Bacterial etiology of pleural effusion with special reference to *Mycobacterium tuberculosis*

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INRODUCTION

Pleural effusion is defined as an abnormal, excessive collection of fluid in the pleural space. Two types of effusions can develop (transudative and exudative). Transudative pleural effusions are caused by fluid leaking into the pleural space and increased hydrostatic pressure or decreased osmotic pressure. Exudative effusions are caused by blocked blood vessels, inflammation, lung injury, and drug reactions which cause damage or disruption of pleural membranes or vasculature (Bartter et al. 1994).

Etiology of the pleural efusion depends on various factors including the geographic region and age of the patient (Esther 1996). Bacterial pleural effusion is commonly found as parapneumonic pleural effusion (Koegelenberg et al. 2008). Pleural effusion occurs in 40% of patients with community-acquired pneumonia (CAP) (Flaguera and Porcel 2002). Light (2008) found that Streptococcus pneumoniae (S. pneumoniae) Streptococcus milleri group, Enterococci, Staphylococcus aureus (S. aureus), Enterobacteriaceae are responsible for parapneumonic pleural effusion (Light 2008). S. pneumoniae is the predominant cause of pneumonia worldwide and its common complication is parapneumonic effusion (Gomez-Go et al. 2012). Tuberculosis usually affects the lung and it has also extra pulmonary effects (Mathur et al. 2006). In high prevalence TB area, pleural effusion is the most common complication of pulmonary tuberculosis (Lima et al. 2003).
*Mycobacterium tuberculosis* (*M. tuberculosis*) is the causative agent of tuberculosis (Reed et al. 2009). It primarily affects the lungs, but it can also affect lymph node, bones, skin and other tissues of the body (Raja 2004). Tubercular bacilli spread by droplet infection, so it can transmit via person to person.

The tubercle bacillus (*M. tuberculosis*) is aerobic, non-motile, non-spore-forming, acid fast bacilli (Levinson 2008). It is revealed that 32% of world population was infected by *M. tuberculosis* and is the leading cause of mortality (WHO 2009). In 1993 The World Health Organization (WHO) declared as global emergency (Wang & Tay, 1999). Orcau et al. (2011) reported that in 2008 more than 9 million new cases occurred worldwide, of which more than one million died (Orcau et al. 2011). In USA 14,511 tubercular cases were found in 2004 (Hayden et al. 2006). In 2009, WHO found 32,939 TB cases in European region (Hass 2011). Reddy and Naidoo (2010) reported that South Africa is the 5th TB burden country, where annually over 2 million TB cases were found and 500,000 deaths due to tuberculosis in 2005 (Reddy & Naidoo 2009). Khan (2006) from Pakistan reported that approximately 2,68,000 new cases of infection and 64,000 deaths occur due to tuberculosis in each year (Khan 2006). WHO reported in 2008, Afghanistan, Bangladesh, Cambodia, Indonesia, Myanmar, Nigeria, Pakistan, Thailand and Uganda as high-burden TB countries (Dye et al. 2008).

Bangladesh Rural Advancement Committee (HAVARD 2011) conducted a national study in 1987-1988 which estimated that adults TB prevalence in Bangladesh was 870 per 100,000 populations. Man were almost twice as likely to have TB as women (1.08% and 0.6% respectively), and TB occurred more frequently in urban areas
(1.61%) than rural (0.8%). In 1997 another community study estimated the prevalence of TB in Bangladesh had dropped to 508 per 100,000 (620,000 cases) and approximately 68,000 individuals died from TB in Bangladesh in 1997 (Havard 2011). In 2004, ICDDR,B found incidence of all cases was (new and relapse) 227 per 100,000, incidence of smear positive was 102 per 100,000 new cases 300,000 per year and 70,000 death per year in Bangladesh (Zaman et al. 2006).

The sixteenth global report on tuberculosis (TB) in 2010 published by WHO found the Incidence, Prevalence and Mortality was 225, 411, 43 per 100,000 population respectively in Bangladesh (WHO 2011).

HIV positive patient has 500 times more chance to be infected with tuberculosis. So TB is the leading cause of death in HIV infected patient (Reddy & Naidoo 2009). In 2007 WHO reported that out of 9 million TB patient 14% patient was HIV positive (WHO 2010). In 2010 WHO reported that 2.1/6.2 million TB patients (34%) had HIV status. The highest rates of HIV co-infection in TB patients are in the African Region, with 44% and then ranked second with 17% cases (WHO 2011).

*S. pneumoniae* is a gram positive, β-hemolytic, bile soluble and capsulated bacterium. This organism was discovered by Louis Pasteur in 1881. *S. pneumoniae* causes invasive infection like septicemia, meningitis and pneumonia, and mild upper respiratory infections (Kaijalainen 2006). It is directly transmitted from contaminated respiratory secretion (Poll and Opal 2009). It is responsible for severe community acquired pneumonia worldwide and is the frequent cause of lower respiratory infection (Poll & Opal, 2009). Most of pneumococcal infection occurs in Africa and Asia (66%) (O’Brien, et al. 2009). Every year 156 million new cases of childhood pneumonia occur worldwide, among them 61 million cases and 3.1 million deaths
occur in South East Asia (Ghimire et al. 2012). In Bangladesh, incidence was 3351/100,000 (O’Brien et al. 2009). ICDDR B reported that incidence of pneumococcal disease was 86/100,000 in 2008 (ICDDR B 2008). Naheed et al. (2008) from Bangladesh reported that death rate was 4% due to pneumococcal disease (Naheed et al. 2008). WHO reported that 50,000 deaths occurred due to pneumonia in Bangladesh in 2000 (Rudan et al. 2008).

In high prevalence HIV infected area pneumococcal pneumonia is increasing with bacteremia specially in young adult (Kadioglu et al. 2006). In case of HIV infected person, extra pulmonary complication is the most common where pleural effusion is the second most (Aljohaney et al. 2012).

In laboratory the presence of an infectious agent can be detected usually by microscopy, culture and immunological test. A Gram stain and Ziehl-Neelsen (Z-N) stain of centrifuged pleural fluid are performed routinely to diagnose pleural space infection (Sahn 1982). Among microscopy methods, Ziehl-Neelsen staining is the rapid and inexpensive method. But it is less sensitive because more than 10,000 organisms must be present in one ml of collected specimen. So the sensitivity of the smear is dependent upon the number of organisms present in the sample (Lima et al. 2003). In conventional method, culture procedure for *M. tuberculosis* is very slow and it takes 4-6 weeks to cultivate. Moreover, microscopy- positive specimens sometimes fail to grow on culture media (Cousins et al. 1992). For parapneumonic effusion gram stain and culture is sometimes useful (Jadavji et al. 1997). For *S. Pneumoniae* overnight culture is done at 35°C with 5% CO₂ on 5% sheep blood agar or chocolate agar.
Among the conventional method Immuno chromatographic test (ICT) for TB is one of the simplest methods but it has low sensitivity. Another serological test for diagnosis of TB is ELISA, where antibody is detected, but its sensitivity and specificity is also very low (Bartoloni et al. 2003 and Zahrani et al. 2000). In case of tuberculin test, it is 30% negative in immune suppressed patients and 59% negative in HIV infected patients on pleural TB (Ferrer 1997). So diagnosis of tuberculosis has several limitations by conventional methods and not really helpful for extra pulmonary tuberculosis because the sensitivity and specificity of these diagnostic tests is very low (Steingart et al. 2007). So rapid and reliable diagnostic methods are needed to overcome the problem and improve the patient care (D’amato 2000).

Diagnosis of pleural TB is mainly based on biochemical, microbiological and cytological study which has limitations. Because, on cytological examination lymphocyte predominant in pleural fluid also found in other than tubercular infection (e.g. malignancy, rheumatoid pleurisy etc.) (Castro et al. 2003). In case of Parapneumonic effusion neutrophils is increased but also increases in other condition such as early tuberculosis, subphrenic abscess etc (Jadavji et al. 1997). As reported by many authors detection of activity of adenosine deaminase enzyme in tubercular pleural fluids appears to be a very sensitive and reliable method, particularly high endemic TB area like Bangladesh (Ghanei et al. 2004, Hauqe 2012). Furthermore it is not that expensive and can easily performed in chemistry analyzers (Laniado-Laborin 2005). So it is be evaluated in high endemic TB area as diagnostic tool.

Another quick method is PCR amplification (Lima et al. 2003). In this process we can detect and amplify the DNA sequence of the TB bacilli and it takes only few hours (Shah et al. 1998). In case of infectious diseases like tuberculosis a rapid and reliable
method is needed for diagnosis and treatment. Though PCR is technically demanding test but still is useful in reference centers (Lima et al. 2003 and Richard 1995).

Having the described background, the present study was carried out to evaluate the efficacy of PCR technique and detection of ADA level in pleural fluid, in the context of Bangladesh in comparison with the conventional methods for laboratory diagnosis of pleural effusion.

**General objective**

To identify bacterial etiology of pleural effusion with special emphasis on *M. tuberculosis*.

**Specific objectives**

1. To diagnose bacterial pleural effusion by cytological, biochemical and bacteriological tests of pleural fluid.
2. To diagnose tubercular pleural effusion by detection of ADA activity from pleural fluid.
3. To evaluate Polymerase Chain Reaction (PCR) for detection of *M. tuberculosis and S. pneumoniae* from pleural fluid.
4. To compare the efficacy and utility PCR and ADA activity for the rapid diagnosis of *M. tuberculosis* from pleural effusion.
Chapter-2

Review of literatures
REVIEW OF LITERATURE

PLEURAL EFFUSION

Definition and classification

The accumulation of serous fluid within the pleural space is termed as pleural effusion. In general, pleural fluid accumulates as a result of either increased hydrostatic pressure or decreased osmotic pressure or from increased micro vascular pressure due to disease of the pleural surface itself, or injury in the adjacent lung. Pleural effusion develop as a result of either transudation or exudation of the fluid on the surface of the pleura, a pleural effusion is a clinical sign of a systemic or pleural disorder. It impairs the lung function in a mechanic way, preventing the lungs to expand and resulting in dyspnea. Chronic presence of pleural effusion induces atelectasis and entrapment of the lungs, as well as intercurrent infections (Davidson 2002 and Koledin, et al. 2001).

