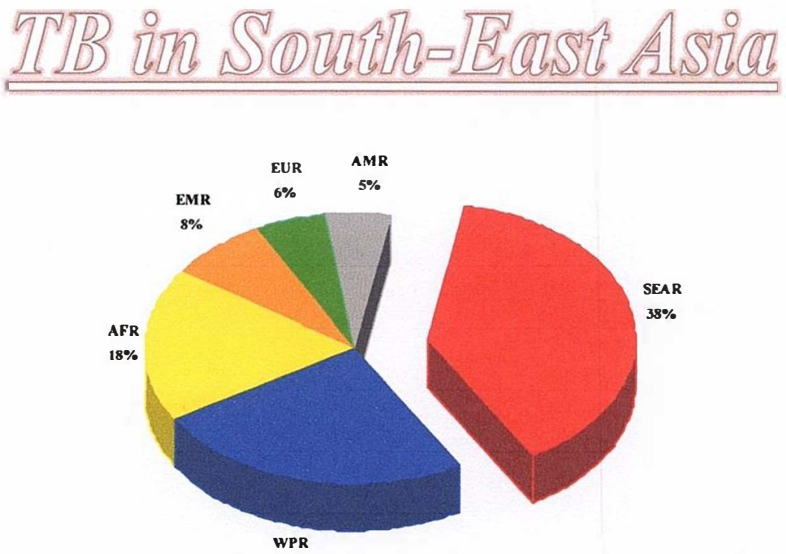


Figure 1. Pie diagram showing[25] TB in South-East Asian region in which India, Bangladesh, Indonesia, Myanmar & Thailand contribute 95% of regional burden.

In Bangladesh, TB remains a major public health problem and major cause of morbidity and mortality. The country ranks sixth in terms of burden of TB, having an estimated 300,000 new cases and 70,000 deaths annually (Hossain *et al* 2007). The WHO estimated that in 2005, there were approximately 576000 TB cases in the country. The number of new cases occurring in 2005 was estimated at approximately 322000. Of these, approximately 145000 were infectious cases transmitting TB in the community. WHO further estimated that about 67000 TB patients, most of not registered, had died of tuberculosis in 2005 (WHO 2007 and Fourth NTP 2007).

Mycobacterium tuberculosis is transmitted from person to person by respiratory aerosol and may occur when pathologists or other laboratory personnel handle infected specimens. The most important source of infection is an undiagnosed person with cavitary and sputum smear positive tuberculosis (Ban *et al* 2012). Infection may also occur after inhaling viable organisms (Haas and Des Prez 1994). After infection, cavitary lesion forms in the lung, transmits the organism through coughing, and causes metastasis

to other organs of the body such as lymph nodes, gastrointestinal system, urinary system etc (Ban *et al* 2012).

Laboratory procedure is essential for diagnosis as well as for treatment of Tuberculosis. The main sure criterion for definitive diagnosis of TB is the demonstration of tubercle bacilli in clinical specimens. The microscopic examination of acid-fast bacilli (AFB) remains the main tool for tuberculosis diagnosis (Aber *et al* 1980, Huebner *et al* 1993). Previous studies showed that the technique sensitivity might vary depending on smearing, staining and smear reading (Corper and Nelson 1949, Kubica 1980). A large number of bacilli, 5,000 to 10,000 per ml of sputum are required to find smear positive for AFB (David 1985, Nicholas *et al* 2006). For proper smearing, N-acetyl-L-cysteine (CH_3-CO-NH-CH-COOH-CH_2SH) with 2% sodium hydroxide (NaOH) is considered better. In these countries, limited resources allow only direct microscopy (un-concentrated sputum) as the available option for tuberculosis diagnosis (Corper and Nelson 1949, Kent and Kubica 1985). In recent years, interest is improving the direct smear microscopy technique in developing countries has arisen (Habeenzu *et al* 1998, Miorner *et al* 1996). Digestion of sputum with sodium hypochlorite (NaOCL, 5.025%) give the best recovery of AFB (Corper and Nelson 1949) and concentration of bacilli by centrifugation of sputum increased the recovery rate of mycobacteria (Ratnam and March 1986). After treatment with NaOCl, it might be attributable to changes in surface properties of mycobacteria and for denaturing of sputum constituents leading to flocculation and subsequent increased sedimentation rate of mycobacteria (Gebre *et al* 1995).

Gebre *et al* (1995) also reported that NaOCl not only increased the sensitivity of sputum but also acts as a potent disinfectant, thus eliminates the risk of transmitting infection especially in laboratories with inadequate safe standards. It is also easily available, inexpensive and requires 20-30 minutes to perform the procedure. Disadvantage of the method is inability of the organism to grow on culture media as they are killed after processing in this method.

Petroffs & N-acetyl L-cysteine-NaOH methods have several disadvantages such as the reagent is expensive, long time needed to process a single specimen and may kill up to 60% of tubercle bacilli in clinical specimens (Gebre *et al* 1995).

Miorner *et al* (1996) also demonstrated that the simple liquefaction and over night sedimentation of sputum with sodium hypochlorite (NaOCl) would significantly augment smear sensitivity up to 70%. So in countries like Bangladesh where the prevalence rate of tuberculosis is high and sputum stained smear for AFB is the major epidemiological tool in the diagnosis of pulmonary tuberculosis, the Bleach sedimentation method may be a valuable tool for detection of acid-fast bacilli.

Other methods for diagnosis of tuberculosis include culture, tuberculin test, chest X-ray, serology, PCR and histological examination etc. Culture techniques are complex and time consuming that requires 4-6 weeks to yield growth of *M. tuberculosis*. Haematological feature, e.g., ESR is raised in tuberculosis but it also increases in multiple myeloma, arthritis and malignancy etc. Mantoux test (MT) is a well-established and widely used test for determining infection with tubercle bacilli. But it may give false negative result in AIDS, immunosuppresion, IV-drug users, malignancy etc. that could not be sufficient for diagnosis.

Molecular diagnostic methods such as Hybridization probe and Polymerase Chain Reaction (PCR) is highly sensitive, 88 to 100% (Schulger *et al* 1994) and will confirm the organism. However, since these methods are costly and time consuming and requires special equipment, it is not applicable in the vast majority of the TB laboratories in the developing countries (Angeby *et al* 2000). This technique may also give false positive results due to contamination with DNA fragments from previous PCRs debris from nonviable bacilli (Noordhock *et al* 1994).

Radiological examination (Chest X-ray) can be useful in the preliminary diagnosis of tuberculosis. However, chest lesions identified by radiograph is not specific and cannot identify the causal agent. Other chests conditions may be accompanied by radiographic abnormalities misleading as tuberculosis and may be improperly treated for tuberculosis (Faulds and O'Brien 1998).

Histopathological and cytopathological examination is an important method for diagnosis of tuberculosis. By this method we can diagnose both pulmonary and extra-pulmonary tuberculosis. In order of frequency, the extra-pulmonary sites most commonly involved in tuberculosis are the lymph nodes, pleura, genitourinary tract, bones, joints, meninges, peritoneum, and pericardium. However, virtually all organ systems may be affected. As a result of haematogenous dissemination in HIV-infected individuals, extra-pulmonary tuberculosis is seen more commonly today than in the past.

The most common presentation of extra-pulmonary tuberculosis (>40% of cases), lymph-node disease is particularly frequent. Once caused mainly by *M. bovis*, tuberculous lymphadenitis is today due largely to *M. tuberculosis* (Ban *et al* 2012). The diagnosis is established only by 'Histopathological and cytopathological' examination from fine-needle aspiration or surgical biopsy specimen. This examination shows granulomatous lesions.

Tuberculosis was the problem in the past, is the problem now, and will be further aggravated in the future if immediate attention is not paid. An essential component of any tuberculosis control program is rapid and accurate identification of new tuberculosis cases. New diagnostic techniques are urgently needed to replace or facilitate microscopy, especially in low-income countries where the disease is endemic and the incidence is high.

Sensitive diagnostic methods including amplification techniques e.g. the polymerase chain reaction (PCR) have been developed, but they are too sophisticated and expensive for routine use in developing countries, where most of the tuberculosis cases occur and where the need for new diagnostic tools is greatest. We need to develop simple, affordable but sensitive diagnostic tools for the detection of tuberculosis, suitable for use in the poorer parts of the world.

Therefore, the present study has been designed to evaluate 'Histopathological and cytopathological' diagnosis of both pulmonary and extra-pulmonary tuberculosis and compare the sensitivity of smear microscopy in NaOCl treated sputum with direct microscopy method.

CHAPTER-2

HYPOTHESIS, AIMS AND OBJECTIVES

2 RATIONAL, HYPOTHESIS, AIM AND OBJECTIVES

2.1. RATIONALE:

Extra-pulmonary tuberculosis continues to be a major health problem in developing countries. Lymphadenopathy is the most common form of extra-pulmonary tuberculosis. In India and South Asian countries in general outpatients 10-20% of new tuberculosis (TB) cases may be extra-pulmonary, while among HIV positive it could be up to 50% (Lalitkant 2004).

The clinical parameters for the diagnosis of TB in lymph nodes are neither specific nor does their absence exclude TB involvement. Conventional Ziehl-Neelsen (ZN) method for acid fast bacilli (AFB) plays a key role in diagnosis and also monitoring the treatment of TB; but has a low sensitivity ranging from 20% to 43% (Balows *et al* 1991).

Mycobacterial culture is the reference method but is time consuming and requires specialized safety procedures in laboratories. Serological techniques lack sensitivity and specificity (Daniel 1989). Newer molecular techniques such as polymerase chain reaction (PCR), although rapid, are costly to be routinely used in developing countries.

Various concentration methods exist for improving sensitivity of direct microscopy for detection of AFB. Bleach concentration method for detection of AFB has been recently described for sputum and extra-pulmonary specimens. Studies have shown that liquefaction of sputum by bleach concentration method improves the sensitivity of detection of AFB.

Determination of tubercular bacilli by bleach concentration method is a simple technique, which requires no expertise and is inexpensive (Annam *et al* 2009).

Thus, the present study was under taken to emphasize the role of Histopathology for diagnosis tuberculosis and role of bleach concentration method over the conventional direct smear microscopy.

2.2. RESEARCH HYPOTHESIS:

1. Bleach sedimentation technique is superior to direct method for microscopic examination of sputum for AFB.
2. Histopathological and cytopathological examination is helpful in the diagnosis of tuberculosis, especially in sputum negative patients and in extra-pulmonary cases.

2.3. RESEARCH OBJECTIVES:

General objective:

To improve the microscopy screening performance for TB case finding.

Specific objective

(1) To compare bleach sedimentation smear microscopy of sputum with direct smear microscopy for AFB detection.

(2) To assess the feasibility of the bleach method in hospitals at the provincial level.

(3) To evaluate/verify the role of Histopathological and cytopathological examination in the diagnosis of tuberculosis, especially in sputum negative patients and in extra-pulmonary cases.

CHAPTER-3

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

3. 1. GLOBAL TUBERCULOSIS MORTALITY:

Tuberculosis, one of the oldest diseases known to affect humans, is a major cause of death worldwide. This disease caused by the bacteria *Mycobacterium tuberculosis* complex, usually affects the lungs, although other organs are involved in up to one-third of cases. If properly treated, tuberculosis caused by drug-susceptible strains is curable in virtually all cases. If untreated, the disease may be fatal within 5 years in 50–65% of cases. Transmission usually takes place through the airborne spread of droplet nuclei produced by patients with infectious pulmonary tuberculosis (Ban *et al* 2012).

More than 5 million new cases of tuberculosis (all forms, both pulmonary and extra-pulmonary) were reported to the World Health Organization (WHO) in 2005; >90% of cases were reported from developing countries. However, because of insufficient case detection and incomplete notification, reported cases represent only 60% of total estimated cases. The WHO estimated that 8.8 million new cases of tuberculosis occurred worldwide in 2005, 95% of them in developing countries of Asia (4.9 million), Africa (2.6 million), the Middle East (0.6 million), and Latin America (0.4 million). It was further estimated that 1.6 million deaths from tuberculosis occurred in 2005, 95% of them in developing countries.

3.2. TUBERCULOSIS SITUATION IN BANGLADESH:

Tuberculosis (TB) has been a major public health problem and is one of the leading causes of adult mortality in Bangladesh. World Health Organization ranks Bangladesh 6th among the world's 22 high-burden TB countries. Translating the estimates of 2007 by WHO on estimated population of 2009, it is found that every year about 66,437 people die due to tuberculosis in Bangladesh. National Tuberculosis Control Programme (NTP)

under Directorate General of Health Services (DGHS), Ministry of Health and Family Welfare (MoHFW) performs tuberculosis control activities and provides service through 800 DOTS centers, 1000 microscopy centers and 35 external quality assurance centers all over the country.

The World Health Organization (WHO) estimated that in 2007 there were approximately 387 TB cases per 100,000 populations of which 223 per 100,000 population new cases were occurring each year in Bangladesh (Table 1).

Table 1. Summary of Tuberculosis situation in Bangladesh

Country ranking among the 22 high burden countries	6th
Estimated incidence of all forms of TB per 100,000 population per year)	223
New smear-positive cases per 100,000 population per year)	100
Estimated mortality from all forms of TB per 100,000 population per year)	45
Estimated prevalence of all forms of TB cases per 100,000 population	387
Proportion of MDR-TB among new cases	3.5%
Proportion of MDR-TB among re-treatment cases	20%
DOTS population coverage	100%
Case detection rate- New smear-positive cases	74 %
Treatment success rate for new smear-positive cases	92 %

Source: Bangladesh Bureau of Statistics and Global Tuberculosis Control WHO Report 2009

Of these, approximately 100 per 100,000 were infectious cases, i.e., they able to transmit TB in the community. It is further estimated that about 45 persons per 100,000 people die of TB every year. Although the HIV prevalence is still low in Bangladesh, HIV poses a threat to TB control. The HIV prevalence in adult TB patients was about 0.1% as revealed

in three limited surveys conducted in 1999, 2001 and 2006-07. The multi-drug resistant tuberculosis (MDR-TB) rate among the new cases of TB was estimated to be 3.5%. This rate among the re-treatment cases was estimated at 20%.

