Prevalence of *Mycobacterium tuberculosis* in prostatic tissues of patients suspected with different prostatic lesion specially cancer

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Prevalence of *Mycobacterium tuberculosis* in prostatic tissues of patients suspected with different prostatic lesion specially cancer.

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF DHAKA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF DHAKA
DHAKA-1000
January, 2016

SUBMITTED BY
EXAMINATION ROLL NO: 04
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SESSION: 2010-2011

Dedicated to……

My beloved parents

Certification

It is hereby certified that student bearing **Roll no: 04, Registration no: 94** has carried out the research work entitled **"Prevalence of *Mycobacterium tuberculosis* in prostatic tissues of patients suspected with different prostatic lesion specially cancer"** for the partial fulfilment of her **Master of Philosophy** Degree in Microbiology from the University of Dhaka, Bangladesh.

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Declaration

I hereby declare that the whole work submitted as a thesis entitled **"Prevalence of *Mycobacterium tuberculosis* in prostatic tissues of patients suspected with different prostatic lesion specially cancer"** in the Department of Microbiology, University of Dhaka, for partial fulfilment of the requirements for the degree of Master of Philosophy in Microbiology under the joint supervision of **Dr. Md. Manjurul Karim**, Professor, Department of Microbiology, University of Dhaka and Prof. **Dr. Md. Tahminur Rahman,** Professor & Head, Department of Pathology, Anwer Khan Modern Medical College, Dhanmondi, Dhaka-1205, Bangladesh.

I, further declare this thesis or part thereof has not been concurrently submitted for the award of any degree or Diploma anywhere.

Mahbuba Ashrafi Mumu

Date:----------

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Abstract

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* is a global health problem. According to the World Health Organization (WHO) 9 million people developed TB and 1.5 million died from the disease in 2013. Bangladesh stands 6th among 22 high TB burden countries. Pulmonary tuberculosis is the most common form of the disease; however, 20-25% of cases are extra-pulmonary in nature. Genitourinary tuberculosis accounts for 5-10% of extra-pulmonary cases in developed countries and 15-20% of cases in developing countries. Tuberculosis of the prostate gland is seen in 2.6% of genitourinary system. Studies have shown that approximately 20% of all human cancers in adults result from chronic infection and inflammatory states. Chronic prostate inflammation accelerates initiation of prostate cancer originating from basal cells and accelerates prostate cancer progression. There are reports describing tuberculosis of testis and prostate mimicking testicular cancer and prostatitis caused by *M. tuberculosis* infection serving as a predisposing factor for prostate cancer. This study aims to investigate whether there is any association between tuberculosis of prostate and development of prostatic lesions especially cancer in a cross section of Bangladeshi population. The study was carried out with 85 prostatic biopsy samples collected by trans-urethral resection of prostate (TURP) from patients and also two known TB positive lymph nodes. In addition to prostatic biopsy, venous blood samples were collected from respective individuals for estimation of prostate specific antigen (PSA) which is an aid for diagnosis of prostatic carcinoma. Histopathological diagnosis of 85 patients revealed nodular hyperplasia with chronic prostatitis (NHCP) in 56%, prostatic intraepithelial neoplasia (PIN) in 30.6%, granulomatous prostatitis (GnP) in 3.5% and cancer in 9.4% patients. The mean age of the cancer patients is 70 years. Most of the cancer patients belong to Dhaka division and smoking habit. None of the 85 biopsy sample revealed the presence of *M. tuberculosis* when analyzed by Ziehl-Neelsen (Z-N) stain and polymerase chain reaction (PCR). The failure of detection of *M. tuberculosis* from formalin fixed paraffin embedded tissue by conventional PCR prompted us to use Gene Xpert MTB/RIF which is a real-time hemi nested PCR test that simultaneously identifies *M. tuberculosis* and detects rifampicin resistance directly from clinical specimens. Two samples from each nodular hyperplasia with chronic prostatitis (NHCP), granulomatous

prostatitis (GnP) and prostatic intraepithelial neoplasia (PIN) states, all 8 prostatic adenocarcinoma tissue samples were tested for presence of mycobacterial genomic DNA by the Gene Xpert MTB/RIF real time PCR. All these samples came out negative for mycobacterial DNA. Very importantly, the two positive control samples (lymph node tissues from confirmed TB cases) were positive in the Gene Xpert MTB/RIF assay. The finding of the positive results coming out from paraffin-embedded formalin-fixed lymph node tissue samples having confirmed *M. tuberculosis* infections validates that the absence of response for *M. tuberculosis* infection in Gene Xpert analysis from paraffin-embedded formalin-fixed prostatic tissues indicates that the prostate tissue samples used in this study did not harbour *M. tuberculosis* indicating that infection of prostate glands by *M. tuberculosis.* These findings indicate that tuberculous prostatitis is rare in a cross section of Bangladeshi population investigated in this study. This finding highlights the need of performing sensitive molecular test such as Gene Xpert in formalin fixed paraffin embedded tissue in ruling out whether a suspected patient is infected with *M. tuberculosis* or not.

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Abbreviations

Symbols	
%	Percentage
≤	Less than equal
≥	Greater than equal
°C	Degree Centigrade
µg	Microgram
µl	Microliter
M	Molar
Ml	Milliliter
Mm	Millimeter
mM	Millimolar
General Terms	
AFB	Acid Fast Bacilli
Bp	Base Pair
BCG	Bacillus Calmette-Guerin
BPH	Benign Prostatic Hyperplasia
BSC	Bio safety cabinet
CIS	Carcinoma In Situ
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DHT	Dihydro testosterone
EPTB	Extra-pulmonary Tuberculosis
et al	With others
GnP	Granulomatous Prostatitis
H37Rv	Reference Strain
ICDDR,B	International Centre For Diarrhoeal Diseases Research, Bangladesh
IFN	Interferon

IL	Interleukin
MTC	*Mycobacterium tuberculosis* complex
MGIT	Mycobacterium Growth Indicator Tube
MOTT	Mycobacteria other than tuberculosis
NHCP	Nodular Hyperplasia with Chronic Prostatitis
NTM	Non Tuberculous mycobacteria
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivatives
PTB	Pulmonary TB
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
PBS	Phosphate Buffer Sulphate
RIF	Rifampicin
Rpm	Rotation Per Minute
RNA	Ribonucleic acid
TB	Tuberculosis
TNF	Tumor necrosis factor
TU	Tuberculin unit
UV	Ultraviolet
WHO	World Health Organization
Z-N	Ziehl- Neelsen

Chapter 1

Introduction & Literature Review

1. Introduction:

1.1 General introduction

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* is globally a major cause of morbidity and mortality, with most cases occurring in developing countries (Murray et al., 1990; Sudre et al., 1992). According to World Health Organization one third of the world population is latently infected with *M. tuberculosis*. In 2013, an estimated 9.0 million people developed TB and 1.5 million died as a consequence. Of them, more than half (56%) were in South East Asia and Western pacific regions. About 60% of TB cases and death occurs among men. TB ranks as the second leading cause of death from an infectious disease worldwide, after HIV (WHO, 2014). Bangladesh ranks 6th position among 22 high TB burden countries (WHO, 2012).

The two types of clinical manifestation of tuberculosis (TB) are pulmonary TB (PTB) and extra pulmonary TB (EPTB). The former is most common (Lee, 2015). Pulmonary tuberculosis comprises of 68.4% of all cases, 20-25% cases are extra pulmonary, while only 27% of the extra pulmonary tuberculosis involves the genitourinary system (Chandra et al., 2012). Extra pulmonary tuberculosis is the occurrence of TB in parts of the body other than the lung. Presentation of extra-pulmonary disease may be atypical or relatively insidious and tuberculosis may not be considered initially in differential diagnosis. Genitourinary tuberculosis accounts for 5-10% of extra-pulmonary cases in developed countries and 15-20% of cases in developing countries (Sáenz-Abad et al., 2008). Tuberculosis of prostate gland is seen in only 2.6% of genitourinary system (Akhtar, 2012). Prostate tuberculosis has mainly been described in immune compromised patients (Gebo, 2002). It is thought that tuberculosis involvement of the prostate is usually a result of haematogenous spread, though it can also occur as a result of descent of the organism from the kidneys, or from local spread from the genital tract (Gorsc, 1985). Sexual transmission of *M. tuberculosis* has been reported, but it is extremely rare (Angus et al., 2001). Although male genital TB seems to be a rare disease, 77% of men who died from TB of all localization had prostate TB, mostly overlooked during their lifetime (Kulchavenya & Krasnov, 2010). Sporer and Auerback (1978) suggested that tuberculosis of prostate is almost always the result of one or perhaps successive haematogenous

seeding. The clinical finding of prostate tuberculosis are often non specific; the symptoms most commonly found are lower genitourinary tract obstruction and painless haematuria (Gebo, 2002).

Prostatic lesions are very common with increasing age. Among prostatic lesions; (i) inflammation of the prostate gland or prostatitis, (ii) benign nodular hyperplasia of prostate, (iii) prostatic intraepithelial neoplasia (PIN) and (iv) prostatic cancer are very common specially of men over 50 years. Inflammation of prostate are usually of four types-1. acute bacterial prostatitis, 2. chronic bacterial prostatitis, 3. chronic abacterial prostatitis and 4. granulomatous prostatitis.

Acute bacterial prostatitis occurs due to urinary tract infection. It is most commonly caused by various strains of *E. coli* and other gram negative enterobacteria. Chronic bacterial prostatitis is difficult to diagnose and treat. The patient may present with low back pain, dysuria, periunial and suprapubic discomfort. Sometimes they may be asymptomatic. It usually occurs due to recurrent urinary tract infection by the same organism. Chronic abacterial prostatitis is much more common form of prostatitis today. It is indistinguishable from chronic bacterial prostatitis. Granulomatous prostatitis may be specific or not. The causes of specific granulomatous prostatitis are BCG vaccination, for treatment of bladder cancer, extension from genitourinary tuberculosis or milliary dissemination of primary tuberculosis by haematogenous spreads. Non specific granulomatous prostatitis may occur from extravasation or secretion from ruptured prostatic ducts or acini. Benign prostatic hyperplasia (BPH) is the most common disorder of the men over 50 years of age. It is characterized by hyperplasia of prostatic stromal and epithelial cells. Resulting in formation of large, discrete nodule in the periurethral region of the prostate. The main initiating event is active metabolites of male hormone testosterone known as 5-dihydrotestosteron (DHT). This leads to proliferation of stromal and epithelial cell leading to nodular hyperplasia. Prostatic intraepithelial neoplasia or PIN is a dysplastic condition which ultimately leads to development of prostatic carcinoma. It is divided into three categories. PIN 1, PIN 2 and PIN 3. PIN 1 and PIN 2 are known as low grade benign as low grade lesion. PIN 3 is a high grade malignant lesion and known as Carcinoma in Situ (CIS). PIN 3 is usually found in 80% of adjacent frank prostatic carcinoma. Prostate cancer is the number one by incidence and number two by mortality among men over 50 years of age throughout the world. It is one of the most remarkable

tumors in term of clinical behavior from very aggressive lethal cancer to incidentally discover clinically in significant cancer. Important etiological and risk factors of prostatic carcinoma are older age, active metabolite of testosterone , 5 DHT, mutation of tumor suppression gene BRCA2, RB gene, PIN (prostatic intraepithelial neoplasia), dilatory factors like increased consumption of fat, decreased lycopene, selenium soya products and vitamin D. Different types of chronic inflammation may also play a part (Robbins &Cotran, 2010).

Tuberculosis is a chronic infection and TB can affect prostate gland and can produce chronic granulomatous prostatitis as well as cancer. Zhang et al., (2010) reported a case of prostatic tuberculosis accompanied by prostate cancer. Studies have shown that approximately 20% of all human cancers in adults result from chronic infection and inflammatory states (De Marzo et al., 2007). Chronic prostate inflammation accelerates initiation of prostate cancer originating from basal cells and accelerates prostate cancer progression (Kwon et al., 2014; Simons et al., 2015). Case studies have shown association of prostatic tuberculosis accompanied by prostatic carcinoma (Zhang et al., 2013; Kulchavenya, & Kholtobin, 2015; Aji et al., 2013). There are reports describing tuberculosis of testis and prostate mimicking testicular cancer (Cho et al., 2013) and prostatitis caused by *M. tuberculosis* infection serving as a predisposing factor for prostate cancer (Kulchavenya, & Kholtobin, 2015).

Co existence of tuberculosis and various cancers are common and reported in different published papers. Stay (1980) reported a case of prostatic adenocarcinoma and genitourinary tuberculosis in a 77 years old male. He concluded that concurrence of these two diseases in the same patient is approximately 1.6 per million population of United States.

In Bangladesh literature search has shown that two case reports of prostatic tuberculosis (Hossain & Alam, 1999; Gafur et al., 2002). Tuberculosis infection is very common in Bangladesh and it follows a long duration of its natural course. For these we thought it will be rationale to see if there is any association between chronic inflammation like tuberculosis infection and development of different prostatic lesions specially cancer.

As Bangladesh is an endemic zone for tuberculosis, prostatic diseases usually occurs after 50 years, people at this stage tend to develop immune compromised state hence

it is highly likely that *Mycobacterium tuberculosis* might be associating promoting cancer in prostatic tissues of elderly patients.

1.2 Literature Review

1.2.1 Tuberculosis- A Global Emergency

Tuberculosis (TB) is globally a major cause of morbidity and mortality, with most cases occurring in developing countries (Murray et al., 1990; Sudre et al., 1992). In 2013, an estimated 9.0 million people developed TB (Figure 1.1) and 1.5 million died from the diseases. Of the estimated 9.0 million people who develop TB in 2013, more than half (56%) were in South East Asia and Western pacific regions. About 60% of TB cases and death occurs among men. TB ranks as the second leading cause of death from an infectious diseases worldwide after HIV (WHO, 2014).

Estimated TB incidence rates, 2013

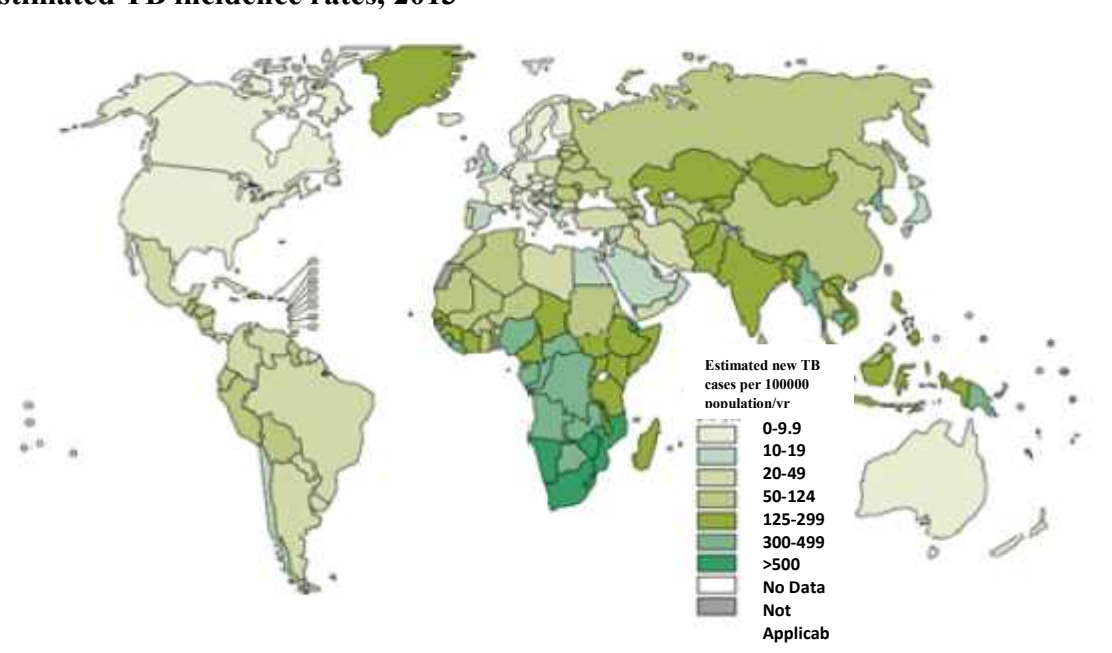


Figure-1.1: **Estimated TB incidence rates by country in 2013 (WHO, 2014).**

1.2.2 Historical Background of Tuberculosis (TB)

Consumption, Phthisis, Scrofula, Pott's disease, and the White Plague are all terms used to refer to tuberculosis throughout the history. Mummies from ancient Egypt,

Greece and Imperial Rome showing signs of spinal tuberculosis and detected by archaeologists known as Pott's diseases were the proof of its presence in ancient times (Hagino et al., 1996).

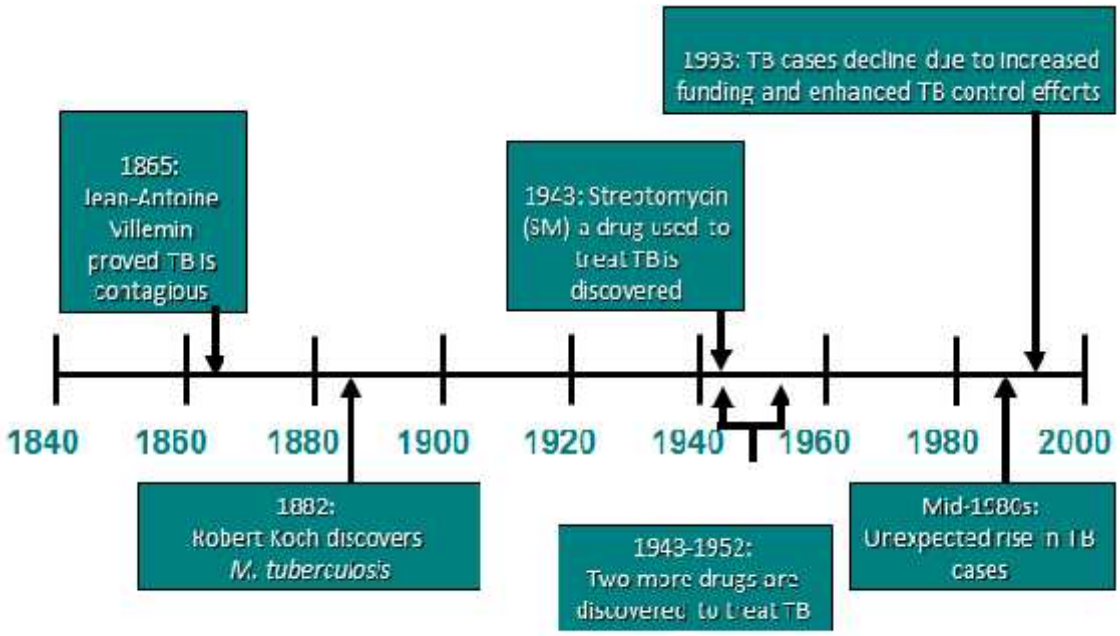


Figure 1.2: Tuberculosis history timeline

Modern history of tuberculosis probably started on December 5, 1865 when the French physician Jean-Antoine Villemin notified the Paris Academy of Medicine that rabbits inoculated with TB developed diseases, thereby demonstrating its infectious nature (Figure 1.2). Later in 1882, Robert Koch isolated *M. tuberculosis* for the first time and conclusively demonstrated in the guinea pig that this slow-growing mycobacterium was the agent of a human disease. He was the first to see *M. tuberculosis* with his staining technique. The first genuine success in immunizing against tuberculosis was developed from attenuated bovine-strain tuberculosis by Albert Calmette and Camille Guérin in 1906. It was called "BCG" (*Bacille Calmette-Guérin*).