A parapneumonic pleural effusion refers to any effusion secondary to pneumonia or lung abscess. Tubercular pleural effusion is one of the common complications of
primary tuberculosis or in conjunction with pulmonary infiltrate typical of post primary tuberculosis (Soe et al. 2010). They are two types, transudative and exudative. A transudative pleural effusion occurs when pleural fluid accumulates because of an imbalance between the hydrostatic and oncotic pressures. The leading causes of transudative pleural effusions are congestive heart failure, cirrhosis of liver and pulmonary embolism. In contrast, an exudative pleural effusion occurs when the local factors influencing the accumulation of pleural fluid are altered. The leading causes of exudative effusions are pneumonia, cancer, and pulmonary embolism (Davidson 2002 and Light 2008).

**Pleural physiology**

The pleura encloses the chest cavity and is derived from the primitive mesoderm. The thoracic cage has two separate individual pleural cavities which are also separated from the pericardial cavity. This arrangement allows flexibility as the organs grow or change in shape and form within the limited space of the thoracic cage. The pleural cavity is a closed space between the visceral and parietal pleura. Both the parietal and the visceral pleura are similar in mesodermal origin and have minor differences in anatomical structure. The pleura extends into the interlobar fissure and may, during inspiration, fill the pleural cavity to form recesses on the surface of the lung. The pleura is essential for the efficient functioning of the lung. It provides protection and allows for a smooth, lubricating, elastic surface for movement of the lung bellows during inspiration and expiration. The most striking finding on examination of the pleural surface via microscopy are the number of microvilli. The microvilli are 0.1 mM in diameter and up to 0.3 mM in length. They exist singly, but often group
together. The density of the microvilli ranges between 100–600 per 100 square microns. There is a higher density of microvilli on the visceral surface than on the parietal surface. The normal amount of pleural fluid is 0.5–1 ml and contains 1–2 g of protein per 100 ml. There are 15-45 cells/cumm of pleural fluid. These are mononuclear cells with occasional lymphocytes. The mononuclear cells are mostly macrophage like. The pleura is a monolayer of mesothelial cells, but also has several layers below the monolayer that include: 1) the basal lamina; 2) an elastic layer; 3) a loose connective tissue layer; 4) a deeper fibro-elastic layer. The nerves, vessels, and lymphatics lie in the loose connective tissue layer. Thus, in disease states where there is inflammation of the pleura, cells have to traverse through the connective tissue layer into the basal lamina and out through the mesothelial surface into the pleural space. There are some well-documented communications between the pleural cavity and the lymphatics. These include stomas, membrane cribriformis, and lacunae (Antony 2003).

**Epidemiology of pleural effusion**

Pleural effusions (PE) can occur as a complication of various diseases. The distribution of etiology of pleural effusion is also changing from a country to another, and from developed to developing countries, according to presence or absence of tuberculosis as an endemic disease in some countries, and also presence or absence of AIDS. Etiological distribution of bacterial pleural effusion depends on geographic region, patient age, and advances in the diagnosis and treatment of the underlying causes (Light 2008, Vaides et al. 1996 and Farghaly et al. 2007).

**Etiology of pleural effusion**
The leading causes of transudative pleural effusions are congestive heart failure, cirrhosis of liver, nephrotic syndrome and pulmonary embolism. Exudative pleural effusion occurs due to bacterial diseases (pneumonia, tuberculosis), viral diseases, fungal diseases, malignancy, drug reactions etc. (Light 2008 and Sahn 1982).

**Causes of pleural effusion** (Medford and Maskell 2005)

<table>
<thead>
<tr>
<th>Transudative</th>
<th>Exudative</th>
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<tbody>
<tr>
<td>Common:</td>
<td>Common:</td>
</tr>
<tr>
<td>Left ventricular failure</td>
<td>Malignancy</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>Parapneumonic effusions</td>
</tr>
<tr>
<td>Hypo-albuminemia</td>
<td>Tubercular pleural effusion</td>
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<tr>
<td>Peritoneal dialysis</td>
<td></td>
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<td>Less common:</td>
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<tr>
<td>Hypothyroidism</td>
<td>Pulmonary infarction</td>
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<tr>
<td>Nephrotic syndrome</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>Mitral stenosis, Pancreatitis</td>
<td>Autoimmune diseases</td>
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<tr>
<td>Pulmonary embolism</td>
<td>Benign asbestos effusion</td>
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<tr>
<td></td>
<td>Pancreatitis</td>
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<td></td>
<td>Pulmonary embolism Post-myocardial infarction syndrome</td>
</tr>
</tbody>
</table>

**Bacterial etiology of pleural effusion**

Parapneumonic pleural effusion are more common in bacterial pleural effusion. Forty percent of pneumonic patients developed pleural effusion. *Mycobacterium tuberculosis* is also responsible for bacterial pleural effusion. In case of tuberculosis (TB) 30% of infected patients developed pleural effusion. Especially overall currently gram positive (*Streptococcus pneumoniae*, *Streptococcus milleri* group, *Staphylococcus aureus* and *Enterococci*) are the most abundant cause and gram negative aerobes (*Escherichia coli*, *Pseudomonas spp.*, *Haemophilus influenzae*, and
Klebsiella spp.) are also responsible. Anaerobes are on the increase (12%–34% of positive fluid culture, 14% alone without aerobes) presenting insidiously, with less fever, greater weight loss, often after aspiration pneumonia or with poor dental hygiene (Kawanami et al. 2010, Koegelenberg, Diacon, & Bolliger, 2008, Light, 2010, Hasaneen et al. 2003 and Medford and Maskell 2005).

### Causes of bacterial pleural effuison (Medford and Maskell, 2005)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumoniae</td>
<td>30–60</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>4–20</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>12–35</td>
</tr>
<tr>
<td>Mycobacterium tularensis</td>
<td>13–64</td>
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### Pathogenesis tubercular pleural effusion

Traditionally, pleural TB has been considered a manifestation of primary TB. Mycobacterial antigens enter the pleural space and interact with T-cells previously sensitized to mycobacterium, resulting in a delayed hypersensitivity reaction and accumulation of fluid. Lymphocytes are the predominant cell type, amounting to more than 70% of the total white cell count, and these cells are mainly of the CD4+ phenotype. So tubercular pleural effusion occurs due to delayed hypersensitivity when tubercular protein access on pleural space. Protein level increased in tubercular pleural effusion due to increase permeability of capillaries by delayed hypersensitivity
reaction. Tubercular pleural effusion occurs after 3-6 month of primary infection due to rupture of ghon focus. Because of this rupture mycobacterial protein acceess on pleural space and causes delayed hypersensitivity. When the delayed hypersensitivity reaction increase the permeability of the pleural capillaries to protein and then the increased protein levels in the pleural fluid result in a much higher rate of pleural fluid formation. In addition, the lymphocytic pleuritis obstructs the lymphatics in the parietal pleura, which leads to decreased pleural fluid clearance from the pleural space. The pleural effusion results from the combination of the increased pleural fluid formation and the decreased pleural fluid removal. CD4+ lymphocytes obtained from tuberculous pleurisy and stimulated in situ or ex vivo with mycobacterial antigens produce significant amounts of IFN-γ. IFN-γ and other cytokines may, in part, be produced by γ or δ T-lymphocytes, which are closely linked to antimycobacterial immunity. The fundamental role of IFN-γ in tuberculous pleural effusions is further supported by the demonstration of high IFN-γ levels in tuberculous pleural effusion. Adenosine deaminase (ADA) and IFN-γ concentrations have been demonstrated to be useful in narrowing the diagnosis since, in malignant and parapneumonic effusions, empyema and transudates, only low values of this IFN-γ can be detected. In addition to IFN-γ, TNF-α, transforming growth factor-β (TGF-β), IL-1β, IL-2, IL-2 receptor (IL-2) and IL-1Ra have been found to be raised. While TNF-β and IFN-γ may participate in the host immune response caused by mycobacterium and may mediate the clinical manifestation of tuberculous pleuritis, such as fever, exudative pleural effusion, and tissue necrosis, TGF-β has been reported to be involved in tissue repair processes in the course of tuberculous inflammation (Light 2010, Chakrabarti & Davies 2006, Seibert Jr, Middleton & Jr 1991 and Kroegel and Antony 1997).
**Tubercular pleural fluid characteristics**

The pleural fluid with tuberculous pleuritis is invariably an exudate. Indeed, the pleural fluid protein level frequently exceeds 5 g/dl and this finding suggests tuberculous pleuritis. Most patients with tuberculous pleuritis have more than 50% small lymphocytes in their pleural fluid and many have more than 90%. The pleural fluid glucose level with tuberculous pleural effusions may be reduced but it usually is similar to the serum level. The pleural fluid pH is usually above 7.30, but it also may be reduced. The pleural fluid lactic acid dehydrogenase (LDH) level is usually higher than the serum LDH level (Light 2010).

**Diagnostic procedures of bacterial pleural effusion**

Once pleural effusion is diagnosed, patient should have many diagnostic procedures needed to reach the etiology of this effusion. For the diagnosis physical, cytological, biochemical, microbiological including immunological and molecular technique is done. All of these tests do not need to be done on all fluids (Farghaly et al. and Sahn 1982).

**Physical examination**

Color, odor and character of the fluid should be noted. The gross appearance of the pleural fluid provides useful information. A bloody appearance of the pleural fluid narrows the differential diagnosis. Turbidity of the pleural fluid can be caused either by cells and debris or by a high lipid level. The odor of the pleural fluid also provides useful information. A putrid odor indicates that the patient probably has an infection due to anaerobic bacteria (Light 2010).

**Cytological examination**
In the exudative pleural fluid leukocyte count is more than 100/cmm of pleural fluid. Conversely, leukocyte counts less than 1,00/cmm of pleural fluid in a transudative one. A predominance of neutrophils in the pleural fluid (more than 50 percent of the cells) indicates an acute process of infection of the pleura. A predominance of lymphocytes indicates a chronic process. The tubercular pleural fluid is usually predominantly lymphocytic. Lymphocytic pleural effusions also indicate malignancies, collagen vascular disease, chylothorax and post-coronary artery bypass graft (CABG) pleural effusion (Sahn 1982 and Light 2010).