Table 2 shows the year-wise tuberculosis case finding situation in Bangladesh.

Table 2. The year-wise tuberculosis case finding situation in Bangladesh (2006-2009).

Year	Area	Smear+ve		Smear-ive	Extra-pulmonary	Total
		New	Relapse	New	New	
2006	Rural/Upazila	89704	2645	16717	9707	118773
	Urban/Metro	9255	1279	5409	3499	19442
	CDC	2806	287	2375	1155	6623
	Total	101765	4211	24501	14361	144838
2007	Rural/Upazila	91606	2517	15852	10861	120836
	Urban/Metro	10264	1049	5449	4164	20926
	CDC	2437	222	1934	1093	5686
	Total	104307	3788	23235	16118	147448
2008	Rural/Upazila	93659	2753	15069	12825	124306
	Urban/Metro	10289	1165	5660	4486	21600
	CDC	2425	220	1463	1048	5156
	Total	106373	4138	22192	18359	151062
2009	Rural/Upazila	96,333	2,692	17,759	15,768	132,552
	Metro	10,390	1,136	5,829	4,872	22,227
	CDC	2,171	150	1,548	1,225	5,094
	Total	108,894	3,978	25,136	21,865	159,873

Source: Communicable Disease Situation - Bangladesh
http://dghs.gov.bd/dmdocuments/Communicable%20diseases%20in%20Bangladesh.pdf

Table 3. shows the estimated number of new smear +ve cases, detected number of new smear +positive cases and estimated incidence new smear +ve cases per 100,000 populations in Bangladesh.

Table 3. Estimated number of new smear+ve, detected number of new smear+ve TB cases and estimated incidence of new smear+ve TB cases / 100,000 population

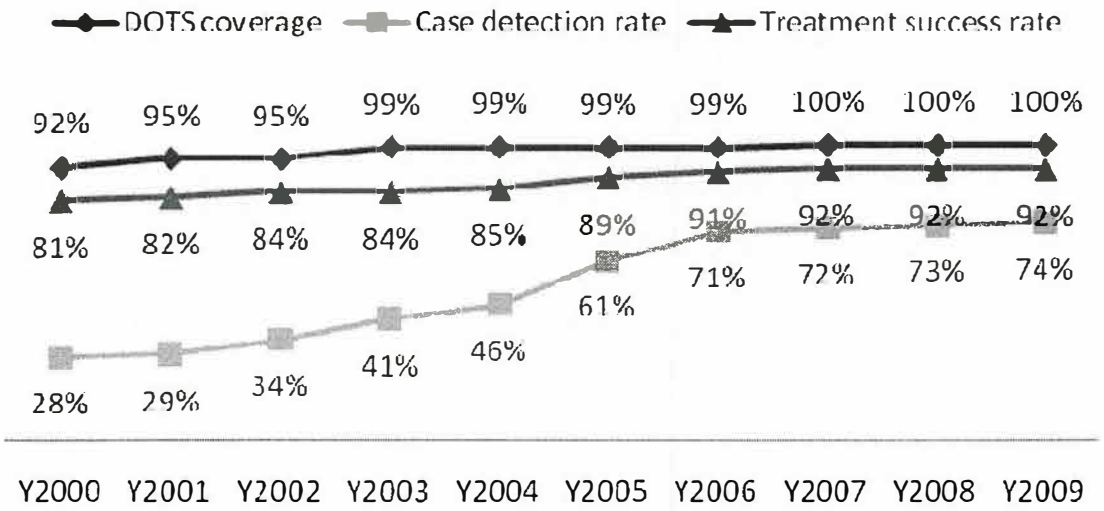
Year	Estimated number of new smear+ve TB cases	Detected number of new smear +ve TB cases	Estimated incidence of new smear+ve cases per 100,000 population
2006	143,514	101,765	102
2007	144,390	104,307	101
2008	145,280	106,373	100
2009	147,640	108,894	100

Source: Communicable Disease Situation - Bangladesh
http://dghs.gov.bd/dmdocuments/Communicable%20diseases%20in%20Bangladesh.pdf

3.3. Tuberculosis treatment success rate in Bangladesh

To quickly understand the progress of Bangladesh on DOTS coverage, tuberculosis case detection rate and tuberculosis treatment success rate, Figure 2 can be seen. The progress is quite impressive as global achievement for case detection and treatment success is 62% and 86% respectively (as of 2007).

Figure 2. Tuberculosis DOTS coverage, case detection rate and treatment success rate by year in Bangladesh.



Source: Communicable Disease Situation - Bangladesh
http://dghs.gov.bd/dmdocuments/Communicable%20diseases%20in%20Bangladesh.pdf

3.4. Multi Drug Resistant Tuberculosis (MDR-TB) in Bangladesh

The emergence of resistance to anti-TB drugs, particularly the Multi-Drug-Resistant Tuberculosis (MDR-TB) has become a significant public health threat globally against effective TB control. WHO reported that there is a half million of new MDR-TB cases emerging throughout the world in 2008 among which approximately 2/3rd of the cases exists in the South East Asia Region. As it is primarily a man-made phenomenon, the threat can be halted through efficient implementation STOP TB STRATEGY by the National TB Control Programs throughout the world.

There are no nationally representative data on drug resistance available in Bangladesh. According to WHO Report 2009 on Global Tuberculosis Control, there were 14,506 number of estimated MDR-TB cases in Bangladesh in 2007 among which 7,694 were smear positive. Recognizing this burden of MDR-TB, Bangladesh Government adopted 5 year DOTS Plus Pilot project approved by the Green Light Committee (GLC) in 2006 for implementation of programmatic Management of MDR-TB cases. Patient enrolment started since august 2008 and total 286 cases have been registered and are being managed by external quality assured 2nd Line Drugs (SLD) till end of 2009.

To coordinate the overall MDR-TB control activities, the National TB Control Programme of Bangladesh formed a DOTS-Plus Coordination Committee. A National Guideline has also been developed to manage all registered patients under DOTS-Plus Project through approved standardized regimen. On the other hand, to diagnose and follow-up of cases a National TB Reference Laboratory has been established and functionalized in National Institute of Diseases of Chest and Hospital (NIDCH). Based on this set up NTP has launched Drug Resistant Survey (DRS) in 2010 to estimate the current resistant burden. According to the health ministry statistics, one person dies of TB every 10 min and one is infected every two minutes in the country. This severe situation has placed Bangladesh in the sixth position (Table 4) in the world in terms of burden of TB patients (Zaman 2007, 2008).

Table 4. High burden countries of Tuberculosis (TB).

1. India	2. China	3. Indonesia
4. Nigeria	5. South Africa	**6. Bangladesh**
7. Pakistan	8. Philippines	9. Russian Federation
10. Ethiopia		

3.5. MYCOBACTERIUM:

(Ban *et al* 2012, Vinay *et al* 2010 and Forbes *et al* 2002)

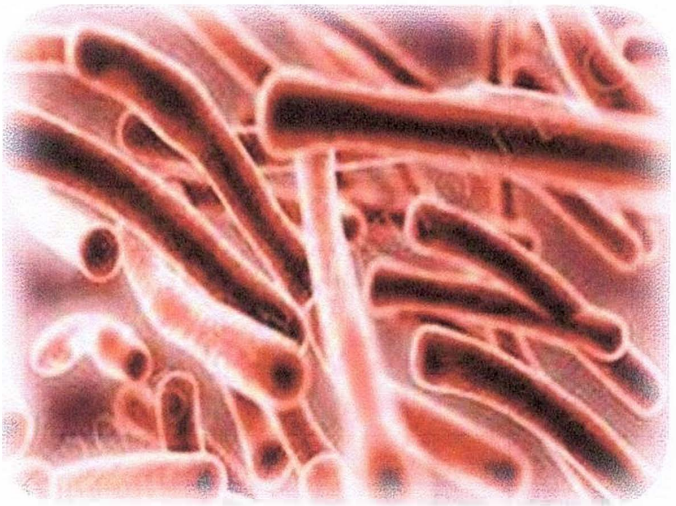


Figure 3. Figure showing *Mycobacterium tuberculosis* organism.

M. tuberculosis is a rod-shaped, non-spore forming, non motile and thin aerobic bacterium measuring 0.2-0.4 × 2-10 um. It is often neutral on Gram's staining. However, once stained, the bacilli cannot be decolorized by acid alcohol; this characteristic justifies their classification as acid-fast bacilli. Acid fastness is due mainly to the organisms high content of mycolic acids, long-chain cross-linked fatty acids, and other cell-wall lipids. Microorganisms other than mycobacteria that display some acid fastness include species of *Nocardia* and *Rhodococcus*, *Legionella micdadei*, and the protozoa *Isospora* and *Cryptosporidium*. In the mycobacterial cell wall, lipids (e.g., mycolic acids) are linked to underlying arabinogalactan and peptidoglycan. This structure confers very low permeability of the cell wall, thus reducing the effectiveness of most antibiotics. (Ban *et al* 2012). Currently, there are 71 recognized or proposed species in the genus *Mycobacterium.* The genus includes pathogens, opportunistic pathogens and saprophytes.

The pathogens are *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis, M. africanum* and *M. microti*), *M. leprae* and *M. ulcerans*.

3.6. Aetio-pathogenesis

M. tuberculosis is most commonly transmitted from a person with infectious pulmonary tuberculosis to others by droplet nuclei, which are aerosolized by coughing, sneezing, or speaking. The tiny droplets dry rapidly may remain suspended in the air for several hours and may reach the terminal air passages when inhaled. There may be as many as 3000 infectious nuclei per cough.

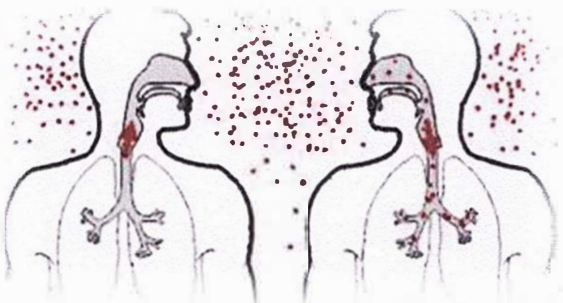


Figure 4. Droplet transmission of TB by coughing, sneezing or speaking.

The probability of contact with a person who has an infectious form of tuberculosis, the intimacy and duration of that contact, the degree of infectiousness of the case, and the shared environment in which the contact takes place are all important determinants of the likelihood of transmission. Several studies of close-contact situations have clearly demonstrated that tuberculosis patients whose sputum contains AFB visible by microscopy are the most likely to transmit the infection. The most infectious patients have cavitary pulmonary disease or, much less commonly, laryngeal tuberculosis and produce sputum containing as many as 10^5–10^7 AFB/mL. Patients with sputum smear–

negative/culture-positive tuberculosis are less infectious, and those with culture-negative pulmonary disease and extra-pulmonary tuberculosis are essentially noninfectious.

Because persons with both HIV infection and tuberculosis are less likely to have cavitations, they may be less infectious than persons without HIV co-infection. Crowding in poorly ventilated rooms is one of the most important factors in the transmission of tubercle bacilli, since it increases the intensity of contact with a case.

Unlike the risk of acquiring infection with *M. tuberculosis*, the risk of developing disease after being infected depends largely on endogenous factors, such as the individual's innate immunologic and non-immunologic defenses and level of function of cell-mediated immunity (CMI). Clinical illness directly following infection is classified as primary tuberculosis and is common among children up to 4 years of age and among immuno-compromised persons. Although primary tuberculosis may be severe and disseminated, it is not generally associated with high-level transmissibility. When infection is acquired later in life, the chance is greater that the mature immune system will contain it at least temporarily. The majority of infected individuals who ultimately develop tuberculosis do so within the first year or two after infection. Dormant bacilli, however, may persist for years before reactivating to produce secondary (or post primary) tuberculosis, which, because of frequent cavitations, is more often infectious than is primary disease. Overall, it is estimated that up to 10% of infected persons will eventually develop active tuberculosis in their lifetime. The risk is much higher among HIV-infected persons. Re-infection of a previously infected individual, which is common in areas with high rates of tuberculosis transmission, may also favor the development of disease. At the height of the tuberculosis resurgence in the United States in the early 1990s, molecular typing and comparison of strains of M. tuberculosis suggested that up to one-third of cases of active tuberculosis in some inner-city communities were due to recent transmission rather than to reactivation of latent infection.