1.2.3 *Mycobacterium tuberculosis*

The most pathogenic mycobacteria for human and animal have been grouped under *Mycobacterium tuberculosis* complex (MTC) which till now comprises of following eight members (Cousins et al., 2003):

i. *M. tuberculosis*

ii. *M. africanum*

iii. *M. bovis*

iv. *M. bovis* subsp. *Caprae*

v. *M. canettii*

vi. *M. microti*, and

vii. A recently described species, *M. pinnipedii* (Cousins et al., 2003), with characteristic animal and/or human epidemiologies (Rastogi et al., 2001).

Mycobacterium tuberculosis, along with *M. bovis*, *M. africanum*, and *M. microti* all cause the disease known as tuberculosis (TB) but *M. tuberculosis* is pathogenic for humans while *M. bovis* is usually pathogenic for animals.

The non tuberculous mycobacteria (NTM)s most frequently involved in diseases cases are *M. avium complex, M. kansasii, M. chelonae, M. abscessus, M. xenopi, M .malmoense, M. scrofulaceum, M. marinum, M. ulcerans, and M. haeomiophilum* (Shinners et al., 1999) affecting immune compromised individuals. The NTM *M. ulcerans* causes the necrotizing skin diseases buruli ulcer.

1.2.4 Evolution of *M. tuberculosis*

The mycobacteria grouped in the MTC are characterized by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences (Sreevatsan *et al.,* 1997) but differ widely in terms of their host tropisms, phenotypes, and pathogenicity. Assuming that they all are derived from a common ancestor, it is intriguing that some are exclusively human (*M. tuberculosis*, *M. africanum*, *M. canettii*) or rodent pathogens (*M. microti*), whereas others have a wide host spectrum (*M. bovis*). The evolution of M. tuberculosis complex and non-tuberculous mycobacteria is given in Figure 1.3.

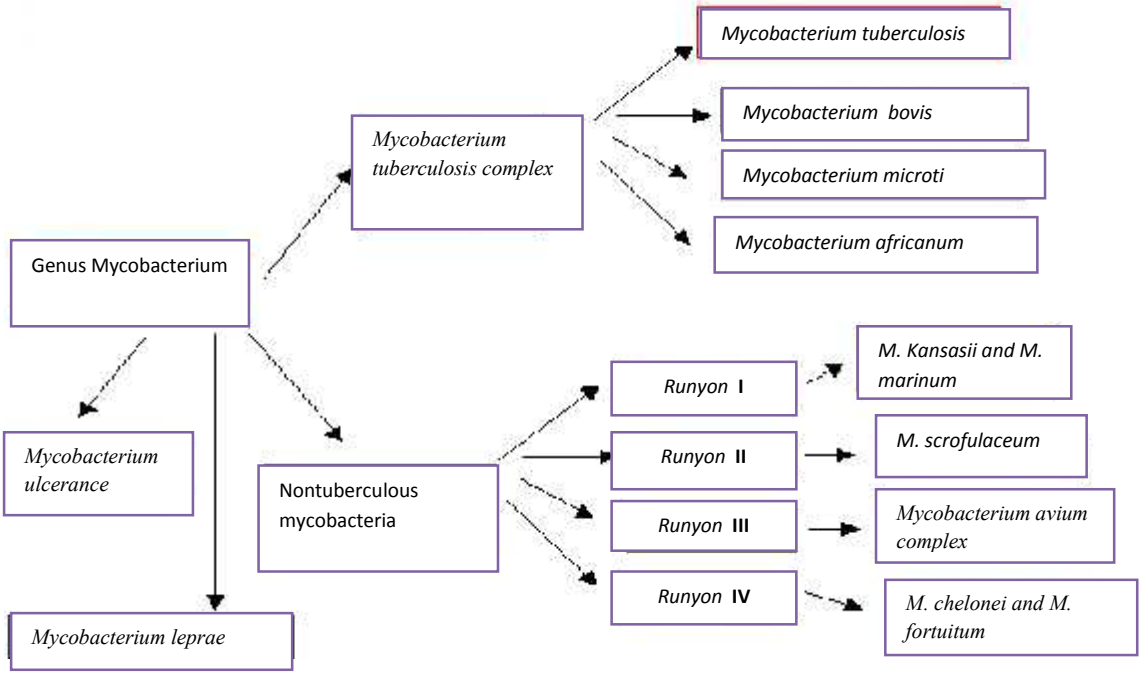


Figure 1.3: Evolution of NTM and *M. tuberculosis* complex.

1.2.5 Unique Characteristics of *M. tuberculosis*

- *M. tuberculosis* is the causative agent of tuberculosis in humans. (Figure 1.4)
- Humans are the only reservoir for the bacterium.
- It is a non-motile rod shaped bacterium.
- The rods are 2-4µm in length and 0.2-0.5µm in width.
- *M. tuberculosis* is a slow-growing obligate aerobe. For this reason, in the classic case of tuberculosis, the MTC are always found in the well-aerated upper lobes of the lungs
- It has slow generation time of 16 to 20 hours, a physiological characteristic that may contribute to its virulence.
- Can withstand weak disinfectants and can survive in a dry state for weeks but, spontaneously, can only grow within a host organism.
- *In vitro* culture of *M. tuberculosis* took a long time to be achieved, usually 8 weeks.

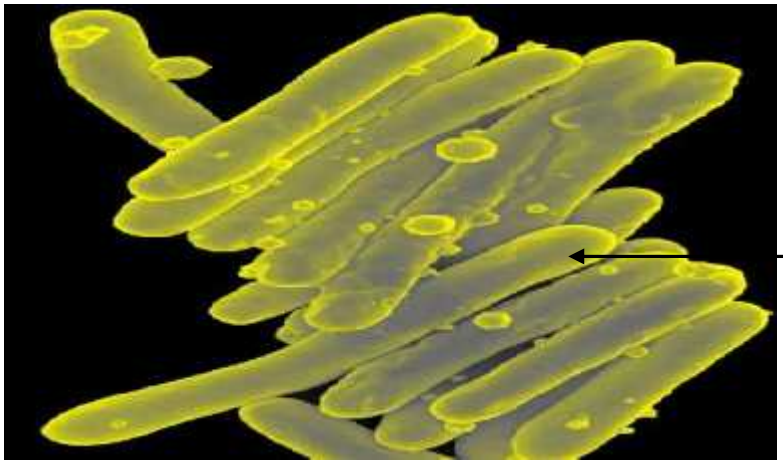


Figure1.4: Scanning electron micrograph of *M. tuberculosis*

1.2.6 Cell Wall Structure

The cell wall structure of *M. tuberculosis* is a major determinant of virulence for the bacterium. The cell wall contains peptidoglycan layer and complex lipid. Over 60% of the mycobacterial cell wall is lipid. The lipid portion of the cell wall contains three major components (Park & Bendelac, 2000) (Figure 1.5).

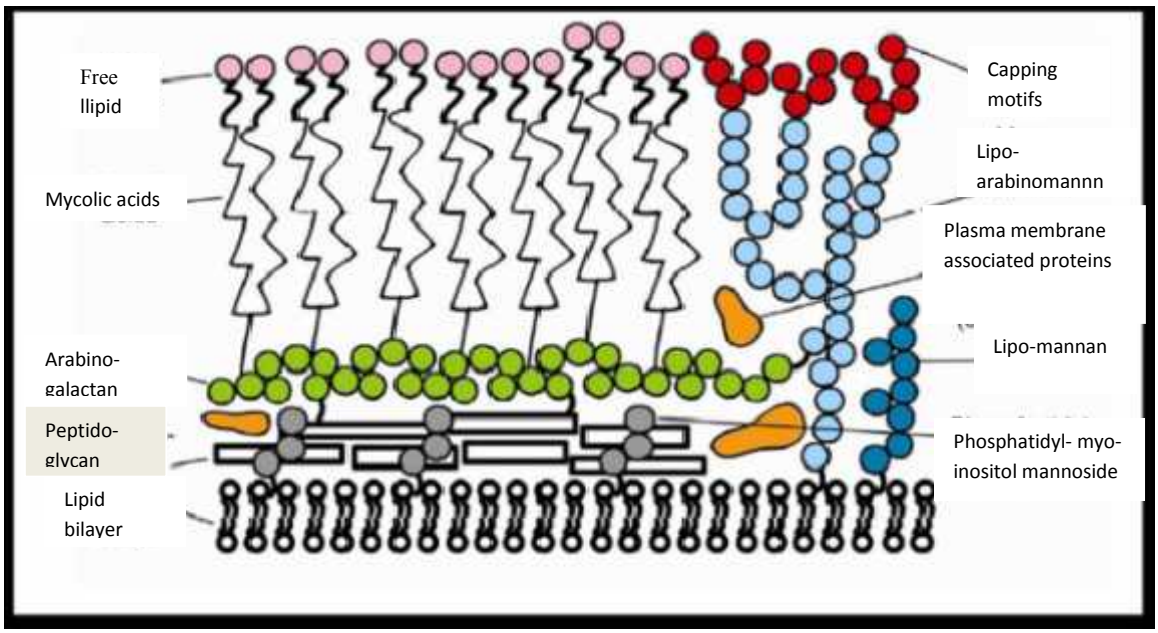


Figure 1.5: **Cell wall structure of *Mycobacterium tuberculosis* complex**

1.2.6.1 Mycolic Acids

- Mycolic acids are alpha-branched lipids found in cell walls of *Mycobacterium.*

They make up 50% of the dry weight of the mycobacterial cell envelope.

- A significant virulence factor of MTB which protect mycobacteria from cationic proteins, lysozyme and oxygen radicals in the phagocytic granule.

1.2.6.2 Cord Factor

- Cord factor is toxic to mammalian cells.
- It is an inhibitor of Poly Morphonuclear Nutrophil (PMN) migration.
- Most abundantly produced in virulent strains of *M. tuberculosis.*

1.2.6.3 Wax-D

Wax-D in the cell envelope is the major component of Freund's complete adjuvant. Finally the high concentration of lipids in the cell wall of *M. tuberculosis* has been associated with following properties of the bacterium such as:

- Impermeability to stains and dyes.
- Resistance to many antibiotics.
- Resistance to killing by acidic and alkaline compounds.
- Resistance to osmotic lysis via complement deposition.
- Resistance to lethal oxidations and survival inside of macrophage.

1.2.7 Tuberculosis - the Disease

Tuberculosis (TB) is a disease caused by the bacterium called *Mycobacterium Sp*. The bacteria usually attack the lungs (Shaw & Taylor, 1998). But, it can attack any part of the body such as the kidney, bone, spine, brain. If not treated properly, TB disease can be fatal.

1.2.8 Predisposing factors for TB infection include

- HIV infection is the first predisposing factor for *M. tuberculosis* infection. Ten percent of all HIV-positive individuals harbour *M. tuberculosis.*
- Close contact with large populations of people, i.e., schools, nursing homes, dormitories, prisons, etc.

- Poor nutrition
- Intravenous drug use
- Alcoholism

1.2.9 Transmission

M. tuberculosis is carried in airborne particles, called droplet nuclei of 1– 5 microns in diameter. Infectious droplet nuclei are generated when persons who have active pulmonary or laryngeal TB disease cough, sneeze, shout, or sing (United States. Centers for Disease Control and Prevention, 2013). Depending on the environment, these tiny particles can remain suspended in the air for several hours. *M. tuberculosis* is transmitted through the air, not by surface contact. Transmission occurs when a person inhales droplet nuclei containing *M. tuberculosis*, and the droplet nuclei travel the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs (United States. Centers for Disease Control and Prevention, 2013) (Figure 1.6).

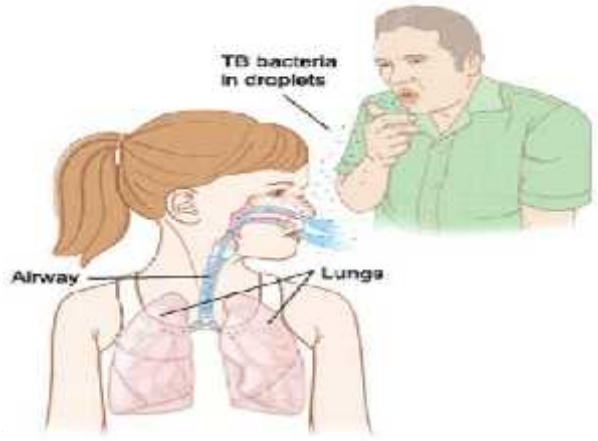


Figure 1.6: Transmission of tubercle bacilli

1.2.10 Types of TB disease

Tuberculosis can be of two types.

1.2.10.1. Active TB infection

TB bacteria become active if the immune system can't stop them from growing. Some people develop active TB disease soon after becoming infected, before their immune

system can fight the TB bacteria. The active bacteria begin to multiply in the body resulting tuberculosis.

1.2.10.2 Latent TB infection (LTBI)

Persons with latent TB infection do not feel sick and do not have any symptoms. They are infected with *M. tuberculosis*, but do not have TB disease. The only sign of TB infection is a positive reaction to the tuberculin skin test or TB blood test. Persons with latent TB infection are not infectious and cannot spread TB infection to others.

1.2.11 Extra pulmonary tuberculosis

The two types of clinical manifestation of tuberculosis (TB) are pulmonary TB (PTB) and extra pulmonary TB (EPTB). The former is most common (Lee, 2015). Extra pulmonary tuberculosis is the occurrence of TB in parts of the body other than the lungs. In EPTB highly vascular areas such as lymph nodes, kidneys, spine, eyes, bones etc are commonly affected (Figure 1.7). Presentation of extra-pulmonary disease may be atypical or relatively insidious and tuberculosis may not be considered initially in differential diagnosis. Inhalation is the most susceptible pathway of *Mycobacterium tuberculosis* infection, pulmonary tuberculosis being the predominant one. Apart from this, Extra Pulmonary Tuberculosis may also arise as a result of the reactivation of tuberculosis focus after dissemination from a chief focus, being a manifestation of tuberculosis infecting about 20% of population worldwide. Extra pulmonary Tuberculosis (EPTB) still constitutes an important clinical problem in Bangladesh. Extra pulmonary forms are more common in immune suppressed persons and in young children.

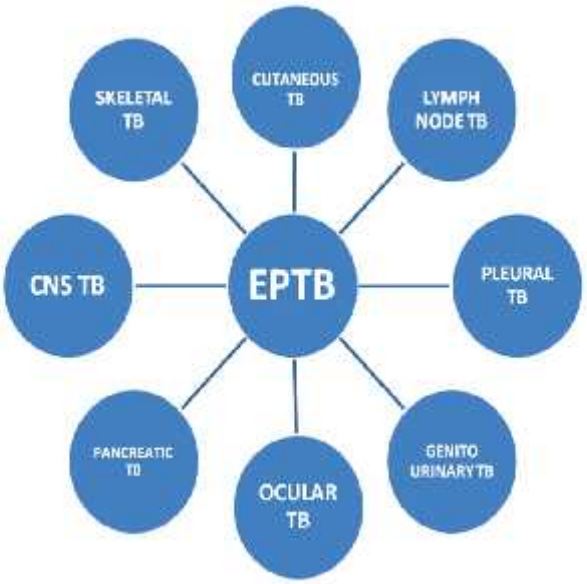


Figure1.7: Representation of various types of extra pulmonary tuberculosis

1.2.12 Symptoms

Symptoms of TB depend on where in the body the TB bacteria are growing. TB bacteria usually grow in the lungs resulting pulmonary tuberculosis (Shaw, 1998) but can also spread outside the respiratory organs affecting the pleura, central nervous system, lymphatic system, genitourinary system, bones and joints etc. These are collectively denoted as "extra pulmonary tuberculosis" (EPTB). Extra pulmonary forms are more common in immune suppressed persons and in young children. Infectious pulmonary TB may co-exist with extra pulmonary TB, which is not contagious (Helb et al., 2010). The symptoms associated with pulmonary and extra pulmonary TB infection is given in Table 1.1.

Table 1.1 Symptoms of pulmonary & extra pulmonary TB

Symptoms of Pulmonary TB Disease (TB disease usually causes one or more of the symptoms)	**Symptoms of Extra pulmonary TB Disease** (Depends on the part of the body that is affected by the disease)
▪ Cough (especially if lasting for 3 weeks or longer) with or without sputum production. ▪ Coughing up blood (hemoptysis) ▪ Chest pain ▪ Loss of appetite ▪ Unexplained weight loss ▪ Night sweats ▪ Fever ▪ Fatigue	▪ TB of the kidney may cause blood in the urine ▪ TB meningitis may cause headache ▪ TB of the spine may cause back pain ▪ TB of the larynx can cause hoarseness ▪ Loss of appetite ▪ Unexplained weight loss ▪ Night sweats ▪ Fever ▪ Fatigue

1.2.13 Pathogenesis

1.2.13.1 Pathogenesis of Pulmonary Tuberculosis

Step 1: Tubercle bacilli in the form of droplet nuclei are inhaled and reached at the terminal alveoli after avoiding entrapment by mucociliary clearance mechanisms (Figure 1.8).

Step 2: Initial infection is most common in the lower lung segments where ventilation is the greatest. The organisms are ingested by alveolar macrophages, but continue to multiply (Fries *et al.,* 1991). A mild local reaction with additional macrophages and lymphocytes develops (Figure 1.8).