Biochemical tests

The first step in the evaluation is to determine whether an effusion is transudative or exudative. If it is exudative, more diagnostic tests are required in order to determine the cause of the local disease by measurement of glucose, protein, lactate dehydrogenase levels, and testing for a pleural-fluid marker of tuberculosis (Light, 2010 and Light 2002).

a) Glucose level

A normal pleural fluid glucose value (more than 60 mg/dl, or a pleural fluid-to-serum ratio of over 0.5) is not particularly helpful; however, a low pleural fluid glucose level (less than 60 mg/dl or a pleural fluid-to-serum ratio of under 0.5) will help narrowing the differential diagnosis of the exudative pleural effusion. The mechanisms responsible for a low pleural fluid glucose level appear to be a combination of enhanced glycolysis by either pleural fluid cells, bacteria or pleural tissue in conjunction with an impairment to transport of glucose from blood to pleural fluid. When pleural fluid is collected for glucose analysis, fluid must either be frozen or
stored with a substance such as sodium fluoride that prevents in vitro glycolysis (Sahn 1982).

b) Protein and lactate dehydrogenase (LDH) level

When inflammation affects the pleura, proteins leak from the pleural capillaries into the pleural space; thus, it would be expected that total protein and LDH levels would increase. If any one of the three following criteria is present (According to Light’s criteria) the fluid is usually an exudate: (1) a pleural fluid to serum protein (more than 30 gm/L in exudative effusion) ratio of over 0.5, (2) a pleural fluid LDH level of more than 200 IU and (3) a pleural fluid-to serum LDH ratio of over 0.6 (Sahn 1982).

c) Adenosine deaminase (ADA)

The diagnosis of pleural TB continues to be a challenge in clinical practice. Traditional diagnostic methods are very useful for the diagnosis of pulmonary TB but have a low yield when applied to pleural fluid (Kaisemann et al. 2004). The analysis of pleural fluid adenosine deaminase (ADA) levels in the diagnosis of tuberculous pleural effusion (TPE) was discovered in 1978 (David et al. 2010). After that ADA activity was found to be high in tuberculous pleural exudates. Now-a-days it has been used in the diagnosis of tuberculous pleural effusions. In short adenosine deaminase (ADA) is an enzyme in the purine salvage pathway that catalyses the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine with the release of ammonia and ADA is found in most cells but plays an important role in the differentiation of lymphoid cells (in particular, active T-lymphocytes). The generic designation ADA is given to a group of enzymes of different molecular weights that have similar chemical functions in the purine metabolism, catalyzing the conversion of adenosine and deoxyadenosine into inosine and deoxyinosine. These ADAs are present in almost all invertebrates, and, in humans. It specially produced during the inflammatory process triggered by the M. tuberculosis. ADA has two principal isoenzymes, ADA-1 and
ADA-2, which have different optimal pH, Michaelis constants and relative substrate specificity patterns. ADA-1 has roughly equal affinities for adenosine and 2'-deoxyadenosine, with a 2'-deoxyadenosine deaminase/ADA activity ratio of approximately 0.75; it is found in many tissues. ADA-2 has much greater affinity for adenosine (2'-deoxyadenosine deaminase/ADA activity ratio approximately 0.25), and is found only in macro-phages, which release it when stimulated by the presence of live micro-organisms in their interior (David song et al. 2010, Kaisemann et al. 2004 and Haque 2012).

Usefulness of adenosine deaminase (ADA) estimation in pleural fluid has been shown as a reliable chemical bio-marker specially when there is suspicion of tuberculosis in endemic areas (Haque 2012). Valdes et al. (1995) reported its sensitivity in diagnosis of pleural TB has been 99% and its specificity is 93%. So Population with a high prevalence of TB, the measurement of adenosine deaminase (ADA) activity in pleural fluid, has been proven to be sensitive and specific for diagnosis of pleural TB (Gandhi et al 2006). In case of tubercular pleural effusion Brazilian Society of Pulmonology and Phthisiology recommends a value more than 40 units per liter (U/L) (David song et al. 2010, Kaisemann et al. 2004 and Haque 2012).

**MICROBIOLOGICAL STUDY**

**Staining of pleural fluid**

A Gram stain and Ziehl-Neelsen (Z-N) stain of centrifuged pleural fluid are performed routinely for diagnosing pleural space infection (Sahn 1982).

**Culture of pleural fluid**

a) **Culture for Streptococcus pneumoniae**

After overnight incubation at 35°C with 5% CO₂ on 5% sheep blood agar or chocolate agar, *S. pneumoniae* colonies appear to be small, grayish, mucoid and are surrounded
by a greenish zone of α-hemolysis. The colonies become centrally depressed ("draughtsman" colonies) after 24–48 hours of incubation (Lund 1960).

b) Culture for *Mycobacterium tuberculosis*

There are three types of culture media: solid media, which includes egg-based media (Lowenstein-Jensen), agar-based media (Middlebrook 7H10 and 7H11), and liquid media (Middlebrook 7H12 and other broths) (Brodie & Schluger 2005). Even with the advent of technological advances such as the BACTEC system (Becton Dickinson Diagnostic Instruments Systems, Cockeysville, Md.) and MB-Chek AFB system (Becton Dickinson Microbiology Systems) and nucleic acid probes. They are considered to be the “gold standard” against which all other methods are measured. Solid media that long the standard for culturing *Mycobacterium* are slower than liquid media. Liquid media are now widely used alongside solid media to increase sensitivity and decrease recovery time. BACTEC medium is the broth medium, here mycobacterium is cultured by radiometric method (D’amato et al. 1995 and Brodie & Schluger 2005).

**SEROLOGICAL TEST OF PLEURAL FLUID**

a) Quellung reaction

The quellung reaction is a more specific method for Pneumococcal capsule detection from pure cultures or sputum samples. After reaction with Pneumococcal anticapsular antisera, the capsule becomes visually enhanced, and the bacterial cell appears to be surrounded by a halo (Figure-1). Although the quellung reaction is generally regarded as being highly specific for Pneumococcus, cross reactions have been reported with other Streptococcal polysaccharides, and noncapsulated strains will produce false-negative results (Werno & Murdoch 2008).
b) C-Reactive protein (CRP)

C-Reactive protein (CRP) is an acute phase protein, synthesized by the liver in response to various stimuli. The induction of CRP synthesis is triggered by a number of cytokines that are released into the inflammatory region, chiefly the pyrogenic cytokine interleukin-6 (IL-6). Fibroblasts, lymphocytes, pro-myelocytes and active macrophages are sources of IL-6. CRP levels are higher in inflammatory pleural effusions than in other types of effusion (Requejo & Cocoza 2003). CRP functions as a pattern-recognition receptor for *S. pneumoniae*. It binds with phosphorylcholine in the pneumococcal cell wall and activates complement. In animal models, human CRP protect against lethal infection with *S. pneumoniae* infection, and in man probably contributes to host defense during bacteremic pneumonia. The basic principle of the test is that when the fluid sample is mixed with the antiserum solution, the CRP reacts specifically with anti-human CRP antibodies to yield insoluble aggregates (Mitchell & Mitchell 2010 and Requejo & Cocoza 2003).

c) Immunochromatographic test (ICT)

The recent development of a rapid immunochromatographic test (ICT) that detects the C polysaccharide cell wall antigen common to all strains of *S. pneumoniae* (NOW *S. pneumonia* urinary antigen test; Binax) has renewed interest in antigen detection. This test has been the only major advancement in pneumococcal diagnosis over recent years (Werno & Murdoch 2008).

d) Latex agglutination test (LAT)

Latex agglutination tests are widely used methods for identification and typing of many clinical important microorganisms. Earlier latex methods have been used for serotyping of Pneumococci, using different combinations of Pneumococcal antisera
for research purposes. Reading of agglutination was taken by mixing 40 µl of boiled body fluid and one drop of test latex on the specified circle of the supplied paper slide and then mixed on a shaker for 3 minutes. The result of LAT was considered positive when agglutination occurred (Alam et al. 2007).

e) ELISA (Enzyme linked immune sorbent assay) for *Mycobacterium tuberculosis*

A quantitative test to detect lipoarabinomannan (LAM) has been developed for the detection of TB in urine specimens. Another test being used in a field trial is the dipstick method (semi-quantitative) for the detection of LAM in both pulmonary and extra-pulmonary specimens (Ramachandran & Paramasivan 2003).

**Special tests of *Mycobacterium tuberculosis***

T cells releasing Interferon-γ (INF-γ) in response to previously encountered mycobacterial antigens. In recent years in-vitro T cell–based IFN-γ release assays (IGRAs) has been developed. These assays use antigens more specific to *Mycobacterium tuberculosis* than the PPD (Purified protein derivatives). Early secretory antigenic target 6 (ESAT-6), and culture filtrate protein 10 (CFP-10) are specific methods for identifying *M. tuberculosis* infection. Several studies have shown that ESAT-6 and CFP-10 are immunodominant T-cell-stimulatory antigens that induce peripheral blood mononuclear cells (PBMC) from TB patients to produce high levels of IFN-γ, a typical Th1 (T helper cell) cytokine produced by T-cells and NK (Natural killer cell) cells. Detection of IFN-γ or IFN-γ-producing cells after antigen stimulation is frequently used as an indicator of cellular effector activity. IFN-γ has a central role in several immunological functions in tuberculosis, including macrophage-stimulated chemokine production. Through their ability to recruit
discrete leukocyte populations, chemokines may enhance antigen specific immune responses (Wang et al 2007 and Pai & Menzies 2007 and Abramo et al. 2006).

**PCR for Mycobacterium tuberculosis**

In microbiology, DNA amplification using PCR has allowed great progress to be made in the rapid and accurate diagnosis of infections due to organisms that are not cultivable by in-vitro means. It requires complex media or cell cultures and prolonged incubation times, or for which culture is too insensitive. Amplification techniques for the diagnosis of tuberculosis have attracted considerable interest, particularly with the hope of shortening the time required to detect and identify *Mycobacterium tuberculosis* in respiratory and non-respiratory specimens. Despite numerous reports in the literature, amplification techniques do not yet have an established role in the laboratory for tuberculosis diagnosis, nor have they replaced traditional techniques, in contrast to diagnostic modalities for other pathogens, like *Chlamydia* or *Mycoplasma* (Honore et al. 2003).