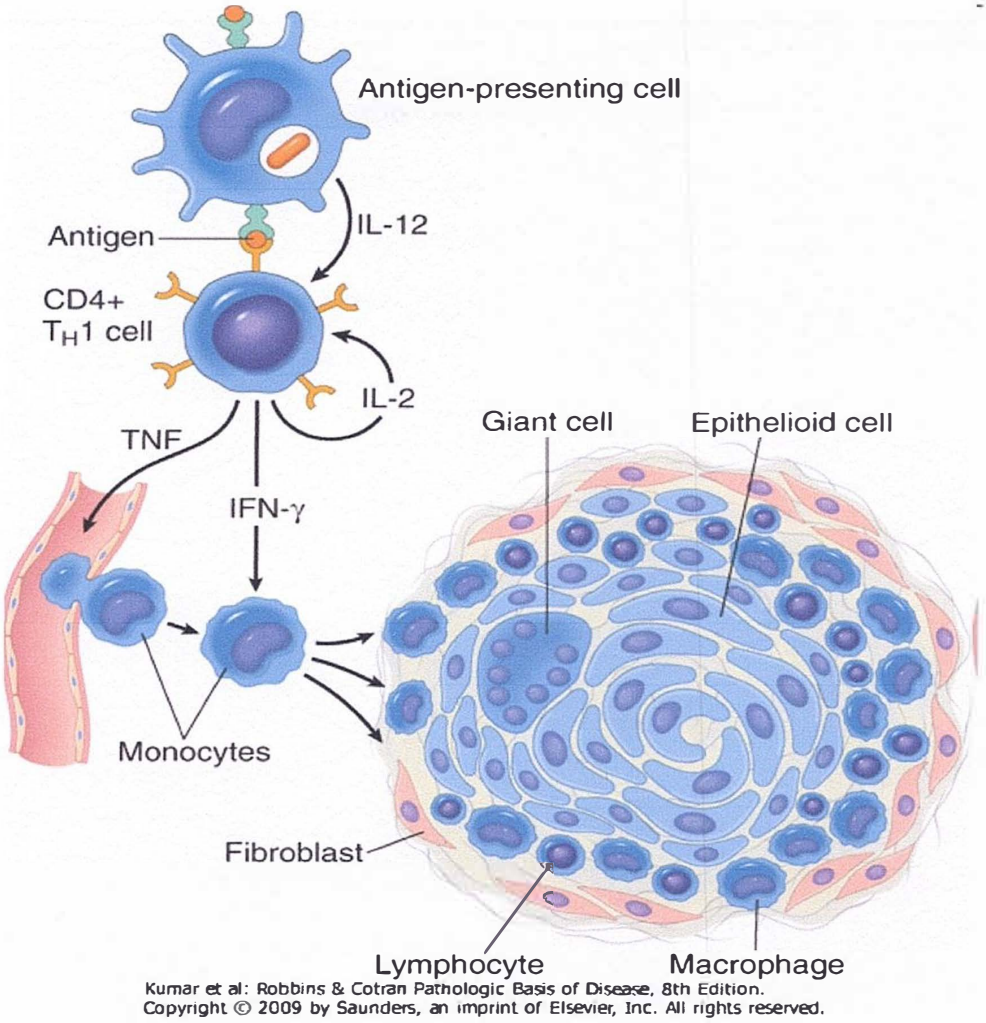


Figure 5. Aetio-pathogenesis of tuberculosis.

Age is an important determinant of the risk of disease after infection. Among infected persons, the incidence of tuberculosis is highest during late adolescence and early adulthood; the reasons are unclear. The incidence among women peaks at 25–34 years of age. In this age group rates among women may be higher than those among men, while at older ages the opposite is true. The risk may increase in the elderly, possibly because of waning immunity and co morbidity.

With the development of specific immunity and the accumulation of large numbers of activated macrophages at the site of the primary lesion, granulomatous lesions (tubercles)

are formed. These lesions consist of accumulations of lymphocytes and activated macrophages that evolve toward epithelioid and giant cell morphologies. Initially, the tissue-damaging response can limit mycobacterial growth within macrophages. As stated above, this response, mediated by various bacterial products, not only destroys macrophages but also produces early solid necrosis in the center of the tubercle. Although *M. tuberculosis* can survive, its growth is inhibited within this necrotic environment by low oxygen tension and low pH. At this point, some lesions may heal by fibrosis, with subsequent calcification, whereas inflammation and necrosis occur in other lesions.

Studies conducted in various countries before the advent of chemotherapy showed that untreated tuberculosis is often fatal. About one-third of patients died within 1 year after diagnosis, and one-half died within 5 years. The 5-year mortality rate among sputum smear–positive cases was 65%. Of the survivors at 5 years, 60% had undergone spontaneous remission, while the remainders were still excreting tubercle bacilli.

With effective, timely, and proper chemotherapy, patients have a very high chance of being cured. However, improper use of anti-tuberculosis drugs, while reducing mortality rates, may also result in large numbers of chronic infectious cases, often with drug-resistant bacilli.

3.7.0. Clinical Manifestations

Tuberculosis is classified as pulmonary, extra-pulmonary, or both. Before the advent of HIV infection, 80% of all new cases of tuberculosis were limited to the lungs. However, up to two-thirds of HIV infected patients with tuberculosis may have either pulmonary and extra pulmonary disease or extra-pulmonary disease alone.

3.7.1. Pulmonary Tuberculosis

Pulmonary tuberculosis can be categorized as primary or post primary (secondary).
Primary Disease

Primary pulmonary tuberculosis occurs soon after the initial infection with tubercle bacilli. In areas of high tuberculosis transmission, this form of disease is often seen in

children. Because most inspired air is distributed to the middle and lower lung zones, these areas of the lungs are most commonly involved in primary tuberculosis. The lesion forming after infection is usually peripheral and accompanied in more than half of cases by hilar or paratracheal lymphadenopathy, which may not be detectable on chest radiography. In the majority of cases, the lesion heals spontaneously and may later be evident as a small calcified nodule (Ghon lesion).

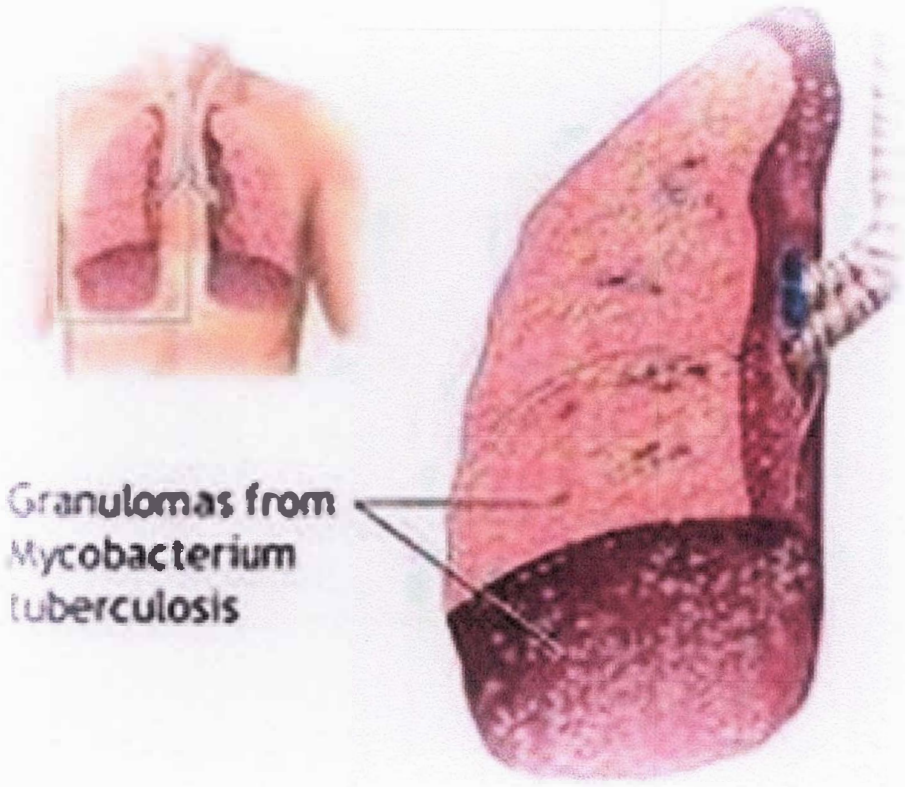


Figure 6. Granulomatous inflammation in lung.

In children and in persons with impaired immunity (e.g., those with malnutrition or HIV infection), primary pulmonary tuberculosis may progress rapidly to clinical illness. The initial lesion increases in size and can evolve in different ways. Pleural effusion, which found in up to two-thirds of cases, results from the penetration of bacilli into the pleural space from an adjacent sub-pleural focus. In severe cases, the primary site rapidly enlarges, its central portion undergoes necrosis, and cavitation develops (progressive primary tuberculosis). Tuberculosis in young children is almost invariably accompanied by hilar or mediastinal lymphadenopathy due to the spread of bacilli from the lung

parenchyma through lymphatic vessels. Enlarged lymph nodes may compress bronchi, causing obstruction and subsequent segmental or lobar collapse. Partial obstruction may cause obstructive emphysema, and bronchiectasis may also develop. Hematogenous dissemination, which is common and often asymptomatic, may result in the most severe manifestations of primary *M. tuberculosis* infection. Bacilli reach the bloodstream from the pulmonary lesion or the lymph nodes and disseminate into various organs, where they may produce granulomatous lesions. Although healing frequently takes place, immunocompromised persons (e.g., patients with HIV infection) may develop miliary tuberculosis and/or tuberculous meningitis.

Post primary Disease

Also called adult-type, reactivation, or secondary tuberculosis, postprimary disease results from endogenous reactivation of latent infection and is usually localized to the apical and posterior segments of the upper lobes, where the substantially higher mean oxygen tension (compared with that in the lower zones) favors mycobacterial growth. In addition, the superior segments of the lower lobes are frequently involved. The extent of lung parenchymal involvement varies greatly, from small infiltrates to extensive cavitary disease. With cavity formation, liquefied necrotic contents are ultimately discharged into the airways, resulting in satellite lesions within the lungs that may in turn undergo cavitation. While up to one-third of untreated patients reportedly succumb to severe pulmonary tuberculosis within a few weeks or months after onset, others undergo a process of spontaneous remission or proceed along a chronic, progressively debilitating course and may continue to discharge tubercle bacilli into the environment.

Early in the course of disease, symptoms and signs are often nonspecific and insidious, consisting mainly of fever and night sweats, weight loss, anorexia, general malaise, and weakness. However, in the majority of cases, cough eventually develops—often initially nonproductive and subsequently accompanied by the production of purulent sputum, sometimes with blood streaking.

3.7.2. Extra-pulmonary Tuberculosis

In order of frequency, the extra-pulmonary sites most commonly involved in tuberculosis are the lymph nodes, pleura, genitourinary tract, bones and joints, meninges, peritoneum, and pericardium. However, virtually all organ systems may be affected. As a result of hematogenous dissemination in HIV-infected individuals, extra pulmonary tuberculosis is seen more commonly today than in the past.

The most common presentation of extra pulmonary tuberculosis lymph-node disease is particularly frequent among HIV infected patients. Once caused mainly by *M. bovis,* tuberculous lymphadenitis is today due largely to *M. tuberculosis*. Lymph-node tuberculosis presents as painless swelling of the lymph nodes, most commonly at posterior cervical and supraclavicular sites. Lymph nodes are usually discrete and nontender in early disease but may be inflamed and have a fistulous tract draining caseous material. Associated pulmonary disease is seen in >40% of cases. Systemic symptoms are usually limited to HIV-infected patients. The diagnosis is established only by fine-needle aspiration or surgical biopsy. AFB is seen in up to 50% of cases, cu lures are positive in 70–80%, and histological examination shows granulomatous lesions.

Among HIV-infected patients, granulomas usually are not seen. Differential diagnosis includes a variety of infectious conditions, neoplastic diseases such as lymphomas or metastatic carcinomas, and rare disorders like Kikuchi disease (necrotizing histiocytic lymphadenitis).

3.8.0. Diagnosis of Tuberculosis

3.8.1. AFB Microscopy

Mycobacterial infection is usually confirmed by microscopy (Ziehl-Neelsen or auramine staining) and culture of samples. It has been estimated that 5000-10000 acid-fast bacilli must be present in sputum for a patient to be smear positive. For patients with suspected pulmonary tuberculosis, three sputum specimens, preferably collected early in the morning, should be submitted to the laboratory for AFB smear (Monica Cheesbrough 2000, Nicholas *et al* 2006).

3.8.2. Mycobacterial Culture

Definitive diagnosis depends on the isolation and identification of *M. tuberculosis* from a clinical specimen or the identification of specific sequences of DNA in a nucleic acid amplification test. Specimens may be inoculated onto egg- or agar-based medium (e.g., Löwenstein-Jensen) and incubated at 37°C. Because most species of mycobacteria, including *M. tuberculosis*, grow slowly, 4–8 weeks may be required before growth is detected. Although *M. tuberculosis* may be presumptively identified on the basis of growth time and colony pigmentation and morphology, a variety of biochemical tests have traditionally been used to speciate mycobacterial isolates.

In modern, well- equipped laboratories, the use of broth-based culture for isolation and speciation by molecular methods or high-pressure liquid chromatography of mycolic acids has replaced isolation on solid media and identification by biochemical tests. These new methods have decreased the time required for bacteriologic confirmation to 2–3 weeks.

3.8.3. Nucleic Acid Amplification

Several test systems based on amplification of mycobacterial nucleic acid are available. These systems permit the diagnosis of tuberculosis in as little as several hours, with high specificity and sensitivity approaching that of culture. These tests are most useful for the rapid confirmation of tuberculosis in persons with AFB-positive specimens but also have utility for the diagnosis of AFB-negative pulmonary and extra-pulmonary tuberculosis (Noordhock 1994).

3.8.4. Radiographic Procedures

The initial suspicion of pulmonary tuberculosis is often based on abnormal chest radiographic findings in a patient with respiratory symptoms. Although the "classic" picture is that of upper-lobe disease with infiltrates and cavities, virtually any radiographic pattern—from a normal film or a solitary pulmonary nodule to diffuse alveolar infiltrates in a patient with ARDS—may be seen. In the era of AIDS, no

radiographic pattern can be considered pathognomonic. CT may be useful in interpreting questionable findings on plain chest radiography and may be helpful in diagnosing some forms of extra-pulmonary tuberculosis e.g., Pott's disease. MRI is useful in the diagnosis of intracranial tuberculosis.