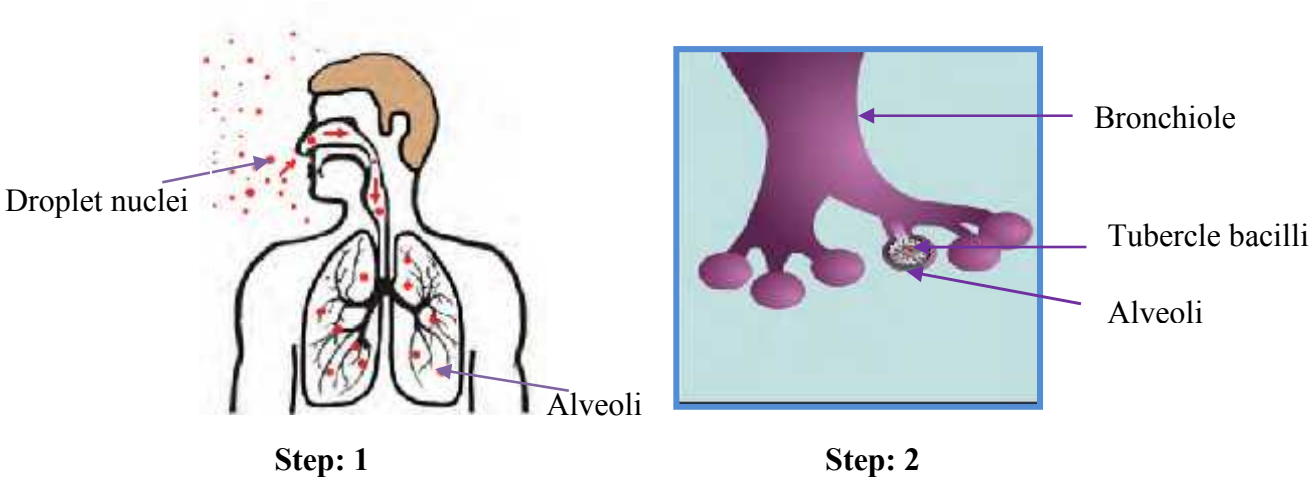
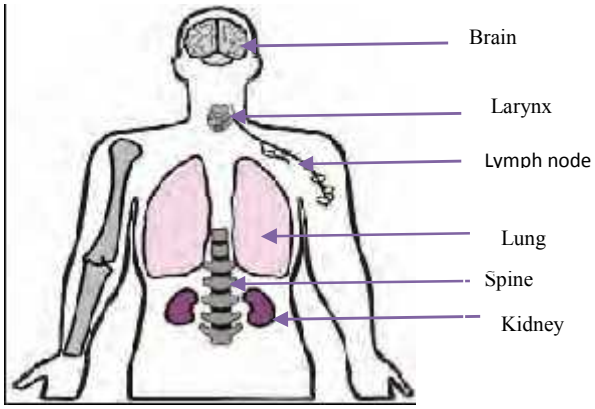


Figure 1.8: Steps involved in the pathogenesis of *Mycobacterium tuberculosis*

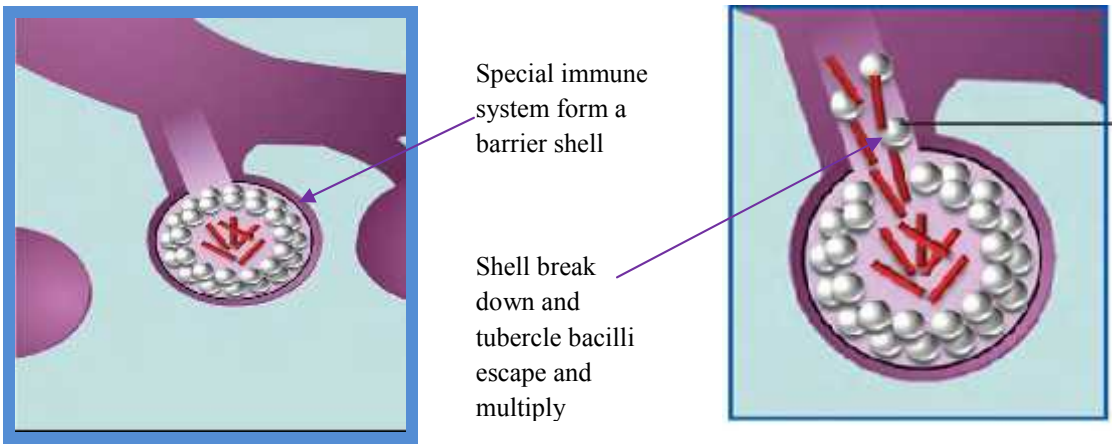
Step 3: A small number of tubercle bacilli enter the bloodstream and spread throughout the body. The tubercle bacilli may reach any part of the body, including areas where TB disease is more likely to develop (such as the brain, larynx, lymph node, lung, spine, bone, or kidney) (Figure 1.9).

Step 4: Within 2-4 weeks, infected macrophages carry the organism to regional lymph nodes (hilar and mediastinal), where multiplication of the organism continues in presence of a minimal inflammatory response. These macrophages form a barrier shell, called a granuloma, that keeps the bacilli contained and under control (Latent TB) (Figure 1.9).

Step 5: If the immune system cannot keep the tubercle bacilli under control, the bacilli begin to multiply rapidly (TB disease). Bacillemia occurs at 4-6 weeks after inhalation of the organism, resulting extension into the bloodstream from the regional nodes (Figure 1.9).



Step 3



Step 4 **Step 5**

Figure 1.9: Steps involved in the pathogenesis of *Mycobacterium tuberculosis*

1.2.13.2 Pathogenesis of extra pulmonary tuberculosis

Isolated peripheral tuberculous lymphadenopathy is usually due to reactivation of disease at a site seeded haematogenously during primary tuberculosis (TB) infection, perhaps years earlier (Alvarez & McCabe, 1984).

In the genitourinary system, the tubercle bacillus lodges in the glomerular and peritubular capillary bed from hematogenous seeding of *M. tuberculosis* from the primary site of inhalation, the lungs (Gibson et al., 2004). Hematogenous seeding of both kidneys occurs, but clinically significant disease is usually limited to one side. Genitourinary TB is commonly reported as a dissemination of the TB infection but the practitioner must also be aware that it may be a localized genitourinary disease (Eastwood, Corbishley, & Grange, 2001).Occasionally, lymphatic spread or secondary spread may occur from TB of the genitourinary tract or bone (Khan,

Chandramohan, & MacDonald, 2004). The kidneys are the most commonly involved organ after the lung but the seminal vesicles, prostate, and testes although rare, may be primarily involved as well.

Abdominal tuberculosis represents the sixth most frequent form of extra-pulmonary tuberculosis after lymphatic, genitourinary, bone and joint, miliary, and meningeal tuberculosis. Tuberculous bacteria reach the gastrointestinal tract via hematogenous spread, ingestion of infected sputum, or contiguous spread from adjacent organs (Aston, 1997).

1.2.14 Prostatic tuberculosis

Genitourinary tuberculosis contribute 20-25% cases of extra pulmonary tuberculosis. Tuberculosis of prostate gland is seen in only 2.6% of genitourinary system (Akhtar, 2012). Prostate tuberculosis is much less common among genitourinary tuberculosis. It is thought that tuberculosis involvement in the prostate is usually the result of haematogenous spread, though it can also occur as a result of descent of the organism from the kidneys, or from local spread from the genital tract (Grosc & Belshe 1985). Sporer et al., (2007) suggested that tuberculosis of prostate is almost always the result of one or perhaps successive haematogenous seeding. Sexual transmission of *M. tuberculosis* has been reported, but it is extremely rare (Angus et al., 2001). The clinical findings of prostate tuberculosis are often non specific; the symptoms most commonly found are lower genitourinary tract obstruction and painless haematuria (Gebo, 2002).

1.2.15 Diagnosis of Extra pulmonary TB

Extra pulmonary tuberculosis can be diagnosed by physical examination, histopathological examination, AFB smear, culture, nucleic acid amplification.

1.2.15.1 Tuberculin skin test

This is a test used to determine presence or absence of TB infection in a person. 5 Tuberculin units (0.1mL) of purified protein derivative (PPD) which is intradermaly injected in the forearm. Presence or absence of induration is measured within 48-72 hours. Induration over 10 mm is considered as +ve and less then 10 mm is negative.

1.2.15.2 AFB – Microscopy

M. tuberculosis is identified microscopically by its staining characteristics. It retains certain stains after being treated with acidic solution, and is thus classified as an "acid-fast bacillus" or AFB (Madison, 2001). In the most common staining technique, the Ziehl-Neelsen stain, AFB are stained bright red which stands out clearly against a blue background. Acid-fast bacilli can also be visualized by fluorescent microscopy, and by an auramine-rhodamine stain.

1.2.15.3 Culture Methods

Solid or liquid media are used in culture methods. Culture provides high sensitivity and specificity but the growth rate is slow (Kallenius et al.,1994; Walker, 2001). Solid culture media are of two types. Agar based 7H11 and egg based Lowenstein Jensen. One of the most widely used broth systems is the non-radiometric mycobacteria growth indicator tube (MGIT) (Bacton Dicknson, Sparks, MD), Which contains a modified Middlebrook 7H9 broth in conjuction with a fluorescence quenching-based oxygen sensor to detect mycobacterial growth. As the mycobacteria grow and deplete the oxygen present, the indicator fluoresces when subjected to ultraviolet light. This liquid culture system BACTEC radiometric system generally detects organism within 9 to 16 days, depending on the number of organisms in the specimen.

1.2.15.4 Polymerase chain reaction (PCR)

Rapid nucleic acid amplification techniques such as polymerase chain reaction (PCR) allow direct identification of *M. tuberculosis* in clinical specimens. Such method detects as few as 10 organisms in clinical specimens, compared with the 10,000 necesary for smear positivity. A variety of PCR methods have been developed for detection of *Mycobacterium tuberculosis* and other mycobacteria (Musial et al., 1988). A large number of PCR assays targeting different gene stretches of *M. tuberculosis* have been described (Sjobring et al.,1990). IS6110 PCR analysis, RD9, TbD1 are most commonly used PCR probes to detect *M. tuberculosis.*

1.2.15.5 Gene Xpert MTB/RIF

The Gene Xpert MTB/RIF is a cartridge-based, automated diagnostic test that can identify *M. tuberculosis* (MTB), together with the strains and resistant to rifampicin (RIF) by real time polymerase chain reaction (RT-PCR). It is based on a platform for rapid and simple-to-use nucleic acid amplification tests (NAAT). The Xpert® MTB/RIF purifies and concentrates *M. tuberculosis* from sputum samples, isolates genomic material from the captured bacteria by sonication and subsequently amplifies RNA polymerase beta (*rpoB*) gene in the *M. tuberculosis* genome in a real time format using fluorescent probes called molecular beacons. Gene Xpert assay uses 3 specific primers and 5 unique molecular probes to ensure a high degree of specificity. Results of Gene Xpert assays are obtained from unprocessed sputum samples in 90 minutes, with minimal risk of biohazard.

1.2.16 Prostate Gland

Prostate gland is an endocrine gland of male genital system located retroperitoneally encircling the neck of the urinary bladder, urethera and devoid of distinctive capsule. It weighs approximately 20 grams (Figure 1.10).

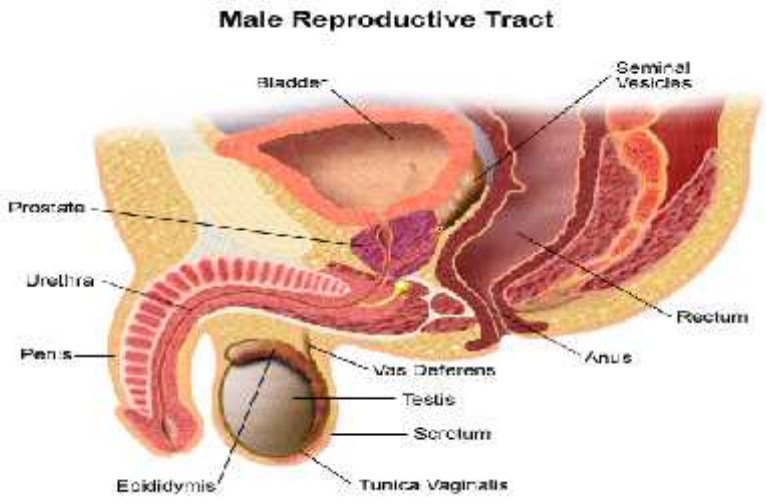


Figure 1.10: Normal anatomy of prostate gland

Histologically prostate is composed of gland lined by two layers of cells. A layer of low cuboidal epithelium covered by a layer of secretory columnar cells. The glands are separated by abundant fibromuscular stroma, Testicular androgen control the growth and survival of prostate cells.

In adult anatomically and biologically prostatic parenchyma is divided into four distinct zones peripheral, central, transitional, anterior fibromuscular zone. Most hyperplasia occurs in transitional zone and most cancers occur in peripheral zone (Figure 1.11) Prostate secret fluid that nourishes and protects sperm. It also act as antibacterial agent.

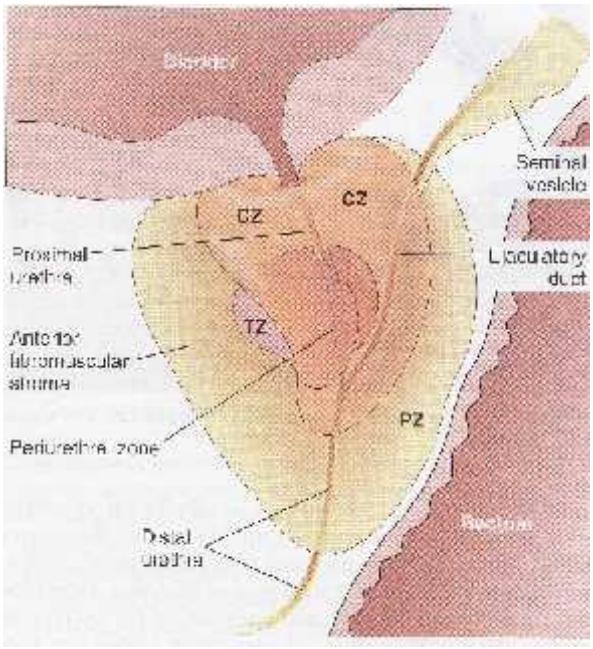


Figure 1.11: Adult prostate. The normal prostate contains several distinct regions, including a central zone (CZ), a peripheral zone (PZ), a transitional zone (TZ), and a periurethral zone. Most carcinomas arise from the peripheral glands of the organ and may be palpable during digital examination of the rectum. Nodular hyperplasia, in contrast, arises from more centrally situated glands and is more likely to produce urinary obstruction early than is carcinoma.

Only three pathologic process or disease occurs in prostate. Inflammation or prostatitis, Benign nodular hyperplasia and cancer (Robbins and Cotran, 2010). Prostatic lesions are the main disease involving men after 50 years. These lesions

include nodular hyperplasia or benign enlargement of prostate accounting for 20% lesions at 40 years. Prostatic cancer is the most common cancer in men and the incidence is 25% worldwide the mortality is prostatic cancer death is 10%. Prostatitis or inflammation of prostate accounts for 50% of prostatic lesion after 40 years. But acute prostatitis is much less common than chronic prostatitis.

Upper left & right of Figure 1.12 shows normal prostate gland. Lower figures showing different stages of prostate cancer. Stage 1 (Figure 1.12) shows-small irregular nodule, grayish white in color & localizes in one lobe. Stage 2 shows multiple irregular grayish white nodule in two lobes. Stage 3 shows multiple irregular nodules throughout the prostate and involvement of lymph node and blood vessel. Stage 4 shows multiple irregular nodules throughout the prostate and involvement of lymph node and blood vessel & it has infiltrated and come outside the prostate gland involving rectum and urinary bladder (Figure 1.12).

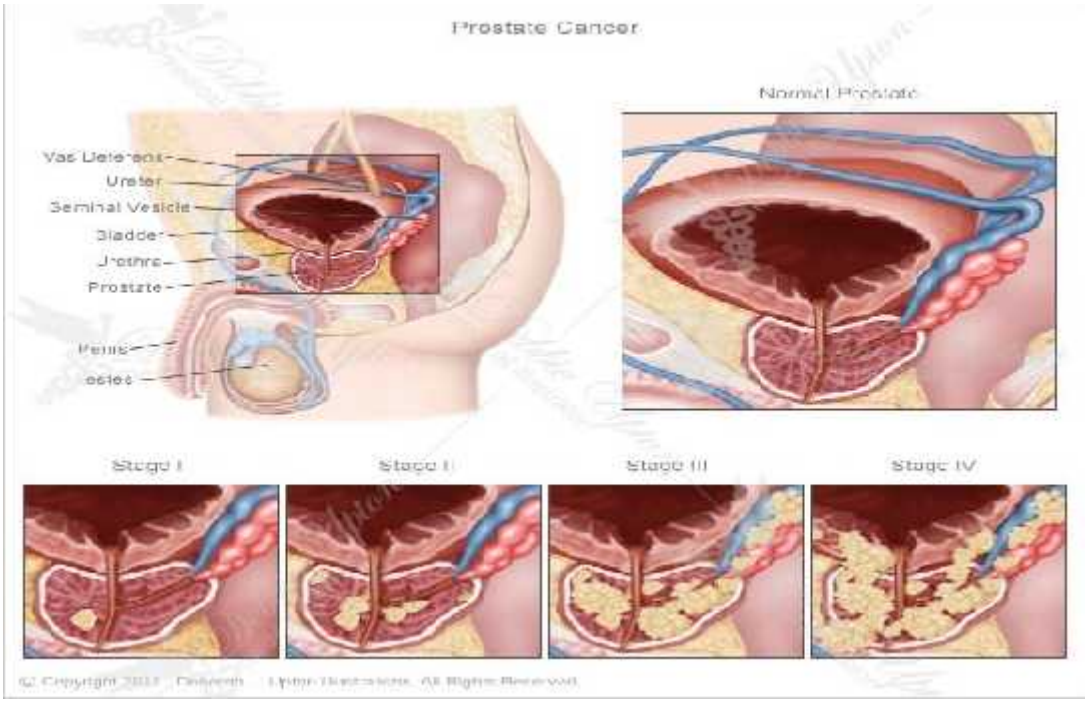


Figure 1.12: N**ormal prostate gland and different stages of cancer**

Experts say that prostate cancer starts with tiny alterations in the shape and size of the prostate gland cells - Prostatic intraepithelial neoplasia (PIN) which is a precancerous stage.

Inflammation is associated with different diseases of prostate including nodular hyperplasia and cancer. One study was carried out by Simons et al., (2015) showed compared to control, infection induced both acute and chronic inflammatory cells with epithelial proliferation and hyperplasia, stromal hyperplasia and inflammatory cell infiltrate. They found in areas of inflammation epithelial proliferation and hyperplasia after persist.

Chronic inflammation has been shown to promote initiation and progression of diverse malignancies by inducing genetic and epigenetic alterations. Using animal model of prostatitis induced by infection from the uropathogenic bacteria. Kwon et al., (2013) showed that acute and chronic inflammation causes tissue damage and create a microenvironment that induced initiation of prostate cancer with a basal cell origin.

Co-existence of tuberculosis and various cancers are common and reported in different published papers. A study was carried out by Dacosta & Kinare., (1991) reported that, two hundred and twenty one consecutive cases of bronchogenic carcinomas were studied histologically for evidence of associated lesions. The most frequent was tuberculosis, seen in 29 patients. This study also claimed that there is significantly high incidence of tuberculosis in association with bronchogenic carcinoma.

Sheikh et al., (1995) reported a 70 year old female was diagnose with both adenocarcinoma with coexistent tuberculous colitis. Moruta et al., (1983) postulated that ulcerative lesions of tuberculosis may be precursor of carcinomas, derived from a chronic inflammatory process with repetition of erosion, ulcer, and consequent regeneration.

Tuberculosis of vertebral column with prostate involvement in a 70 years old male was described by Aji et al., (2013). The patient presented with low back pain,

progressive weakness of lower limbs and lower urinary tract symptoms. His prostate gland enlarged nodular, PSA 6ngm/ml and MT positive. Prostate biopsy reveled tuberculosis and lumbosecral X ray reveled features of pott' diseases (tuberculosis of the spine).