The polymerase chain reaction (PCR) can specifically amplify discrete fragments of DNA in which target material is present in only picogram quantities. It is now a well-developed technique and has been used extensively for the diagnosis of numerous infectious diseases. The major benefits of this rapid diagnostic test are improved patient care, reduced medical costs (Nagesh et al. 2001). Diagnostic assays based on nucleic acid amplification methods such as polymerase chain reaction (PCR) dramatically decreased the time required to identify an organism in clinical specimens. This method has been tested extensively for the detection of *M tuberculosis* in sputum specimens, cerebrospinal fluid, pericardial fluid and biopsy specimens. PCR has also been used to detect *M tuberculosis* in pleural fluid samples with highly variable sensitivity (11 to 81%) (Hasaneen et al. 2003).
*Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* was discovered by Robert Koch on 24th March 1882 (Al-shamah et al. 2003).

**TAXONOMY AND DESCRIPTION OF THE GENUS**

Family is Mycobacteriaceae, genus is *Mycobacterium*, species is *M. tuberculosis* and other species of Mycobacterium are *M. bovis, M. africanum, M. microti, M. canettii, M. caprae, M. pinnipedi*. (Wikipedia 2012)

**Morphology and characteristics**

The tubercle bacillus (*M. tuberculosis*) is aerobic, non-motile, non-spore-forming, high in lipid content, acid and alcohol-fast organism. Cell wall of mycobacterium is chemically very complex and unlike of gram positive or gram negative organism. Here, lipid accounts for approximately 60% of dry weight of the cell wall. The cell wall is composed of two polymers (peptidoglycan & arabinogalactan) conveniently linked by phosphodeister bonds. Peptidoglycan (PG) is covalent attached to arabinogalactan (AG), which in turn is attached to the mycolic acids with their long meromycolate and short α-chains. Mycobacterial PG forms the backbone of the mAGP (mycolyl arabinogalactan–peptidoglycan) complex, composed of alternating N-acetylglucosamine (GlcNAc) and modified muramic acid (Mur) residues, linked in a β(1→4) configuration. Arabinogalactan(AG), the unique heteropolysaccharide, is covalently tethered to the PG via a phosphodeister bond to approximately 10-12% of the muramic acid residues of PG. Collectively, PG and AG, form a covalently linked network positioned between the plasma membrane and the mycolic acid layer, resulting in an exceptionally cell wall. Glycolipids also bound to the Peptidoglycan,
other important lipid substance present on the cell surface are cord factor, sulfatides and mycosides (Jolkik et al. 1992). On Loewenstein-Jensen (LJ) medium colonies are dry, wrinkled and tenacious with buffy colored. Time taken for growth is 4-6 weeks. On liquid media twisted rope like colonies are found and time taken 10-17 days (Brennan 2003, Waren Levinson 2008, Jolkik et al 1992 and Birch 2011 and Kubica GP 1973).

**Virulence factors**

Pathogenic mycobacteria are facultative intracellular and they have ability to survive within the macrophage. Virulence factor is needed for their intracellular survival. Usually bacteria are rapidly degraded within phagolysosomes but pathogenic mycobacterium have evolved to block lysosomal delivery and they active their protein kinase by their lysosomal maturation (Bartos, Falkinham, & Pavlik, 2004 and Scherr, et al. 2007).

**a) Catalase-peroxidases and superoxide dismutases**

After phagocytized mycobacteria release toxic forms of oxygen including H$_2$O$_2$, superoxide, HOCl, OH and singlet oxygen. H$_2$O$_2$ is linked to virulence factor of *Mycobacterium tuberculosis* because low virulence isolates were shown to be more susceptible to H$_2$O$_2$. The catalase peroxidase activities of *M. tuberculosis, M. bovis*, BCG and *M. avium* were the only agents resulting in the destruction of H$_2$O$_2$. Therefore, it is likely that protection against other reactive oxygen (ROI) and nitrogen (RNI) intermediates is mediated by additional enzymes. *Mycobacteria* are resistance to reactive nitrogen (RN1) because of catalase and peroxidase. RN1 is bactericidal agent. Because of this resistance mycobacteria are not destroy within the alveolar macrophage (Bartos et al. 2004).
b) Cord factor and trehalose-6,6’-dimycolate (TDM)

Gardner Middlebrook was first reported the cord factor in 1947. Later Hubert Bloch observed the formation of serpentine cords was due to a lipid on the surface when it dissolve in oil surface. After that he extracted viable organisms with gentle organic solvents and recovered a glycolipid from their surface called ‘cord factor,’ because its removal caused disruption of cords. This cord factor was later identified as trehalose-6,6’-dimycolate (TDM).

TDM is structurally lipid and produced by all *mycobacterium* species. It is non-toxic and it protects the organism from host defense. Here its quantity and mycolic acid structure make the difference from non virulent *mycobacterium*. TDM prevent lysosome/phagosome fusion, also prevent acidification, on this way it protects the organism from killing by macrophage. Recent data suggest that TDM on MTB also impedes antigen presentation, probably as a result of altered trafficking within phagosomes (Hunter et al. 2006).

c) Phosphatidylinositol 3-phosphate and glycolipid

The mycobacterium has unique cell wall from others bacteria. Its thick cell wall is made up of unique lipid and glycolipid moieties. Glycolipids of *M. tuberculosis* can interfere with phagosome lysosome fusion through blocking a normal host trafficking event that is regulated by phosphatidylinositol 3-phosphate (PI3P). *M. tuberculosis* interferes with the activity of the PI3 kinase hVP34, thereby preventing the generation of PI3P on the phagosomal membrane and blocking phagosome-lysosome fusion.
This inhibitory activity was proposed to be mediated by the mycobacterial cell-wall component lipoarabinomannan (LAM) (Pieters, 2008).

Fig - MTB can resistant the hydrolysis of lysozyme

**d) Sulfolipids**

Sulfolipid-1 (SL-1), the most abundant sulfatide, is a tetra-acylated trehalose-based glycolipid located on the mycobacterial outer membrane of MTB. Provocatively, SL-1 is uniquely expressed in pathogenic mycobacteria, and its amounts have been positively correlated with strain virulence. The linkage between SL-1 and MTB virulence inspired a search for functions of SL-1, which over the span of 50 years has resulted in attribution of numerous, sometimes conflicting, biological activities to this sulfatide. For example, in cell culture models purified SL-1 has been proposed to alter phagosome lysosome fusion, disrupt mitochondrial oxidative phosphorylation, and activate as well as suppress the production of cytokines and reactive oxygen species produced by human leukocytes (Gilmore, et al. 2012).

**EPIDEMIOLOGY OF TUBERCULOSIS**

**Worldwide incidence**
More than 2 billion people (about one-third of the world population) are estimated to be infected with Mycobacterium tuberculosis. The global incidence of tuberculosis (TB) peaked around 2003 and appears to be declining slowly. According to the World Health Organization (WHO), in 2010, 8.8 million individuals became ill with TB and 1.4 million died (Lönnroth K and Raviglione M 2008, WHO 2008, WHO 2012). TB remains a major global health problem. In 1993, the World Health Organization (WHO) declared, TB a global public health emergency, at a time when an estimated 7–8 million cases and 1.3–1.6 million deaths occurred each year (WHO 2004).

Sixteenth global report on tuberculosis (TB) published in 2010 by WHO in a series that started in 1997. It provides a comprehensive and up-to-date assessment of the TB epidemic and progress which is implementing and financing TB prevention, care and control at global, regional and country levels using data reported by 198 countries (it accounts for over 99% of the world’s TB cases). In 2010, there were 8.8 million (range, 8.5–9.2 million) cases of TB infection, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB. HIV testing among TB patients reached 34% globally of which 59% in the African Region and 75% in 68 countries (WHO 2011).

**TB in South-East Asia region**

WHO South-East Asia (SEA) region has an estimated 5 million prevalent and about 3.3 million incident cases of tuberculosis annually. It carries more than one third of the global burden of tuberculosis. 5 of the 11 members countries in the region are among the 22 high-burden countries. India alone accounting for more than 20% of the world’s cases. Most cases continue to occur in the most reproductive age group of 15–54 years, with males being disproportionately affected. The male-to-female ratio
in the region is 2:1 among new smear-positive TB cases detected in 2009. Though the death rates due to TB have declined after the introduction of DOTS (Directly observed treatment, short course) in the region, there are still about half a million deaths occurring among TB patients each year (WHO 2010).

**Tuberculosis in Bangladesh**

According to sixteenth global report on tuberculosis (TB) published in 2010 by WHO found the mortality, prevalence and incidence were 43 (32–57, 411 (188–671), 225 (184–269) per 100,000 population respectively in Bangladesh (WHO 2011). In 2010 WHO estimated Multidrug resistance (MDR) – TB cases in Bangladesh and it was 2.2% among MDR new cases and 14.7% among MDR previously treated cases (WHO 2011).

Zaman et al (2006) found 300,000 new cases and around 70,000 deaths annually in Bangladesh. This data was collected from directly observed treatment, short-course (DOTS) provided by the government of Bangladesh (WHO 2011).

In 2011, Bangladesh Rural Advancement Committee reported 340,000 TB cases and 75,000 TB deaths annually in Bangladesh (Havard 2011).

**Epidemiology of MDR-TB**

In 2008, an estimated 390 000–510 000 cases of MDR-TB emerged globally (best estimate, 440 000 cases). Among all incident TB cases globally, 3.6% (95% confidence interval (CI): 3.0–4.4) are estimated to have MDR-TB. These estimates, which lie in the same range as the previous ones, are based on more data and a revised methodology. Almost 50% of MDR-TB cases worldwide are estimated to occur in China and India. In 2008, MDR-TB caused an estimated 150, 000 deaths. Resistance
to 1st line anti TB drugs was found in 109 out of 114 countries during the year 1994 to 2009 when data on resistance occurring among new TB causes reported (WHO 2010). In 2009 at global level, the treatment success rate among new cases of smear positive pulmonary TB was 87%. Between 1995 and 2010, 55 million TB patients were treated in programmers that had adopted the DOTS/Stop TB Strategy, and 46 million were successfully treated. These treatments saved almost 7 million lives. Alongside these achievements, diagnosis and appropriate treatment of multidrug-resistant TB (MDR-TB) remain major challenges. Less than 5% of new and previously treated TB patients were tested for MDR-TB in most countries in 2010. The reported number of patients enrolled on treatment has increased 46,000 in 2010. However, MDR TB cases was equivalent to only 16% of total 290,000 cases among notified TB patients in 2010 (WHO 2010).