3.8.5. Histopathological and cytopathological examination

Diagnosis of tuberculosis is often difficult when no AFB seen in sputum and in extra pulmonary tuberculosis. In such situations, Histopathological and cytopathological examination is helpful. These examinations shows many granulomas composed of epithelioid cells, occasional Langhan's type of giant cells, rim of lymphocytes and surrounded by fibroblast. Areas of caseation necrosis may be seen (Vinay *et al* 2010).

3.8.6. Serologic and Other Diagnostic Tests for Active Tuberculosis

A number of serologic tests based on detection of antibodies to a variety of mycobacterial antigens are marketed in developing countries but not in the United States. Careful independent assessments of these tests suggest that they are not useful as diagnostic aids, especially in persons with a low probability of tuberculosis. Various methods aimed at detection of mycobacterial antigens in diagnostic specimens are being investigated but are limited at present by low sensitivity (Van Pinxtern *et al* 2000, Wilkins 1998).

3.8.7. Tuberculin Skin Testing

Skin testing with tuberculin-PPD (TST) is most widely used in screening for latent *M. tuberculosis* infection (LTBI). The test is of limited value in the diagnosis of active tuberculosis because of its relatively low sensitivity and specificity and its inability to discriminate between latent infection and active disease. False-negative reactions are common in immuno-suppressed patients and in those with overwhelming tuberculosis. False-positive reactions may be caused by infections with non-tuberculous mycobacteria and by Bacille Calmette-Guérin (BCG) vaccination (John Bernard 1996).

CHAPTER-4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

A total of 115 patients were involved in this study. Among them, we have compared microscopy of smear made directly from sputum, with microscopy of smear made after liquefaction and concentration of sputum with bleach. We also revealed the effectiveness of Histopathological and cytopathological diagnosis of both pulmonary and extra-pulmonary tuberculosis either sputum positive or negative.

4. 1. Type of study: Cross sectional type.

4.2. Period of study: From July 2009 to June 2011

4.3. Ethical clearance: Permission to use patient materials and laboratory medical records for this study was sought from the institutional review committee prior to study initiation. Ethics approval was granted by the Human Research Ethics Committee Institute of Biological Sciences, University of Rajshahi. The ethics clearance certificate number is 'Memo No. 21/320-IAMEBBC/IBSc', and it is included in appendix-I of this dissertation.

4.4. Place of the Study:

Samples collected from the clinically suspected tuberculosis patients attending in the inpatient and outpatient departments of Khwaja Yunus Ali Medical College Hospital (KYAMCH). Examinations of clinical specimens were conducted in the Department of Laboratory Services in KYAMCH.

4.5. Patient selection criteria:

Patients were selected only by clinically suspicion of tuberculosis. These patients usually showed low-grade irregular evening rise of temperature, malaise, cough, lymphadenopathy and other features according to site of involvement.

4.6. Exclusion criteria:

Patients on antituberculosis treatment and known case of malignancy were excluded from the study.

4.7. Collection of samples:

Considering the ethical issues, samples were collected according to the site of involvement.

4.7.1. Samples for Histopathological and cytopathological examination: Biopsy materials from the site of infection (e.g. lymph node, tissue from abdomen etc.) were taken in a sterile container along with preservatives and cytopathological specimens were collected in the laboratory by fine needle aspiration from clinically suspected patients.

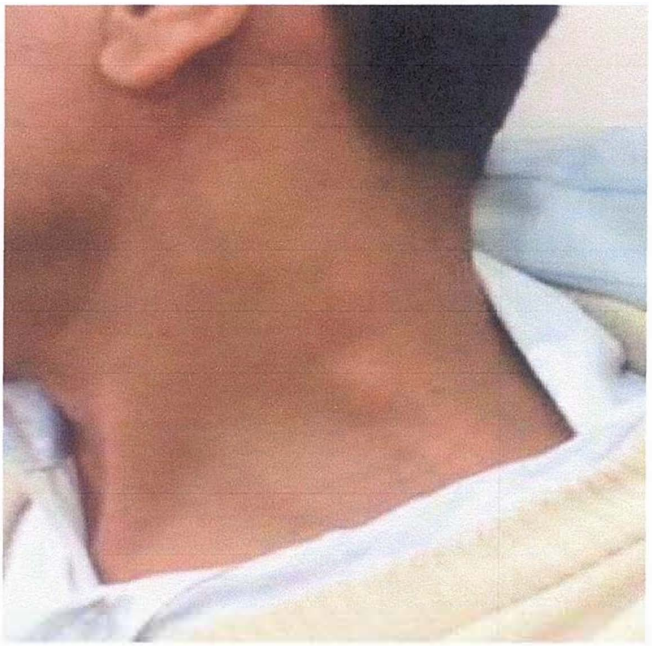


Figure 7. Enlarged cervical lymph node from which FNA was done.

4.7.2. Sputum for bacteriological examination: Sputum samples were collected from the study cases in a clean, dry, wide-necked, leak-proof container by deeply coughing. Sputum was collected in the morning soon after the patient wakes and before any mouthwash is used. A total of three specimens were collected on three consecutive days from each patient. During collection, adequate safety precautions were taken to prevent the spread of infectious organism and to avoid contaminating the outside of the container. After collection, the container was labeled with the date, time of collection and patient's name etc. A correctly completed request form was accompanied each specimen.

4.8. Laboratory procedure

4.8.1. Specimen processing:

(John and Marilyn 2002)

4.8.1.1. Histopathological and cytopathological specimens:

- Histopathological processing:

Gross examination

↓

Proper sectioning

↓

Fixation

↓

Paraffin blocking

↓

Microtome cutting and then

↓

Examined after haematoxylin and eosin staining.

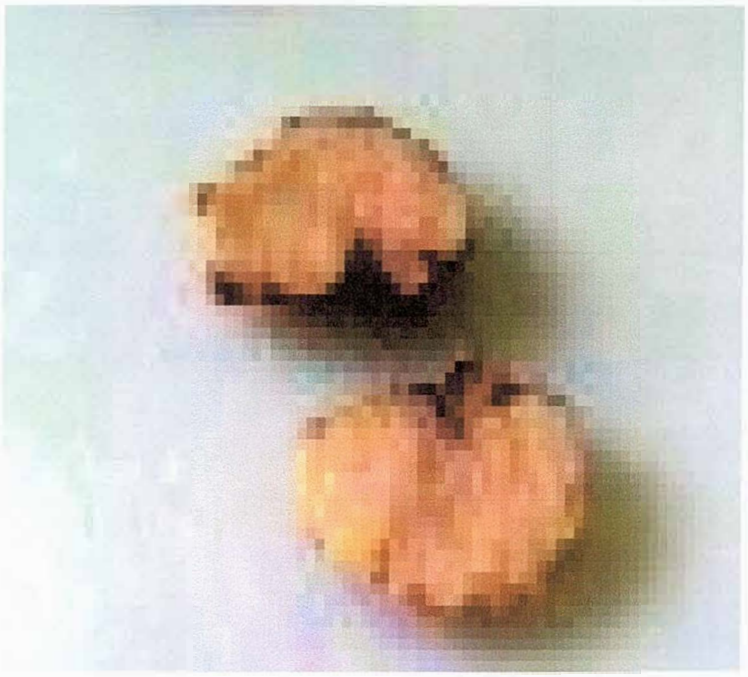


Figure 8. Macroscopic finding (caseous surface) of tuberculous lymph node.

- Cytopathological (e.g. Fine needle aspiration) specimens were at first fixed with 95% ethyl alcohol after collection and then examined after Pap's staining.

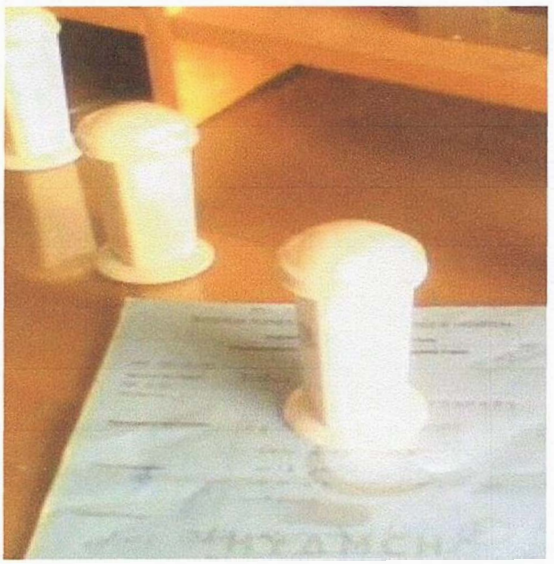


Figure 9. Photomicrograph showing fine needle aspiration (FNA) smears fixation jar with request form.

4.8.1.2. Sputum processing:

Studies have shown that the chances of detecting AFB in sputum smears are significantly increased if the organisms are first concentrated by centrifugation. Sodium hypochlorite (bleach) recommended for liquefying the sputum because it kills *Mycobacterium tuberculosis*, making the handling of specimens safer for laboratory staff (Monica Cheesbrough 2000).

Figure 10. Photomicrograph showing sputum staining procedure.

A direct smear from each specimen was prepared and stained with Ziehl-Neelsen's stain (Appendix-II). A portion of the remainder sputum was processed with bleach method. Then smear was made from the processed sputum and ZN staining was performed.

4.8.1.3. Bleach (Sodium hypochlorite) sedimentation method to concentrate AFB:

(Monica Cheesbrough 2000).

- A portion of sputum (1-2 ml) was taken particularly that contained any yellow caseous materials to a screw-cap tube of 15-20 ml capacity. The specimen was

opened with care and at arms length to avoid inhaling infectious aerosols. When available should handle the specimen inside a safety cabinet.

- Then was added an equal volume of concentrated sodium hypochlorite (household bleach, 5.25 %) solution and mixed well.
- Then was left at room temperature for 10-15 minutes, shaked at intervals to breakdown the mucous in the sputum.
- Then about 8 ml of distilled water was added and mixed well.
- Then centrifugation was done at 3000 g for 15 minutes. When centrifugation was not possible, was left the NaOCL treated sputum to sediment overnight.
- By using a pipette remove and discarded the supernatant fluid. Then after mixing the sediment, transferred a drop of the well-mixed sediment to a clean scratch-free glass slide and spread to make a thin preparation and then allow air-drying.
- Then the smear was heat-fixed and stained using Ziehl-Neelsen technique.
- Finally the stained slide was examined microscopically for acid-fast bacilli (AFB).

4.8.2. Advantage of bleach sedimentation:

- This reagent is very cheap.
- Easily available and non-toxic.
- Kills the AFB, so we can easily handle the specimen.
- Liquefy the concentrated and thick portion of the specimen.
- Due to sedimentation, organisms are accumulated and can easily be found by microscopic examination after ZN staining.
- This method can be used in all peripheral laboratories of the country without special equipments.

4.8.3. Histopathological and cytopathological examination findings:

(Vinay *et al* 2010)

Microscopic examination shows many granulomas composed of epithelioid cells, caseation necrosis, occasional langhan's type of giant cells, and rim of lymphocytes and surrounded by fibroblast.

Diagnosis**:** Granulomatous inflammation compatible with tuberculosis.

4.8.4. Microscopic examination of sputum for acid-fast bacilli (AFB):

Microscopic examinations were prepared from both direct samples and processed sputum. The reporting of the smear was done as in the following table.

Table 5. Acid-fast smear reporting (According to Kent's rule): (John Bernard 1996)

Number of AFB	Fields	Report
No AFB	Per 100 oil immersion field	No AFB seen
1-9 AFB	Per 100 oil immersion field	(+)
1-9 AFB	Per 10 oil immersion field	(++)
1-9 AFB	Per every oil immersion field	(+++)
> 9 AFB	Per every oil immersion field	(++++)

CHAPTER-5

RESULTS

5. RESULTS AND OBSERVATIONS

All the results have been described in successive page using suitable tables, graphs and diagrams. A total of 115 clinically suspected tuberculosis patients were screened and among them 73 cases were found to be tuberculosis positive cases.

5.1. Prevalence of tuberculosis in different age groups of the study population

Under the present study, ages of the patients were between 7-85 years with mean age 36.71 and SD 15.83. As shown in Table 6 and Figure 11, the prevalence of tuberculosis was highest among patients belonging to the age group of 20-40 years.

Table 6. Incidence of tuberculosis in different age groups (n=115)

Age in years	No of subject	Percentage (%)	Mean age (M±SD)
<20	16	13.9	36.71 ± 15.83
21-30	34	29.6	
31-40	20	17.4	
41-50	21	18.3	
51-60	15	13.0	
61-70	5	4.3	
71-80	3	2.6	
>80	1	.9	

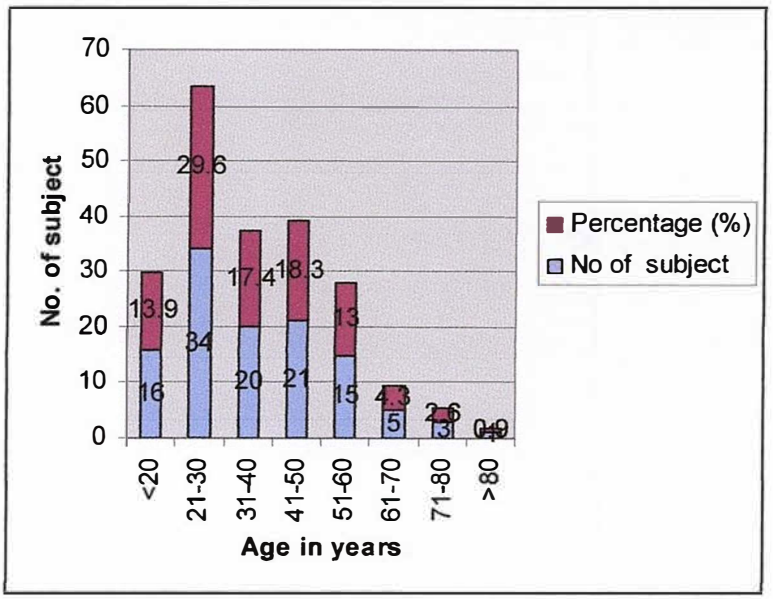


Figure 11. Bar diagram showing the age group wise incidence of Tuberculosis.