Stay (1980) reported a case of prostatic adenocarcinoma and genitourinary tuberculosis in a 77 years old male. He concluded that concurrence of these two diseases in the same patient is approximately 1.6 per million population of United States.

In Bangladesh, literature search has shown that two cases have been report of prostatic tuberculosis. The first study by Hossain and Alam (1999) had showed that the 32 old man complains lower abdominal discomfort , weakness. Low grade fever, dysuria and treated with different antibiotic but without any result. An USG biopsy of the prostate was done t and the biopsy report showed tuberculosis. Gafur et al., (2002) also repoted a case of prostatic tuberculosis.

Zhang et al., (2010) reported a case where a 74 year male patient was diagnosed with both granulomatous inflammation and prostate carcinoma. A case report of prostatic tuberculosis accompanied by prostate cancer. He reported a 74 years male complained 10 kg weight loss , frequency, ugency dysuria. He has past history of taking treatment of pulmonary tuberculosis. Prostatic biopsy revealed both granulomatous inflammation and prostate carcinoma. His PSA was 83.2 ngm/ml.

Kulchavenya & Kholtobin (2015) reported a 72 years male who was cured from pulmonary and prostate tuberculosis. Seven years after recovery he presented with dysuria and elevated PSA level (11.0 ng/ml). Prostate biopsy revealed prostate cancer. He opined that prostate TB like any other chronic infectious inflammation may predispose to prostate cancer.

1.3. Aims and objectives

Aims

This study aims to investigate whether there is any association between tuberculosis of prostate and development of prostatic lesions especially cancer in a cross section of Bangladeshi population.

Objectives

1. To determine prostate specific antigen (PSA) in patient's sera.
2. To observe socio-economic condition in development of prostate cancer.
3. To observe the epidemiology of prostatic disorders in Bangladesh.
4. To observe the prevalence of *Mycobacterium tuberculosis* in prostatic tissues.

Chapter 2

Materials & Methods

2. Materials and Methods

The investigation attempts to find out any association of *Mycobacterium tuberculosis* in promoting cancer in prostatic tissues of elderly patients with different prostatic lesions. The clinically suspected prostatic tissues were processed for routine H&E stain for detection of different prostatic diseases. Then serological analysis (total PSA) was done for the correlation of prostatic diseases. Then the AFB stain of tissue sections were done for detection of *M. tuberculosis*. For confirmation, we did conventional PCR and some few cases of Gene Xpert MTB/RIF. All chemicals and reagents, apparatus used in this study are given in the appendix I, II, III respectively.

2.1 Study place and period

Prostatic tissue samples were collected from the suspected prostatic patients of different hospitals of Dhaka city. The samples were collected from August, 2013 to December 2014.

Ethical approval

This study was reviewed by ethics committee of the Anwar Khan Modern Medical College (AKMMC) **(reference number AKMMC/15/2385)**

2.1.1 Study subjects

2.1.1. a. Prostatic tissues: The study was conducted with different prostatic patients having complained fever, haematuria, incomplete micturation etc. A total 85 prostatic tissue samples were collected from the prostatic disorder patients.

2.1.1 .b. Known TB positive lymph node tissue

These study subjects are 2 known TB positive lymph nodes. These patients were used as control.

2.2 Type of the study

The study was designed as a cross sectional retrospective study. The overall study design is outlined in Figure 2.1.

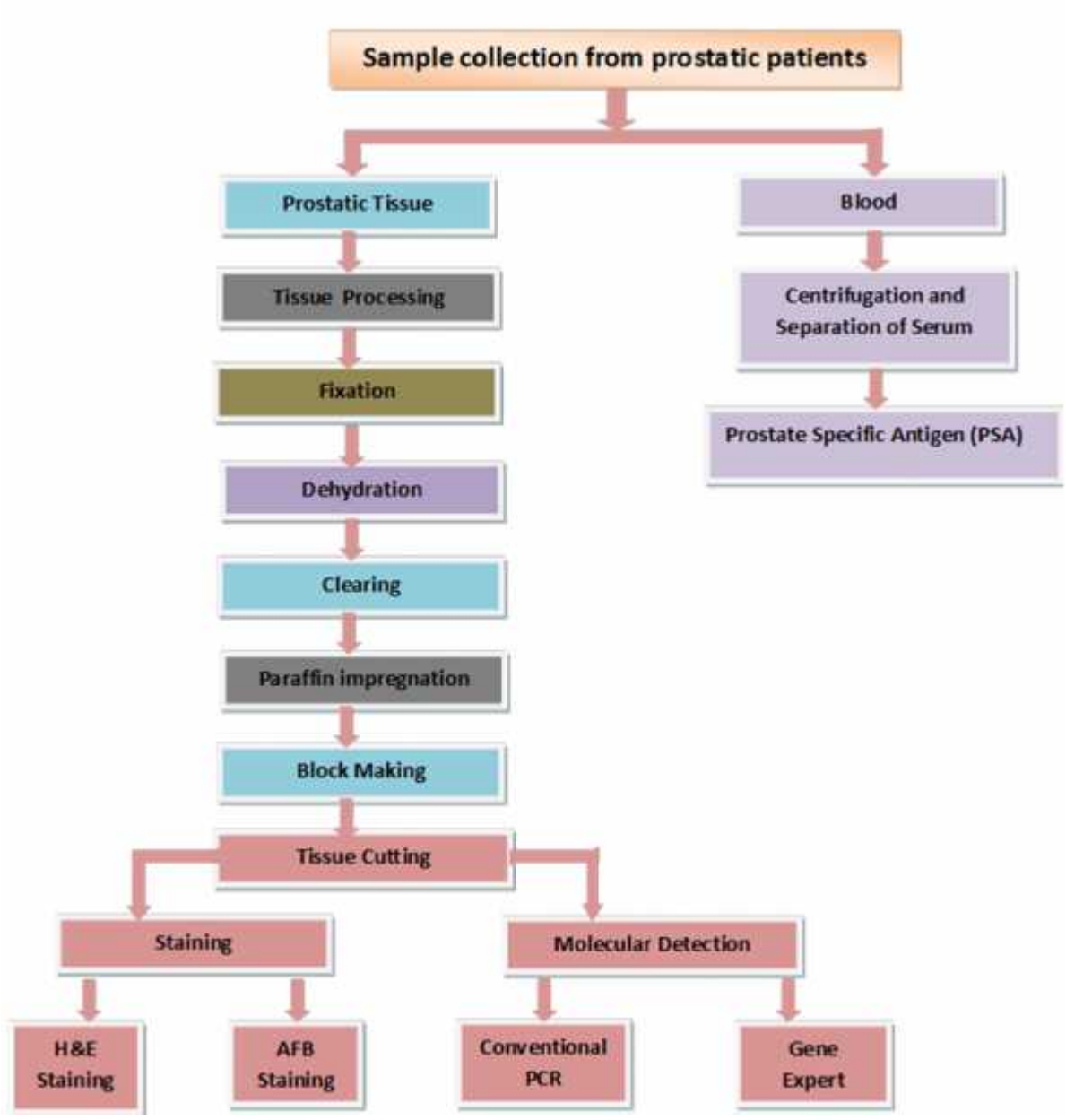


Figure 2.1: Diagrammatic presentation of workflow of investigation

2.3 Patients history taking

A questionnaire was used for data collection. Data on age, sex, occupational status, clinical features, smoking habit etc were collected. The details questionnaire is given in Appendix II.

2.4 Collection and transportation of specimen

Prostatic tissues were collected from prostatic patients and blood from anti-cubital vein.

2.4.1 Collection of prostatic tissue

The patients were admitted in BIRDEM and different clinics of Dhaka city. The biopsies of the patients were taken by an Urologist from patients having suspected prostatic disease after clinical and other relevant investigations. After anaesthetics, assistant surgeons performed the operation known as TURP (Trans urethral resection of prostate) and took out the prostatic tissues from the patients and preserved it in 4% formalin preservative. These TURP samples were sent in the laboratory.

Figure 2.2: Collection of prostatic tissue

Figure 2.3: Transportation of the tissue

2.4.2 Transportation of prostatic tissue

After collection, the prostatic tissue samples were immersed in a transport container. The samples were properly labelled and transported to the Anwer Khan Modern Medical College & Hospital (AKMMC), Dhaka.

2.4.3 Collection of blood

Under all aseptic precautions (cleaning the area with povidone iodine followed by adequate rubbing by alcohol pad containing 60% isopropyl) 5 ml of venous blood was collected from median ante-cubital or appropriate veins by gentle suction. The blood samples were allowed to clot at 15-24^{o}C. The clotting time was minimally 30 minutes to maximally one hour.

(a) Blood samples collection **(b) Serum separation**

Figure: 2.4: Procedure of (a) Blood sample collection (b) Serum separation

2.4.4 Blood sample transportation:

Immediately after collection the blood samples with proper labelling were carried to the laboratory.

2.5 Serum Preparation and storage

For serum preparation, blood samples were spin for 10 minutes at 1500g. After centrifugation, the tubes were inspected carefully in order to recognize possible haemolysis. The serum was promptly separated from clots or cells and transferred to a clean eppendorf tube and was carefully marked with sticker or other method with identification number.

2.6 Histopathology

Different tissue samples were immersed in 4% formalin and embedded in paraffin. Paraffin embedded tissue sections were cut 5/6 μm, mounted on glass slides and kept at room temperature for further histopathological study.

2.6.1 Techniques

(a) Introduction: In modern medical practice histopathological investigations are regarded as essential tools in patient care in order to make meaningful interpretations of tissue preparations and a dependable diagnosis.

(b) Documentation: Once a tissue sample is received in the laboratory a serial number was given to the sample with name of the patient, age, sex, nature of the tissue, nature of biopsy, name of the referring physician and any either be entered in a register or in a computer.

(c) Storage: The histology laboratory should have a specified space where paraffin blocks and slides can be stored. There are specially designed cabinets available or can be prepared. Blocks and slides should be kept serially.

(d) Discard: At the end of the year unimportant slides and blocks were discarded.

2.6.2 Fixative and tissue processing

In order to prevent putrefactions and autolysis the tissue samples must be preserved in appropriate fixatives. This is the first step in tissue processing.

(a) Fixatives: The main function of fixatives is to stabilize the protein by cross linking. Soluble proteins are linked to structural protein and thus made insoluble. The main purpose of fixation is to preserve the tissue structure to normal stage. Fixation usually takes place in aqueous solutions.

(b) Temperature: Fixation usually carried out at room temperature. However, the process of fixation is enhanced at 36^{o}C.

(c) Penetration of fixatives: Penetration of tissues by fixatives is an important matter. All fixative cannot penetrate tissue blocks with equal speed.

(d) Concentration of fixatives: This is variable for different fixatives. For formalin it is 10% to 4% formaldehyde.

2.6.3 Tissue processing

This refers to various treatments to which tissue blocks are subjected before paraffin wax impregnation. This is the aim for cutting suitable sections and staining. Steps to be ensured before processing can be started. These include assignment of proper identification number and satisfactory fixation.

- **Stages of processing:** Included in the stages are dehydration, clearing and paraffin wax impregnation.
- **Dehydration:** This is achieved by the process of tissue graded of alcohols.
- **Clearing:** After satisfactory dehydration, clearing of tissue blocks are to be done. This step is essential before paraffin impregnation. The usual clearing agent is xylene.
- **Paraffin impregnation:** In order to achieve satisfactory paraffin impregnation good fixation, dehydration and clearing are prerequisites. For Bangladesh the melting point of paraffin wax is 60^{o}- 65^{o}C.

2.6.4 Block making

On completion of paraffin impregnation blocks of proposed tissue are held in solidified paraffin blocks. In order to obtain desired section embedding has to be obtain desire section embedding has to be made in such a way that sections are made perpendicular to the surface. This can be achieved readily when the orientation is known. Blocks can be made in paper boats, metallic “L”s or in cassettes and stainless steel moulds. Whatever the method is identification number must always be affixed.

2.6.5 Section cutting (Microtome)

For cutting sections two types of microtome's were used. The most commonly used one was rotary microtome. The other one was sliding microtome. The microtome should be placed on a heavy table. Properly trimmed paraffin block with the contained tissue is fixed with the clump of the microtome in a secure manner. At this stage care should be taken in tightening the screw block holder to avoid vibration during section cutting. Then the microtome knife is put in place and secured tightly with the help of the screws are available for the purpose. Before starting the actual sectioning, the block is trimmed properly so that an appropriate cutting surface is exposed. At this stage geometry of the knife such as, the angle of slope, the clearance angle and the angle between rake and knife should be properly adjusted.

(a) Cutting the sections in rotary microtomes

(b) Equipments: 1) a pointed forcep 2) a small hair brush 3) scalpel 4) ice cubes 5) a warm waterbath for floatation of sections and 6) slides.
The trimmed block is sectioned at 5-6 μm thickness. The ribbions were lifted by means of brush and the forceps or the scarples and floated in the waterbath. The temperature is usually 50^{o}C. The suitable sections are lifted on the appropriately numbered glass slide. The slides were then kept in a slanting positions for drying. The drying is completed overnight. Now the slides are ready for stain.

2.6.6. Paraffin embedding and microtomy procedure

(a) Processing of tissue

Following overnight fixation in 4% formalin, the tissue blocks were gradually dehydrated in ascending concentration of ethyl alcohol. Then it was cleared in xylene, impregnated in paraffin for making blocks and sectioning.

The following stages were followed during processing:

(A) Receiving: Patient name and address were resisted.

(B) Fixation: Fixation in 4% formalin- overnight.

(C) Dehydration: 50% alcohol	1 hour
70% alcohol	1 hour
80% alcohol	1 hour
Absolute alcohol	1 hour
Absolute alcohol	1 hour or overnight
(D) Clearing:	xylol I 1 hour
	Xylol II 1 hour

(E) Paraffin impregnation: A paraffin bath with a temperature of 60^{o}-70^{o} C was used. Melting point of the paraffin used was 58^{o}-62^{o}C. The tissues were treated with paraffin for 1 hour or 2 hour.

(F) Paraffin embedding: Metallic moulds were used for embedded the tissue in melted paraffin. The moulds were first lubricated with liquid paraffin. Melted paraffin was poured onto it. The tissues were carefully embedded in proper plane. The paraffin was then allowed to harden at room temperature. After hardening the moulds were removed and the blocks were trimmed properly to mount on a block holder. The blocks were kept in the ice chamber of a refrigerator for sometime before for section cutting.

(G) Microtomy or section cutting: Each block of the tissue on the holder was fitted in the microtome machine. A properly sharpened microtome knife was used for cutting sections. A water bath with a temperature of 45^{o} to 50^{o}C was used for flotation for sections. Sections were cut at 5-6 μm thickness. Ribbons of the sections were selected and placed on the water bath. The sections were taken on glass slides. The slides were kept in slanting position for some time to drain out the water and then allowed to dry at room temperature. Then the slides were ready for staining.

2.7 Harris Haematoxylin and Eosin (H&E) staining method

The procedure of Harris Haematoxylin and Eosin (H&E) stain is given in Figure 2.5

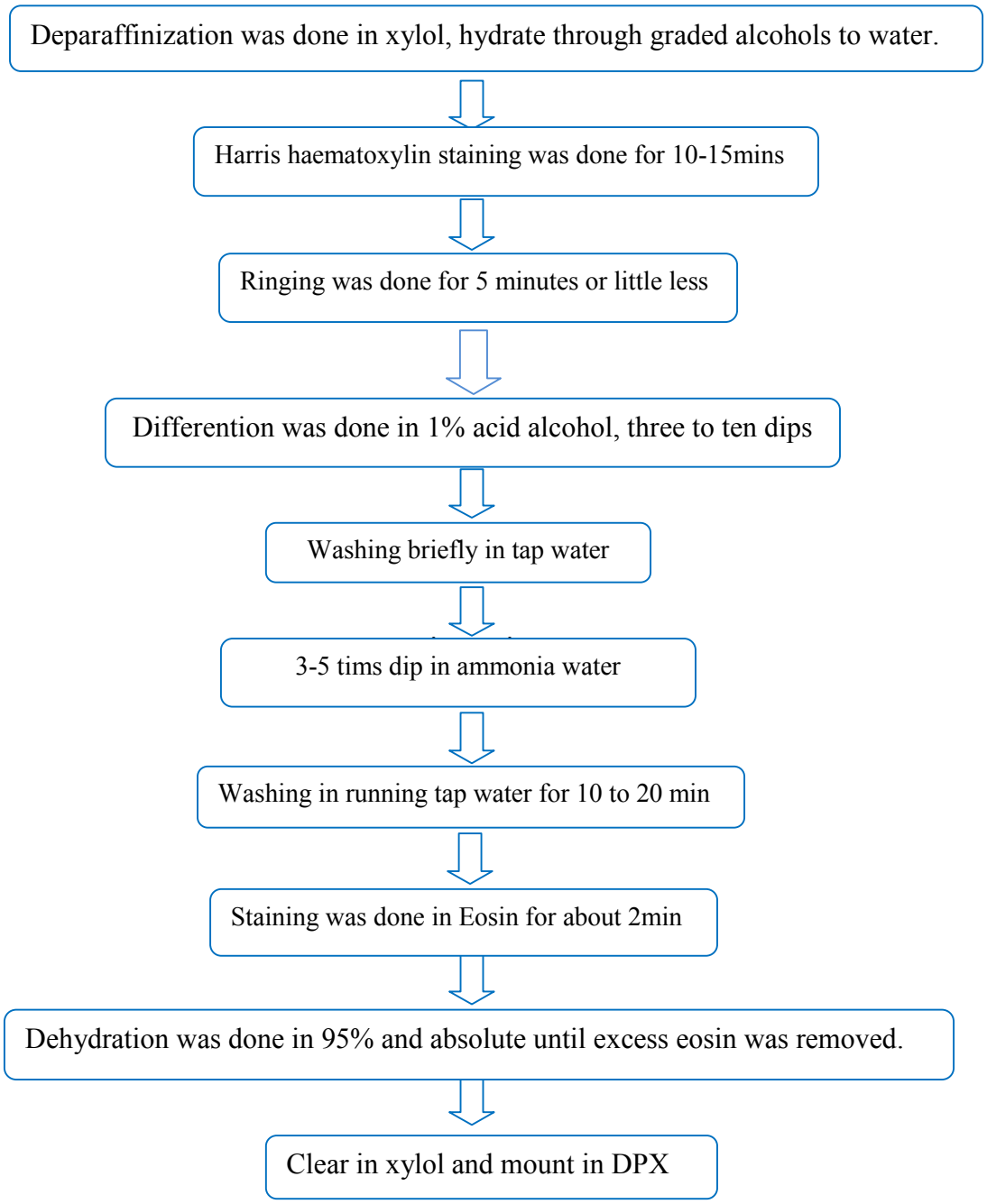


Figure 2.5: Flowchart showing the H&E staining procedure.