**Streptococcus pneumoniae**

Microbiologists, George M. Sternberg from United States and Louis Pasteur from France, independently first described about *pneumococcus* (Wqatso et al 1993). But Louis Pasteur was the first microbiologist who could isolate this organism in 1881 (Kaijalainen, 2006).

**Morphology and characteristics**

*Streptococcus pneumoniae* is a gram positive, β-hemolytic, bile soluble and capsulated bacteria (Kaijalainen, 2006). It has three major layers (plasma membrane, cell wall, and capsule) .The thickest layer of capsule is composed of high molecular ploymers (oligosaccharides) and cell wall is composed of triple layer peptidoglycan.Typical Pneumococci are lanceolet shaped diplococci which are often
occurring in short chains and growing with typical glistening, dome-shaped colonies. The colony shows central autolysis with time. The isolates are optochin sensitive, bile soluble and react with anti-C polysaccharide antiserum (DeVelasco et al 1995 and Henrichsen, 1995).

**Virulence factors**

The pathogenicity of Pneumococci depends on its various surface structures (DeVelasco et al 1995). e.g.- Polysaccharide capsule, surface proteins, enzymes, and the toxin pneumolysin (PLY) (Mitchell & Mitchell, 2010).
Chapter-3
Materials and methods
Materials and methods

Place of the study:

The study was carried out in the Department of Microbiology, Mymensingh Medical College, Mymensingh.

Period of the study:

The study was carried out during the period of January 2011 to January 2013.

Type of study:

The study was designed as cross sectional one.

Sample

Pleural fluid

Collection of specimen: Patient admitted on indoor medicine of Mymensingh Medical college hospital.

Procedure of pleural fluid aspiration: Firstly patient will be allowed to sitting position with arms and head resting supported on a bed side adjustable table. Next the skin will be anesthetized by 1% lidocaine using 5 cc syringe with 25 gauge needle. Then the site will be confirmed by counting the ribs based on chest x-ray percussing out the fluid level. After that insertion needle (50cc) will be inserted on the posterior aspect of the back over the diaphragm but under the fluid level. The top of the dullness will be marked by washable ink. Lastly 50 ml fluid will be collected for the laboratory procedure.
Sample size determination:

\[
n = \frac{Z^2pq}{d^2} = \frac{(1.96)^2 \times 0.32 \times 0.68}{(0.05)^2} = \frac{0.833}{0.0025} = 334
\]

\[
n = \text{Sample size} \\
Z = 95\% \text{ confidence level } Z = 1.96 \\
P = \text{Prevalence of occurrence} \\
q = (1-p) \\
d = \text{acceptable error 5\% (0.05)} \\
\text{Sample size} = 329
\]

Study population:

A total of 100 clinically diagnosed patients of pleural effusion from medicine wards of MMCH, Mymensingh were included in this study.

Inclusion criteria:

Patients with pleural effusion given consent for study were included in the study.

Exclusion criteria:

Pleural effusion patients who refused to give consent or from whom fluid could not be collected.

Study population was divided into categories according to the following criteria
On the basis of cytological and biochemical tests of pleural fluid the study subjects were categorized into three groups, viz. tubercular, parapneumonic and others.

A. Tubercular pleural effusion:

1. Appearance: Purulent or cloudy or red

2. Total count of WBC (cmm): 100–500 or more; Predominant cells are Lymphocytes

3. Glucose (mg/dl): 40-60 mg/dl

4. Protein (gm/dl): High usually 3-5 gm/dl

5. ADA (IU/L): >40 IU/L

6. Organisms usually identified by Ziehl-Neelsen stain

B. Parapneumonic pleural effusion:

1. Appearance: Cloudy or slightly turbid

2. Total count of WBC (cmm): 100–500 or more; Predominant cells are PMNs.

3. Glucose (mg/dl): <40 mg/dl

4. Protein (gm/dl): Normal or increased.

5. ADA: <35 IU/L

C. Others

Which did not fulfill either of the above two categories.

Data collection and analysis:
All the relevant history, clinical findings and laboratory records of every case were systematically recorded in a pre-designed data sheet (Appendix-I) for each patient and analyzed using computer based program Excel 2007.

**Storage:** Specimens were kept in micro-centrifuge tubes (1.5ml) (Eppendorf) and kept at -20°C for PCR. All other tests were (except PCR) performed immediately.

**Procedures done:**

1. Cytological study for the pleural fluid.
2. Ziehl-Neelsen staining and gram staining of deposit of centrifuged pleural fluid
3. Culture in Lowenstein-Jensen medium and other bacteriological medium.
5. Biochemical study – glucose and protein
6. PCR

**Cytological study**

**Physical examination or macroscopic examination:**
After collection, Pleural fluid was observed for appearance (clear or slightly turbid or cloudy or definitely purulent), color (contain blood or not) and clots. Normal pleural fluid appears clear, bright and colorless.

**Microscopic Examination:**

**Wet preparation:**

A drop of uncentrifuged pleural fluid was transferred to a slide and was covered with a cover glass. This preparation was examined by using the 10X and 40X objectives, with the condenser closed sufficiently to give good contrast and the findings were recorded accordingly.

**Cell count:**

Total count of WBC was done by improved Neubauer counting chamber. A clean dry improved Neubauer counting chamber with a clean dry cover glass was carefully charged (poured) with an uncentrifuged pleural fluid by micropipette and was waited for 2-3 minutes.

Counting was done by using 10x objectives and 40x objectives. The number of white cells that was found in 4 large squares corner was counted. If N number of cells were found in 4 large squares, it was then made an average $\frac{N}{4}$ (0.04 mm$^2$) which was multiplied by depth (0.1mm) of the fluid. So the count was $\frac{N}{4} \times 10$ cells/ mm$^3$ (1/10 is depth of the fluid).

**Differential count of WBC in Pleural Fluid:**
The smear was prepared from the centrifuged deposits of Pleural fluid and then the smear was stained with Leishman stain. After air dry of the smear it was examined under microscope using high power and oil immersion.

**Culture of pleural fluid specimen:**

One loop of pleural fluid was inoculated at bed side immediately in Blood agar, Chocolate agar and MacConkey agar media. Then these plates were incubated at 37°C for 24 hrs. For *Mycobacterium tuberculosis*, after centrifugation of the fluid, sediment was inoculated on Lowenstein - Jensen Medium and incubated at 37°C for 8 weeks.

**Isolation and identification of bacteria:**

The Isolates from the collected specimens were identified on the basis of colony morphology, Gram’s stain and appropriate biochemical tests.

a) *Streptococcus pneumoniae*:

Isolates of *Streptococcus pneumoniae* were identified on the basis of translucent or mucoid colonies of 1-2 mm in diameter with alpha hemolysis on blood agar media. On gram stained smear from colonies were appeared as Gram positive elongated (lance late) diplococcus and in short chain. Biochemically the isolates were catalase negative and sensitive to optochin (ethylhydrocureine hydrochloride).

b) *Mycobacterium tuberculosis*

The Isolates from the collected specimens were identified on the basis of colony morphology, Ziehl-Neelsen and appropriate biochemical tests. The inoculated Lowenstein-Jensen medium was incubated at 37°C and examined for growth twice
weekly for the first 2 weeks and once weekly thereafter up to 8 weeks, after which a definitive result was obtained.

**Lowenstein - Jensen Medium** was use with fresh egg and glycerol for the isolation and differentiation of *Mycobacterium* spp. (Appendix-3).

**Measurement of ADA level**

An ADA kit set (catalogue number DZ117A-K) manufactured by Diazyme Laboratories (USA) was used for this test. The reagent kit contains one R1 and one R2 and another was ADA control. ADA activity was measured by automated analyzer on the department of biochemistry Mymensingh Medical College (Appendix-4).

**Other Biochemistry:**

Glucose and protein level was measured by automated analyzer on the department of biochemistry Mymensingh Medical College.

**PCR:**

**DNA EXTRACTION:**

About 300 µl pleural fluid was added into 300 µl Nucleic acid Lyses solution in a 1.5 ml micro centrifuge tube and vortexed. Then the solution was incubated at 65°C for 15 min. After that 3 µl RNase was added to it and the solution was mixed inverting.
the tube 2-5 times. Again the mixture was incubated at 37°C for 15 min. The sample was allowed to cool to room temperature for 5 minutes. After that 200 μl Protein Precipitation solution was added to the RNase-treated cell lysate and was vortexed vigorously at high speed for 20 seconds. After that the mixture was centrifuged for 4 minutes at 13000-16000 x g. The supernatant containing DNA was carefully removed and transferred it to a clean 1.5 micro centrifuge tube containing 600 μl of isopropanol. The solution was mixed gently and centrifuged at 13000-16000 x g for 1 minute at room temperature. The supernatant was again carefully decanted and 70% ethanol added at room temperature and gently inverted the tube several times of wash the DNA. The solution again was centrifuged at 13000-16000 x g for 1 minute at room temperature. The ethanol was carefully aspirated. The tubes inverted into clean absorbent paper and air-dried pellet for 15 minutes. Lastly 100 μl DNA re-hydration solution was added and dehydrated DNA by incubation at 65°C for 1 hour. Then DNA was stored at 2-8°C until use.

**PREPARATION OF REACTION MIXTURE:**

Sterile micro-centrifuge tubes (1.5ml) (Eppendorf) were taken and the tubes were labeled with date and reaction number. Master mixture including all reagents was prepared for PCR reaction except Taq polymerase and the sample. The amount of master mixture was prepared according to the number of reaction. The master mixture was aliquoted and stored at -20°C.
PCR for detection of *LytA* gene for diagnosis of Pneumococcus

**Master mixture of detection of *LytA* gene of *Streptococcus pneumoniae*:**

**Preparation of Primer mixture:**

- Primer-1 - 20 µl
- Primer-2 - 20 µl
- Distilled Water - 40 µl

**Preparation of master mixture for each reaction (50 µl) contained:**

**Preparation of Master mixture:**

- 10x buffer - 5µl
- 200 micromole deoxy nucleoside triphosphate -4µl
- Primer mixture - 1µl
- 2units of Taq DNA polymerase - 0.5µl
Protocol of Thermal cycles:

Initial denaturation at 94°C for 2 minutes followed by

Denaturation at 94°C for 30 seconds.

Primer annealing at 55°C for 30 seconds.

Extension at 72°C for 30 seconds. 30 cycles

Final extension at 72°C for 1 minutes.