5.2. Sex-wise incidence of tuberculosis of the study population:

Among the 115 cases of the present study, 64 were male (55.65%) and 51 (44.35%) were female shown in Table 7 and Figure12. The present study demonstrated that the prevalence rate of tuberculosis in the study population was higher among males than the females i.e. males are more suffer of tuberculosis than females.

Table 7. Sex distribution of study cases (n=115)

Sex	No of subjects	Percentage (%)
Male	64	55.7
Female	51	44.3
Total	115	100

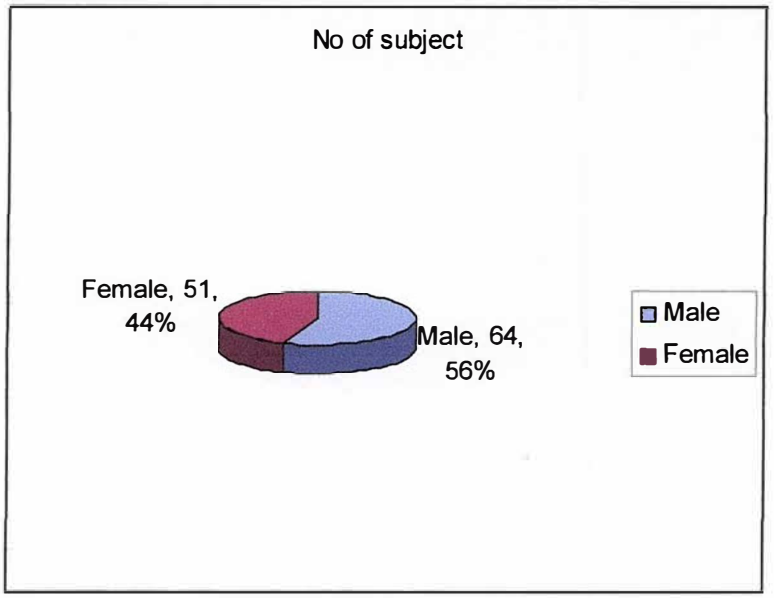


Figure 12. Pie diagram showing the sex distribution of study population.

5.3. Findings of Erythrocyte Sedimentation Rate (ESR) of the study population (n=115)

ESR values of the study cases were found to range between 06-140 mm in 1st hour with mean ESR 57.09. Among them, ESR value <30 mm in 1st hour was recorded in 18 (15.7%) subjects, 31-60 mm in 1st hour in 48 (41.7%) patients, 61-90 mm in 36 (31.3%) subjects and > 120 found in 1 case as shown in Table 8 and Figure 13.

Table 8. ESR values of the study cases (n=115)

ESR	No of subject	Percentage (%)	Mean ESR (M±SD)
<30	18	15.7	57.09 ± 26.74
31-60	48	41.7	
61-90	36	31.3	
91-120	12	10.4	
>120	1	0.9	

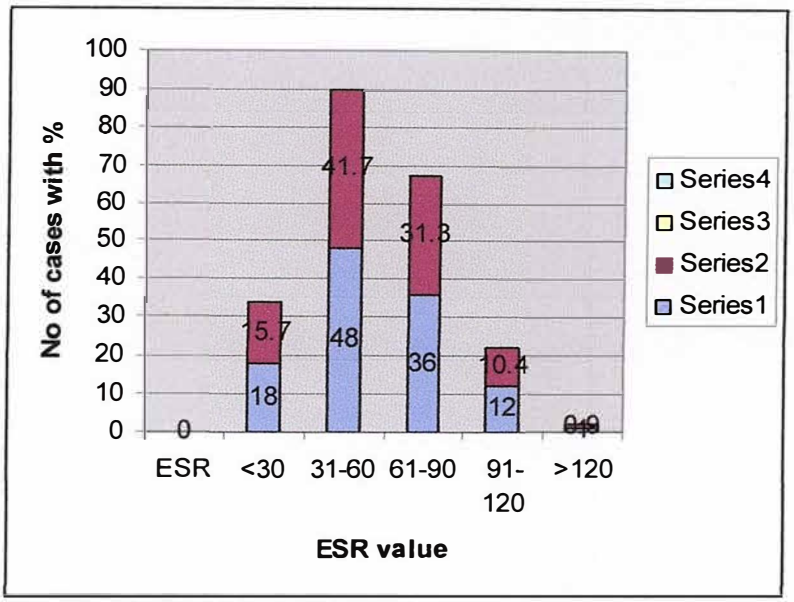


Figure 13. Bar diagram showing ESR values of the study cases.

5.4. Mantoux test (MT) findings of the study population (n=115)

Among the study population Mantoux test (MT) values were found between 2 to 30 mm after 72 hours with mean 14.62. MT value of < 5 mm was found in 3 (2.6%) cases, 6-10mm found in 19 (16.5%) cases, 11-15 mm found in 41 (35.7%) cases, 16-20 mm found in 42 (36.5%) cases, 21-25 mm found in 9 (7.8%) cases and > 25 mm found in 1 cases as shown in Table 9.

Table 9. MT values of the study population (n=115)

MT value	No of subject	Percentage (%)	Mean MT (M±SD)
< 5	3	2.6	14.62 ± 4.64
6-10	19	16.5	
11-15	41	35.7	
16-20	42	36.5	
21-25	9	7.8	
> 25	1	0.9	

5.5. Determinations of optimum bleach concentration for detection of AFB:

Optimum bleach concentration for the detection of AFB was determined by performing several *ad hock* experiments. Different concentrations of bleach (1.25%, 2.5%, 5%, 7.5% and 10%) were prepared from stock bleach (Sodium hypochlorite) solution by serial dilution technique and bleach (Sodium hypochlorite) sedimentation method to concentrate sputum was performed and specimen observed under microscope for the presence of AFB. Of the different bleach concentrations tested in AFB detection, 5% concentration appeared to be optimum. Increasing or decreasing the bleach concentrations caused markedly reduction in the delectability of AFB as shown in Table 10.

Table 10. Determination of optimum bleach concentration for detection of AFB.

Concentration of bleach (%)	Delectability	Comment
1.25	Partial	
2.5	Partial	
5.0	Clearly detectable	Optimum concentration
7.5	Partially with fragmented	
10.0	Not detectable	

5.6. Findings of positive and negative tuberculosis cases

Among the 115 clinically suspected tuberculosis patients screened under the present study, 73 (63.5%) cases were found to be tuberculosis positive and the remaining 42 (36.5%) cases negative as shown in Table 11 and Figure14.

Table 11. Data of tuberculosis positive and negative cases (n=115).

Positivity / Negativity	**No. of cases**	**Percentage (%)**
TB positive cases	73	63.5
TB negative cases	42	36.5
Total	115	100

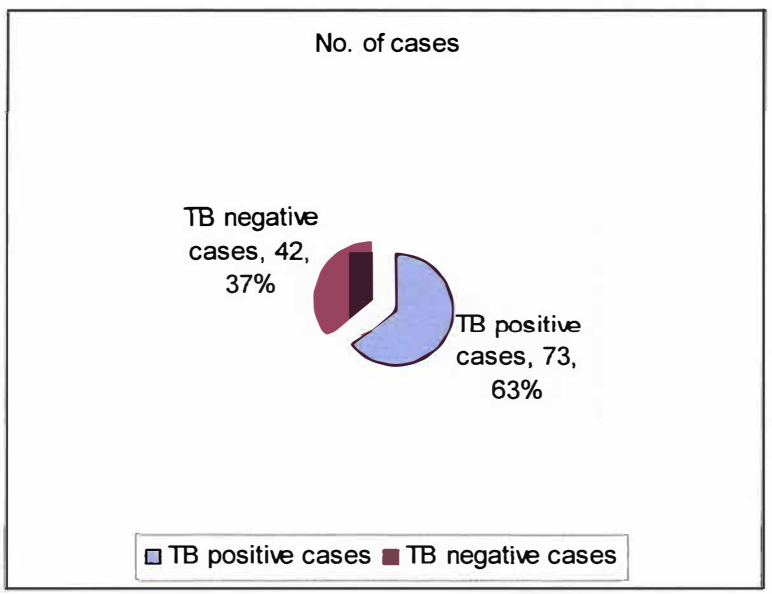


Figure 14. Pie diagram showing tuberculosis positive and negative cases.

5.7. Findings of Tuberculosis detection by the different methods

Direct smear microscopy, Bleach sedimentation microscopy and Histopathological and cytopathological examinations were performed among all the study cases. The findings showed that 35 (30.4%) cases were found TB positive on Direct method, 42 (36.5%) cases TB positive by Bleach method and 73 (63.5%) cases were found to be Tuberculosis positive by Histological and Cytopathological examinations (Table 12 and Figure 16). These results indicate that Histopathological and Cytopathological examination was more significant in the detection of Tuberculosis compared to the other two methods as shown in Table 12 and Figure 15.

Table 12. Data of findings of Tuberculosis detection by different methods (n=115).

Method	No. of positive cases	Percentage (%)
Direct	35	30.4
Bleach	42	36.5
Histo. & cytopathology	73	63.5

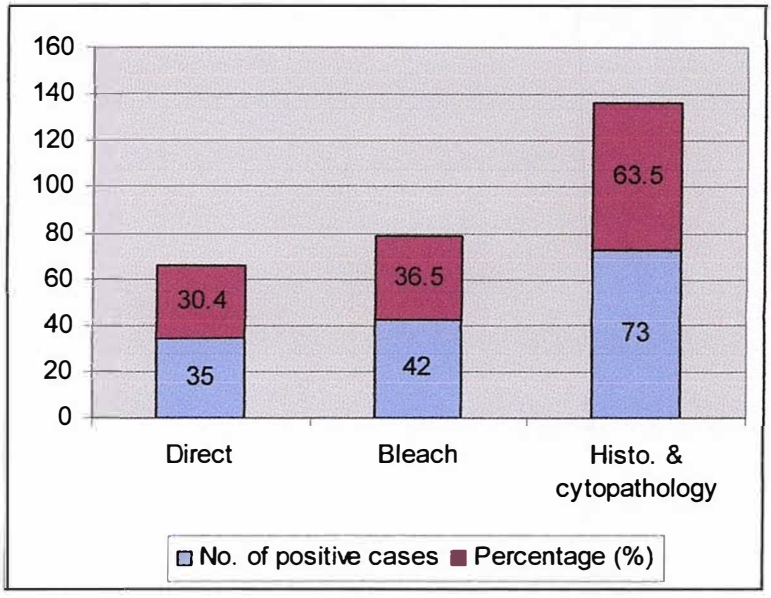


Figure 15. Bar diagram showing findings of different method.

5.8. Comparative positive-smear delectability of AFB by Direct smear microscopy and Bleach sedimentation microscopy.

Under the present study, sputum specimen was collected from 115 patients with suspected tuberculosis and processed for detection of AFB by Direct smear microscopy and Bleach sedimentation microscopy. The results of positive-smear delectability of AFB by Direct smear microscopy and Bleach sedimentation microscopy has been presented in Table 13.

Bleach sedimentation microscopy yielded significantly more positive smears than did direct smear microscopy. 42 (36.5%) of positive smears were detected by bleach sedimentation microscopy, compared with 35 (30.4%) positive smears detected by direct smear microscopy. The results clearly shows that Bleach sedimentation microscopy is superior than Direct smear microscopy in detecting AFB as 07 additional positive smears were detected by bleach sedimentation microscopy which were missed by Direct smear microscopy.

Table 13. Comparative positive-smear delectability of AFB by Direct smear microscopy and Bleach sedimentation microscopy (n=115).

Specimen	Microscopy			
Sputum	Direct smear microscopy (n=115) No. of positive specimens (Percentage positive)		Bleach sedimentation microscopy (n=115) No. of positive specimens (Percentage positive)	
	35 (30.4%)		42 (36.5%)	
	Day-1	Day-2	Day-1	Day-2
	27 (23.4%)	35 (30.4%)	34 (29.6%)	42 (36.5%)
Note: No positive smear found on third day that were not found on 1st and 2nd day.				

Again, data presented in Table 13 shows that of the 35 positive smears detected by direct smear microscopy, 27 (23.4%) cases were AFB positive using direct Ziehl-Neelsen's (ZN) staining on 1st day but after processing of sputum with bleach and staining with ZN, the number of positive cases increased from 27 (23.4%) to 34 (29.6%). Then on 2nd day, 35 (30.4%) cases were AFB positive by direct smear microscopy. On the other hand, bleach sedimentation microscopy alone on the first day could detect 34 (29.6%) smear-positive cases and on the 2nd day, 8 more i.e. totally 42 smear-positive cases were detected. Bleach sedimentation microscopy therefore detected significantly more smear-positive cases than did direct smear microscopy.

5.9. Tuberculosis in different organs of the body of the respondents:

Among the study population, Tuberculosis cases were detected in different organs of the body. The highest 48 (65.7%) cases were found in the lung, 20 (27.4%) cases found in lymph

nodes and remaining were found in other organs of the body as shown in Table 14 and Figure 16.

