THE EFFECT OF THE EMBALMING FLUID, USED BY THE DEPARTMENT OF BASIC MEDICAL SCIENCES (UFS), ON THE VIABILITY OF *MYCOBACTERIUM TB* IN HUMAN CADAVER LUNG TISSUE

by

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DECLARATION

I certify that the dissertation hereby submitted by me for the Magister in Medical Science at the University of the Free State is my independent effort and had not previously been submitted for a degree at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

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J.C. Correia

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LIST OF ABBREVIATIONS

AFB	Acid-fast bacilli
BCG	Bacilli calmette-gluerin
DNA	Deoxyribonucleic acid
CFU	Colony-forming unit
LJ	Lowenstein-Jensen
MDR-TB	Multi-drug resistant tuberculosis
MGIT	*Mycobacterium* growth indicator tube
MOTT	*Mycobacterium* other than tuberculosis
MTB	*Mycobacterium tuberculosis*
NaOH	Sodium Hydroxide
PANTA	Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, and Azlocillin
PCR	Polymerase chain reaction
PPD	Purified Protein Derivative
PTB	Pulmonary tuberculosis
TB	Tuberculosis
UFS	University of the Free State
UVGI	Ultraviolet germicidal irradiation

WHO	World Health Organization
ZN	Ziehl-Neelsen

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1. INTRODUCTION

Embalming fluid contains substances such as formalin, ethanol, phenol, and other solvents to temporarily prevent decomposition. These agents disinfect, preserve, and/or sanitise (Pretorius 1995). An embalming fluid formula should have the following requirements: fixative, bactericide, fungicide, humectant, and colour enhancer (Pretorius 1995). Formalin and ethanol are used as fixatives by denaturing the cellular proteins, meaning the proteins cannot act as a source of nutrients for the bacteria. Phenol is used as a bactericide to destroy a broad spectrum of organisms and promotes good colour retention to muscle tissue (Pretorius and Brune 1992).

The risk of contracting a disease such as tuberculosis (TB) among persons who are in close contact with recently deceased people, is high (Gerston, Blumberg, Tshabalala and Murray 2004) and the risk varies according to occupation. Workers at anatomy departments and embalmers are some of those people who are at a greater risk of contracting tuberculosis carried by cadavers. This increased risk may be due to the exposure to this organism during the embalming process, which involves the inhalation of infectious aerosol (Sterling, Pope, Bishai, Harrington, Gershon, and Chaisson 2000).

Embalming does not guarantee that the growth of TB bacteria is halted. In 1949, Meade and Steenken already reported the growth of *Mycobacterium* from embalmed cadavers although not much detail was given. Sterling *et al.* also reported a case where an embalmer contracted TB after handling an infected cadaver (Sterling *et al.* 2000). Another study by Gerston *et al.* (2004) attempted to culture TB from formalin-fixed lungs. Of the 138 cases, *Mycobacterium* was grown from 12 cases (9%). Gerston *et al.* (2004) also found that *Mycobacterium tuberculos*is (MTB) was isolated from formalin-fixed tissue up to 45 days after fixation.

Nevertheless, in medical circles it is a perception that once tissue is fixed in formalin, the risk for contracting TB is greatly reduced or eliminated (Gerston *et al.* 2004). A subsequent study by Weed and Baggenstoss showed that MTB remains infectious for at least 24 – 48 hours after embalming (Weed and Baggenstoss 1951). Demiryürek, Bayramoglu, and Ustaçelebi stated that formalin is an effective tuberculocidal (Demiryürek *et al.* 2002).

MTB causes granuloma (a mass of inflamed granulation tissue) and the effective penetration of embalming agents into these sites raises questions. The granuloma that forms typically appears cheese-like (caseation) and this is

known as a tubercle (Thomas 1983). MTB infection rarely occurs in the anterior segments of the lungs, but commonly in the apical segments of the upper and lower lobes as well as the hilus region of the lung (Brink and de Kock 1973). MTB is an aerobic bacterium and thus multiplies better in the apex of the lung where oxygen concentration is higher.

There also seem to be a lack of guidelines for the effective and safe handling of formalin-fixed autopsy tissue that has been infected by MTB. Both the Centres for Disease Control and Prevention (CDC 1994) and the Occupational Safety and Health Administration (OSHA 1996) lack to mention these guidelines. Based on this, the disinfection properties of fixatives for MTB infected tissue thus remain unclear.

1.1 AIM AND OBJECTIVES

The **aim** is to investigate if MTB is still viable in human cadaver lung tissue and especially in granulomatous tissue after embalming has taken place.

Thus, the **objective** of this research study is to test the efficacy of the embalming fluid used at the department of Basic Medical Sciences (University of the Free State) on eliminating *Mycobacterium tuberculosis* in human cadaver lung tissue.

1.2 CADAVERS

The department of Basic Medical Sciences (UFS) receives approximately 80 – 100 human cadavers per year for research and educational purposes (Table 1.1). The cadavers are obtained as human donations and unclaimed bodies. If bodies are not claimed within 14 days after death, the Inspector of Anatomy is notified. The department is according to The Human Tissue Act, Act 65 of 1983, the only authorised institution at the UFS to receive human cadavers (Pretorius and Brune 1992) and is authorised by the Inspector of Anatomy to perform research on these cadavers.

According to the number of MTB infected cadavers recorded during 2010, approximately 25% is expected to have TB as seen in Table 1.1.

Table 1.1 Number of cadavers received from 2009 – 2011 at the department of Basic Medical Sciences (UFS) and the number of deceased due to *Mycobacterium tuberculosis* according to the death certificates.

Year	Total number of cadavers received	Number of cadavers with TB (according to death certificate)	% of cadavers with TB (according to death certificate)
2009	90	22	24%
2010	103	26	25%
2011	81	11	14%

1.3 EMBALMING FLUID

A simple definition for embalming is the disinfection, temporary preservation, and restoration by way of injection of embalming fluid into the radial artery of the cadaver (Pretorius 1995). According to Creely (2004), embalming is also to restore a more life-like appearance for presentation. According to Pretorius and Brune (1992), embalming fluid formula should have the following requirements: fixative; bactericide; fungicide; humectant and colour enhancer.

A fixative is a stabilizing or a preservative agent. Fixation methods fall in two classes: cross-linking agents and organic solvents. The result of adding a fixative such as formalin to embalming fluid is to react with proteins to create "cross-links". These "cross-links" cause the firmness of embalmed tissue and inactivate the enzymes that are responsible for post-mortem autolytical processes. Autolysis (self-digestion) is the process of self-destruction that starts soon after cell death creating an enzyme attack that causes protein breakdown and eventually liquefaction of the cells. In the absence of embalming, putrefaction (stage of decomposition) will take place (Pretorius and Brune 1992). Autolysis can be halted by disabling the enzymes by adding a fixative. By changing the pH level in the body or by placing the body in

temperatures well below normal body temperature are also ways of stopping autolysis (Pretorius and Brune 1992). The microorganism involved in the process of decomposition is not pathogenic (Morgan 2004) and is mostly normal flora found in the gastro-intestinal tract. The putrefactive stage of decomposition is mainly driven by anaerobic bacteria within the gastro-intestinal tract. Organic solvents such as alcohol (e.g. ethanol) remove lipids and dehydrate the cells. The dehydrated firm tissue is less attractive for bacterial consumption (Pretorius and Brune 1992).

A bactericide is an agent or a substance capable of destroying bacteria or inhibiting the growth of bacteria. These agents need to be effective against a broad spectrum of bacteria. Bactericides can work in various ways, either by stopping cell division of the bacteria or by interfering with the formation of the cell walls or even by stopping the bacteria to multiply (Ajmani 1998).

A fungicide is an agent or a chemical substance capable of killing fungi or inhibiting the growth of fungi. Fungicide thus prevents the spread of fungi on cadaver tissue. Most fungal infections on human cadavers are saprophytic and multiply in untreated or poorly embalmed cadavers (Ajmani 1998). A fungal infection will appear as blue, green, or purple “fur” on the surface of the body.

A humectant (moistening/wetting agent) is a hygroscopic substance. Hygroscopic refers to the ability to absorb water from the air. A humectant is added to prevent hardening and distorting and help restore the tissue dehydrated by the formalin to a more natural hydrated condition (Pretorius and Brune 1992).

A colour enhancer gives the cadaver a more natural look. Certain chemicals act on the blood pigment to restore the red colour after fixation. Other chemicals give a better colour contrast between the different types of tissue (Pretorius and Brune 1992).

The department of Basic Medical Sciences (UFS) uses a standardised embalming formula for the embalming of the cadavers. **Table 1.2** shows the recipe for the embalming fluid for 1 cadaver.

Table 1.2 Embalming fluid recipe (Pretorius and Brune 1992).

20 L 96% Ethanol
1 L 40% Formalin
1.5 L Pine Oil
7.5 L Water
1.5 L 80% Liquid Phenol

The formal name for formalin is formaldehyde (HCHO), the British Chemist; August Wilheld Von Hofmann (Dixit 2008) discovered it. According to Pretorius and Brune (1992), formalin is the best fixative but it has the following disadvantages: slow in penetrating tissue; the fumes may irritate mucous membranes of air passages; limits suppleness of tissue and does not promote good colour retention (Pretorius and Brune 1992). It is a colourless, highly flammable gas at room temperature with a distinct odour. Commercially it is available as formalin, which is formaldehyde gas in water. A study conducted by Coleman and Kogan (1998) found that 0.5 L 37 – 40% formaldehyde per body is adequate for embalming purposes. Formalin usually also contains methanol, which inhibits polymerisation. Formalin acts as a high-level germicide (Demiryürek *et al*. 2002).

The fumes of the formaldehyde are intensely irritating to mucous membranes of the eye, nose, and upper respiratory tract. There are also indications that formaldehyde has a carcinogenic effect (Pretorius and Brune 1992). According to Pretorius and Brune (1992), asthma and allergies are more prevalent in humans if they are exposed continuously to fumes of formaldehyde.

Ethanol (Table 1.2) acts as a fixative, bactericide, and fungicide but is highly flammable and may dehydrate tissue (Pretorius and Brune 1992). Ethanol is however ineffective against prions, endospores and nonenveloped viruses (Demiryürek *et al.* 2002). Pine oil (Table 1.2) acts as a humectant and a colour enhancer. It also improves the smell of the embalming fluid by giving it a "pine" odour (Pretorius and Brune 1992).