2.8 Ziehl-Neelsen staining method

The procedure of Ziehl-Neelsen staining is given in the Figure 2.6

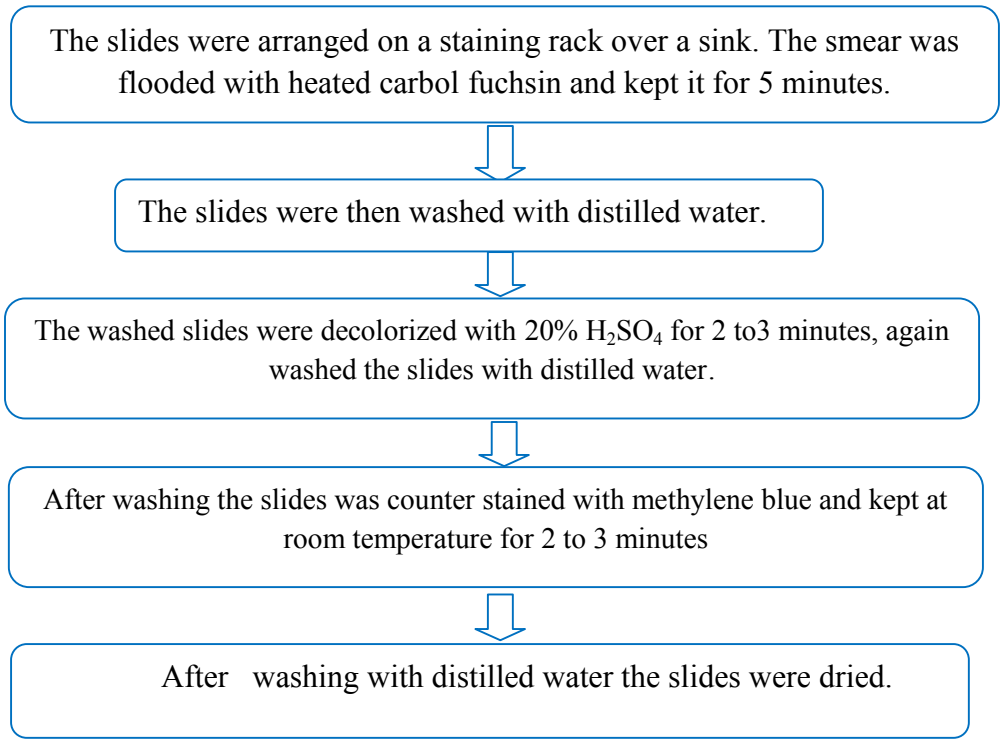


Figure 2.6: Flowchart showing the Z-N staining procedure

2.9 Molecular Study

2.9.1 Polymerase Chain Reaction

This study includes three major steps:

I. DNA extraction from samples
II. DNA amplification in a thermal cycler
III. Gel electrophoresis and visualization under UV light

2.9.1.1 Extraction of DNA from samples

The DNA was extracted by using Maxwell® 16 FFPE Tissue LEV DNA purification kit (Promega, USA) automatically by Maxwell16 instrument with low elution volume.

2.9.1.2 Sample processing protocol.

1. One to ten 5µm sections from the FFPE sample of interest was scraped into a single microtube.

2. The samples were centrifuged briefly at full speed to collect the sample at the bottom of the tube. The samples were overlayed with 20µl of Proteinase K solution and 180 µl of Incubation Buffer.

3. The tube cap was closed and incubated the samples at 70°C overnight.

4. 400 µl of Lysis Buffer was added to each sample.

5. The sample and Lysis Buffer was vortexed briefly.

6. The lid of the microtube was and saved until ready for automated DNA extraction using the Maxwell 16 LEV instrument.

2.9.1.3 Preparation of Samples for Maxwell 16 LEV Cartridges

1. Each cartridge was placed in the rack with the label side facing away from the Elution Tubes. The cartridge was pressed down to snap it into position.
2. Place an LEV Plunger in well #8 of each cartridge. Well #8 is the well closest to the Elution Tube.
3. Elution Tubes were placed in the front of the Maxwell 16 LEV Cartidge Rack. 50 µl of Elution Buffer was added to the bottom of each Elution tube.

After that DNA was extracted according to the manufacturer's instruction.

2.9.1.4 Primers

The primers that are used in the study are given in Table 2.1

Table 2.1 Primers used for amplication of *IS6110* region

Primers	Sequences
Forward Primer	**5'-CCTGCGAGCGTAGGCGTCGG-3'**
Reverse Primer	**5'-CTCGTCCAGCGCCGCTTCGG-3'**

2.9.1.5 Preparation of reaction mixture

The PCR reaction was prepared by mixing the components at given volumes described in Table 2.2. A master mix was prepared simultaneously using the amounts mentioned below. For individual test, separate primer set was used. Finally the PCR tube containing reaction mixture was capped and centrifuged briefly to spin down the contents. The PCR tubes were then placed in a thermal cycler (MJ Rsearch, PTC-200 Peltier thermal cycler).

Table 2.2 Components of PCR reaction mixture

Component	Component volume
5x PCR green buffer (promega)	5.0µL
25 mM $MgCl_2$	0.2 µL
10 mM dNTPs	0.4 µL
Primer (F) (2µM)	1.5 µL
Primer (R) (2µM)	1.5 µL
Taq polymerase, 5u/ µL	0.085µL
Template DNA	5.0 µL
Nuclease free water	11.31 µL

2.9.1.6 PCR conditions:

All the PCR tubes containing the appropriate mixtures were heated at 94^{o}C for 5 minutes in the thermal cycler to ensure the denaturation of all DNA templates. The PCR reaction was then contained with the following programme:

Segment -1	Initial heating at 94^{o}C for 5 min then containued with the denaturation at 94^{o}C for 45 second.
Segment-2	Annealing at 68^{o}C for 45 seconds.
Segment- 3	Extention at 72^{o}C for 2 minute for a total of 31 cycles followed by a final extention of 7 min at 72^{o}C.

After this, PCR tubes were stored at -20^{o}C until further analysis.

2.9.1.7 Post- PCR Detection of Amplified DNA by Electrophoretic Analysis

Efficient amplification was confirmed by gel electrophoresis on 1.5% agarose (Agarose, Promega) gel. Then 10 minutes at 100 volt was set and run. After that 45 minutes was set at 90 volts. Agarose was dissolve in 1X Tris –acerate EDTA buffer to give a final concentration of 1.5% agarose and was heated to dissolve in a microwave oven for about 2.5-3 minutes. When the temperature came down to 50^{o}C the gel was poured onto the gel tray already mixed with appropriate combs. Following solidification of the gel, it was submerged in 1X TAE buffer in a gel running tank. 6 µl PCR product was loaded into slots of the gel with the aid of a micropipette. Electrophoresis was continued until DNA fragments were separated. The gel was stained with gel red. The gel red stained DNA bands were observed on a UV transmitter. Photographs were taken using Gel doc system attached to a computer and bands were analysed. A standard (100bp ladder) was also run on the agarose gel for confirmation of efficient amplification. The band obtained for *IS6110* region was 123 bp long.

2.10 Gene Xpert System

The Xpert MTB/RIF test for use with the Cepheid Gene Xpert ®System is a semi-quantitative nested real-time PCR in-vitro diagnostic test for:

i. the detection of *Mycobacterium tuberculosis* complex DNA from concentrated sediments of TB suspected patient's specimen and

ii. the detection of rifampicin resistance associated mutations of the *rpoB* gene in samples from patients at risk for rifampicin resistance.

The Xpert MTB/RIF assay uses 3 specific primers and 5 unique molecular probes to ensure a high degree of specificity (Figure 2.7). This assay targets the *rpoB* gene, which is critical for identifying mutations associated with rifampicin resistance.

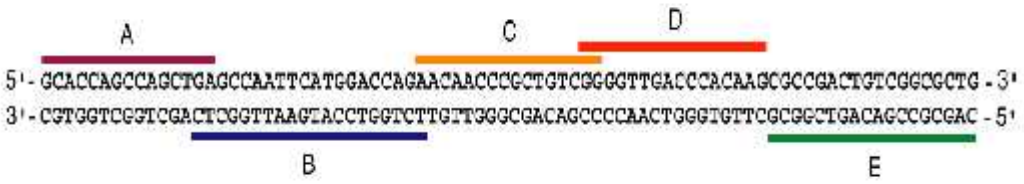


Figure 2.7: *rpo*B gene 81 bpRIF resistance determining region

2.10.1 Sample processing for Gene Xpert from formalin fixed paraffin embedded tissue

Sample processing protocol for Gene Xpert from formalin fixed paraffin embedded tissue is given in Figure 2.8.

The tissue was taken from formalin fixed paraffin embedded block by cutting the block using a sterile surgical blade in a class ii biosafety cabinet.

⇩

This tissue was cut into small pieces

⇩

The small pieces of tissue was taken in a 50 ml falcon tube containing 2.0 ml PBS and placed in a water bath at 95^0C to dissolve any remaining paraffin within the tissue.

⇩

The supernatant PBS was discarded remaining the tissue at the bottom of the tube.

⇩

This tissue was then homogenized with additional 2.0 ml PBS using tissue homogenizer ,vortexed and allowed to stand for 5 minutes to settle down the course particle.

⇩

Then 1.0 ml of the supernatant was transferred very carefully in a new labelled 15 ml falcon tube.

⇩

Cont...

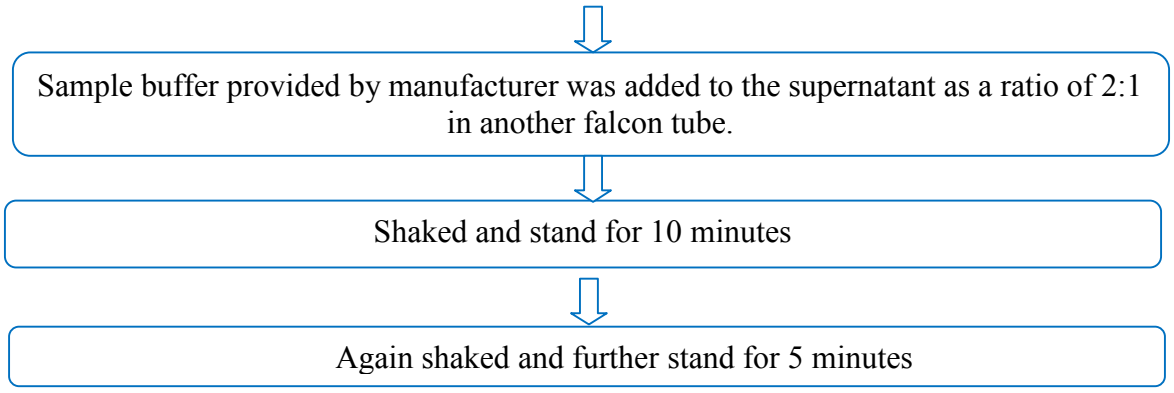


Figure 2.8: Flowchart showing sample processing protocol for Gene Xpert from formalin fixed paraffin embedded tissue.

2.10.2 Preparing the cartridge and sample loading

- Each Xpert MTB/RIF cartridge was labelled with the sample ID. It should be concerned not to put the label on the lid of the cartridge or obstruct the existing 2D barcode on the cartridge.
- The sterile transfer pipette which provided by the manufacturer was used, the liquefied sample was aspirated into the transfer pipette until the meniscus is above the minimum mark.
- The cartridge lid was opened and the sample was transferred into the open port of the Xpert MTB/RIF cartridge.
- Then the cartridge lid was closed. It was made sure that the lid snapped firmly into place.

2.10.3 Starting the test

Important: Before starting the test, it was ensured that the system was equipped with the GX software, and the Xpert MTB/RIF assay was imported into the software.

- The computer was turned on, and then the Gene Xpert Dx instrument was turned on.
- On the Windows desktop, the Gene Xpert Dx shortcut icon was double-clicked.
- The Gene Xpert Dx System software was logged on using user name and password.
- In the Gene Xpert Dx System window, Create Test button was clicked.

- When the Scan Cartridge Barcode dialog box was appeared the barcode on the Xpert
- MTB/RIF cartridge was scanned. The Create Test window appeared. Using the barcode information, the software automatically filled the boxes for the following fields: Assay, Reagent Lot ID, Cartridge SN, and expiration date were selected.
- In the Sample ID box, the sample ID was scanned or typed. It was made sure that the correct sample ID was typed. The sample ID is associated with the test results and was shown in the "View Results" window and all the reports.
- "Start Test" button was clicked.
- Then the instrument module door was opened with the blinking green light and the cartridge was loaded.
- After cartridge loaded the door was closed. The test was started and the green lightstops blinking. When the test was finished, the light was turned off.
- It was waited until the system releases the door lock at the end of the run, then the module door was opened and the cartridge was removed.
- Used cartridges were disposed of in the appropriate specimen waste containers according to Bio-safety regulation (Figure 2.9).

Figure 2.9: Workflow of Gene Xpert MTB/RIF

2.10.4 Procedure of result analysis in Gene Xpert

Each Xpert MTB/RIF cartridge includes reagents for the detection of MTB complex and RIF resistance as well as a sample processing control (SPC) to control for adequate processing of the target bacteria and to monitor the presence of inhibitor(s) in the PCR reaction. The Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability. The primers in the Xpert MTB/RIF assay amplify a portion of the *rpo*B gene containing the 81 base pair "core" region. Five differently colored fluorogenic nucleic acid hybridization probes, called molecular beacons, interrogate the entire 81-bp core. Each molecular beacon was designed to be so specific that it does not bind to its target if the target sequence differs from the wild-type *rpoB* sequence by as little as a single nucleotide substitution. Since molecular beacons fluorescence only when they are bound to their targets, i.e. wild-type *rpoB* sequence, the absence of any one of the five colors in the assay differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance (Helb et al., 2010). The Sample Processing Controlss should be positive in a negative sample and can be negative or positive in a positive sample. The test result will be "Invalid" if the SPC is not detected in a negative test. Before the start of the PCR reaction, the Gene Xpert Dx System measures the fluorescence signal from the probes to monitor bead rehydration, reaction-tube filling, probe integrity and dye stability.

2.11 Analysis of blood samples for prostate specific antigen (PSA)

Reagent : ARCHITECT Total PSA reagent kit (7k70)

Microparticles: Anti PSA (mouse,monoclonal) coated microparticles in TRIS buffer with protein (bovine).

Conjugate: Anti PSA (mouse,monoclonal) acridium-labeled conjugate in MES buffer with protein (bovine).

The ARCHITECT total PSA assay is a two-step immunoassay to determine the presence of total PSA in human serum, using chemiluminescent Microparticle immune assay technology.

In the first step, sample and anti PSA coated paramagnetic microparticles are combined. PSA present in the sample binds to the anti PSA coated microparticles. After washing, anti PSA acridinum labelled conjugate is added to the second step. Pre-Trigger and Trigger solutions are then added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units RLU. A direct relationship exists between the amount of total PSA in the sample and the RLU detected by architect operating system.

Chapter 3

Results

3. Results

Mycobacterium tuberculosis remains a serious health issue due to it's risk of person-to-person transmission and high level of morbidity and mortality. Now-a-days, extra-pulmonary tuberculosis is becoming increasingly common. The genitourinary tract is one of the most common sites of extra pulmonary tuberculosis (30-33%). On the other hand, Prostatic diseases including cancer usually affect men after fifty years and it is the second leading cause of death after lung cancer. This study attempts to find out any association of *M. tuberculosis* in promoting cancer in prostatic tissues of elderly patients with different prostatic lesions. The purpose of this study was to estimate the level of prostate specific antigen (PSA) in patients' sera, socio-economic relation in developing prostatic disorders, epidemiology of prostatic disorders in Bangladesh, establish a reliable method for detection of *M. tuberculosis* from formalin-fixed paraffin-embedded tissues, and to observe the prevalence of *M. tuberculosis* in prostatic tissues.

A total of eighty seven samples, composed of eighty five prostate tissues and two TB positive lymph nodes as control were included in this study.

3.1 Clinical history of patients

The clinical histories of patients suspected with different type of prostatic disorder were taken and tabulated accordingly (Table 3.1). It appears that maximum patient's were in the age group of 60-69 years.

Table 3.1 Distribution of patient's characteristic

Analyzed parameters	Percentage (%)
Number of patients / individuals	85
Mean age of the patients	66.5 years
Family history of pulmonary tuberculosis	0%
Family history of cancer	3%
Maximum patient' s location	59% (Dhaka Division)
Frequency of micturation	85 (100%)
Urgency of micturation	61 (72%)
Low back pain	85 (100%)
Haematuria	72 (85%)
Frequncy of disurea	77 (90%)

3.2 Patient's sample

A total of eighty five prostatic tissue samples as well as respective blood samples were collected from patients suspected with different prostatic lesions. After successful collection and processing of samples, each of them was then subjected to investigation as per following order:

(A) Histopathological analysis

(B) Serological assay

(c) Mantoux test (MT)

(D) Microscopy (AFB)

(E) Molecular diagnosis

a. Conventional PCR

b. Gene Xpert MTB/RIF

3.3 Histopathological analysis

Histopathology involves detail microscopic study of diseased tissue after use of special techniques for preparation of specimens. According to the Haematoxylin & Eosin stain the total samples were categorized into four groups (Table 3.2)**.**

Table 3.2: Histopathological analysis

Group	Microscopic observations	Remarks	Identified group
1.	In this, glands are increased in number in relation to stroma, variation in size shape and huge amounts of inflammatory cells like lymphocytes are present.	This is a benign and inflammatory state	NHCP (Nodular hyperplasia with chronic prostatitis).
2.	Here, there is presence of granulomas in prostate obliterating its gland and stroma. Focal collections of epithelioid cells, Multinucleated giant cells, and lymphocytes are present.	Granulomatous inflammation is suggestive of TB. But may be due to AMTB,Foreign body, BCG vaccination	Granulomatous prostatitis (GnP)
3.	Nucleus is hyperchromatic, nuclear and cellular pleomorphsm are present.	Precancerous stage.May also be found beside a cancer.	PIN (Prostatic intraepithelial neoplasia)
4.	Features of anaplasia is present.Like large hyperchromatic nuclei,irregular nucear membrane,prominent nucleoli,irregular dense clump chromatin.Poorly formed glands. Lining cells atypia Increased numbers of glands scanty or absent stroma.	Frank carcinoma.	Prostatic carcinoma