Table 14. Data of TB on different organs of the body (n=73)

Name of organ	No. of cases	Percentage (%)
PT	48	65.7
LN	20	27.4
Skin & ST	3	4.1
Bone	1	1.4
Abdominal	1	1.4
Total	73	100

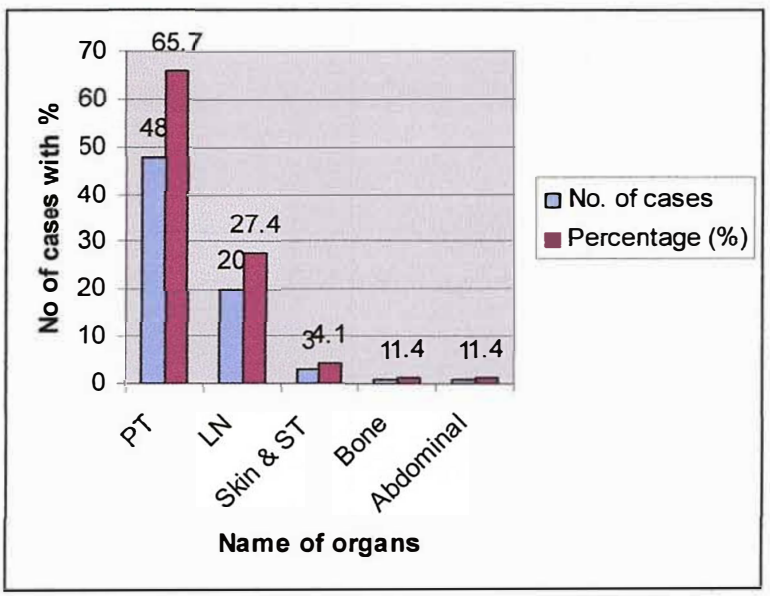


Figure 16. Bar diagram-showing data of different sites of TB.

5.10. Clinical features of the study cases:

Common clinical features of the study population were mainly cough, low-grade evening rise of temperature, weight loss, lymph node swelling, haemoptysis etc. as shown in Table 15 and Figure 17.

Table 15. Main clinical features of the study cases (n=115)

Clinical feature	No of cases	Percentage (%)
Cough	78	67.8
Low grade fever	105	91.3
Weight loss	85	73.9
Lymph node swelling	40	34.8
Haemoptysis	18	15.6

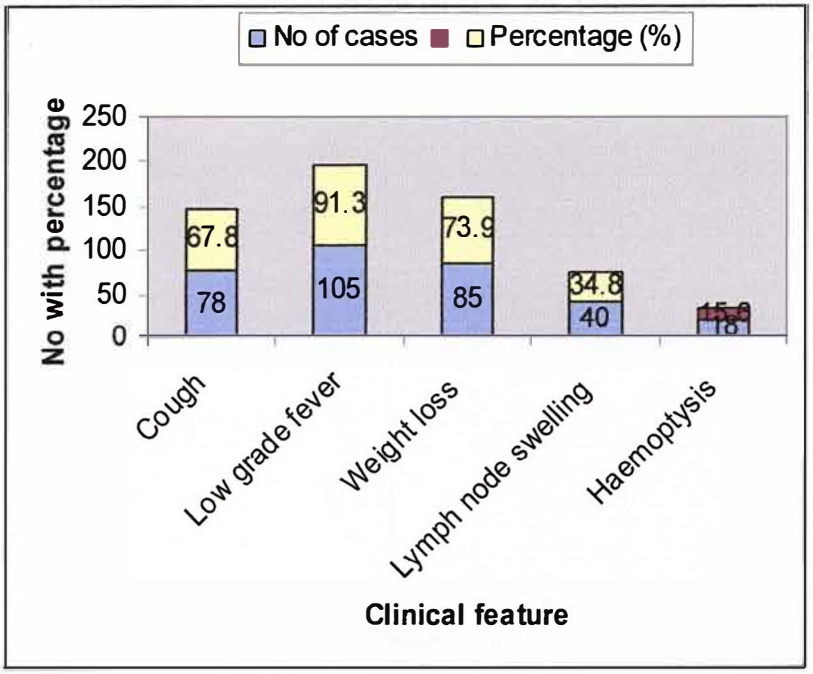


Figure 17. Bar diagram showing the clinical features of Tuberculosis.

5.11. Social statuses of the study population:

Among the 115 study cases of the present study, 49 were poor (42.6%), 52 were from middle class family (45.2%) and 14 (12.2%) rich people as shown in Table 16 and Figure 18. The study demonstrated that the incidence of tuberculosis was higher among the poor and middle class family.

Table 16. Data showing social status of the study cases (n=115)

Social status	No of subjects	Percentage (%)
Poor	49	42.6
Middle	52	45.2
Rich	14	12.2
Total	115	100

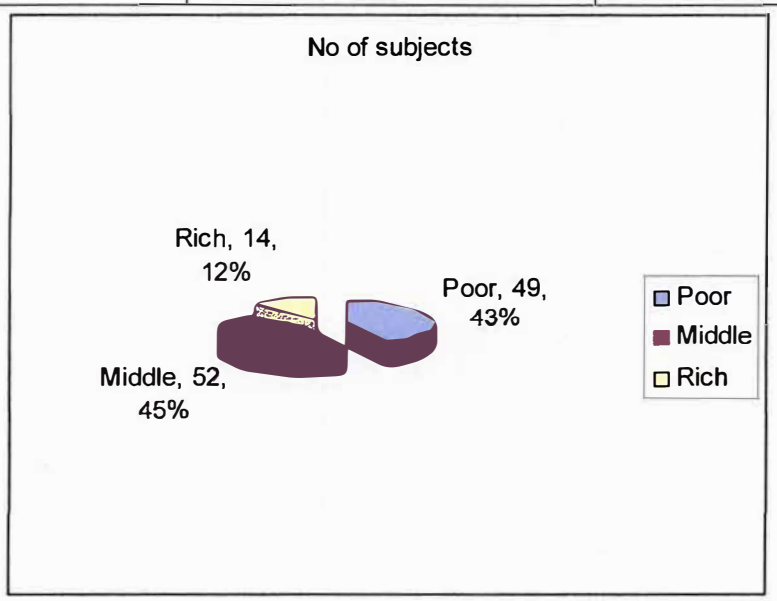


Figure 18. Pie diagram showing the social status of study population.

5. 12. Data of pulmonary tuberculosis:

Pulmonary tuberculosis was detected in 48 cases out of total 115 positive cases. Of them 42 (87.50%) cases were sputum positive and 06 (12.50%) cases were smear negative but diagnosed by cytopathological examination shown in Table 17 and Figure 19.

Table 17. Data of pulmonary tuberculosis (n= 48).

Positive or negative	No of subject	Percentage (%)
Sputum positive	42	87.5
Sputum negative	06	12.5
Total	48	100

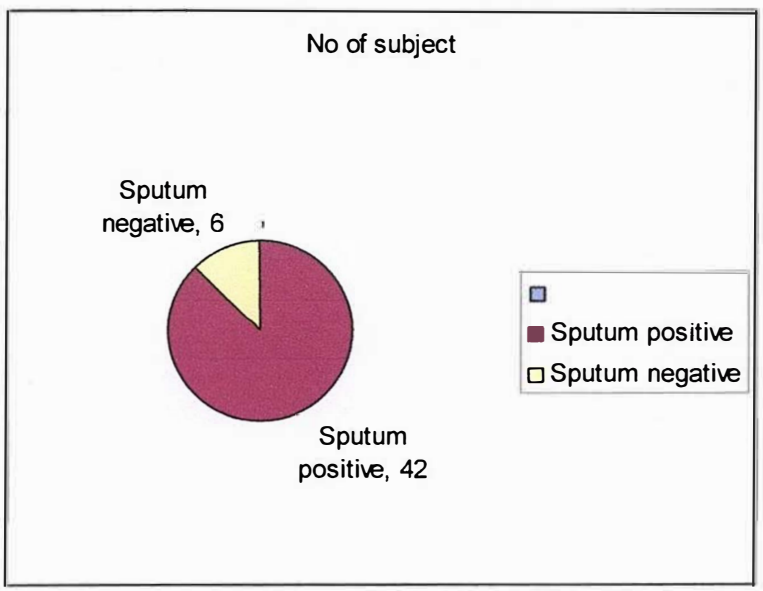


Figure 19. Pie diagram showing sputum positive and negative pulmonary TB in study group.

5.13 Data of significance of AFB findings between Direct smear microscopy and Bleach sedimentation microscopy:

Microscopic findings revealed that maximum AFB positive patients 14 (33.33%) were found as (++) followed by (+) (28.57%), (+++) (19.05%) and (++++) (2.38%) by direct smear preparation. When using Bleach sedimentation technique, 21 (50.00%) sputum positive patients were detected as (++), 14 cases detected as (+++), 4 cases detected as (++++) and only 3 sputum positive cases detected as (+) shown in Table 18.

Table 18. Data of significance of AFB findings between bleach and direct method

Method	Number of patient according to AFB positivity				Total positive
	(+)	(++)	(+++)	(++++)	
Direct	12 (28.57%)	14 (33.33%)	08 (19.05%)	01 (2.38%)	35 (83.33%)
Bleach	03 (7.14%)	21 (50.00%)	14 (33.33%)	04 (9.52%)	42 (100.0%)

5.14. T-TEST FOR DIRECT AND BLEACH METHOD:

T-test was done for sputum examination between Direct and Bleach method. For convenience of the calculation '+' was count as 1, '++' was count as 2, '+++' as 3 and '++++' as 4 and mean was calculated accordingly as shown in the bellow tables.

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair	Direct	.59	115	1.008	.094
	Bleach	.90	115	1.273	.119

Paired Samples Correlations

		N	Correlation	Sig.
Pair	Direct-Bleach	115	.896	< 0.001

Paired Samples Test: Differences between Direct and Bleach method

Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		*t*	df	*p* value
			Lower	Upper			
-.304	.580	.054	-.411	-.197	-5.627	114	< 0.001

This paired samples *t* test analysis indicates that for the 115 subjects. The mean score on the bleach method (M==1.273) was significantly greater at the p <0.001 level than mean score on the direct method. These results also indicates that a significant correlation exists between this two methods (r=0.896, p <0.001).

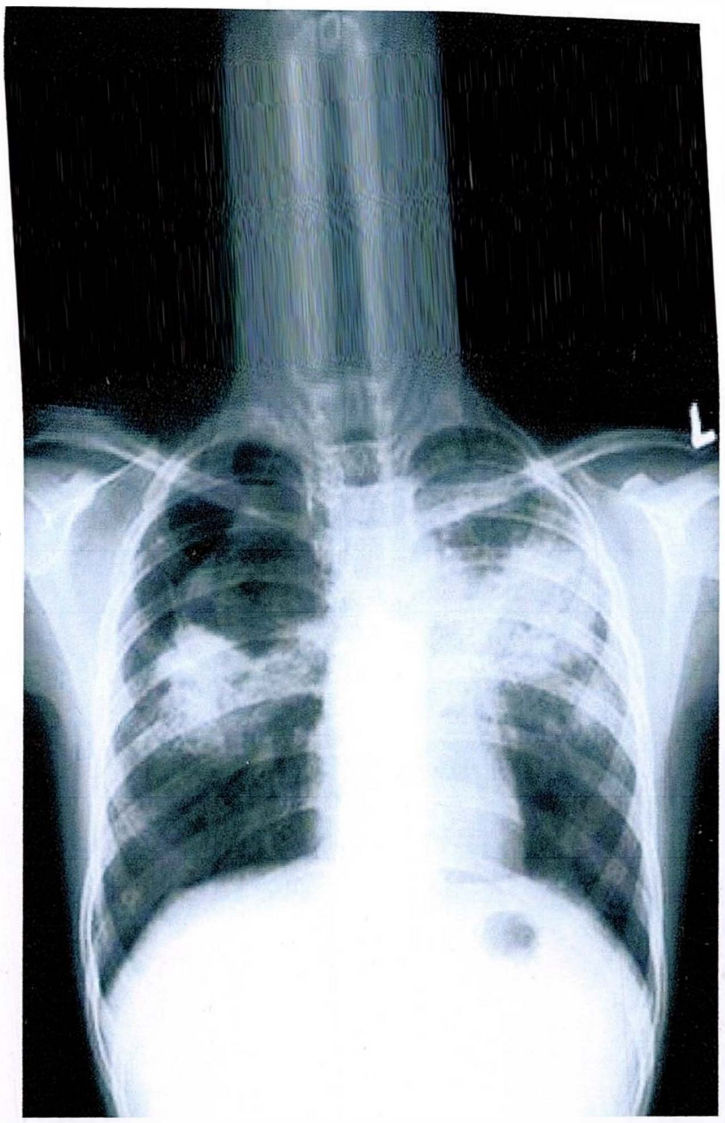


Figure 20. Chest radiograph of a pulmonary tuberculosis patient showing extensive infiltrates with evidence of cavitation in both lobes.