Phenol (carbolic acid) acts as a bactericide and fungicide but has a few disadvantages: it is expensive, toxic, has an unpleasant smell, and if splashed on skin it can cause serious burns (Dixit 2008). At the right concentrations, phenol is used as a colour enhancer of muscle tissue giving muscle tissue a suitable brown colour (Pretorius and Brune 1992).

According to Dixit (2008), the toxic effects of the fumes of formaldehyde during embalming may be reduced by installing good ventilation systems in the embalming rooms. Embalmers should be educated about the potential health hazard of the fumes, wear protective clothing and masks during the embalming process, and avoid spillage of the embalming fluid.

Six months after embalming, the cadavers are ready for dissection purposes (Pretorius and Brune 1992). In the dissection hall, the cadavers are wrapped in cloths and plastic to prevent the cadavers from drying out and discolouring. The cloths are dipped in a wetting fluid consisting of a mixture of 250 ml pine oil and 250 ml 80% liquid phenol.

1.4 PULMONARY TUBERCULOSIS

Pulmonary TB (PTB) is a contagious, bacterial lung disease but it can also affect the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, bones, joints, and even the skin (Bloom and Murray 1992). PTB is caused by *Mycobacterium tuberculosis*, a rod-shaped bacterium (Figure 1.1) with a lipid-rich cell wall. *Mycobacterium* infections are intracellular and cause granulomatous lesions (Strohl, Rouse, and Fisher 2001). MTB has the following characteristics: non-motile; non-spore forming; acid-fast and aerobic.

MTB was first isolated by Robert Koch in 1882 (Bloom and Murray 1992) and today it is the leading cause of infectious disease according to the World Health organisation (WHO 2011) second to HIV. An estimated 1.3 million people died of TB in 2008. In 1993, the WHO declared TB a global health emergency (WHO 1993). Eight to ten million new cases of TB are reported each year, according to the WHO (2011). In South Africa TB has been the leading cause of death since 1997 accounting for about 13% of the deaths in the country according to Statistics South Africa (2008). The emergence of multi-drug resistant TB (MDR-TB) in recent years has added to the control of

TB (Leung 1999). MDR-TB is strains that are resistant to one or more of the primary drugs (Strohl *et al.* 2001).

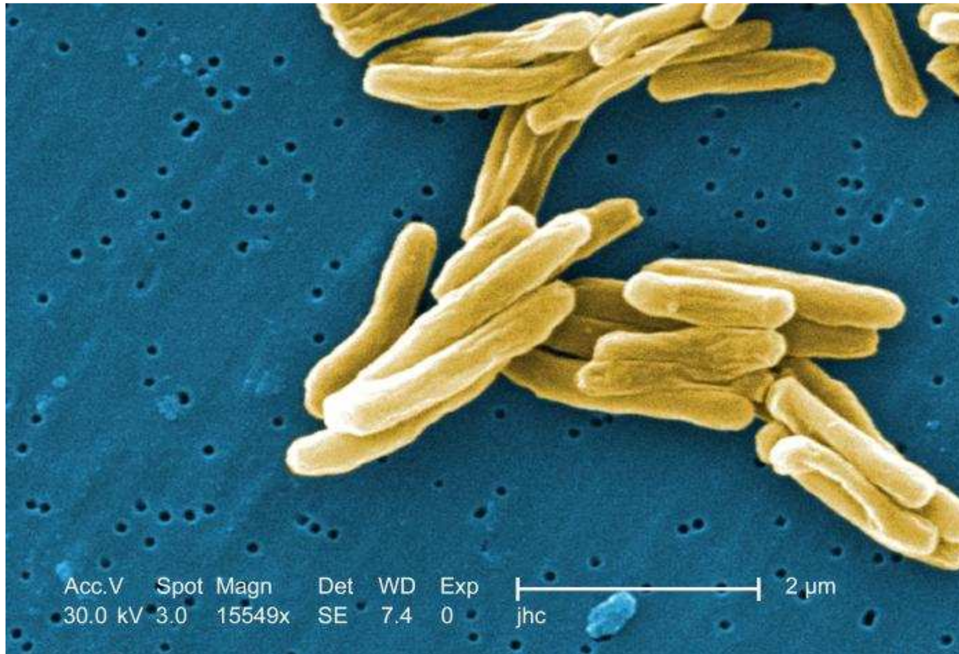

Figure 1.1 Scanning electron micrograph of *Mycobacterium tuberculosis* bacilli (CDC 2006).

1.4.1 Mode of transmission

Transmission of the bacillus is human-to-human. TB is spread by the inspiration of aerosol infected with large numbers of the organism (Leung 1999). MTB can remain viable in the environment due to the resistance to desiccation (Strohl *et al.* 2001). Infected aerosol droplets are produced when persons with pulmonary TB cough, sneeze, speak, or sing. A single cough can

produce about 3000 droplet nuclei that contain the tubercle bacilli (CDC 1994).

1.4.2 Pathogenicity

After the tubercle bacilli have been inhaled, MTB multiplies slowly in the terminal alveoli of the lungs and in the lymph nodes of the corresponding drainage areas, this is known as the primary infection (Strohl *et al.* 2001). The Ghon complex is a lesion that consists of calcified focus of infection and an associated lymph node as seen in Figure 1.2. The Ghon complex is the characteristic appearance associated with primary infection. The bacilli are ingested by alveolar macrophages but also by alveolar epithelial type II pneumocytes (Smith 2003). If the alveolar macrophage cannot destroy or inhibit the MTB, the bacilli multiply within its intracellular environment, causing the host macrophage to burst (Leung 1999). Within 2 – 4 weeks, many of the macrophages die and release the bacilli. When MTB multiplies within the alveolar macrophages, this causes more macrophages to migrate to the area, causing an early tubercle (Strohl *et al.* 2001). The released bacilli form a caseous centre in the middle of the tubercle.

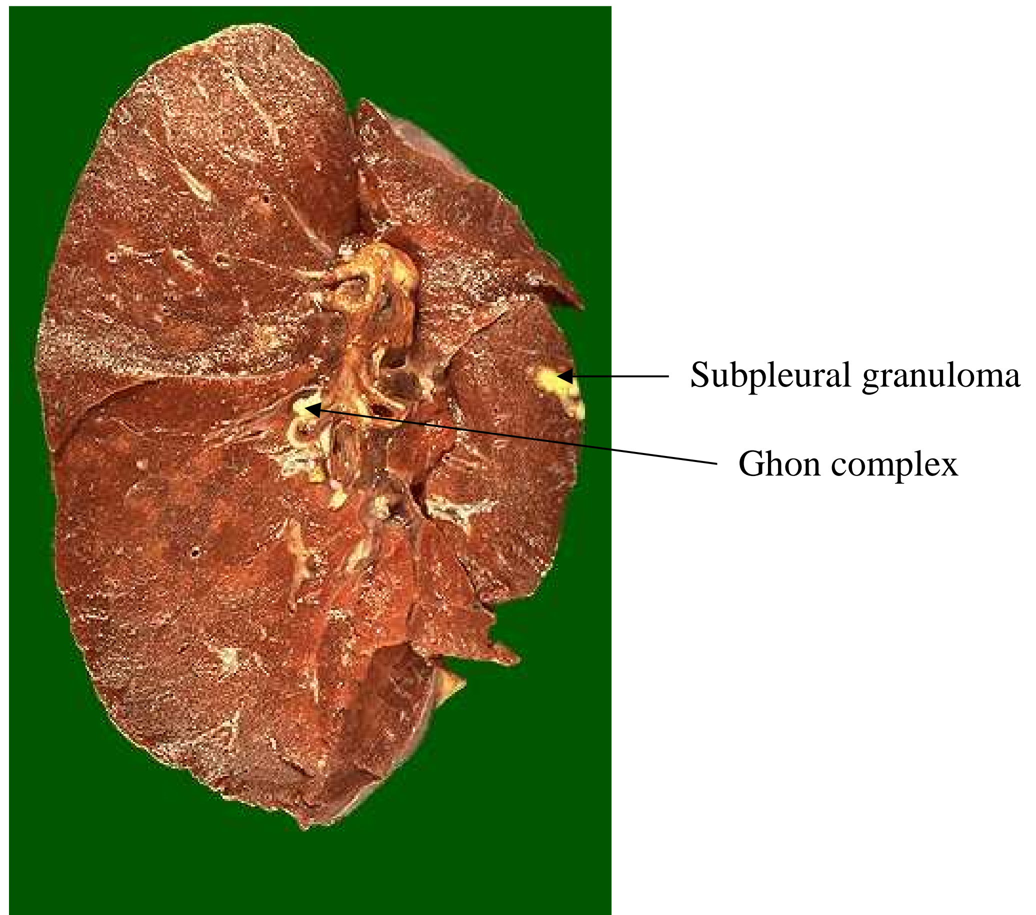

Figure 1.2 In the hilus is the Ghon complex, a small yellow granuloma in a hilar lymph node next to the bronchus (Klatt n.d.).

A dormant phase may occur after this stage. In the majority (90 - 95% in HIV negative patients) of the cases, the pulmonary lesions will gradually heal (Leung 1999). The lesion may stop and become fibrotic and calcify, if not, a mature tubercle is formed and the caseous centre enlarges and forms a cavity in which the bacilli multiply. The centre can burst and spread to other body systems via lymph to the hilar or mediastinal lymph nodes and through the

bloodstream to more distant sites in the body (Leung 1999). Other known risk factors for the development of active TB include conditions that are associated with defects in T-lymphocyte and/or macrophage function, such as malnutrition, drug and alcohol abuse, coexistent medical conditions and corticosteroid or other immunosuppressive therapy.

MTB has the ability to grow in immunologically activated macrophages and remain viable by inhibiting the fusion of phagocytic vesicles to lysosomes. MTB produces no endotoxins or exotoxins. MTB stimulates a humoral and cell-mediated immune response (Strohl *et al.* 2001). Tubercle bacilli are immunogenic meaning they enhance immunonologic responsiveness non-specifically.

Miliary (disseminated) TB is a form of tuberculosis that spreads through the entire body through blood and lymph vessels (Strohl *et al.* 2001). This can take place during primary and active infection. Common sites for this extrapulmonary infection are the meninges, lymph node, bones, joints, and the genitourinary tract. Miliary TB occurs in about 15% of TB patients (Bloom and Murray 1992).