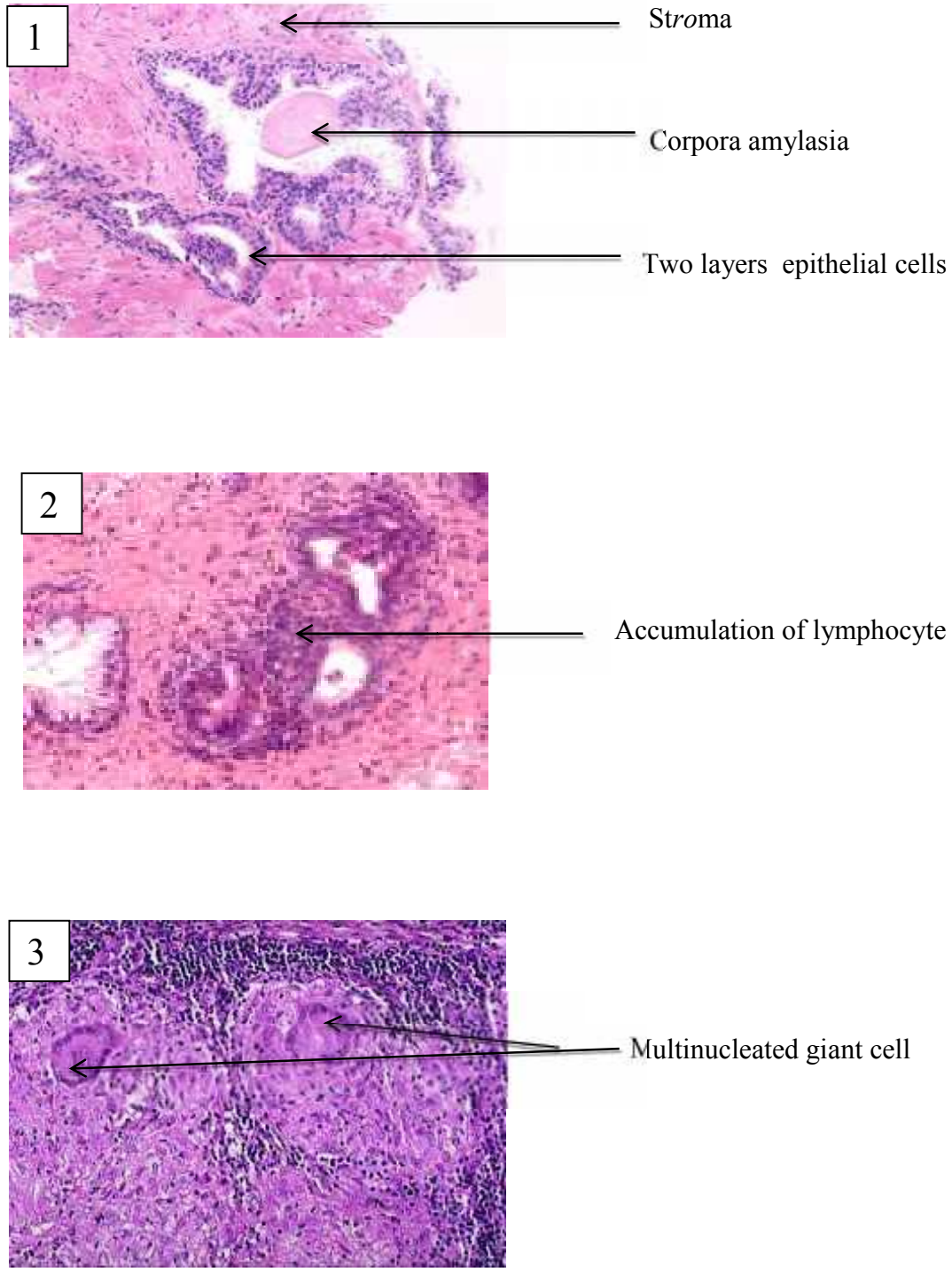


Figure 3.1: Histopathological investigation. (1) Normal prostate tissue (2) Nodular Hyperplasia of Chronic Prostatitis (NHCP)-Accumulation of lymphocytes suggestive of chronic infection, (3) Granulomatous Prostatitis-Giant cells within a granuloma

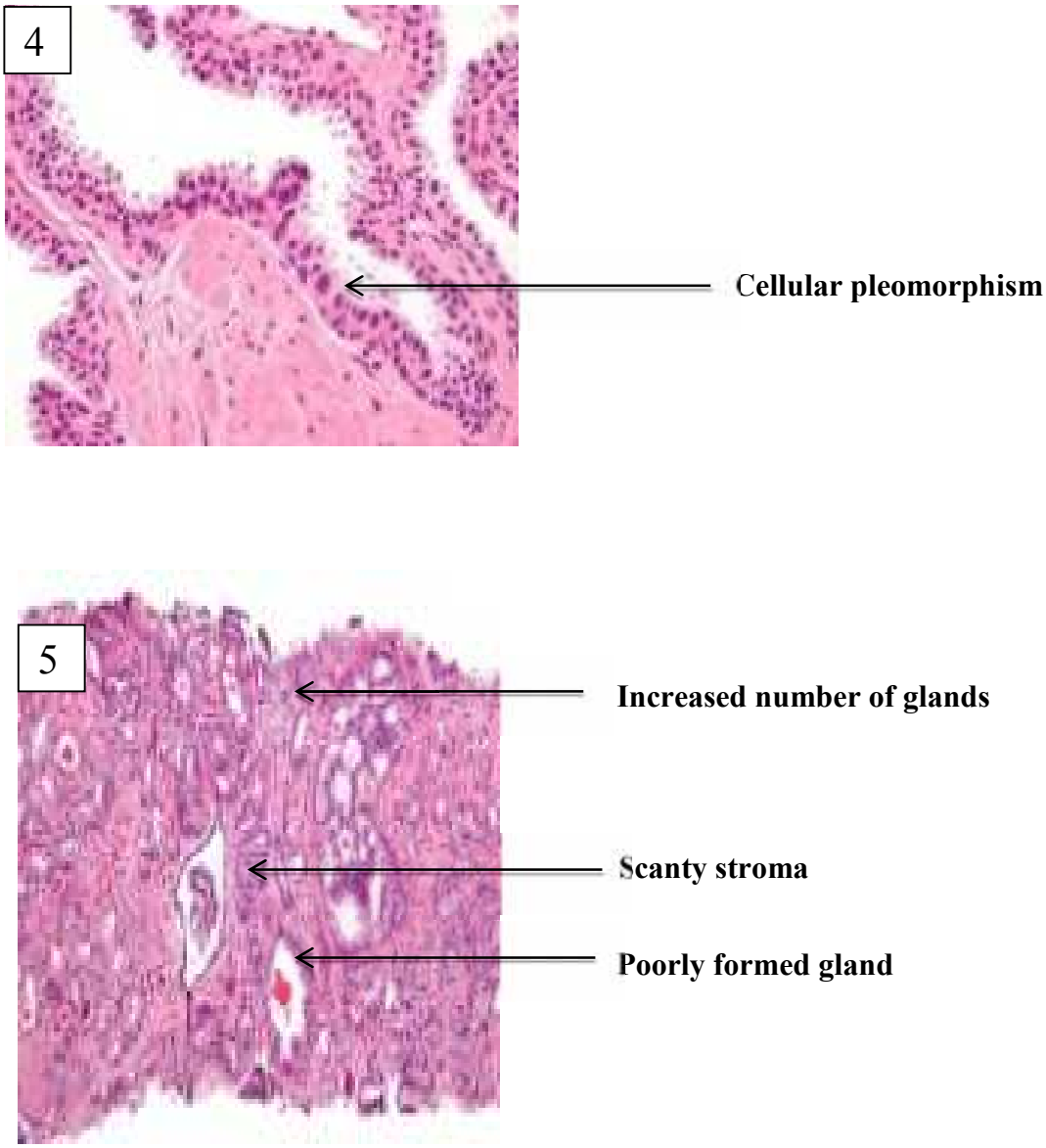


Figure 3.2: Histopathological investigation. (4) Prostatic intraepithelial Neoplasia (PIN)- Nuclear and cellular pleomorphism, (5) Adenocarcinoma- Increased number of glands, poorly formed glands, nuclear atypia, scanty stroma

3.4 Histopathology-based prostatic disorders

Out of 85 patients, 48 (56%) were NHCP, 26 (30.6%) were PIN, 3 (3.5%) were granulomatous prostatitis and 8 (9.4%) were cancer (Figure 3.3).

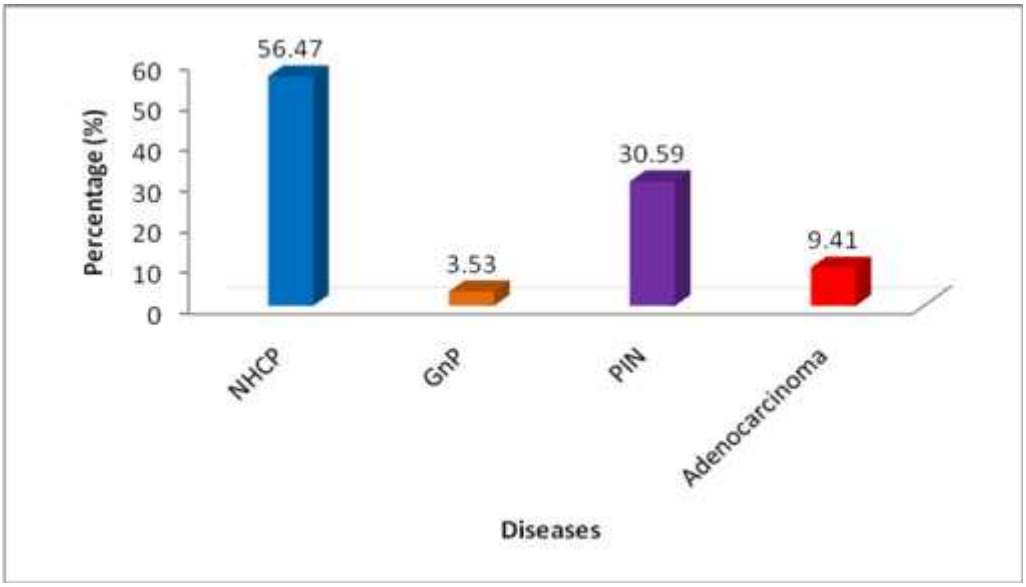


Figure 3.3: Diagrammatic representation of percentage distribution of different types of prostatic disorder.

3.5 Demographical data of prostatic patients

This study was conducted on 85 prostatic patients from different hospitals of Dhaka city. A questioner based survey was done for understanding the demography of the patients.

1. Categorization of prostatic patients according to age

Different age group ranging from less than or equal to 40 years to greater than or equal to 91 years of age were included in this study. One PIN patient and one one GP patients with NHCP were observed in the age group > 40 years. A large number of patients with NHCP were found in the age range from 41 years to 90 years. Number of patients with PIN were also found in the age ranges from 51 years to >91 years. However, patients with adenocarcinoma were only observed in the age group ranges from 51 years to 80 years. In conclusion the age group between 61-70, is the most vulnerable range to be affected with prostatic disorder (Figure 3.4).

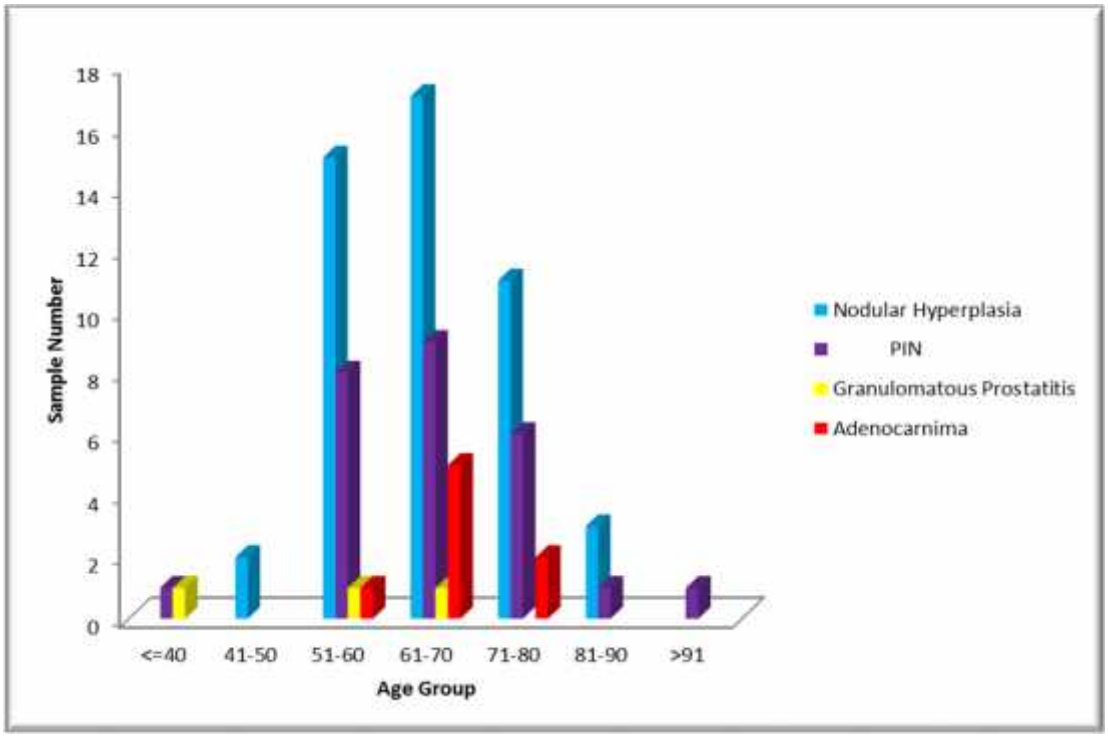


Figure 3.4: Diagrammatic representation of age group among the prostatic diseases patients.

The mean age of NHCP, GP, PIN and cancer is 63, 51, 66 and 70 years. It can be concluded that, the elderly patients are more susceptable to cancer (Figure 3.4).

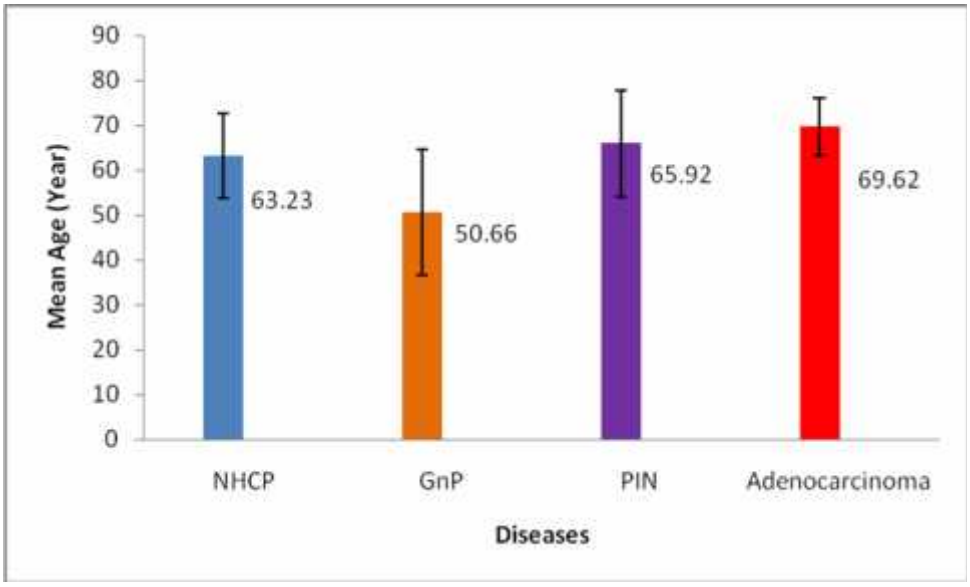


Figure 3.5: Age of four categories of prostatic disease.

2. Categorization of prostatic patients according to smoking habit

Out of 48 NHCP patients it was observed that 60% were non-smoker and 40% were smoker. The following Figure (3.6) is representing that out of 26 PIN patients 54% were smoker and 46% were nonsmoker. Out of total 8 cancer patient, 75% patient had smoking history and rest 25% patient had no history of smoking. From the above demography it can be concluded that smokers are more susceptible to cancer (Figure 3.6).

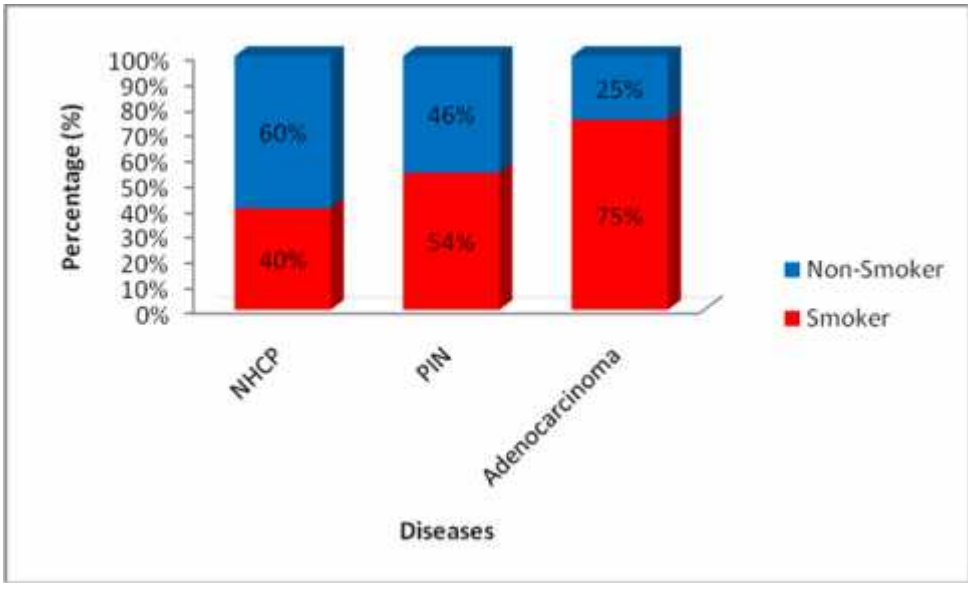


Figure 3.6: Diagrammatic representation of percentage distribution of smoking habit among NHCP, PIN and cancer patients.

3. Categorization of prostatic patients according to occupation:

The categories of occupation of 85 prostatic patients were divided into 6 groups (Service, Service retired, labour, teacher, businessman and farmer), where it is observed that business group were higher especially in cancer (62%) and NHCP (40%). In cancer patients 62% patients are businessman, the second prevalent group are service retired (25%), followed by labour (13%). In case of NHCP patients, the most prevalent group are business group (40%), followed by service retired (32%), 8% service holder, farmer, 6% farmer and teacher respectively. From our study it can be said that, people of business community had greater risk of cancer (Figure 3.7).