Above steps were repeated for 30 cycles in an automated DNA thermal cycler (Eppendorf, Germany). Final extension was done at 72°C for 10 minutes. The PCR products were run in 1.5% agarose gels stained with ethidium bromide. Then that were visualized under the UV transilluminator.

Primer used for PCR:

The sequences from 5’ to 3’ ends of these primers were as follows:

Primer -1 (5’-TGAAGCGGATTATCACTGGC-3’)

Primer -2 (5’-GCTAAACTCCCTGTATCAAGCG-3’).
Above primers was used for amplify 273-bp sequence of LytA gene (Saha et al. 2005).

**Agarose gel electrophoresis:**

The PCR product was analyzed by 1.5% agarose gel electrophoresis to detect specific band which was 273-bp.

**PCR FOR Mycobacterium tuberculosis IS6110 SEQUENCE**

The most common 20-bp oligonucleotides were found in whole *M. tuberculosis* genomes. The Mtb1 (5’-CCT GCGA GCG TA GGCG TCGG- 3.) sequence was found and was subsequently used for comparison to the whole genome. Repeated oligonucleotides were subsequently extended by successive additions of single nucleotides from the 5’ and 3’ ends until the repeat number was not lower than 30. Independent searching for at least 20-bp repeats in the genome of *M. tuberculosis*. Sequence (5’-CTCG TCCA GCG CCG CTT CGG-3.) containing the 20-bp sequence of the Mtb2 oligonucleotide, but to the best of our knowledge its use as a primer for PCR typing of *M. tuberculosis* in combination with primers designed within IS6110 inverted repeats. IS6110 sequence is present in all *M. tuberculosis* strains.
Table-1.

**Preparation of Primer mixture:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mtb1 (200mM)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Mtb2 (200mM)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

Table-2.

**Preparation of Master mixture:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>39 µl</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>05 µl</td>
</tr>
<tr>
<td>dNTP</td>
<td>04 µl</td>
</tr>
<tr>
<td>Primer mixture</td>
<td>01 µl</td>
</tr>
<tr>
<td>Extracted DNA</td>
<td>01 µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>
Table-3

Primers used for the PCR:

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS6110</td>
<td>Mtb1</td>
<td>5.-CCCTGCCAGCGTAGGCGTCGG-3.</td>
</tr>
<tr>
<td></td>
<td>Mtb2</td>
<td>5.-CTCGTCCAGCGCGCTTCGG-3.</td>
</tr>
</tbody>
</table>

Total                                                                  50 µl
AMPLIFICATION

Amplification was carried out in the thermal cycler (Eppendorf). 50µl amplification mixture was dispensed in thermal cycler tubes 0.5µl of Taq polymerase was added to each tubes and mixed with pipette tips. Amplification was carried out according to the following directions.

94 °C.......30 seconds
65 °C........30 seconds
72 °C........1 minute

Cycles 32

72 °C........5 minutes (Final extension)

Agarose gel electrophoresis

The PCR products were analyzed by 1% agarose gel electrophoresis to detect specific band for IS6110 sequence.

Preparation of agarose gel

One percent agarose gel was prepared by melting 2.0 gm agarose in 200 ml of diluted TBE buffer (1:4) using a microwave oven or gas burner. The melted agarose was allowed to cool to about 50 °C and was poured into gel electrophoresis unit with spacers and comb. After solidification of the gel, the comb was removed and wells
were formed. During electrophoresis, the gel was placed in a solution containing ethidium bromide in the electrophoresis chamber.

**Loading and running of the sample**

5 µl of amplified PCR product was mixed with 2.0µl of gel loading dye. The mixture was slowly loaded into the well using disposable micropipette tips. Marker DNA of known size (100 bp ladder) was loaded in one well to determine the size of the amplified PCR products. Electrophoresis was carried out at 100 volts for 35 minutes.

**Staining and visualization of the gel**

The bands (123 bp) of the sample of the study and were visualized by transilluminator and the gel was photographed by a digital camera (Canon PC 1234) and transferred data to computer for further documentation.
Chapter-4

Results
Results

A total 100 pleural effusion patients were enrolled in the study from Mymensingh Medical College and Hospital (MMCH) during the period from January 2011 to January 2013 and the following tests were done from pleural fluid includes cytology test, biochemical test, Gram’s stain, Z-N stain, culture, Polymerase chain reaction (PCR) and level of ADA. For Pneumococcus optochin sensitivity was done.
Table-I Cytological test of pleural fluid (n=97)

<table>
<thead>
<tr>
<th>Test Cases</th>
<th>Total count of WBC</th>
<th>Differential count of WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Neutrophil (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Tubercular pleural effusion</td>
<td>327.50±114.75</td>
<td>19±0.9</td>
</tr>
<tr>
<td>Parapneumonic pleural effusion</td>
<td>1712±1167</td>
<td>812±12</td>
</tr>
<tr>
<td>Undiagnosed</td>
<td>264±115</td>
<td>20±1</td>
</tr>
</tbody>
</table>

Table-I shows the cytological study of the pleural fluid in different groups. Here lymphocytes are predominant in tubercular pleural effusion, undiagnosed effusion and in parapneumonic effusion neutrophils are predominant. The Mean±SD of TC of tubercular pleural was 327.50±114.75, parapneumonic pleural effusion was 1712.00±1167.80 and undiagnosed pleural effusion was 264.12±115.85. The Mean±SD of DC (neutrophil and lymphocyte) of tubercular pleural effusion was and 19±1, parapneumonic effusion was 81±1 and 21±1, undiagnosed effusion was 21±1 and 81±1 respectively.
Table-II Biochemical study of pleural fluid (n=100)

<table>
<thead>
<tr>
<th>Test Cases</th>
<th>Glucose (mg/dl) Mean±SD</th>
<th>Protein (gm/dl) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubercular pleural effusion</td>
<td>51.69±4.07</td>
<td>3.25±0.82</td>
</tr>
<tr>
<td>Parapneumonic pleural effusion</td>
<td>49.40±2.46</td>
<td>3.25±0.50</td>
</tr>
<tr>
<td>Undiagnosed</td>
<td>50.40±5.14</td>
<td>3.23±0.63</td>
</tr>
</tbody>
</table>

Table-II and fig-1 reveals that glucose level (Mean±SD in mg/dl) for tubercular pleural effusion was 51.69±4.07, for parapneumonic effusion was 49.40±2.46 and for undiagnosed effusion was 50.40±5.14. Whereas the protein level (Mean±SD) was 3.25±10.82, 3.25±0.50 and 3.23±0.63 gm/dl for tubercular pleural effusion, parapneumonic pleural effusion and undiagnosed cases respectively.
Table-III Identification of bacterial etiology by staining methods (n=100)

<table>
<thead>
<tr>
<th></th>
<th>Gram stain</th>
<th>Ziehl-Neelsen stain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Tubercular pleural fluid</td>
<td>0</td>
<td>100(100)</td>
</tr>
<tr>
<td>Parapneumonic pleural fluid</td>
<td>05(5.0)</td>
<td>97(97.0)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage

Table –III describes that gram stain was positive in 3% pleural fluid and Ziehl-Neelsen stain was not positive for any fluid sample.

Table IV: - shows the result of culture of pleural fluid

<table>
<thead>
<tr>
<th>Total no. of pleural fluid cultured</th>
<th>No. positive</th>
<th>M. tuberculosis</th>
<th>S. pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>06 (6.0%)</td>
<td>03 (3.0%)</td>
<td>03 (3.0%)</td>
</tr>
</tbody>
</table>

In Table IV shows the result of culture of pleural fluid. A total of 06 were culture positive of which 03(3.0%) were S. pneumoniae and 03(3.0%) were M. tuberculosis.
Table V PCR of pleural fluid for detection of tubercular and parapneumonic pleural effusion

<table>
<thead>
<tr>
<th>Cases</th>
<th>Result</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=100 (all cases )</td>
<td></td>
<td>06(6.0)</td>
<td>94(94.0)</td>
</tr>
<tr>
<td>n=10 (only Parapneumonic pleura effusion)</td>
<td></td>
<td>05(50.0)</td>
<td>05(50.0)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage

Table-V Shows in PCR 6% cases were positive for tubercular pleural effusion and 50% for parapneumonic pleural effusion.
Table VI: ADA level of pleural fluid among the study population

<table>
<thead>
<tr>
<th>Test ADA(IU/L)</th>
<th>Tubercular pleural effusion (n=52)</th>
<th>Mean±SD</th>
<th>Parapneumonic pleural effusion (n=10)</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>49.72±15.18</td>
<td></td>
<td>31.100±5.09</td>
</tr>
<tr>
<td>0-09</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td></td>
<td></td>
<td>2(20.0)</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td></td>
<td></td>
<td>8(80.0)</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td>34(65.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td>14(26.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60-79</td>
<td>3(5.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80&gt;</td>
<td>1(1.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage

Table VI and fig-1 Shows ADA level of tubercular pleural effusion were >40 IU/L with a Mean±SD of 49.72±15.18. Whereas Mean±SD of parapneumonic pleural effusion was 31.100±5.087.

At level of 95% significance P value is <0.05. So ADA level was significant for the diagnosis of tubercular pleural effusion.
Fig-1 Detection of ADA level in tubercular and parapneumonic effusion
Table VII: Comparison of ADA, cytological and biochemical examination of pleural fluid of the study population.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Parapneumonic pleural effusion</th>
<th>Tubercular pleural effusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Total count of WBC/cmm</td>
<td>1712.00</td>
<td>1167.80</td>
</tr>
<tr>
<td>Protein (gm/dl) of pleural fluid</td>
<td>3.25</td>
<td>0.50</td>
</tr>
<tr>
<td>ADA (IU/L) of pleural fluid</td>
<td>31.10</td>
<td>5.09</td>
</tr>
</tbody>
</table>

Table VII shows Mean±SD (Total count of WBC/cmm) of parapneumonic pleural effusion and tubercular pleural effusion was 1712.00±1167.80 and 327.50±114.74 respectively. Mean±SD of protein (gm/dl) of parapneumonic pleural effusion and tubercular pleural effusion was 3.25±0.50 and 3.98±0.82 respectively. Mean±SD of ADA (IU/L) of parapneumonic pleural effusion was 31.10 ± 5.09 and Mean±SD of ADA (IU/L) of tubercular pleural effusion was 49.72±15.18.