1.4.3 Clinical signs and symptoms of pulmonary tuberculosis

Commonly, symptoms of infection of pulmonary tuberculosis include chest pain, coughing up blood, fever, anorexia, fatigue, night sweats, and weight loss that may persist for weeks to months (Leung 1999). These symptoms are illustrated in Figure 1.3. Extrapulmonary symptoms depend on the site of infection.

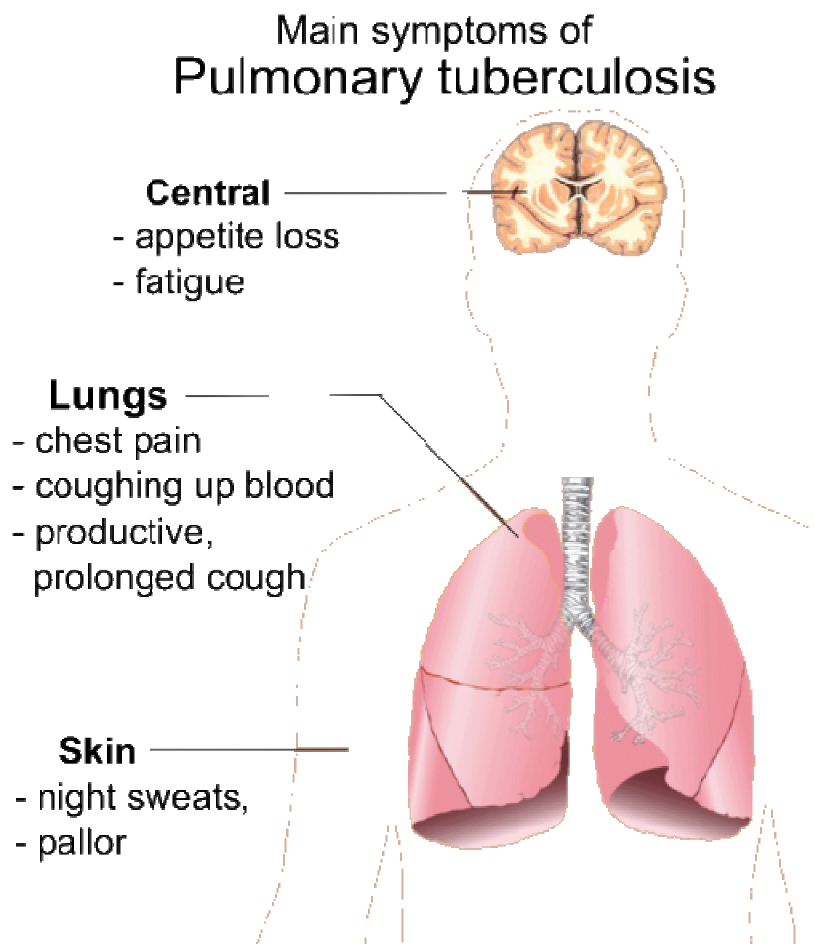

Figure 1.3 Main symptoms of pulmonary tuberculosis (Häggström 2012).

1.4.4 Tuberculin reaction test

This is also known as the Mantoux test or Purified Protein Derivative (PPD) test. The tuberculin skin test is an expression of delayed-type hypersensitivity to protein antigens of the bacterium. Tuberculin is a glycerol extract of the tubercle bacilli (Strohl *et al.* 2001).

The Mantoux test entails the following according to Strohl *et al.* (2001); a standard dose of tuberculin units is injected intradermally in the forearm and is read 48 – 72 hours later. The reaction is read by measuring the diameter of palpable raised hardened area across the forearm in millimetres. If there is no induration, the result should be recorded as "0 mm". An induration of 5 mm or greater than 5 mm is interpreted as positive if the person was in contact with persons infected with TB; persons with abnormal chest X-rays; HIV infected and other immunosuppressed persons. An induration of 10mm or more is interpreted as positive in the following populations: Recent arrivals (less than 5 years) from high-prevalence countries; prisoners, residents of nursing homes, and other institutions; health care workers; injection drug users; mycobacterial laboratory personnel; persons with clinical conditions that place them at high risk; children less than 4 years of age, or children and

adolescents exposed to adults in high-risk categories. An induration of 15 mm or more is positive in persons with no known risk factors for TB. The Tine test is not quantitative and is useful for population screening (Strohl *et al.* 2001).

1.4.5 Laboratory identification of *Mycobacterium tuberculosis*

1.4.5.1 Direct microscopy and staining

This is usually done as a first line screening because it is fast and allows for rapid illumination of infective patients (Mackie, Duguid, Marmion, McCartney and Swain 1978). The sensitivity is directly proportional to the number of acid-fast bacilli present. Direct microscopy can be performed on tissue fluid obtained through aspiration of granulomatous tissue as a method of rapid diagnosis. Sensitivity for the detection of acid-fast bacilli is significantly lower than with culture techniques. 10 000 organisms per ml sample is needed to obtain a positive result and the sensitivity is about 60 - 70% (Blumberg 1995). Variations of this method are the Ziehl-Neelsen (ZN) stain and the fluorescent microscopy method.

In the ZN technique, also known as the acid-fast bacillus (AFB) stain, the MTB is stained with carbol fuchsin and then washed with alcohol or acid (Strohl *et al.* 2001). MTB, however, retain the carbol fuchsin stain, appear pink, and slightly curved (Strohl *et al.* 2001). *Mycobacterium* species contain large amounts of lipid substances in their cell walls, which resist staining by ordinary methods unless the dyes are combined with phenol (Strohl *et al.* 2001). When these organisms are stained with a basic dye, e.g. carbol fuchsin, with phenol added, and heat is applied, the stain can penetrate the cell wall and reach the cell cytoplasm. Once the cytoplasm is stained, it resists decolourisation with acid-alcohol, which cannot dissolve and penetrate beneath the cell wall. Under these conditions of staining, the bacilli are said to be acid-fast (Strohl *et al.* 2001). Other bacteria whose cell walls do not contain high concentrations of lipid are readily decolourised and are non-acid-fast. A counterstain (e.g. methylene blue or malachite green) is used to show structures and cells and to form a contrast with the red-stained bacilli.

Fluorochrome stain is used in the fluorescent microscopy method that allows for easier identification of fluorescent bacilli (Strohl *et al*. 2001). The identification of *Mycobacterium* with auramine dye is possible due to the affinity of the mycolic acid in the cell walls for the fluorochromes. The dye

will bind to the *Mycobacterium*, which appears as bright yellow, luminous rods against a dark background. Slides, which are stained with auramine, may be restained with ZN stain directly. This helps confirming and differentiating the morphology of the positive or query slides. The fluorochrome stains are recommended for specimen examination because of the increased sensitivity and speed (Mackie *et al.* 1978).

1.4.5.2 Lowenstein-Jensen method

This method is the traditional method; it consists of an egg-based medium, and takes about 2 - 6 weeks to culture (Strohl *et al.* 2001). Only 100 – 1000 bacilli per ml of sample need to be present to detect MTB using the Lowenstein-Jensen (LJ) method (Blumberg 1995). Positive results indicate that the MTB is viable, but further growth and identification is needed for a definitive diagnosis (Blumberg 1995). Middlebrook and Cohn (1958) described an agar-based medium for more rapid detection, but it still requires 3 – 4 weeks to culture.

1.4.5.3 *Mycobacterium* growth indicator tube culture method

Mycobacterium growth indicator tube (MGIT) is an automated method to detect the production of an indication substance released from specific reactions when active growth of acid-fast bacilli takes places. The purpose thus is to detect the viability of the tubercle bacilli. This method is the most widely used method of detecting MTB infection (Leung 1999) and will be the mainstay detection method for MTB in this study. Although culture according to this method extends over 6 weeks before it can be regarded as negative, the greater majority (probably more than 90%) of positive results are obtained within 2 weeks (Leung 1999). This method has a shorter time of detection than that of the LJ method (Blumberg 1995).

The BACTEC MGIT 960 instrument is an *in vitro* diagnostic instrument designed and optimised for the rapid detection of *Mycobacterium* in clinical samples other than blood. Specimens are collected, processed, and inoculated into ***M****ycobacterium* **g**rowth **i**ndicator **t**ubes. These tubes contain a liquid broth medium consisting of modified Middlebrook broth base and when supplemented with MGIT Growth Supplement and an antimicrobial mixture

(PANTA), it provides optimum medium for growth (Siddiqi, Libonati, Carter, Hooper, Baker, Hwangbo and Warfel 1988).

The decontamination recommendation is based on a procedure using sodium hydroxide (NaOH) with N-acetyl L-cysteine (Siddiqi *et al.* 1988). NaOH is bactericidal for contaminating bacteria but is to a much lesser extent harmful to *Mycobacterium*. N-acetyl L-cysteine has no decontamination properties. NaOH and N-acetyl L-cysteine helps in liquefying the specimen.

A fluorescent compound is embedded in the silicone on the bottom of the tubes and is sensitive to the presence of oxygen dissolved in the broth. Initially the large amount of dissolved oxygen quenches emissions from the compound. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected. The tubes entered into the system are continuously incubated at 37°C and monitored for increasing fluorescence (Siddiqi *et al.* 1988). Analysis of the fluorescence is used to determine if the tube contains viable organisms. The intensity of the fluorochrome is directly proportional to the extent of oxygen depletion. In case of MTB, at the time of positivity, there is approximately 10^5 – 10^6 colony forming units (CFU) per ml

of medium. A tube is declared negative if it remains negative for 6 weeks (42 days).

1.4.5.4 Polymerase chain reaction method

Polymerase chain reaction (PCR) method is for testing the presence of genetic material of MTB. This method is extremely useful but lack sensitivity in some specimens with very low bacterial counts and is not able to determine whether the bacilli are viable or not. Consequently, false positive results can be obtained (Blumberg 1995). The method forms the basis for differentiating MTB from other *Mycobacterium* species (Mackie *et al.* 1978).

According to a study done by Salian, Rish, Eisenach, Cave and Bates (1998), this method can be used to detect MTB DNA in formalin-fixed tissue. With this method deoxyribonucleic acid (DNA) is extracted from a sample using an automated DNA extraction system. A small portion of a predetermined target region of MTB DNA is amplified to differentiate between MTB complex and *Mycobacterium* other than TB (MOTT).