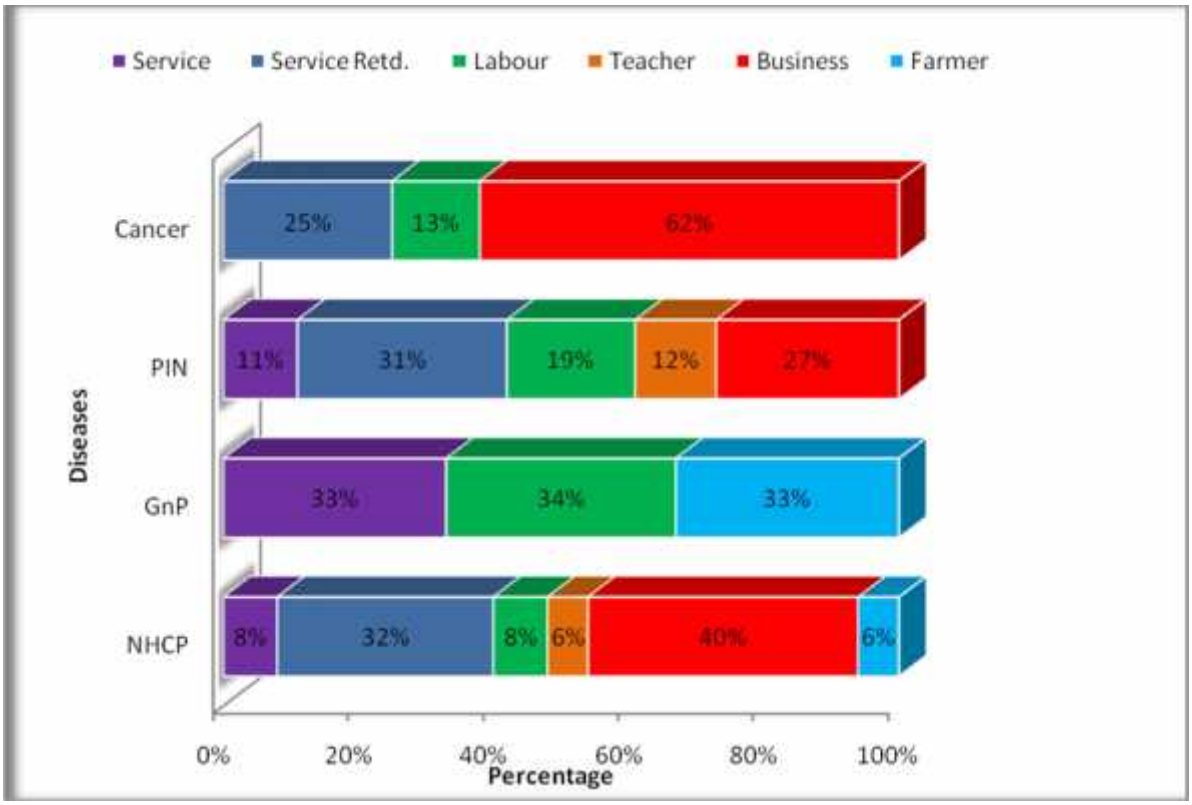


Figure 3.7: Diagrammatic representation of percentage distribution of different occupation among prostatic patients.

4. Categorization of patients according to Epidemiology

In Bangladesh, among the 85 cases diagnosed, 50 cases were from Dhaka, 16 from Chittagong, 10 from Rajshahi, 7 from Barisal and remaining 2 from Sylhet division.

In Dhaka division, 48%, 34%, 4%, 14% cases were NHCP, PIN, GnP and cancer respectively (Table 3.3).

The second largest samples were from Chittagong division and it was 16. Out of 85 samples, 10 were from Rajshahi division and 7 were from Barishal division. From Sylhet division, 50% samples were NHCP and rest 50% were PIN. In Bangladesh, the cases of prostate cancer are more prevalent in Dhaka district (Figure 3.8).

Table 3.3: Epidemiology of collected samples

Division	No. of Sample	NHCP	PIN	Granulomatous	Cancer
Dhaka	**50**	**24 (48%)**	**17 (34%)**	**2 (4%)**	**7 (14%)**
Chittagong	**16**	**11(69%)**	**4(25%)**	**1(6%)**	**0**
Rajshahi	**10**	**6(60%)**	**4(40%)**	**0**	**0**
Barisal	**7**	**6 (86%)**	**0**	**0**	**1(14%)**
Sylhet	**2**	**1(50%)**	**1(50%)**	**0**	**0**
Total	**85**	**48 (56%)**	**26(31%)**	**3(4%)**	**8(9%)**

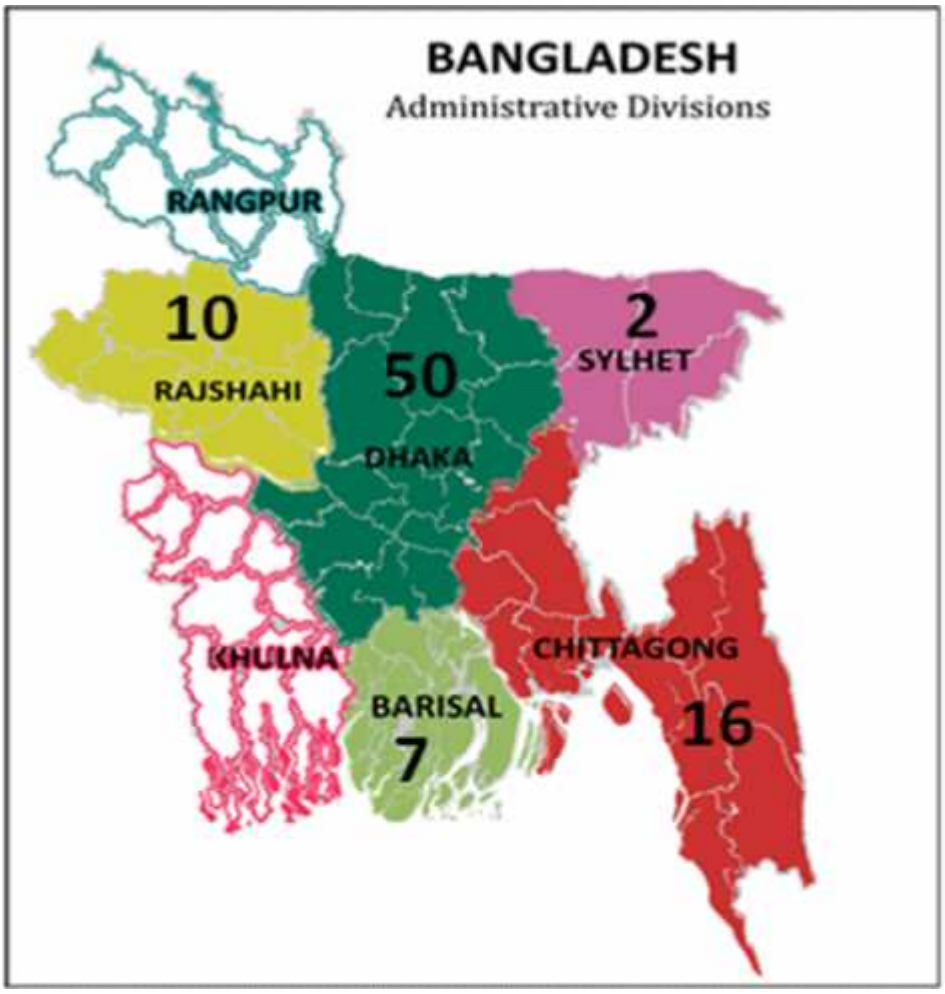


Figure 3.8: Epidemiology of collected samples

3.6 Serological analysis (PSA estimation)

Serum samples from eighty five subjects were taken and tested for total prostate specific antigen level. It is normal to secrete small amount of PSA into the blood stream that measures 4 ng/ml or lower. (http://www.cancer.gov/types/prostate/psa-fact-sheet). Large amount of PSA in the bloodstream usually signal that the prostate gland is enlarged, infected or malignant. In this study, we observed elevated levels of PSA in patients with NHCP, PIN and adenocarcinoma (Figure 3.9) ranging from 2.1 ng/ml to 50.56 ng/ml. Except granulomatous prostatitis all these disorders harbor excessive than normal PSA level.

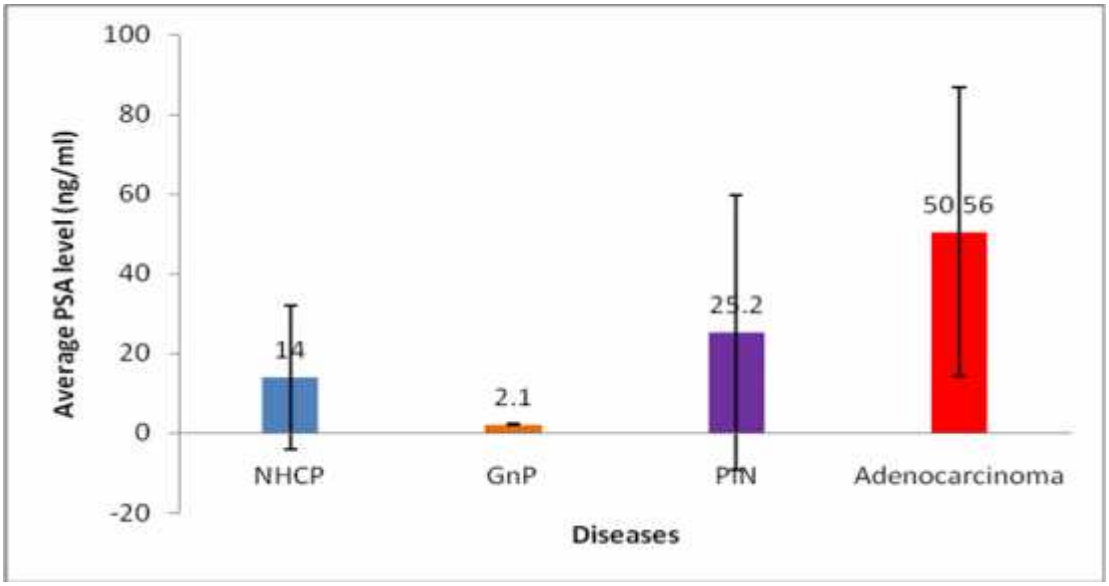


Figure 3.9: Average PSA level of different prostatic disorders.

3.7 Mantoux test result:

The Mantoux test is a standard method of determining whether a person is infected with *M. tuberculosis* or not. 22 patients were subjected to Mantoux tests and the findings are illustrated based on their prostatic disorders (Figure 3.10). Altogether, 55% patients (12 out of 22) indicated that they were already exposed with TB.

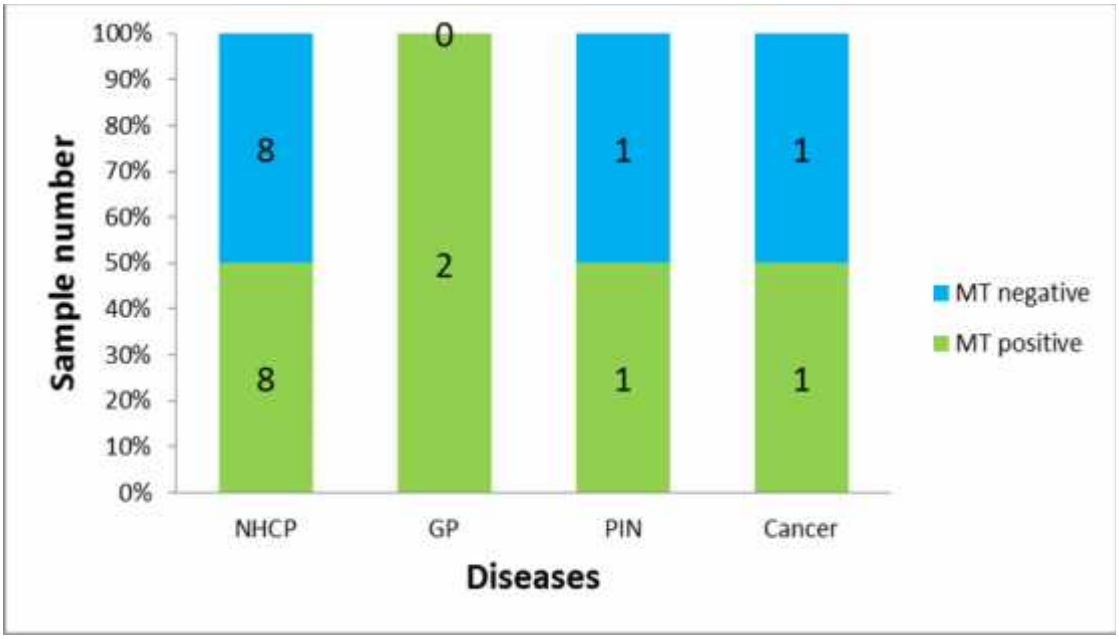


Figure 3.10: Diagrammatic representation of numerical distribution of Mantoux test result among NHCP, GP, PIN and cancer patients

3.8 Microscopy (AFB)

The tissue samples from control and patients were observed under microscope for acid-fast bacilli. None of the specimen, be it test or control showed acid fast bacilli under microscope (Figure 3.11). Details result is given in the Table 3.4 & Table 3.5.

Table 3.4: Microscopy (AFB) (Test samples)

Sample type (Test sample)	Microscopy positive	Microscopy negative	Total
Prostatic tissue	0	85	85
Total	0	85	85

Table 3.5: Microscopy (AFB) (Control samples)

Sample type (Control sample)	Microscopy positive	Microscopy negative	Total
Lymph node tissue (TB positive)	0	2	2
Total	0	2	2

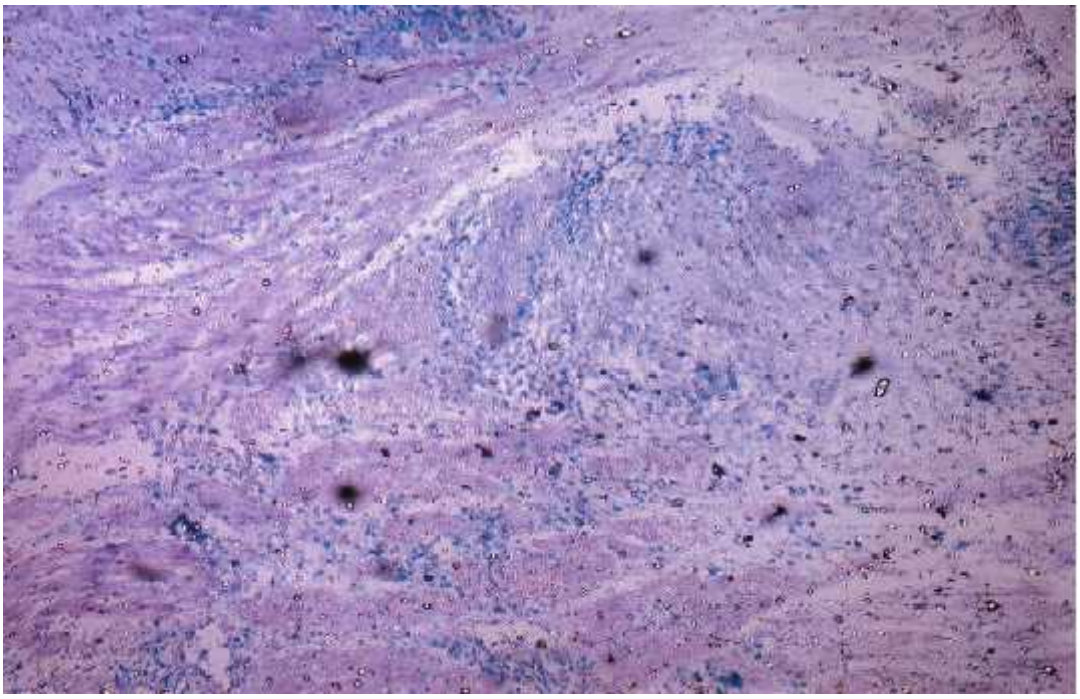


Figure 3.11: Negative for *Mycobacterium tuberculosis* in Z-N stain at 100X (oil immersion) lens under microscope.

3.9 Molecular detection

3.9.1 PCR amplification of test and control samples

Conventional PCR (IS6110) has been done with samples from both paraffin-embedded formalin-fixed tissues and control (TB positive lymph node) samples. As revealed on a 1.5% agarose gel, no PCR product was observed originated from all test sample (Figure 3.12). However, the positive control from TB positive lymph node also failed to produce any amplicon. The PCR experiment was ok as the amplicon of 123 bp using *M. tuberculosis* H37Rv was found positive.

Table 3.6 : Conventional PCR (Test samples)

Sample type (Test sample)	Conventional PCR positive	Conventional PCR negative	Total
Prostatic tissue	0	85	85
Total	0	85	85

Table 3.7 : Conventional PCR (Control samples)

Sample type (Control sample)	Microscopy positive	Microscopy negative	Total
Lymph node tissue (TB positive)	0	2	2
Total	0	2	2

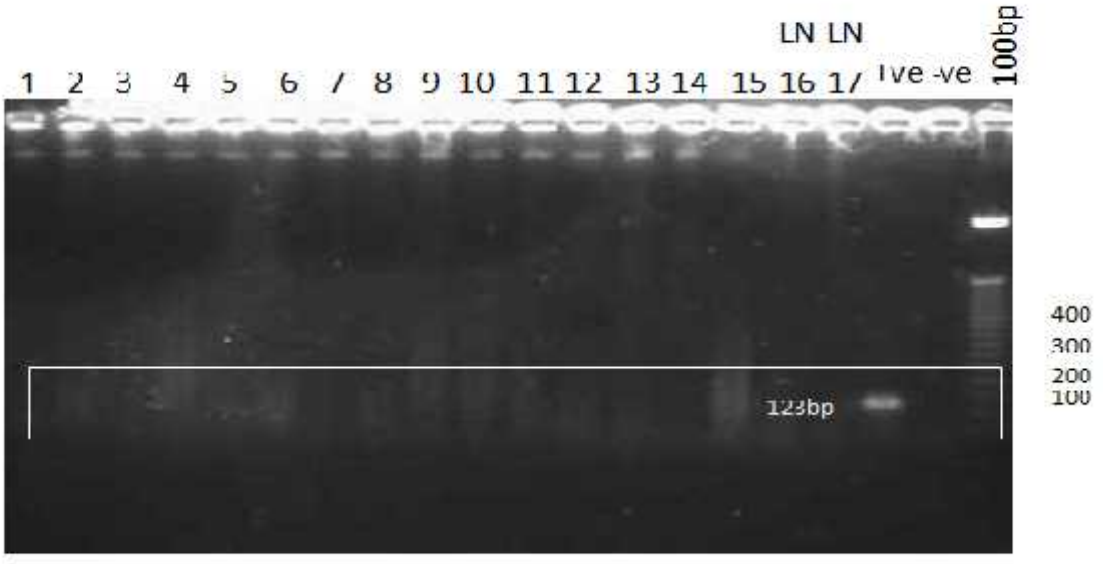


Figure 3.12: Agarose gel electrophoresis to detect PCR product, 123 bp *Mycobacterium tuberculosis* IS6110. DNAs from prostatic tissue samples (lanes 1

to 15), two TB positive lymph node tissue samples (lane 16, 17), and *M. tuberculosis* H37Rv (lane labeled +ve), Nuclease free water (lane labeled –ve), and 100bp DNA ladder were loaded as indicated.