In parapneumonic effusion ranges TC of WBC (/mm$^3$ of pleural fluid), ADA (IU/L) and protein (gm/dl) was 225-3250, 20-37 and 3.1-3.9 respectively.

The coefficient of variations of WBC, ADA and protein of parapneumonic effusion (calculate from appendix-1) was 68%, 19% and 15% respectively (Appendix-1).

The range of WBC (/mm$^3$ of pleural fluid), ADA (IU/L) and protein (gm/dl) was 50-500, 40-138 and 3.2- 4.7 respectively in tubercular pleural effusion.

The coefficient of variations of WBC, ADA and protein in tubercular pleural effusion was 36%, 30% and 15% respectively.
Table VIII A - Sensitivity and Specificity of ADA level detect in *Mycobacterium tuberculosis* infection considering culture as a gold standard method (n=100)

<table>
<thead>
<tr>
<th>Test result</th>
<th>Disease status</th>
<th>Total</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture positive</td>
<td>Culture negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADA (&gt;40IU/L)</td>
<td>03 (a)</td>
<td>49 (b)</td>
<td>52</td>
<td>100%</td>
</tr>
<tr>
<td>ADA (&lt;40IU/L)</td>
<td>00 (c)</td>
<td>48 (d)</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>03</td>
<td>97</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

The table VIII A showing sensitivity and specificity of ADA detect were 100% and 66.41 % respectively taking Culture as gold standard.

a = True positive, b = False positive, c = False negative and d = True negative.
Table VIII B - Sensitivity and Specificity of PCR in *Mycobacterium tuberculosis* infection considering culture as a gold standard method (n=100)

<table>
<thead>
<tr>
<th>Test result</th>
<th>Disease status</th>
<th>Total</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture positive</td>
<td>03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR Positive</td>
<td>03 (a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Culture negative</td>
<td>03 (b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR Negative</td>
<td>0(c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>94 (d)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Total       | 03                  | 97    | 100         | 100%        | 96.91 %    |

The table VIII B showing sensitivity and specificity of PCR were 100% and 66.41 % respectively taking Culture as gold standard.

a = True positive, b = False positive, c = False negative and d = True negative.

**Formula for sensitivity**

\[
\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive + False Negative}} \times 100
\]

**Formula for Specificity**

\[
\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative}} \times 100
\]
OBSERVATIONS (Table-IX)

To reach the objectives of this experiments few observations were found as very important and helpful. With the help of these observations this research was directed to the final conclusion. These observations are not directly related to result but important parameter to be taken care of.

Firstly all the study samples were categorized in to groups which are given in the table – IX

Table IX: Categories of study population on the basis of findings of pleural effusion

<table>
<thead>
<tr>
<th>Study population (N=100)</th>
<th>Parapneumonic pleural effusion</th>
<th>Tubercular pleural effusion</th>
<th>Malignant</th>
<th>Undiagnosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 (100)</td>
<td>10 (10)</td>
<td>52 (52.0)</td>
<td>03 (3)</td>
<td>35 (35.0)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage

Table- IX shows, among 100 cases, 10 (10%) were diagnosed as parapneumonic pleural effusion, 52 (52.0%) as tubercular pleural effusion, 03 (03%) were malignant pleural effusion and 35 (35%) as undiagnosed pleural effusion.
Table X: Age distribution of the study population (N=100)

<table>
<thead>
<tr>
<th>Age group</th>
<th>Parapneumonic pleural effusion (n=10)</th>
<th>Tubercular pleural effusion (n=52)</th>
<th>Malignant (n=03)</th>
<th>Undiagnosed (n=35)</th>
<th>No of patient reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-40</td>
<td>06 (60)</td>
<td>41 (78.8)</td>
<td>0 (0.0)</td>
<td>20 (57.1)</td>
<td>67</td>
</tr>
<tr>
<td>&gt;40-60</td>
<td>04 (40)</td>
<td>11 (21.2)</td>
<td>0 (0.0)</td>
<td>15 (42.9)</td>
<td>30</td>
</tr>
<tr>
<td>&gt;60</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (100)</td>
<td>0 (0.0)</td>
<td>03</td>
</tr>
<tr>
<td>Total</td>
<td>10 (100)</td>
<td>52 (100)</td>
<td>3 (100)</td>
<td>35 (100)</td>
<td>100</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage.

Table X shows most of the cases of study population were in the age group 20 to 40 years (67.0%). Among 67 patients, 41 were tubercular, 6 were parapneumonic and undiagnosed were 20. A total of 03 patients were found of malignant origin and all of them were > 60 years of age.

Table XI: Sex distribution of the study population (N=100)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Parapneumonic pleural effusion (n=10)</th>
<th>Tubercular pleural effusion (n=52)</th>
<th>Malignant (n=03)</th>
<th>Undiagnosed (n=35)</th>
<th>Total (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>06 (60)</td>
<td>49 (94.2)</td>
<td>02 (66.6)</td>
<td>29 (82.8)</td>
<td>86 (86)</td>
</tr>
<tr>
<td>Female</td>
<td>04 (40)</td>
<td>3 (5.8)</td>
<td>01 (33.3)</td>
<td>06 (17.2)</td>
<td>14 (14)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage.

Table XI shows the distribution of pleural effusion among male and female patients and it was found 86% among male and 14% among female patients.
Table- XII: Clinical features of pleural effusion in the study population (N=100)

<table>
<thead>
<tr>
<th>Clinical findings of study subjects</th>
<th>Parapneumonic pleural effusion (n=10)</th>
<th>Tubercular pleural effusion (n=52)</th>
<th>Malignant (n=03)</th>
<th>Undiagnosed (n=35)</th>
<th>Total (N=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>10 (100)</td>
<td>52(100)</td>
<td>01(33.3)</td>
<td>35(100)</td>
<td>98(98.0)</td>
</tr>
<tr>
<td>Cough</td>
<td>10 (100)</td>
<td>42(80.8)</td>
<td>02(66.6)</td>
<td>30(85.7)</td>
<td>84(84.0)</td>
</tr>
<tr>
<td>Respiratory distress</td>
<td>08(80)</td>
<td>52(100)</td>
<td>03(100)</td>
<td>35(100)</td>
<td>98(98)</td>
</tr>
<tr>
<td>Progressive weight loss</td>
<td>-</td>
<td>52(100)</td>
<td>03(100)</td>
<td>18(51.4)</td>
<td>73(73)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage

Table- XII shows the clinical features of pleural effusion were suffering from fever (98.0%) followed by cough (84%), Respiratory distress (98%) and progressive weight loss (73%).

Fig 2- Clinical findings of pleural effusion
Table- XIII: Physical findings of pleural effusion in the study population (N=100)

<table>
<thead>
<tr>
<th>Findings of physical appearance of pleural fluid</th>
<th>Parapneumonic pleural effusion (n=10)</th>
<th>Tubercular pleural effusion (n=52)</th>
<th>Malignant (n=03)</th>
<th>Undiagnosed (n=35)</th>
<th>Total (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbid</td>
<td>07 (70.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>07 (7.0)</td>
</tr>
<tr>
<td>Yellow</td>
<td>03 (30.0)</td>
<td>39 (75.0)</td>
<td>0 (0.0)</td>
<td>22 (66.9)</td>
<td>66 (66.0)</td>
</tr>
<tr>
<td>Hemorrhagic</td>
<td>0 (0.0)</td>
<td>13 (25.0)</td>
<td>03 (100)</td>
<td>13 (37.1)</td>
<td>29 (29.0)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentage

Table- XIII shows the physical findings of pleural fluid, in Parapneumonic pleural effusion 07 (70.0%) were turbid and 03(30.0%) were yellow. In tubercular pleural effusion 39 (75.0%) were yellow, 13 (13.0%) were red and turbidity was not found. In malignant pleural effusion 03(100%) were red.

Fig-3 Physical findings of pleural fluids
Chapter-5
Discussion


Discussion

Pleural effusion is defined as accumulation of excessive pleural fluid (> 10ml) in pleural space. It occurs due to primary manifestation of disease or its complication (Farghaly et al. 2007). Pleural effusion is of two types, exudative and transudative (Davidson 2007). Transudative pleural effusion occurs due to imbalance between hydrostatic and oncotic pressure (e.g. – congestive cardiac failure, nephrotic syndrome). Exudative pleural effusion occurs due to various diseases like pneumonia, tuberculosis and malignancy (Light 2002). It is a common medical problem. It causes death worldwide (Medford and Maskell 2005). Mesothelial cells are involved, when pleura is inflamed (Antony 2003). Predominance of polymorph cells indicates acute process (pneumonia) affecting the pleura and lymphocyte indicates chronic disease like tuberculosis, malignancy (Maskell et al. 2012). The etiology of pleural effusions depends on the geographic region, patient’s age and advances in diagnosis and treatment of underlying causes (Lima et al. 2003). Tuberculosis is the major health problem in developing countries and pleural effusion occur upto 30% of TB infected patient (Hasaneen et al. 2003). According to WHO Bangladesh is the 6th TB burden country in the world (WHO 2004). From extra pulmonary site TB diagnosis is often non specific or inappropriate. In some cases it also shows negativity on histological examination and culture (Shah et al. 1998). Because of this etiology of pleural effusion is from unknown origin in 20% cases (Lima et al 2003). Diagnosis of tuberculosis is based on clinical feature, X-ray finding and laboratory results. At present, in developing countries tuberculosis diagnosis relies heavily on the microscopic demonstration of tubercle bacilli in appropriate clinical specimen in laboratory. The presence of an infectious agent can be detected by means of direct identification of an organism by microscopy and culture, serological testing, which is
to measure specific antibodies against the organism, measuring a specific product of the organism, which are bacterial antigens or DNA and biochemical test which measure the level of ADA (Ti Ti 1996).

Conventional methods for the diagnosis of pleural TB had proven inefficient. Direct examination of pleural fluid and Ziehl-Neelsen staining requires bacillus concentrations of 10,000/ml and, therefore, has a low sensitivity. Culture is more sensitive but it requires 2-6 weeks due to high doubling time (Villegas et al. 2000). In case of culture 50 to 1000 bacilli per milliliter is needed (Lima et al. 2003).

In this study conventional methods (cytology, gram stain, Ziehl-Neelsen stain and culture of pleural fluid) and modern methods (detection of ADA level and PCR) both were done.