1.4.6 Treatment and prevention of *Mycobacterium tuberculosis*

Some strains are resistant to drug therapy, but several chemotherapeutic agents are effective against MTB (Blumberg 1995). Isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin are first-line anti-tuberculosis drugs (Strohl *et al.* 2001) and needs to be taken for 2 months. The reason for this extensive course is that the bacilli are located intracellular, the caseation blocks the penetration of the agents, and the bacilli grow slow and can become metabolic inactive (Strohl *et al.* 2001).

Bacilli calmette-gluerin vaccine (BCG) is available and mostly given to tuberculin-negative individuals with a high risk of infection (Strohl *et al.* 2001). Isoniazid is used prophylactically for individuals who are tuberculin positive but asymptomatic.

1.5 GROSS ANATOMY OF THE LUNGS

The lungs are found in the thorax within the pulmonary cavities. The pulmonary cavities are situated laterally to the mediastinum and are lined with parietal pleura, whereas the lungs are covered by visceral pleura. Each pulmonary cavity (left and right) is lined by the parietal pleura as well as the superior surface of the diaphragm and the lateral surfaces of the mediastinum (Moore, Dalley, and Agur 2009). The potential space between the parietal and the visceral layers is the pleural space containing serous pleural fluid. At the hilus of the lung (Figure 1.4), on the medial area on the lung where the bronchus and pulmonary vessels enter and leave the lung, the visceral pleura is continuous with the parietal pleura. Cadaveric lungs are shrunken, hard, and discoloured (Moore *et al*. 2009).

The parietal pleura consists of the costal, mediastinal, diaphragmatic, and the cervical parts. The costal pleura lines the internal surface of the thoracic wall. The mediastinal pleura covers the lateral parts of the mediastinum (Moore *et al*. 2009). The diaphragmatic pleura covers the thoracic part of the diaphragm. The cervical pleura (pleural cupola) covers the apices of the lungs and is the superior continuation if the costal and mediastinal part of the

parietal pleura (Leonard 1995). The cervical pleura is strengthened by an extension of the endothoracic fascia that attaches on the internal border of the first rib and transverse process of C7; the suprapleural membrane or Sibson's fascia (Moore *et al.* 2009).

The lungs are the organs of respiration, roughly cone-shaped and each has an apex a base (Figure 1.4), lobes, three surfaces (costal, mediastinal and diaphragmatic) and three borders (anterior, inferior, and posterior). The anterior, posterior, and lateral surfaces are in contact with the ribs and known as the costal surface (Figure 1.4). The apex is covered by cervical pleura and found above the level of the first rib in the root of the neck. The base lies in relation to the diaphragm (Moore *et al.* 2009). The anterior border (Figure 1.4) of the right lung is straight but the anterior border of the left lung has an angular indentation known as the cardiac notch (Drake, Mitchell, and Vogl 2005). The lingula is formed in the superior lobe by this cardiac notch, presented as a tongue-like projection inferior to the notch (Moore *et al*. 2009).

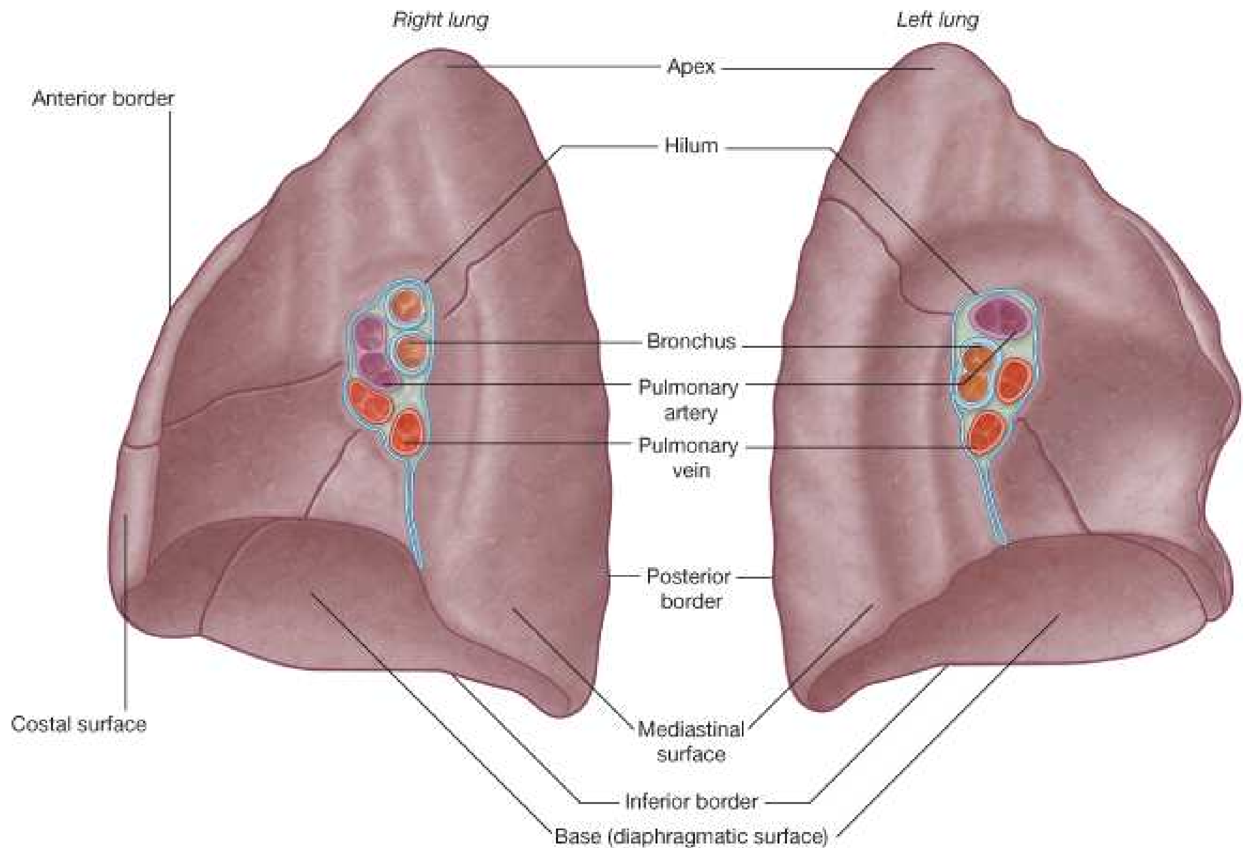

Figure 1.4 Medial view of right and left lung (Drake *et al.* 2005).

The right lung consists of three lobes divided by the oblique and horizontal fissure (Figure 1.5) whereas the left lung consist of two lobes divided by the oblique fissure (Moore *et al*. 2009). The three lobes of the right lung include the superior, middle, and inferior lobe. The left lung is divided into a superior and inferior lobe (Moore *et al*. 2009).

Figure 1.5 Embalmed and plastinated lungs (Personal photograph by author: 02 June 2011).

Bronchopulmonary segments are a pyramidal shaped segments of the lung covered by visceral pleura, separated by septa from adjacent segments, supplied by a segmental branch of the pulmonary artery and is named according to the segmental (tertiary) bronchus that supplies it. The trachea bifurcates at the level of 4th or 5th thoracic vertebrae into left and right main bronchi. The main bronchi further divide into lobar bronchi and then into segmental bronchi (Moore *et al.* 2009). Each segmental bronchus supplies a specific bronchopulmonary segment. The right lung has 10 bronchopulmonary

segments while the left lung has 8 to 10 bronchopulmonary segments as seen in Table 1.3.

Table 1.3 List of bronchopulmonary segments of the left and right lung (Moore *et al.* 2009).

<table>
<tr><th>Right lung</th><th>Left lung</th></tr>
<tr><td>Superior lobe:
• Apical
• Posterior
• Anterior</td><td rowspan="2">Superior lobe:
• Apical } Apicoposterior
• Posterior
• Anterior
• Superior lingular
• Inferior lingular</td></tr>
<tr><td>Middle lobe:
• Lateral
• Medial</td></tr>
<tr><td>Inferior lobe:
• Apical basal
• Medial basal
• Anterior basal
• Lateral basal
• Posterior basal</td><td>Inferior lobe:
• Apical basal
• Medial basal } Anteromedial
• Anterior basal
• Lateral basal
• Posterior basal</td></tr>
</table>

1.5.1 Neurovascular supply

The parietal pleura is supplied by the intercostal nerves and vessels except the inner portion of the diaphragmatic and mediastinal pleura which is supplied by the phrenic nerve and the pericardiophrenic vessels (Moore *et al.* 2009).

The pulmonary artery conveys deoxygenated blood to the lungs from the right ventricle of the heart (Drake *et al.* 2005). Each pulmonary artery gives rise to lobar and segmental arteries and end in pulmonary capillaries around the alveoli. The pulmonary veins commence in the pulmonary capillaries and carry the oxygenated blood to the left atrium of the heart.

The bronchial arteries (which supply the lung tissue) are branches of the thoracic aorta (left bronchial artery) or from the superior posterior intercostal artery (right bronchial artery). The bronchial veins are formed at the root of the lung and drain into the azygos or hemiazygos vein (Drake *et al.* 2005).

The lungs are supplied by the pulmonary nervous plexus, which is found anterior and posterior to the root of the lung (Drake *et al.* 2005). The parasympathetic fibers originate from the vagus nerve (cranial nerve X): motor to the smooth muscle of the bronchial tree (bronchoconstrictor), inhibitory to the pulmonary vessels (vasodilator) and secretory to the glands of the bronchial tree (secretomotor). The sympathetic fibers (from from T1 - T4 via the sympathetic trunk) are bronchodilators, vasoconstrictors and inhibit glandular secretion (Drake *et al.* 2005).

1.5.2 Lymphatic drainage of the lungs

The lymphatic drainage of the lungs consists of the superficial lymphatic draining the visceral pleura (**subpleural network**) and the deep lymphatic accompanying the bronchi and the pulmonary veins (**peribronchial network**). These two networks communicate at the pulmonary hilum and convey lymph mainly to the tracheobronchial lymph nodes. The tracheobronchial lymph nodes are also known as the carinal nodes because the nodes are associated with the carina of the trachea found at the bifurcation of the trachea (Moore *et al.* 2009).

The subpleural network drains (Singh 2009) the visceral pleura and the superficial lung. The superficial lymphatic vessels course around the borders, surfaces and fissures of the lungs and convey lymph mainly to the bronchopulmonary lymph nodes and then to the tracheobronchial lymph nodes.