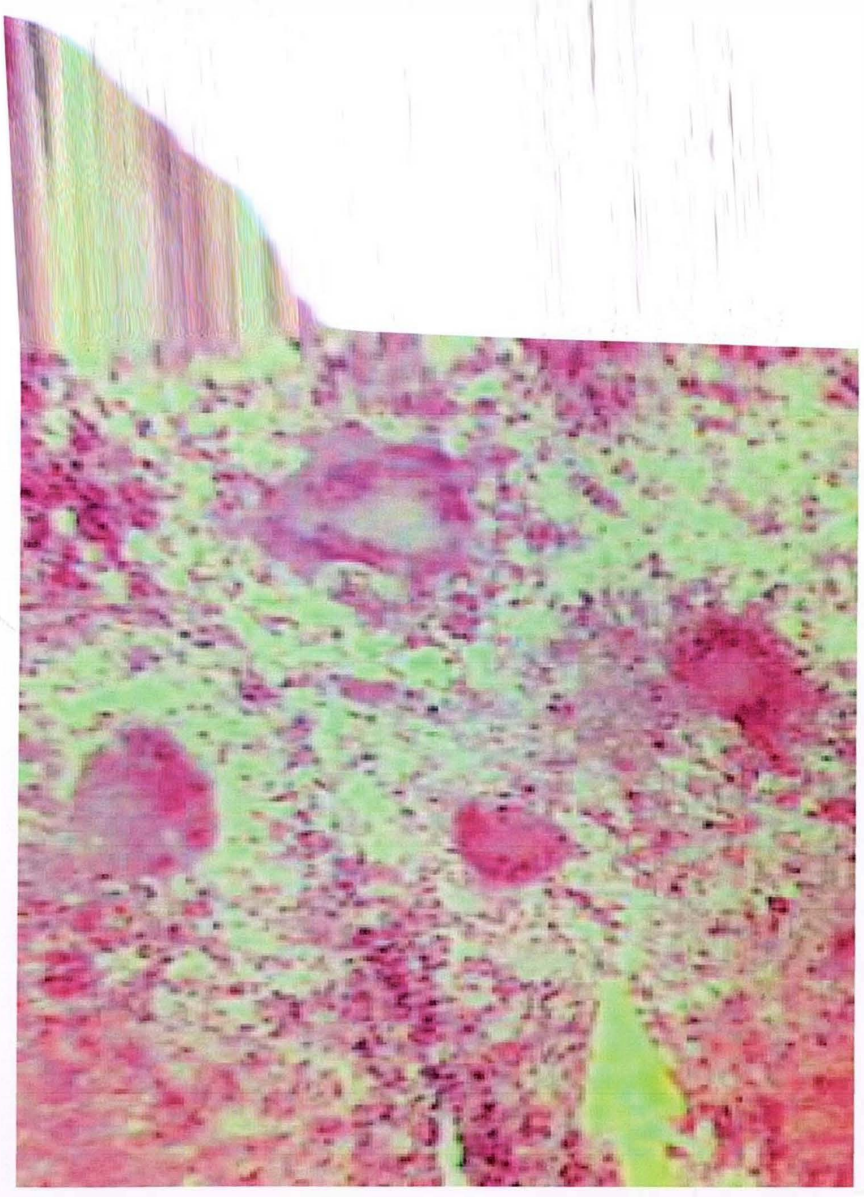
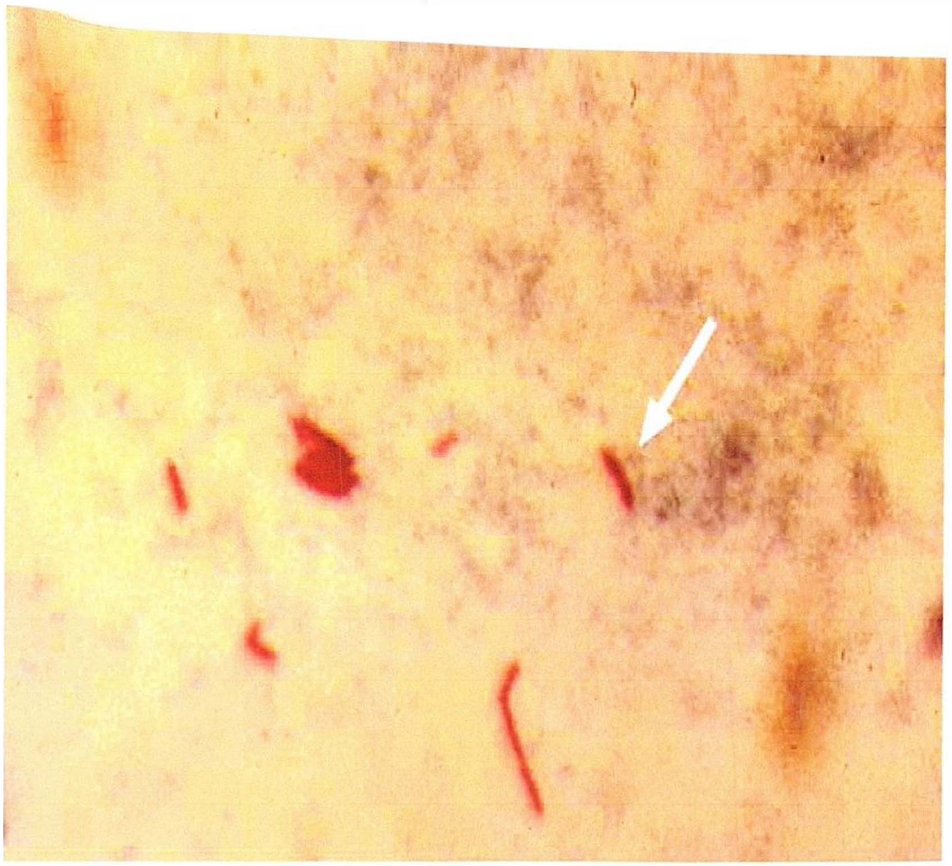


Figure 21. Photomicrograph of lymph node tissue showing Histopathological finding of tuberculosis (H & E staining). **Magnification: 10x × 40**

Staining name: Papanicolaou staining Magnification: 10x × 40

Figure 22. Photomicrograph showing Cytopathological finding of tuberculosis.



Magnification: 10x × 100

Figure 23. Ziehl-Neelsen Stain showing several characteristic red-staining acid-fast bacteria (*M. tuberculosis*) shown by arrow.

CHAPTER-6

DISCUSSION

6. DISCUSSION

Despite all advances made in the treatment and management of tuberculosis, it continues to be a major public health problem in Bangladesh as well as in other developing countries. Laboratory procedure is essential for diagnosis as well as for treatment of tuberculosis. In low-income and middle-income countries, direct (un-concentrated) sputum smear microscopy is the primary method for diagnosing pulmonary tuberculosis. The method is fast, inexpensive, and specific for *Mycobacterium tuberculosis* in high incidence areas. The main limitations of direct microscopy are its relatively low sensitivity and require the presence of 5000-10000 organisms per ml, especially in individuals co-infected with HIV, and variable quality of the test in programme conditions.

Thus, reliance on smear microscopy may cause missed or delayed tuberculosis diagnosis, potentially increasing morbidity, mortality and tuberculosis transmission. Increasing the sensitivity of tuberculosis diagnostic testing is a public health priority and there is an urgent need to identify methods to improve the sensitivity of direct microscopy. Liquefaction and concentration of sputum before Ziehl Neelsen staining improves yield and also makes examination of smears rapid and convenient. Physical and chemical sputum processing methods, including centrifugation, sedimentation, and bleach, have been studied and found to show promise.

Previous studies have shown that concentration and liquefaction of sputum significantly improves the sensitivity of direct microscopy (Corper and Nelson 1949). Digestion of sputum and concentration of bacilli by centrifugation increases the recovery rate of mycobacteria (Ratnam and March 1986). Improved recovery of mycobacteria after treatment with NaOCl might be attributable to changes in surface properties of the mycobacteria (i.e. charge and hydrophobidcity), and for denaturing of sputum

constituents leading to flocculation and subsequent increased sedimentation rate of mycobacteria (Gebre *et al* 1995).

Gebre *et al* (1995) also reported that the use of NaOCl method increased the numbers of samples positive for acid fast bacilli by more than 100%. In the study by Habeenzu *et al* (1998), the use of NaOCl was found to increase the smear sensitivity from 43.4% to 76.3% with the specificity of 100%.

The purpose of the current study was to evaluate the performance and feasibility of tuberculosis diagnosis by sputum microscopy after bleach sedimentation, compared to conventional direct smear microscopy. We have compared microscopy of smear made directly from sputum with microscopy of sputum made after liquefaction and concentration of sputum with household bleach (5.25%, NaOCl).

Of the different bleach concentrations tested in AFB detection in the present study, 5% concentration appeared to be optimum (Table-10, Results and observation section). This finding of ours are almost similar to an evaluation study of the sputum smear concentration technique for the laboratory diagnosis of pulmonary Tuberculosis by Kamga *et al* (2011) who found 5% bleach concentration to be optimum. We also observed that increasing or decreasing the bleach concentrations caused markedly reduction in the delectability of AFB which are in agreement with the findings of Rusheng Chew (2011) who also reported that changes in bleach concentrations decreased the concentration of visible acid-fast bacilli, especially in specimens with higher concentrations of acid-fast bacilli and caused occasional false-negative microscopy results. However, Githui *et al* (2007) reported that overnight sedimentation using 3.5% NaOCl significantly improved diagnoses of ZN smear negative TB and there was a significant increase of 8.7% smear positivity.

In our study, sputum specimens were collected from patients suspected of having tuberculosis and processed for detection of AFB by Direct smear microscopy and Bleach sedimentation microscopy. 35 (30.4%) patients were found to be TB positive by routine (Direct microscopy) method where as, by bleach concentration method 42 (36.5%) were found positive; diagnosing 07 additional patients. The rise of 16.7% in sputum positivity by Bleach sedimentation microscopy over direct smear microscopy was found to be statistically significant (p= <0.005). Saxena *et al* (2001) reported that sputum samples were studied by direct staining and after sodium hypochlorite treatment and centrifugation. Use of sodium hypochlorite method increased the number of positive samples from 52 to 96.

T-test (paired samples *t* test) was performed for sputum examination between Direct smear method and Bleach sedimentation method for the 115 subjects of our study. The results showed that the mean score on the bleach method (M==1.273) was significantly greater at the p <0.001 level than mean score on the direct method. These results also indicated that a significant correlation exists between this two methods (r=0.896, p <0.001).

Diagnosis of tuberculosis is often difficult specially when no acid-fast bacilli (AFB) seen in sputum and no pulmonary lesion seen radiologically. In such situations, Histopathological or cytopathological examination is helpful for diagnosis of tuberculosis. These methods are reliable and reasonably safe and have increased value in terms of diagnostic accuracy, sensitivity and specificity.

By Histopathological & cytopathological examinations, 73 (63.5%) cases had tuberculosis (Table-12, Chapter 5 and Page 37). This indicates that additional 31 positive cases were found which were missed by Direct and Bleach method. These are performed by simple fine needle aspiration (e.g. from lymph node) or excision followed by

examination of the stained slide. Therefore, we can diagnose tuberculosis patients by these methods without any special laboratory settings.

The results of findings of tuberculosis detection by the different methods in the present study clearly demonstrated that the use of Histopathological and Cytopathological examination was the most potent method in the detection of tuberculosis as the highest number of 73 (63.5%) positive cases were detected while 35 (30.4%) cases were TB positive by direct method and 42 (36.5%) cases positive by Bleach method.

In the present study, the highest prevalence of tuberculosis was noticed among patients belonging to the age group of 20- 40 years. This finding of ours is almost similar to that reported by WHO regional office for South East Asia most Tuberculosis cases occur in the age group of 15-54 years. (http://www.searo.who.int/en/Section10/Section2097/Section2100_10639.htm). However, our finding differs from a study conducted by Zaman *et al* (2007) in our country on the prevalence of smear-positive tuberculosis in persons aged ≥15 years; where prevalence of TB was found to be highest in the 55–64 years age group (201/100 000) and lowest in 15–24 years age group (43·0/100 000).

The results of sex-wise incidence of tuberculosis of our study showed that out of the total 115 subjects; 64 (55.65%) were male and 51 (44.35%) were female. The prevalence of tuberculosis therefore, was higher among males than the females i.e. males are more suffer of tuberculosis than females. This finding of ours agree in good agreement with the findings of Zaman *et al* (2007, 2008) who worked on the prevalence of sputum smear-positive tuberculosis in Matlab, a rural area in Bangladesh. In their study, they reported that population-based rate of smear-positive TB was almost six times higher in males than females.

Another study in Bangladesh conducted by Salim (2004) also reported about three times higher sputum-positive cases in males compared to females (35.4 vs. 12 . 3/100 000). The male predominance for persistent cough and for AFB-positive sputum is consistent with data from other countries and could reflect occupational, behavioural or immunological contributions to risk (Hudelson 1996, Borgdorff 2000 and Yamasaki-Nakagawa 2001). Begum (2001) in her study in Bangladesh suggested that women have less access to public health clinics, and they are less likely to undergo sputum smear examination when they present with chronic cough.

The normal Erythrocyte Sedimentation Rate (ESR or Sed-Rate) for male is 1 - 13 mm/hr and for Female 1 - 20 mm/hr. In tuberculosis very high ESR levels (up to 100+mm/h or higher) are noticed. ESR values of the Tuberculosis positive patients of the study subjects were also found to be elevated than the normal values and it ranged between 06-140 mm in 1st hour and the mean ESR being 57.09.

The results of socio-economic demography of the present study revealed that the incidence rate of tuberculosis was higher among the poor and middle class. Tuberculosis is usually considered a disease of poor and downtrodden and such association between socioeconomic status and tuberculosis was found during this study.

Chowdhary *et al* (1967) in their epldemiologlcal investigation of pulmonary tuberculosis reported that people belonging to the lower Socio-economic group were the worst sufferers. Similarly Tiwari *et al* (1969) in Mali Village (Lucknow, India) found that those belonging to social Lower middle class, poor and very poor class suffered maximum from tuberculosis.