Among the conventional methods cytological test was done for all pleural fluid. On the basis of cytological study, pleural fluids were categorized into tubercular, parapneumonic and undiagnosed case (Table-1). In tubercular pleural effusion lymphocytes are predominant (>80%) (castro et al. 2002). But lymphocytic pleural effusion also common in cancer, rheumatoid pleurisy, lymphoma etc (Light 2002 and Sahn 1982). From Spain Castro et al. (2003) also reported that lymphocytic exudate which was seen with tubercular pleuritis, also occur with other diseases such as malignancy and collagen vascular diseases (Castro et al. 2003). In this study lymphocytes were predominant in 90% pleural fluid samples and among this only 52% were tubercular and rests were undiagnosed (35%) and malignant cases (3%).

In the present neutrophils were predominant in 10 pleural fluids. Usually large numbers of neutrophils are present in parapneumonic pleural effusion. Predominance
of neutrophils also present in various pleural diseases like pancreatitis, subphrenic abscess and early tuberculosis. So for diagnostic purpose its significance is limited (Kroegel and Antony 1997).

Exudative pleural fluid (protein >3gm/dl) usually present in various diseases. In exudative pleural fluid glucose level is less than 60 mg/dl (Maskel et al. 2003 and Light 2002). In this study concentration of protein and glucose in tubercular and parapneumonic fluids were >3gm/dl and glucose level were in less than 60 mg/dl respectively.

In this study gram staining and Ziehl-Neelsen stain was done for all pleural fluids. Lima et al. from Brazil reported that, the microscopic examination of the pleural fluid was negative in all of the samples (Lima et al. 2003). Burnes et al from USA also found AFB stain negative in all pleural fluid samples (Burnes et al. 2005). Hasaneen et al. (2003) from Egypt also found sensitivity of AFB stain of pleural fluid was 0% (Hasaneen et al. 2003). In our study no tubercular pleural fluid samples (Table-III) were microscopically positive. Soe et al. (2010) from Malaysia found smear positive pleural fluid only in 0.9% cases. The present study well correlate with these study (Soe et al. 2010).

In this study 5 samples were gram stain positive which was 5% of the total cases (Table-III). Burnes et al. (2005) from USA also found gram stain positive is 2.5% cases (Burnes et al. 2005). Boersma et al. (1993) found gram stain positive in 2 cases out of total 9 cases (Boersma et al. 1993). In the present study 5 cases were gram stain positive from a total of 10 parapneumonic cases. Maskell et al. (2006) found 2%
pleural fluids as gram positive. Which well correlate with the present study (Maskell et al. 2006).

In this study 3(3.0%) samples (Table-IVA) were culture positive in Lowenstein-Jensen egg based solid medium for *Mycobacterium tuberculosis*. Soe et al. (2010) from Malaysia found culture positive only in 5.6% cases (Soe et al. 2010). Culture method is gold standard but sometimes microscopic positive samples are failed to grow on culture medium (Nagesh et al. 2001). Hasaneen et al from Egypt found no *Mycobacterium* growth in culture medium (Hasaneen et al. 2003). This study results well correlate with the study results mentioned.

In this study *S.pneumoniae* was culture positive in 3% cases of the total (Table VI A). From USA Burnes et al. (2005) also found 3 % is culture positive. Kawanami et al. (2011) reported that rate of detection of bacteria on cultivation method always <20% (Kawanami et al. 2011). Boersma et al. (1993) found culture positive in 1 case out of total 9 parapneumonic cases (Boersma et al. 1993). In the present study 3 cases were culture positive from total 10 parapneumonic cases. Eastham et al. (2005) found 94% pleural fluids were culture negative in ordinary bacteriological media (Eastham et al 2005). In this present study 97% pleural fluids were culture negative in ordinary bacteriological media which well correlate with this study.

Adenosine deaminase (ADA) is an enzyme in the purine salvage pathway that catalyses the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine with the release of ammonia. ADA is found in most cells but plays an important role in the differentiation of lymphoid cells (in particular, active T-lymphocytes). These ADAs are present in almost all invertebrates, and, in human. It
specially produced during the inflammatory process triggered by the *M. tuberculosis* (Song et al 2010, Kaisemann et al 2004 and Haque 2012).

In this study level of ADA was >40 IU/L in 52 (52%) pleural fluids (Table-VI). Here its sensitivity and specificity was 100% and 66.44% respectively (Table- VIII) considering direct pleural fluid culture as gold standard. By considering pleural biopsy culture as gold standard Lima et al. (2003) from Brazil found sensitivity and specificity of ADA level (>40 IU/L) as 68.8% and 72.2% respectively, Ahmed et al. (2011) from Bangladesh also detected higher ADA level (>40 IU/L) in 60% pleural effusion cases with a sensitivity and specificity 94% and 88% respectively, Villegas et al. (2000) from Colombia reported that measurement of ADA activity (>40 IU/L) was the single most sensitive method with the sensitivity 88.1% and specificity 85.7% specificity for the diagnosis of pleural TB (Lima et al. 2003, Ahmed et al. 2011 and Villegas et al. 2000). Pleural biopsy culture is more sensitive than direct pleural fluid culture (Lima et al. 2003). This may be the reason why the present study had low specificity. The sensitivity of this study well correlate with the mentioned above.

In this study only 06 (6.0%) samples were PCR positive from a total 100 pleural fluids samples (Table-V) with sensitivity 100% and specificity 96.91% (Table-VII) comparing with culture as gold standard. By considering culture as gold standard, Lima et al. (2003) found the sensitivity of PCR as 31.3% and specificity 96.6%, Gopi et al. (2007) from India reported that PCR sensitivity as 20 to 90% and specificity from 78 to 100%, Ferrer (1997) from Spain also found sensitivity of PCR was 20-81% and specificity 78-100% (Lima et al. 2003, Gopi et al. 2007 and Ferrer1997). So present study well correlate with these studies mentioned above. The level of ADA was >40 IU/L in all the 06 PCR positive samples. Villegas et al. (2000) from
Colombia reported that extraction of DNA from frozen samples was less efficient than extraction of DNA from fresh samples (Villegas et al. 2000). In this study DNA extraction was done from all frozen samples. Lima et al. (2003) from Brazil found that 04 hemorrhagic samples from 45 samples had negative results for PCR. These 04 samples were diagnosed as tubercular cases. Hemorrhagic samples became negative may due to purulent materials are present in hemorrhagic fluid. These purulent materials contain nucleases that degrade DNA (Lima et al. 2003). In our study 13 samples (TableXIII) were hemorrhagic had ADA level >40 IU but PCR negative. Villegas et al. (2000) also found that sometimes extraction method may be able to vary the result (Villegas et al. 2000).

In this study 5(50%) samples were PCR positive for a total 10 parapneumonic effusion cases (Table – V). Flaguera and Porcel (2002) from USA found PCR positive parapneumonic effusion was 12% (Flaguera and Porcel 2002). Utine et al. (2008) found the presence of \textit{S. pneumoniae} in 3 pleural fluid samples from 4 gram stain positive smear by PCR analysis (Utine et al. 2008). In this study presence of \textit{S. pneumoniae} in 5 pleural fluid samples from 5 gram stain positive smear by PCR analysis. Which well correlate with this study.

In this present study all the pleural TB cases were between the age ranges of 21-60 years (Table-X). Tubercular infection rate was high in young adult because of high transmission rate due to outdoor exposure and in older adult may be due to reactivation of latent infection (Dye and Brogdroff 2008). In 2001 WHO reported that, tubercular infection can affect the all age group in developing country. But its incidence and mortality was higher in age group 18-59 years of age (WHO 1991).
Valdes et al. (2000) from USA reported age group was 21-57 years (Valdes et al. 2000).

In this study Male: Female ratio was about 6:1 (Table-XI). Valdes et al. (1996) found male and female ratio as 6:1 (Valdes et al. 1996) which well correlate with the present study. Haque (2011) from India reported the male: female ratio as 3:1 (Haque 2011). Yildiz et al. (2011) found 144 male and 52 female from a total 196 patients (Yildiz et al. 2011). In the present study 86 were male and 14 were female from a total 100 patients. WHO (2003) reported that in South India progression of pulmonary tuberculosis infection in male and female was 8.6% and 3.1% respectively. Probable cause was, most of male were alcoholic and smokers, which is the predisposing factors for tuberculosis (WHO 2003). WHO (2003) also reported that, female shows less productive cough than male. Because of this, their sputum is less examined. Female get less health facilities due to difficulties in accessing health care, embarrassment, fear of stigma in developing countries like Bangladesh, Vietnam Thailand etc (WHO 2003).

The present study showed that PCR is the rapid and sensitive method but it is highly technical and expensive which requires expert manpower. Alternative method is measurement of ADA activity from pleural fluid is simple and also rapid and no skilled manpower is needed for its estimation as it is done by analyzer.

Now a days PCR is available in a few govt. medical college and analyzer machine is also available in many govt. medical colleges, private medical colleges and most of private clinics. So early diagnosis of tubercular pleural effusion can be done by PCR and measurement of ADA activity. In this study we detect the bacterial etiology of
pleural fluid. This technique can be applied for other body fluids (Ascitic fluid, CSF) also.
Conclusion and recommendations

Pleural effusion is a common disease in medical practice. By conventional method sometimes diagnosis of bacterial cause of pleural effusion is difficult. In developing country like Bangladesh tuberculosis is a common disease. In tubercular infection one third of the patients develop pleural effusion. Conventionally, most of the time microscopy may fail to detect the causative organism. Cytological examination of pleural fluid is an ineffective procedure, because predominant of lymphocyte and neutrophil in pleural fluid do not indicate the tubercular/parapnumonic effusion only. By culture method *Mycobacterium tuberculosis* takes 6-8 weeks. Detection of *Mycobacterium tuberculosis* and *S. Pneumonae* from pleural fluid by molecular technique like PCR is the rapid method but expensive. Measurement of ADA from pleural fluid is a rapid and reliable method for extrapulmonary tuberculosis and can be done by analyzer. So it may be recommended that measurement of ADA level can be used together with conventional methods as an extra diagnostic tool where PCR is not available. Measurement of ADA activity as well as PCR can be done simultaneously on its availability.
Limitation

1. In this study the sample size was smaller than the estimated sample size due to limitation of budget, time and resource.
Chapter-6

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