The peribronchial network consist of intrapulmonary and bronchopulmonary nodes. The intrapulmonary nodes are found in the lung tissue at the divisions of the segmental bronchi and lie in the bifurcation of the smaller branches of

the pulmonary artery. The bronchopulmonary lymph nodes are further divided into two groups, the interlobar nodes found at the division of the lobar bronchi, and the hilar nodes found at the lower portions of the main bronchi.

Lymph flows from the tracheobronchial lymph nodes to the paratracheal (Figure 1.6) lymph nodes (situated in the superior mediastinum) and to the bronchomediastinal trunks. There are three groups of tracheobronchial nodes: superior, inferior and anterior. The superior tracheobronchial nodes (Figure 1.6) are found in the angle between the trachea and the left and right bronchi. The inferior tracheobronchial nodes (Figure 1.6) lie in the angle of the bifurcation of the trachea (Singh 2009) and the anterior nodes are anterior to the distal end of the trachea.

The main lymphatic flow from the superior lobe of the left lung, and the superior and middle lobes of the right lung (the superior region) is to bronchopulmonary, superior tracheobronchial and paratracheal lymph nodes; the inferior lobes of both lungs (the inferior region) drain to bronchopulmonary, inferior tracheobronchial and pulmonary ligament lymph nodes as seen in Figure 1.6. Pulmonary ligament nodes lie within the pulmonary ligament (Topol and Masłoń 2009).

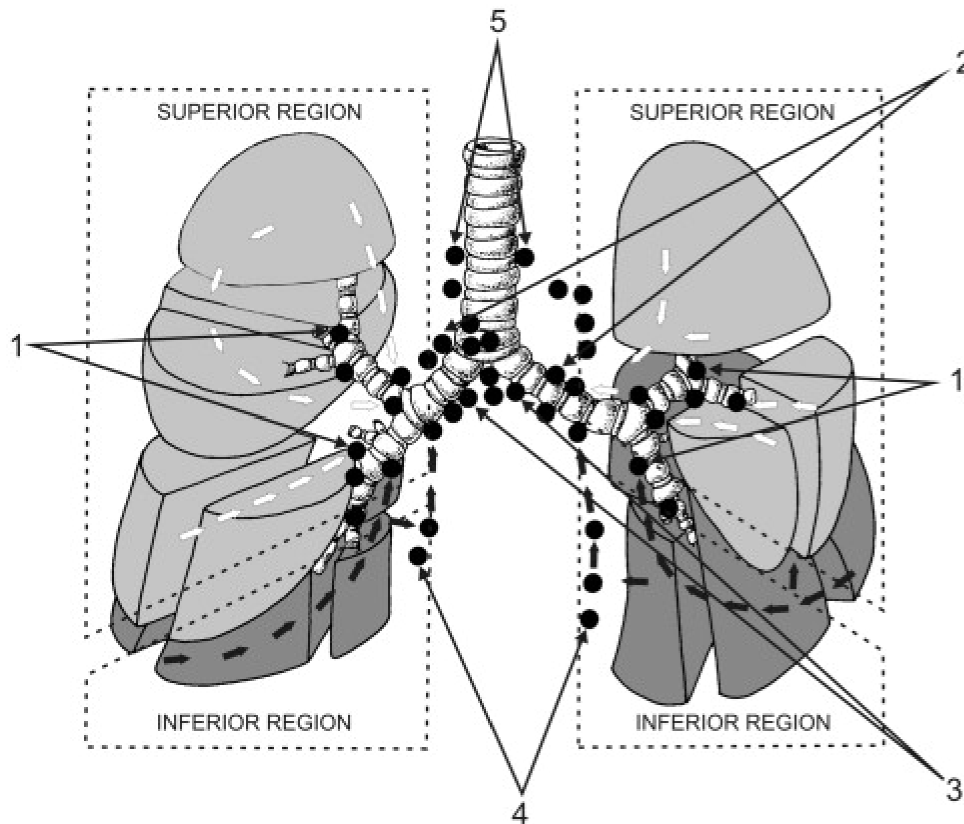

Figure 1.6 The diagram of the lymphatic drainage of the superior and inferior regions of lungs. 1—bronchopulmonary lymph nodes, 2—superior tracheobronchial lymph nodes, 3—inferior tracheobronchial lymph nodes, 4—pulmonary ligament lymph nodes, 5—paratracheal lymph nodes (Topol and Masłoń 2009).

The left and right bronchomediastinal trunks are found in the superior mediastinum. Efferent vessels of the tracheobronchial, parasternal and brachiocephalic nodes unite to form the trunks (Drake *et al.* 2005). The right bronchomediastinal trunk may drain into the right lymphatic duct and the left trunk may drain directly into the thoracic duct. The trunk may also drain into the deep veins at the root of the neck.

1.5.3 Surface anatomy of the lungs

The lines of reflection of the parietal pleura mark the extent of the pleural cavities and consist of the sternal, costal, and diaphragmatic parts. Posterior to the superior part of the sternum the sternal lines of pleural reflection approach each other in the midline. On the right side, the line descends in the midline to the xiphoid process (6th costal cartilage) where it turns laterally. On the left side, at the level of the 4th costal cartilage, the line moves laterally to the sternum (6th costal cartilage) to make space for the heart (cardiac notch) as seen in Figure 1.7. The costal lines of pleural reflection are continuations of the sternal lines from the 6th costal cartilage. The costal line of pleural reflection crosses the 8th rib in the midclavicular line, the 10th rib in the midaxillary line and the 12th rib posteriorly (Moore *et al.* 2009).

Peripheral areas where the diaphragmatic and the costal pleura come in contact are known as costodiaphragmatic recesses (Drake *et al.* 2005) and the areas where the costal and the mediastinal pleura come in contact are known as costomediastinal recesses (Figure 1.7).

The cervical pleura and apices of the lungs are found 2 – 4 cm superior to the medial third of the clavicle in the supraclavicular fossa, but do not extend above the neck of the 1st rib (Drake *et al.* 2005). The hilum of the lungs is found at the level of the 3rd and 4th costal cartilage (vertebral level T5 – T7).

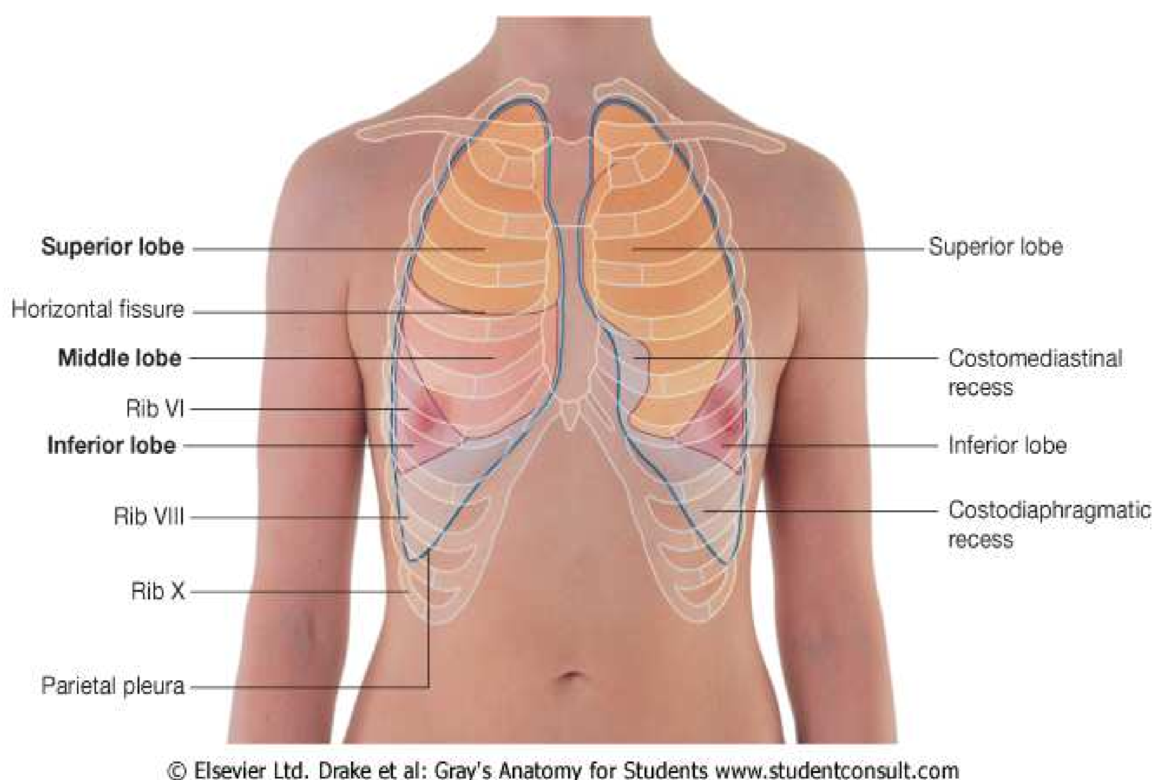

Figure 1.7 Surface anatomy of the right and left lungs, parietal pleurae are indicated by the blue line (Drake *et al.* 2005).

The oblique fissure begins at the level of the spinous process of T2 vertebra posteriorly and runs anteriorly to the level of the 6th costal cartilage. The right horizontal fissure begins at the oblique fissure at the level of the 4th rib and costal cartilage (Moore *et al.* 2009). The lower limit of the lungs are found 2

costal spaces above the line of pleural reflection as illustrated in Figure 1.7, 6th rib in the midclavicular line, the 8th rib in the midaxillary line and the 10th rib posteriorly.

1.5.4 The lungs and *Mycobacterium tuberculosis*

MTB occurs in the anterior segments of the lungs, but commonly in the apical segments of the upper (superior) and lower (inferior) lobes as well as the hilus region of the lung (Brink and de Kock 1973). MTB is a strictly aerobic bacterium and therefore multiplies better in pulmonary tissue, in particular at the apex, where oxygen concentration is higher.