3.9.2 Gene Xpert MTB/RIF

The failure of detection of *M. tuberculosis* from formalin fixed paraffin embedded tissue by conventional PCR prompted us to use Gene Xpert MTB/ RIF which is a real-time hemi nested PCR test that simultaneously identifies *M. tuberculosis* and detects rifampicin resistance directly from clinical specimens. Two samples from each nodular hyperplasia with chronic prostatitis (NHCP), granulomatous prostatitis (GnP) and prostatic intraepithelial neoplasia (PIN) states, all 8 prostatic adenocarcinoma tissue samples were tested for presence of mycobacterial genomic DNA by the Gene Xpert MTB/RIF real time PCR. All these samples came out negative for mycobacterial DNA. Very importantly, the two positive control samples (lymph node tissues from confirmed TB cases) were positive in the Gene Xpert MTB/RIF assay (Figure 3.13). The finding of the positive results coming out from paraffin-embedded formalin-fixed lymph node tissue samples having confirmed *M. tuberculosis* infections validates that the absence of response for *M. tuberculosis* infection in Gene Xpert analysis from paraffin-embedded formalin-fixed prostatic tissues indicates that infection of prostate by this pathogen is not common in prostate cancer patients in Bangladesh. This finding also highlights the need of performing sensitive molecular test such as Gene Xpert in formalin fixed paraffin embedded tissue in ruling out whether a suspected patient is infected with *M. tuberculosis* or not.

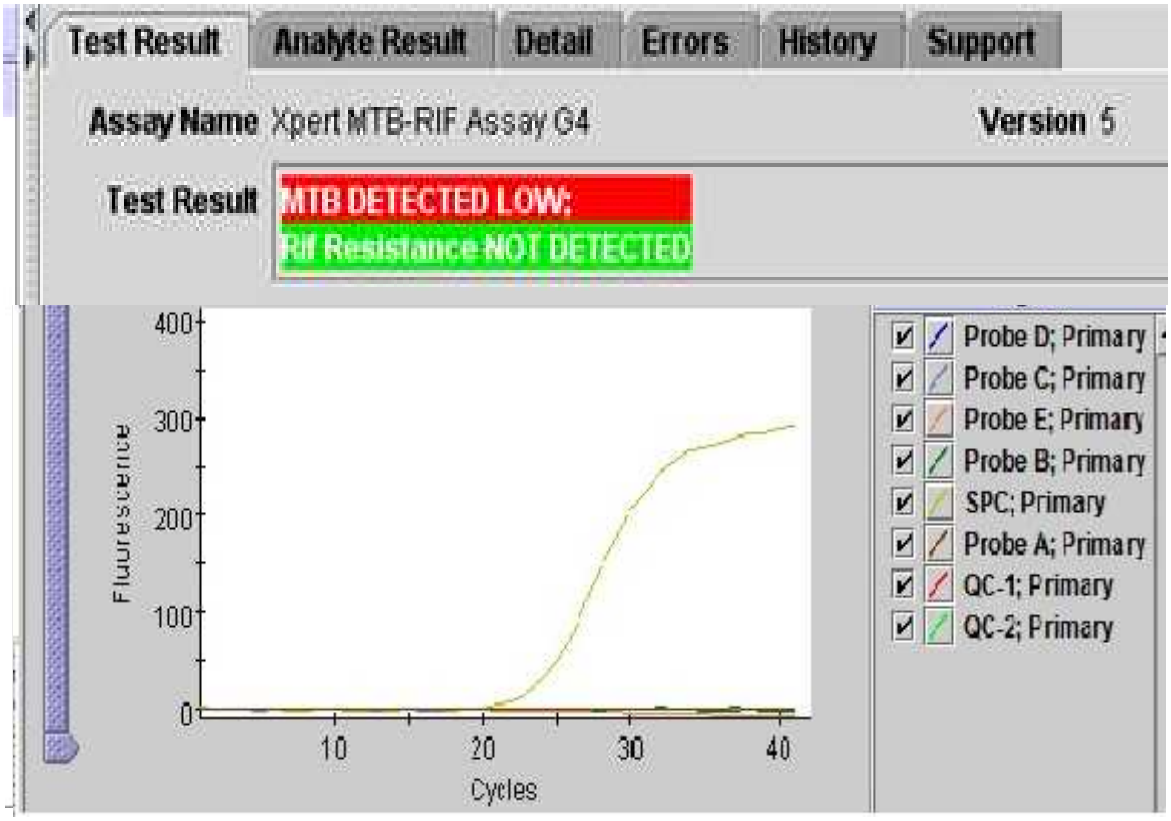


Figure 3.13: Gene Xpert result of lymph node tissue samples. The yellow colored curve indicates the presence of *M. tuberculosis.*

Chapter 4

Discussion

4. Discussion:

Tuberculosis (TB), remains a major global public health problem. It is estimated that about one-third of the world's population is infected with *Mycobacterium tuberculosis*. There were an estimated 9 million new cases of TB resulting in 1.5 million deaths, with the greatest burden of diseases in developing nations (WHO ,2014). A group of mycobacterium species called *M. tuberculosis* complex comprise *M. tuberculosis, M. bovis, M. africanum*, is one of the most clinical importance since it causes tuberculosis in humans worldwide. *Mycobacterium* species other than those of the tuberculosis complex, also called nonmycobacteria, are widely distributed in the environment and may colonize and occasionally cause infections in human (Esquivel et al., 2009).

Tuberculosis infection can be pulmonary or extra pulmonary type. After primary infection, TB may reactivate anytime and anywhere in the body (Sreeramareddy et al., 2008). Extra pulmonary tuberculosis can occur alone or in combination with the pulmonary variety. It is usually confined to a single site, but disseminated form may also occur (Wiener el al., 2005). Extra pulmonary tuberculosis comprises 20-25% of all tuberculosis, however only 27% of the extra pulmonary tuberculosis involves the genitourinary system (Chandra et al., 2012). Tuberculosis in prostate gland is seen in only 2.6% of genitourinary system (Akhtar, 2012). Although male genital TB seems to be a rare diseases, 77% of men who died from TB of all localizations had prostate TB, mostly overlooked during their lifetime (Kulchavenya & Krasnov, 2010).

Prostate cancer is one of the most common type of cancer and it is the second commonest cause of cancer-related death among men in western world (Silberstein et al.,2013). Some recent studies suggested that prostate TB like any other chronic infectious inflammation may predispose for prostate cancer (Kulchavenya & Kholtobin, 2015). There is very limited number of literature published on association of prostatic tuberculosis and prostatic cancer. This study attempts to find out any association of *Mycobacterium tuberculosis* in promoting cancer in prostatic tissues of elderly patients with different prostatic lesions in Bangladeshi subjects. This study also demonstrated the role of demography in development of prostatic cancer. In this study a total of 85 clinically suspected prostatic patients and 2 known TB positive lymphadenitis patients were also included. Different clinical samples were collected

from both case and control were subjected to histopathological analysis, serological assay, Mantoux test, microscopy (AFB) and molecular assay (a) conventional PCR and (b) Gene Xpert MTB/RIF.

In the present study, among 85 patients histopathology revealed chronic inflammation in 56%, prostatic intraepithelial neoplasia 30.6%, granulomatous prostatitis is 3% and prostatic cancer is 9.4% patients. Almost similar finding were also found in other study which revealed inflammation in 94% cases, intraepithelial neoplasia in 9.7% cases and cancer in 5.4% patients (Kulchavenya & Krisnov 2010).

The findings of this study demonstrates that demographic factors influence in the development of prostatic cancer. This study found that prostatic patients more than 70 years of age are more susceptible to cancer. Similar findings were also reported by other authors which explained that prostate cancer incidence rates have levelled off in men aged 65 years and older (Stangelberger et al., 2008).

This study showed that among cancer patients 75% are smokers. Almost similar findings were also found in many studies such as smoking is associated with prostate cancer (Robert et al., 1993). Plaskon et al., (2003) hypothesized the mechanism of prostate cancer development and cigarrate smoking. Cigarette smoking may alter circulating levels of steroid hormones. In particular, cigarette smoking has been associated with higher levels of bioavailable testosterone and lower levels of bioavailable estradiol in men (Ferrini & Varrett-Connor, 1998). Studies found significant positive correlations between cigarette smoked/day and serum total androstenedione as well as total and free testosterone in men (Dai et al., 1988). This is significant because testosterone and its more potent metabolite DHT are necessary for normal prostate development and growth and also appear to enhance cell proliferation in the prostate, which potentially could be associated with malignant transformation. In addition, cigarettes contain significant levels of cadmium, which has been linked to prostate carcinogenesis. So from the above findings, it may be concluded that, people with smoking habit bear more risk for development of prostate cancer.

In this study it is found that Dhaka division is more cancer prone compare to other districts in Bangladesh. Out of 85 patients 8 patients were suffering from cancer of which 7 patients were from Dhaka division. Prevalence of cancer also observed high

in Dhaka division in the Cancer Registry Report of the National Institute of Cancer Research and Hospital (NICRH, 2009).

The level of PSA appeared to increase in prostatic disorders ranging from 2.1 to 50.56 ngm/ml. Except granulomatous prostatitis all these disorders harbour more than normal PSA level. Brawer et al., (1989) reported an association between PIN (prostatic intraepithelial neoplasia) and high serum PSA level. Serum PSA level of metastatic prostatic cancer patient was also found high in a case study carried out by (Iwamura et al., 2014) and which was 2036 ngm/ml. Therefore it can be said that serum PSA level may be markedly high in PIN and prostate cancer patients.

None of the 85 specimen were light microscopically positive for acid-fast bacilli in this study. Two known TB positive lymph node samples are also negative for acid-fast bacilli. This is may be due to lower number of bacilli in the sample, non mycobacterium tuberculosis and other infectious agents like Treponema pallidum, viruses and fungi and BCG inoculation (Nassaji et al., 2014; Gupto et al., 2008). Despite low sensitivity, this method can be performed in patients suspected to extra pulmonary tuberculosis especially in developing counties where new modality is not routinely available (Nassaji et al., 2014).

Microscopic examination of mycobacterial lesions frequently results in few or no bacilli seen, even if the lesions appear active histologically (Fukunaga et al.,2002). This might be due to the effects of the fixative fluid and/or organic solvent, both of which are conventionally used to make tissue sections for histopathology on the acid fast staining of bacteria. The bacilli are frequently missed or underestimated with acid-fast microscopy on formalin fixed paraffin embedded tissue (Fukunaga et al., 2002).

Moreover, some researchers have suspected that the organic solvents, which are used to make paraffin-embedded tissue samples, might affect the stainability of mycobacteria by acid-fast staining. This hypothesis seems reasonable because the molecular target of the acid-fast staining dyes (fuchsin, auramine, or rhodamine) is the mycolate on the bacterial surface (Harada el al., 1976; Cserni, 1995). Mycolate is soluble in organic agents (Minnikin et al.,1984) and might be more or less extracted from the cell surface into the liquid.

Molecular diagnosis of mycobacteria is considered to be particularly helpful if histochemical demonstration of acid-fast bacilli bacteria in formalin fixed, paraffin embedded tissue is not successful and suspicion based on histologic or clinical features needs to be confirmed (Heinmöller et al., 2001). Detection of mycobacteria of the *Mycobacterium tuberculosis* complex with PCR based techniques are necessary to clarify the presence of these microorganisms in clinical specimens particularly when the histochemical demonstration of acid fast bacteria was negative (Condos et al 1996, Qian et al 1999). In this study none of the specimen of test sample are positive for conventional PCR. Moreover two known TB positive lymph node samples were also negative for conventional PCR. It may be due to fragmentation of DNA. Fragmentation of DNA extracted from clinical samples on the one hand and the rigid cell wall of mycobacteria on the other are obstacles in the detection by PCR in paraffin embedded tissues (Gupta et al., 2003).

The failure of detection of *M. tuberculosis* from formalin fixed paraffin embedded tissue by conventional PCR prompted us to use Gene Xpert MTB/ RIF which is a real-time hemi nested PCR test that simultaneously identifies *M. tuberculosis* and detects rifampicin resistance directly from clinical specimens. Two samples from each nodular hyperplasia with chronic prostatitis (NHCP), granulomatous prostatitis (GnP) and prostatic intraepithelial neoplasia (PIN) states, all 8 prostatic adenocarcinoma tissue samples were tested for presence of mycobacterial genomic DNA by the Gene Xpert MTB/RIF real time PCR.. All these samples came out negative for mycobacterial DNA. Moreover, the two positive control samples (lymph node tissues from confirmed TB cases) were positive in the Gene Xpert MTB/RIF assay (Figure 3.13). The finding of the positive results coming out from paraffin-embedded formalin-fixed lymph node tissue samples having confirmed *M. tuberculosis* infections validates that the absence of response for *M. tuberculosis* infection in Gene Xpert analysis from paraffin-embedded formalin-fixed prostatic tissues indicates that the Gene Xpert MTB/RIF assay is more sensitive for detection of *M. tuberculosis* from formalin fixed paraffin embedded tissues. Our findings are in agreement with previous findings that Gene Expert assay is more sensitive than conventional PCR in detecting mycobacteria directly from tissue samples (Zeka et al, 2011; Denkinger et al, 2014). In summary, the prostate tissue samples used in this study did not harbour

M. tuberculosis indicating that infection of prostate by this pathogen is not common in prostate cancer patients. Further study with larger number of subjects will be needed to come to a definite conclusion. The important finding of this study is that positive control samples included in this study (lymph nodes tissues from confirmed TB cases) were negative in Z-N staining and conventional PCR in detection of mycobacterial infection in prostate tissues, but were in fact positive as detected by Gene Expert System. This finding highlights the need of performing sensitive molecular test such as Gene Xpert in ruling out whether a suspected patient is infected with TB or not.

Concluding remarks

This study attempts to find out any association of *Mycobacterium tuberculosis* in promoting cancer in prostatic tissues of elderly patients with different prostatic lesions. None of the 85 prostatic biopsy samples revealed the presence of *M. tuberculosis* when analyzed by Ziehl-Neelsen (Z-N) stain and polymerase chain reaction (PCR). Two samples from each nodular hyperplasia with chronic prostatitis (NHCP), granulomatous prostatitis (GnP) and prostatic intraepithelial neoplasia (PIN) states, all 8 prostatic adenocarcinoma tissue samples were tested for presence of mycobacterial genomic DNA by the Gene Xpert MTB/RIF real time PCR.. All these samples came out negative for mycobacterial DNA. Very importantly, the two positive control samples (lymph node tissues from confirmed TB cases) were positive in the Gene Xpert MTB/RIF assay (Figure 3.13). The finding of the positive results coming out from paraffin-embedded formalin-fixed lymph node tissue samples having confirmed *M. tuberculosis* infections validates that the absence of response for *M. tuberculosis* infection in Gene Xpert analysis from paraffin-embedded formalin-fixed prostatic tissues indicates that the prostate tissue samples used in this study did not harbour *M. tuberculosis* indicating that infection of prostate glands by *M. tuberculosis* appears to be not common in Bangladesh. In addition to this observation the finding of the present study concluded as follows:

- Four different lesions in collected samples of prostatic tissues were identified. The cancerous state was observed in patients with age range of 61-70 years, where smoking habit could be attributed as a risk factor.

- In Bangladesh, the cases of prostate cancer were more prevalent in Dhaka district, and people of business community had a greater incidence of cancer.
- PSA level was gradually increased as the prostatic disorders complicated, and the highest value was recorded in adenocarcinoma.
- Using formalin-fixed paraffin-embedded tissues, Gene Xpert MTB/RIF appears to be more convenient than that of conventional PCR for detection of *M. tuberculosis*.

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Appendix I

Solution and Reagents used

Jiehl-Neelsen Staining

Carbol Fuschsin (1L)

Basic Fuschsin 3.0 gm

95% ethanol 100ml

Dissolved basic fuschsin in ethanol

Phenol crystal 45gm
D/W 900.0 ml

Combine 100 ml of solution A with 900.0 ml of solution B and store in another bottle. Label bottle with name of reagent as well as preparation and expiry date. Keep in a cool place for upto three months.

Methylene blue (100 ml)

Methylene blue 0.30gm

D/W 100 ml

Dissolve 1.0 gm of methylene blue chloride in 100 ml of distilled water. Label bottle with name, date of preparation and expire date. Store at room temperature for upto three months.

25% H_2SO_4

H_2SO_4 (Concentrate) 25 ml

D/W 75 ml

Harris haematoxylin & Eosin staining solution

1. Harris haematoxylin powder : 5.0 gm

   Ethyl alcohol: 50 ml

   5.0 gm harris haematoxylin dissolve in 50 ml ethyl alcohol.....(1)

2 .Potassium alam :100gm

   Distilled water : 950ml

   Potassium alam dissolve in boiling water. It should be until the potassium dissolve.....(2)

   Solution 1 and Solution 2 dissolve properly.

Murcury oxide red 2.5 gm mixed with the solution. It is a preservative. It should be kept in dark room in amber bottle.

Eosin powder: 1.0 gm

Disstiled water: 100 ml

1.0 gm eosin powder dissolve in 100 ml distilled water. This is stock solution.

Working:

75 absolute alcohol + 25ml stock solution

Preparation of PBS (ph 6.8)

Sodium phosphate dibasic 9.47gm/L

Potassium phosphate monobasic : 9.0 gm/L

Volume 4 L

1. Na_2HPO_4: 25.56gm
2. D/W: 2700ml

1 KH_2PO_4 :11.75 gm

3. D/W: 1300ml

Mixing of these two solutions and adjust ph by ph meter then autoclave.

Appendix II

Instruments & apparatus

The important instruments and apparatus used through the study are listed below:

Autoclave, priorclave Limited	London
Class II biological safety cabinet	Singapore
PCR cabinet PCR 3 A1	Singapore
Waterbath-Leuda E 100,	Germany
Pipette (100-1000µl), Model LPiooL	Franch
Gel Doc XR system, Boi-RAD	Italy
PTC-200, Peltier Thermal Cycler	USA
Microwave oven, Sharp	Japan
Micro centrifuge tube	Eppendorf, Germany
Architect, Model CI4100	USA
Microtome leica, Model RM 2125& RMRM 2125 RT	Germany
Centrifuge machine, Rotofix 32A	China
Precision electric balance	USA
Vortex machine, ISE Mix, VM-10	USA
Refrigerator, Sharp, SJEK 2625	Japan
Gene Xpert machine, M-GX IV R2	USA

Appendix III

Questionnaire form and Ethical clearance certificate

Questionnaire form

Pt ID Date

1. Name
2. Address
3. Age
4. Sex
5. Occupation
6. History of patient illness
   - A. Low back pain
   - B. Haematuria
   - C. Frequency of micturation
   - D. Urgency of micturation
   - E. Frequency of dysurea
   - F Weight loss
7. Family history of pulmonary TB
8. Family history of cancer
9. Place where patient live
10. Total Prostate Specific Antigen (PSA) level

Anwer Khan Modern Medical College

Memo No.AKMMC/15/2585 Dated 21/5/2015

To
Mahbuba Ashrafi Mumu
M.Phil(Thesis Part)
Department of Microbiology
University of Dhaka

Subject: Ethical Clearence.

The ethical review committee of Anwer Khan Modern Medical College has reviewed your submitted protocol entitled " Prevalence of Mycobacterium Tuberculosis in prostatic tissue of patients suspected with different prostatic lesions specially cancer" and approved it.

Prof.Dr.Md. Mahfuzar Rahman 21/05/2015

Member Secretary,

Ethical Review Committee,AKMMC

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