In conclusion, it is evident from the results of the present study that after processing of sputum by bleach sedimentation technique followed by staining with Ziehl-Neelsen (ZN)

stain, 07 additional cases were found to be positive which were missed by direct microscopic method. This study suggests that digestion and liquefaction of sputum with bleach and concentration by centrifugation increases the sensitivity of direct microscopy with improved laboratory safety. Sodium hypochlorite (bleach) is easily available, cheaper and it kills *Mycobacterium tuberculosis*, making the handling of specimens safer for the laboratory staff. This method is simple and can be used in all peripheral laboratories across the country.

On the other hand, by Histopathological & cytopathological examinations 73 (including sputum positive 42 cases) cases were confirmly diagnosed of having tuberculosis although some of them were sputum negative. This indicated that Histopathological & cytopathological examinations were able to diagnose additional 31 positive cases found which could not be diagnosed by Direct and Bleach method. These are performed by simple fine needle aspiration (e.g. from lymph node) or excision followed by examination of the stained slide. Therefore, Histopathological & cytopathological examinations also play a very important role in diagnosing sputum negative tuberculosis (extra-pulmonary TB) patients without requiring any special laboratory settings

Finally, the information emerging from the present study is believed to be helpful in the diagnosis of both pulmonary and extra-pulmonary tuberculosis either sputum positive or negative.

CHAPTER-7

CONCLUSION

7. CONCLUSION AND RECOMMENDATIONS

Conclusion:

Tuberculosis is a very common disorder. This study has been carried out to evaluate (1) the usefulness of Histopathological and cytopathological examination for diagnosis of tuberculosis, especially in sputum negative patients and in extra-pulmonary cases and (2) performance of Bleach sedimentation technique to detect AFB in sputum smear by comparing it from direct microscopy.

It is evident from the results of the present study that after processing of sputum by bleach sedimentation technique followed by staining with Ziehl-Neelsen (ZN) stain, 07 additional cases were found to be positive which were missed by direct microscopic method. This study suggests that digestion and liquefaction of sputum with bleach and concentration by centrifugation increases the sensitivity of direct microscopy with improved laboratory safety. Sodium hypochlorite (bleach) is easily available, cheaper and it kills *Mycobacterium tuberculosis*, making the handling of specimens safer for the laboratory staff. This method is simple and can be used in all peripheral laboratories across the country.

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play a very important role in diagnosing sputum negative tuberculosis (extra-pulmonary TB) patients without requiring any special laboratory settings

Finally, the information emerging from the present study is believed to be helpful in the diagnosis of both pulmonary and extra-pulmonary tuberculosis either sputum positive or negative.

Recommendations:

- The NaOCl method could significantly improve the effectiveness of sputum microscopy.
- It is security for laboratories, inexpensive, widely available, and easy to use.
- It would be of great value to the national program against TB to increase the case detection rate.
- Its implementation could be considered in all field laboratories of TB units in developing countries where the culture is unavailable.

CHAPTER-8

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8. REFERENCES

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CHAPTER-9

APPENDICES

9. APPENDICES

Appendix-I

SAMPLE INFORMED PATIENTCONSENT FORM:

KHWAJA YUNUS ALI MEDICAL COLLEGE & HOSPITAL
SIRAJGONJ, BANGLADESH

RESEARCH INFORMED CONSENT FORM

TITLE OF THE RESEARCH PROJECT: **ROLE OF HISTOPATHOLOGY, CYTOPATHOLOGY AND BLEACH SEDIMENTATION TECHNIQUE IN THE DIAGNOSIS OF TUBERCULOSIS**

SUPERVISOR'S NAME: **PROFESSOR DR. PARVEZ HASSAN**

INVESTIGATOR: **MD. MAHBUR RASHID SARKER** (MBBS)

PURPOSE OF RESEARCH:

I have been explained about the reason for doing the study and selecting me as a subject of the study. This study is for the better understanding of the role of bleach concentration method over conventional direct smear microscopy for detection of tubercular bacilli in FNAC material of lymph nodes.

RISK AND DISCOMFORTS:

I understand that I may experience some pain or discomfort during my examination or during my treatment. This is mainly the result of my condition and the procedure of the study is not expected to exaggerate these feeling which are associated with the usual course of treatment.

BENEFITS:

I understand that my participation in the study will have no direct benefits to me other than potential benefit of treatment.

ALTERNATIVES:

Even if you decline the participation in the study, you will get the routine line of management.

CONFIDENTIALITY:

I understand medical information produced by this study will become part of my hospital record and will be subject to the confidentiality and privacy regulations of the said hospital.

If the data are used for publication in the medical literature for teaching purposes, no names will be used, and other identifiers, such as photographs and audio or videotapes, will be used only with my special written permission. I understand I may see the photographs and videotapes and hear the audio tapes before giving this permission. For this purpose every effort will be made by publishing person to contact me in the address furnished by me through postal communication. If no response is received within a reasonable time, all the identities will be removed from the photographs and case report before being submitted for publication.

REQUEST FOR MORE INFORMATION:

I understand that, I may ask more questions about the study at any time. Researcher is available to answer my questions or concern in this research period. I understand that I will be informed of any significant new findings discovered during the course of this study, which might influence my continued participation.

REFUSAL OR WITHDRAWL OF PARTICIPATION:

I understand that my participation is voluntary and I may refuse to participate or my withdraw consent and discontinue participation in the study at any time without prejudice to my present or future care at this hospital. I also understand that researcher may terminate my participation in the study at any time after I have been explained the reasons for doing so and has been helped to arrange for my continued care by my own physician, if this is appropriate.

INJURY STATEMENT:

I understand that in the unlikely event of injury to me resulting directly from my participation in this study. If such injury were reported promptly, then medical treatment would be available to me, but no further compensation would be provided. I understand that my agreement to participate in the study I am not waiving any of my legal rights.

I have explained to _____

(Patient/Guardian Name)

The purpose of research, procedures required the possible risk and benefits to the best of my ability.

Investigator **Date:** / /

I have been explained clearly about the reason for doing this study, reason for selecting me as a subject in the study. I also have been explained about the risks, benefits and confidentiality of the study. Alternative procedures that might be used in the treatment of my disease also explained to me. I am willing to attend any follow up requested to me at a future date. Freedom is given to me for the participation in the study or discontinues participation at any time without prejudice.

All the above explained in detail to me clearly in my own language. I am giving consent voluntarily for inclusion of me in the study as a subject.

Participant/Guardian

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(Approved in the Resolution No. 21 of the 71th meeting of the Board of Governors of the Institute of Biological Sciences and Resolution No. 57 of the 433th meeting of the Syndicate of the University of Rajshahi)

Memo No. 21/320-IAMEBBC/IBSc 07 January 2012

Certificate

This is to certify that the project title "Role of histopathology, cytopathology and bleach sedimentation technique in the diagnosis of tuberculosis" submitted by Md. Mahbur Rashid Sarker, M. Phil Fellow, Institute of Biological Sciences, University of Rajshahi has been approved by the IAMEBBC in its resolution no. 09 of the 2nd meeting held on 4th January 2012.

Name of Chairman: Prof. Dr. M. Khalequzzaman

Signature with Date

Appendix-II

PATIENT PRO FORMA

I.P. / O.P. No:

Unit: **Referred by:**

Date: **Case No:**

Personal data collection sheet

1. Serial no……Registration no……Date……..

2. Particulars of the patient:

   Name…… Age…….. Sex... (Male / Female)

   Dwelling- Urban / Rural

   Address:

   Village…… Post……...

   Thana……... District…….

   Educational status: ……..

   Occupation: ……

3. Chief Complaints:

4. Clinical Findings/ Presentation

   Fever ……... days

   Cough ……. days

   Sputum …… Yes / No

   Haemoptysis ….. Yes / No

   Chest pain …… Yes / No

   Weight loss …… Yes / No

   Others ….. Enlarged gland / Infertility

5. History of illness:

Previously suffered from/treated for TB- Yes / No

BCG vaccination- Yes / No

Any other disease: AIDS / DM / COPD etc.

6. Personal History:

Smoking- Yes / No
Drug abuse- Yes / No
Living condition- Healthy / Unhealthy
Alcoholic- Yes / No

7. Family History: Family history of TB- Yes / No

8. Present investigation:

   a. X-ray findings: Positive for TB / Negative
   b. Tuberculin test: Positive / Negative / Not done
   c. Haematological findings: ESR, TC, DC etc.

9. Sputum examination:

   a. Visual appearance: Mucopurulent / Blood stained / Saliva
   b. Sputum for AFB: Positive (+, ++, +++, ++++) / Negative

10. Histopathological and Cytopathological examination:
(FNAC, ZN Stain, Bleach Method)
Positive for TB / Negative

11. Others:

Diagnosis: **Signature**

Appendix-III

Ziehl-Neelsen method

(Monica Cheesbrough 2000)

1. Reagents

a) Ziehl-Neelsen's Carbol fuchsin stain

Fuchsin:

Basic fuchsin..... 3 gm
95% ethanol..... 100 ml
Dissolved basic fuchsin in ethanol..... (Solution-1)

Phenol:

Phenol crystals---------- 5gm
Distilled water---------- 100 ml
Dissolved phenol in distilled water--------- (Solution-2)

Working solution:

10 ml of solution-1 was combined with 90 ml of solution-2 and then stored in an amber bottle at room temperature for 6-12 month and filtered before use.

b) Decolorizing agent: (20% Sulfuric acid)

Concentrated sulfuric acid---------- 25 ml
Distilled water ---------- 100 ml

Carefully added concentrated sulfuric acid to water. Always acid is added slowly to water, not vice versa. The mixture heat up and stored in an amber bottle. The bottle was labeled with name of reagent and date of preparation and expiry and stored at room temperature for 6-12 months.

c) Counter stain: (Methylene blue)

Methylene blue chloride---------- 0.3 gm
Distilled water ----------100 ml

Methylene blue chloride was dissolved in distilled water and stored in an amber bottle. The bottle was labeled with name of reagent and date of preparation and expiry. Stored at room temperature for 6-12 months.

2. Procedure:

a) After fixation, the numbered slides were placed on a staining in batches (maximum 12). Ensured that slides did not touch each other.
b) Entire slide was flooded with carbol fuchsin stain.
c) The slides were heated slowly until steaming. Not boiled. Steaming maintained for three to five minutes by using low or intermittent heat.
d) Each slide was rinsed individually in a gentle stream of running water until all free stain was washed away.
e) The slide was flooded with the decolorizing solution (acid alcohol) for five minutes or until the smear was sufficiently decolorized, i.e. pale pink.
f) Washed well with clean water.
g) The slide was covered with counter stain (Methylene blue) for 1-2 minutes.
h) Washed off the stain with clean water.
i) Then wiped the back of the slide clean and placed it in a draining rack for the smear to air-dry.
j) Finally examined the smear microscopically, using 100 x oil immersion objective.

Appendix-IV

Histopathological method:

(John and Marilyn 2002)

a) Tissue processing techniques for thin slices

Container	Fluid	Time, minutes
1	Carnoy's fluid	45
2	100% Alcohol x 6	15 each
3	Xylene	10
4	Xylene	15 (or until cleared)
5	Wax	20
6	Wax	45

Agitation was done at each stage frequently to assist transfer of fluid.

b) Standard haematoxylin and eosin staining method for paraffin sections

1. The sections were dewaxed and hydrate through graded alcohols to water.
2. Removed fixation pigments if necessary.
3. Stained in an alum hematoxylin for a suitable time.
4. Washed well in running tap water until sections 'blue' for 5 minutes or less.
5. Differentiated in 1% acid alcohol for 5-10 sec.
6. Washed well in tap water until sections are again 'blue' (10-15 min).
7. Stained in 1% eosin Y for 10min.
8. Washed in running tap water for 1-5 min.
9. Dehydrated through alcohols, cleared and mounted.

Cytopathological method:

Papanicolaou stain (Pap's stain)

(John and Marilyn 2002)

a) Papanicolaou formula

Harris's hematoxylin

Haematoxilin---------- 5 g

Ethanol---------- 50 ml

Potassium alum---------- 100 g

Distilled water ---------- 1000 ml

Mercuric oxide ---------- 2-5 g

Glacial acetic acid ---------- 40 ml

Orange G 6

Orange G (10% aqueous) --------- 50 ml

Alcohol ---------- 950 ml

Phosphotungstic acid ---------- 0-15 g

EA 50

0.04 M light green SF ---------- 10 ml

0.3 M eosin Y ---------- 20 ml

Phosphotungstic acid ---------- 2 g

Alcohol ---------- 750 ml

Methanol ---------- 250 ml

Glacial acetic acid ---------- 20 ml

All stains filtered before use

b) Staining method

1. (a) Polyethylene glycol fixative was removed in 50% alcohol for 2 min. (b) Hydrating in 95% alcohol, 2 min, and 70% alcohol, 2 min.
2. Rinsed in water, 1 min.
3. Stained in Harris's haematoxylin, 5 min.
4. Rinsed in water, 2 min.
5. Differentiated in 0.5% aqueous hydrochloric acid, 10 seconds.
6. Rinsed in water, 2 min.
7. 'Blue' in Scott's tap water substitute, 2 min.
8. Rinsed in water, 2 min.
9. Dehydrated, 70% alcohol for 2 min.
10. Dehydrated, 95% alcohol, 2 min.
11. Dehydrated, 95% alcohol, 2 min.
12. Stained in OG 6, 2 min.
13. Rinsed in 95% alcohol, 2 min.
14. Rinsed in 95% alcohol, 2 min.
15. Stained in EA 50, 2 min.
16. Rinsed in 95% alcohol, 1 min.
17. Mounting and cover slipping.