2. MATERIALS AND METHODS

Closed needle biopsies were performed on 20 cadavers to obtain lung tissue from the apical and hilar areas. With the use of a pro-cut biopsy needle a sample of lung tissue was obtained by inserting the needle through the 3rd intercostal (hilar sample) and the costoclavicular space (apical sample). The first sample was taken before embalming. The second sample 3 weeks after embalming. Tissue was then retrieved and deposited into a sterile specimen container and transported to the pathology laboratory (Pathcare: Drs Dietrich, Voigt, Mia, and partners). MTB samples were then tested using the ZN stain, Mycobacterium growth indicator tube (MGIT) culture method and identification using PCR techniques. The study took place over a 3-year period from 2009 to 2011.

2.1 STUDY DESIGN

The study design was a before-after study.

2.2 STUDY PARTICIPANTS

The human cadavers were accompanied by their death certificates indicating the cause of death. Only cadavers whose certificates indicated that the cause of death was TB were selected to be included in the study. Twenty cadavers were tested during this time for MTB. Two of the cadavers were claimed before the second sample could be taken. The first 5 cadavers were used to perform a pilot study and were included in the sample size of the main study.

2.3 ETHICAL ASPECTS

The protocol was submitted to the Ethics committee (Faculty of Health Sciences) of the UFS for approval before commencement of the study (ECUFS nr. 05/08). Consent was obtained from Dr. S. van Zyl, Head of the department of Basic Medical Sciences, in order to do research on the cadavers.

The cadavers' identities were not disclosed but only reference numbers were used to distinguish between them. This ensured that they stayed entirely anonymous. The results of the test will remain confidential and only a limited amount of people handled the samples as well as the results. If any of the information or results from this study were to be published or used at a seminar or congress, no specific information regarding the cadavers or their identity will be revealed. The information gathered during this study is the property of the department of Basic Medical Sciences.

2.4 PILOT STUDY

A pilot study was done on 5 cadavers to determine the feasibility of the study. The 5 cadavers were included in the main study. The method of the pilot study did not differ from the main study and the cadavers could thus be included in the main study. The samples were taken, like in the main study, before embalming and 3 weeks after embalming but a third sample was taken 6 months after embalming. Only the first 2 cadavers of the pilot study were tested after 6 months. Samples were taken just before the cadavers were released to the medical students for dissection purposes.

Two different samples were taken from the hilus area of the lung at different depths and 2 different samples at different depths in the apical area. It was recorded as Hilus 1 and 2 and Apex 1 and 2. In the pilot study the needle was inserted until soft lung tissue could be felt in the apex and hilus, this was Hilus 1 and Apex 1. This depth was noted and another sample was taken 1 cm deeper and marked Hilus 2 and Apex 2. The follow up samples (3 weeks after embalming) were taken from exactly the same locations as the initial samples to prevent inaccurate and not comparable results.

2.5 CLOSED NEEDLE BIOPSY

Closed needle biopsies using a pro-cut biopsy needle (Figure 2.1) were performed on the cadavers to obtain lung tissue. Two samples, hilar and apical samples, were taken from each cadaver during different stages of the routine embalming process:

- The **first sample** was taken before embalming.
- The **second sample** 3 weeks after embalming to determine whether the embalming was sufficient to eliminate the MTB.

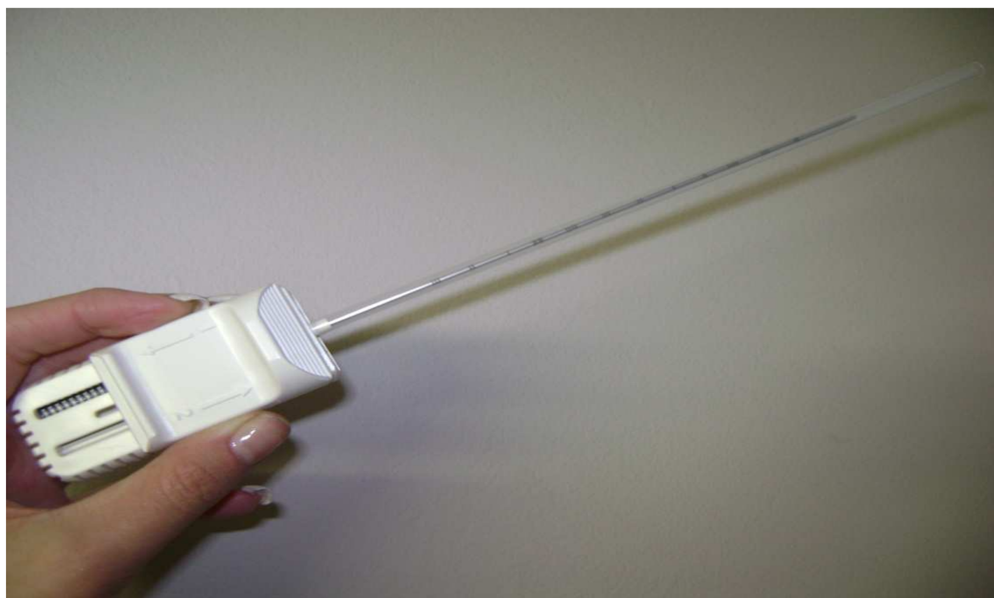

Figure 2.1 Pro-cut biopsy needle (Personal photograph by author: 07 June 2011).

The procedure was performed using standard precautions. Protective gloves and a mask, specifically for the prevention MDR-TB, were worn. The cadaver was placed in a supine position. A pro-cut biopsy needle was used to obtain a sample of lung tissue by inserting the needle through the third intercostal and the supraclavicular spaces respectively.

The **hilar sample** was obtained by inserting the needle in the third intercostal space as seen in Figure 2.2. The needle was inserted 1 cm lateral from the body of the sternum.

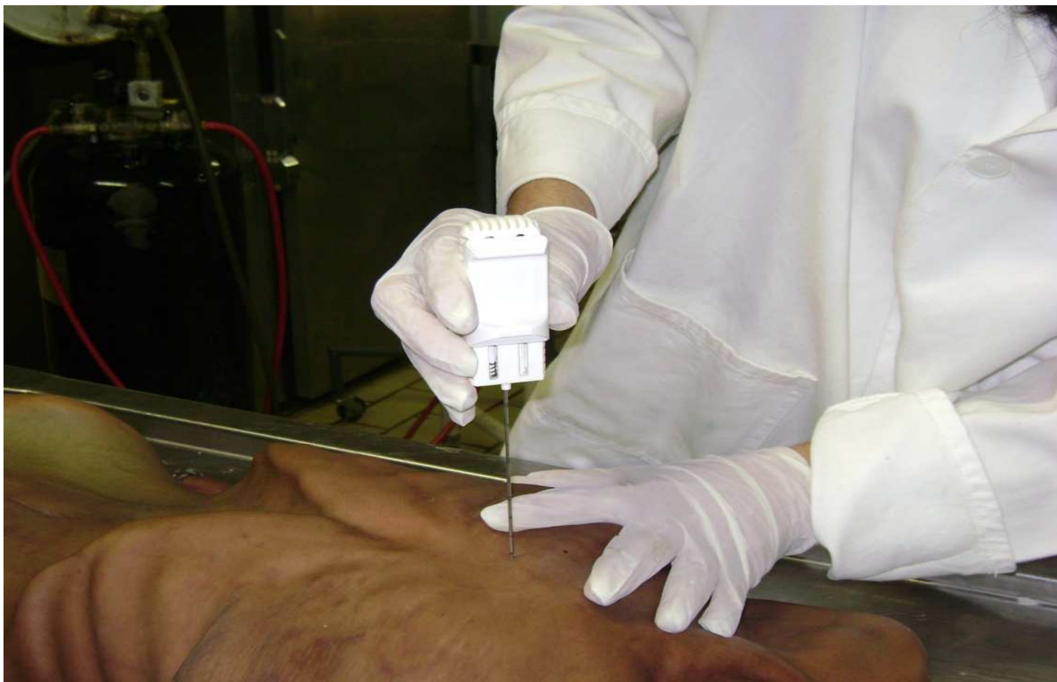

Figure 2.2 Obtaining hilar samples from the 3rd intercostal space (Personal photograph by author: 07 June 2011).

The needle was inserted directly inferiorly, making a 90-degree angle with the table. Figure 2.3 illustrates the direction of the needle. The needle depth of each sample was recorded for each cadaver to ensure consistency. The sample that was taken 3 weeks after embalming was inserted in the same puncture opening used to get the first sample and at exactly the same depth.

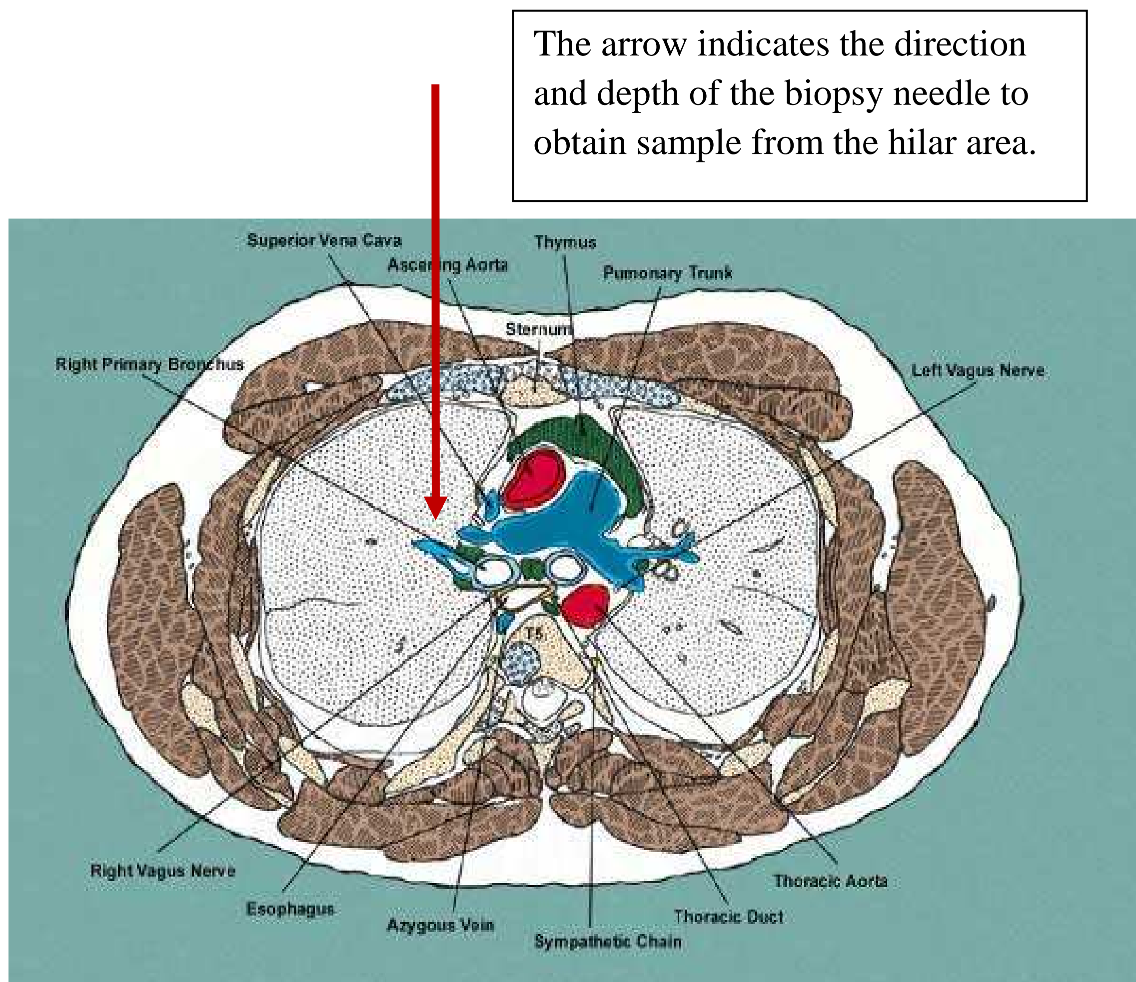

Figure 2.3 Cross section of the thoracic cavity at the level of the third costal cartilage (T5) illustrating the direction and depth of the needle (Author unknown 2006).

The **apical sample** was obtained by inserting a needle through the supraclavicular space as seen in Figure 2.4. The entrance of the needle was superior to the medial third of the clavicle. The needle was inserted posterior to the clavicle and the first rib as illustrated by the arrow in Figure 2.5 through the superior thoracic inlet. Cadaver lungs are shrunken and deflated; the needle was inserted until the soft lung tissue could be felt of the apical segment of the superior lobe. As with the hilar sample, the needle depth and direction was recorded.

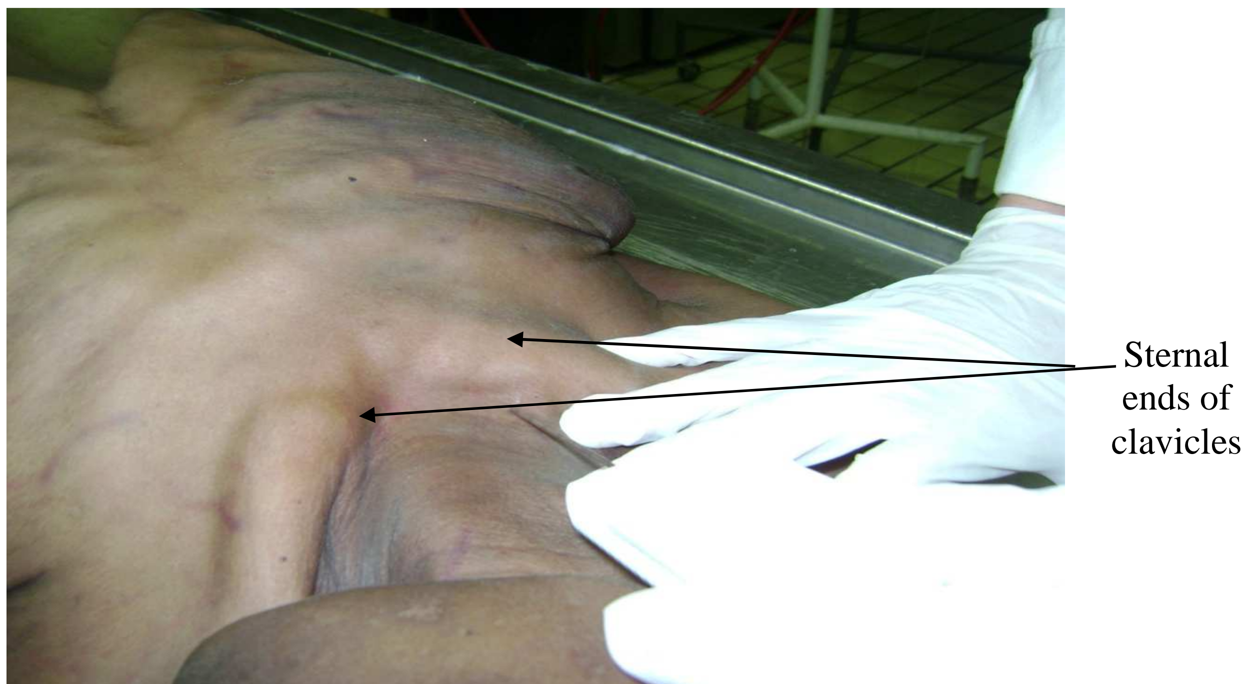

Figure 2.4 Obtaining tissue sample from apex of lung through the supraclavicular space, view from superior looking into the right thoracic inlet (Personal photograph by author: 07 June 2011).

The arrow indicates the direction and depth of the biopsy needle to obtain sample from the apical area.

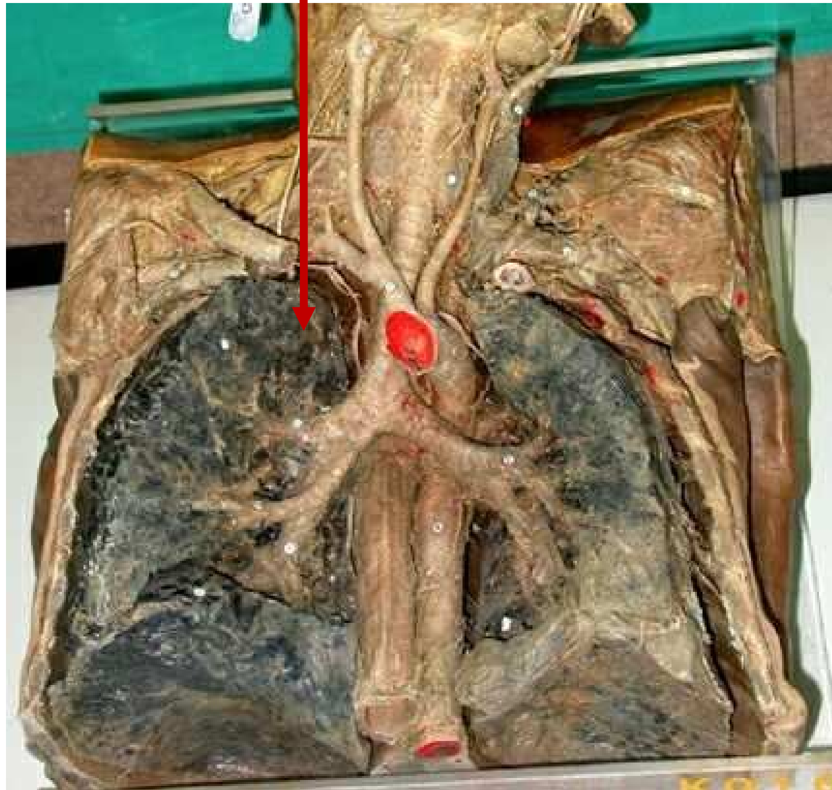

Figure 2.5 Anterior view of a plastinated specimen of the thorax. The anterior thoracic wall has been removed and the arrow indicates the direction and depth of needle to obtain the apical sample (Personal photograph by author: 02 June 2011).

The hilar and apical samples were taken either on the left side or on the right side. When the cadavers arrived at the department, rigor mortis had already set in, and their necks were usually either to the left or right side. The

supraclavicular space that was exposed was used to obtain the apical as well as the hilar sample.

Tissue was then retrieved from the instrument and deposited into a sterile specimen container, with saline as transport medium. The specimen containers were provided by the laboratory (Pathcare: Drs Dietrich, Voigt, Mia, and partners) with a specific volume of normal saline. The container was then transported to the pathology laboratory within 4 hours of the specimen being obtained. The container (Figure 2.6) was pre-labelled, and pre-printed request/information forms (see Appendix A) accompanied the specimen to the laboratory. The cadaver's identity was not disclosed, only a reference number was used for identification purposes. Leakage from the specimen containers was prevented through the usage of parafilm sealant.

Figure 2.6 Pre-labelled container with saline as transport medium (Personal photograph by author: 07 June 2011).

2.6 THE EMBALMING PROCESS

A standardised embalming formula (see Table 1.2) is used at the department of Basic Medical Sciences at the UFS for the embalming of the cadavers as discussed previously (Pretorius and Brune 1992). This formula is injected by the workshop technicians (embalmers) into the radial artery of the cadaver under pressure (1 – 1.5 kPa), until the cadaver is completely filled (Pretorius 1995) as seen in Figure 2.7. More or less 30 L of the embalming fluid is used for a 70kg cadaver. The embalming fluid is injected into the radial artery, the blood flows into the veins, and the empty arteries are injected with Latex 4 - 6 weeks after embalming.

The cadaver is stored in a suitable polyethylene bag (Figure 2.8) after the embalming process. The cadavers are stored in the mortuary of the department of Basic Medical Sciences each on a stainless steel body tray and stored in a body cabinet (Figure 2.8) at room temperature between 16 - 18°C. The cadavers are then stored for a minimum of 6 months until they are taken to the dissection hall for dissection purposes.

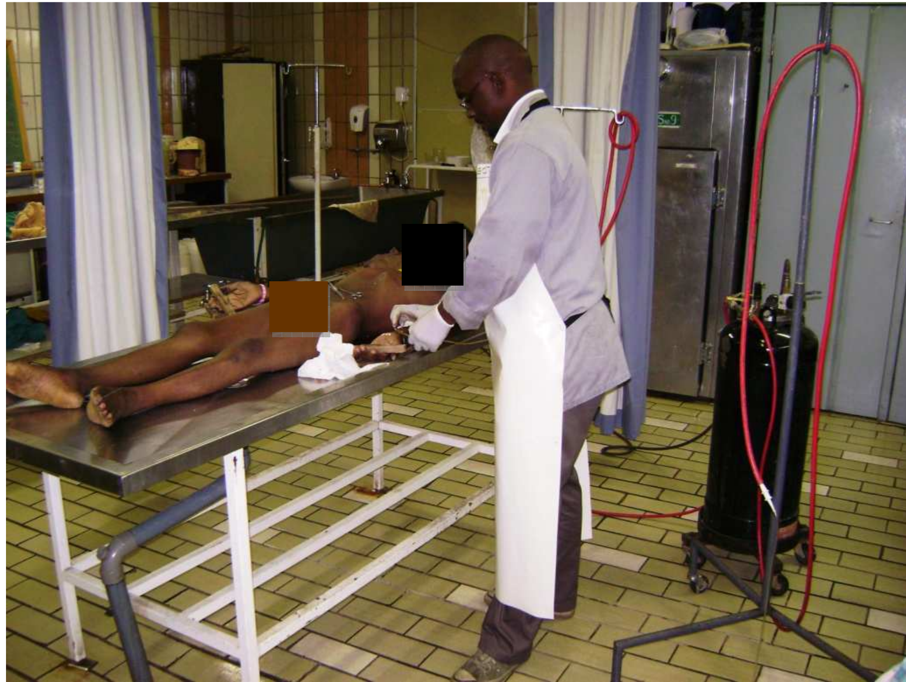

Figure 2.7 Workshop technician embalming a cadaver through the radial artery (Personal photograph by author: 21 May 2012).

Figure 2.8 Body cabinets in the mortuary where the cadavers are kept for 6 months (Personal photograph by author: 21 May 2012).

2.7 DIAGNOSTIC LABORATORY METHODOLGIES

Taken into consideration the sensitivity, specificity, and technical shortcomings of the methods available (as discussed in 1.4.5), the following diagnostic laboratory methodologies were used by Pathcare Laboratory (Drs Dietrich, Voigt, Mia, and partners), to test the samples for MTB:

- **Ziehl-Neelsen staining** and **direct microscopy** from aspirates (lungs in the case of PTB or from granulomatous lesions).
- **MGIT culture method.**
- Identification using **PCR techniques,** if tested positive with the MGIT culture method.

2.7.1 Standard operating procedures for Ziehl-Neelsen stain

A smear from the yellow purulent portion was made in a bio-safety cabinet and allowed to dry. A smear should be 2 cm by 3 cm in size and neither to thin or too thick. The slide was then passed through a flame 3 - 4 times or by placing the smear on a hotplate (heat fixation) and covered with concentrated carbol fuchsin. The smear was allowed to stain for 5 min while heat was applied 3 times at 1-minute intervals. The slide was washed with distilled

water and then covered with 3% acid alcohol. The red colour of the smear will change to yellowish brown. After about 1 min in the acid, the slide was washed with distilled water. The film will appear faintly pink after decolourisation. The slide was then washed with distilled water and counterstained with methylene blue or malachite green for 15 – 20 s. The slide was then washed (distilled water), blotted and allowed to dry.

The smears were examined with a 100x oil immersion objective and a 10x eyepiece (1000x total magnification for ZN smears). A series of systematic sweeps over the length of the smear was made and examined. Each field should be examined thoroughly. A useful field is one in which cellular bronchial elements (leucocytes, mucous fibres and ciliated cells) are observed. The fields with no such cells should not be included in the reading. At least 100 fields were examined before reporting the smear negative. After microscopic examination, the result was reported.

The immersion lens should be cleaned between examinations of different clinical specimens to prevent contamination. Smears are graded according to the WHO guidelines (Table 2.1).

Acid-fast bacilli stain bright red, while the cells and other organisms stain blue or green according to the counterstain used. Tubercle bacilli look like fine red rods, slightly curved and more or less granular. It can appear isolated, or in pairs or groups, and stand out clearly against the blue background. Individual bacilli may show heavily stained areas, referred to as “beads” and areas of alternating stain may produce a banded appearance. *Mycobacterium* other than tuberculosis (MOTT) appears pleomorphic (capable of assuming different shapes). Ranging from long rods to coccoid forms, with more uniform distribution of staining properties.

Table 2.1 Reporting results for Ziehl-Neelsen staining according to the WHO guidelines (2006).

Number of acid fast bacillus	**Fields**	**Report**
0	Per 100 fields	Negative
1 - 9	Per 100 fields	Scanty (Low Positive)
10 - 99	Per 100 fields	Positive
1 - 10	Per field	Positive
>10	Per field	Positive

2.7.2 Standard operating procedure for *Mycobacterium* growth indicator tube culture method

The tissue was homogenized in a tissue grinder with a small quantity of sterile saline or water (2 - 4 ml) in a bio-safety cabinet. The specimen was then decontaminated by using 4% NaOH with N-acetyl L-cysteine, mixed by gentle inversion, placed in a vortex and allowed to stand for 15 min. The specimen was spun at 2500 – 3000g for 20 min using a refrigerated centrifuge, and the supernatant poured off. Phosphate buffer (5 ml) was added to the deposit, placed in vortex and spun for 20 min. After resuspension of the sediment with the phosphate buffer, 0.5 ml of the concentrate was inoculated into the MGIT 960 tube. Contamination was further reduced by adding 0.8 ml PANTA (**P**olymyxin B, **A**mphotericin B, **N**alidixic Acid, **T**rimethoprim, and **A**zlocillin) prior to the inoculation. Once inoculated, the MGIT tubes were placed in the instrument within 12 hours of inoculation. Smears (ZN) were prepared from all processed specimens before inoculation into the medium (as described in 2.7.1). The temperature of the instrument was maintained at 37°C; the instrument read all the tubes every hour for a maximum period of 6 weeks.

The MGIT medium was prepared per 1000 ml of purified water by adding modified 5.9 gm Middlebrook broth base and 1.25 g Casein pepton. The MGIT growth supplement contains: Bovine albumin, dextrose, catalase, oleic acid and polyoxyethylene stearate (Siddiqi *et al.* 1988), and was added in a bio-safety cabinet to avoid contamination of the medium.

When growth is detected, the instrument will illuminate the positive light and an alarm will sound. The relevant positive tube indicated by a red light was removed and observed visually. Mycobacterial growth will appear granular and not very turbid (clear). In a liquid broth medium, MTB will appear granular. Once a MGIT tube is positive by fluorescence or by visual observation, a smear can be prepared to help with the tentative differentiation of MTB from other mycobacteria.

2.7.3 Standard operating procedure for Tuberculosis polymerase chain reaction identification

Two different procedures for the identification from a liquid culture and a solid culture were used. A screw top tube (2 ml) was labelled and a vertical line was made with a marker on the smooth side of the tube, to ensure that the

technologist knows where the pellet will settle. The tubes were then placed in an Eppendorf centrifuge, the labelled marked facing to the front.

For a liquid culture, the MGIT tube was placed in a vortex and the liquid taken out by using a pastette (Pasteur pipette). The stem of the pipette was filled until just below the bulb and the liquid transferred to the already labelled screw top tube and centrifuged at 14k rpm for 5 min. The supernatant was taken off with a pastette without disturbing the pellet. Water (200 µl) was added, placed in a vortex to resuspend the pellet, and incubated at 100°C for 30 min in a heating block. The specimen was left to cool for 5 min and centrifuged at 14k rpm for 5 min. 100 µl was transferred into a Magna pure LC sample cartridge and covered with optical adhesive cover. DNA was extracted from the secondary sample using an automated DNA extraction system. The region of interest was amplified using the sequence specific primers to differentiate between MTB complex and MOTT.

For a solid culture, a sterile plastic disposable loop was used to pick up as many colonies as possible, and the loop was transferred to an already labelled screw top tube containing 200 µl of water. The loop was swirled until clean and incubated at 100°C for 30 min in a heating block. Left for 5 min to cool

and centrifuged at 14k rpm for 5 min. 100 μl was transferred into a Magna pure LC sample cartridge and cover with optical adhesive cover. DNA was extracted from the secondary sample using an automated DNA extraction system. Region of interest was amplified using the sequence specific primers to differentiate between MTB complex and MOTT.

A copy of the sample form with the results was returned to the TB lab where a technologist will enter the results on the TB sheet and then into a computer. Results were either TB PCR positive, which is MTB complex positive or TB PCR negative, which is MOTT positive. All positive specimens were kept for 3 months in the fridge. The standard operating procedures was supplied by Pathcare (Drs Dietrich, Voigt, Mia, and partners).

2.8 STATYSTICAL ANALYSIS OF RESULTS

Microsoft Excel was used to perform the descriptive statistical analysis to decisive frequencies (number of positive and negative for the tests) and percentages (percentage of positive and negative for the tests).

3. RESULTS

3.1 PILOT STUDY

From the outcome of the pilot study it is clear that between the first (before embalming) and second (3 weeks after embalming) sample, using the **first line screening technique or the ZN stain**, the same percentage of samples returned a positive and negative result with the exception of Apex 2 as seen in Table 3.1. In the first sample, 40% of the Apex 2 samples tested positive with the first line screening method, while the second sample 0% of the samples tested positive with the first line screening method (Table 3.1).

The pilot study also demonstrates that with the first sampling round, for **TB growth,** 3 of the 4 sampling areas tested positive for TB (Table 3.2). However, with the second sampling round none of the 4 sample areas returned a positive result as seen in Table 3.2. The first 2 cadavers tested in the study, were also tested 6 months after embalming to ensure that before the cadavers are released for dissection purposes, no viable MTB is present. After 6 months, the first line screening and the TB growth tested negative for both these samples.

Table 3.1 Percentage of samples with a positive and negative TB result, with the first line screening technique in the pilot study *(n=5)*.

	1st Sample (Before embalming)		**2nd Sample (3 weeks after embalming)**	
n=5	*Negative*	*Positive*	*Negative*	*Positive*
Apex 1	80	20	80	20
Apex 2	60	40	100	0
Hilus 1	80	20	80	20
Hilus 2	100	0	100	0

Table 3.2 Percentage of samples with a positive and negative growth in the pilot study (*n=5*).

	1st Sample (Before embalming)		**2nd Sample (3 weeks after embalming)**	
n=5	*Negative*	*Positive*	*Negative*	*Positive*
Apex 1	40	60	100	0
Apex 2	60	40	100	0
Hilus 1	60	40	100	0
Hilus 2	100	0	100	0

3.2 MAIN STUDY

We conducted the main study and examined all the cadavers with the first line screening technique and for TB growth. The results of the pilot study (Table 3.2) indicated that one sample should be taken from in the apex (Apex 1) and one sample in the hilus (Hilus 1). The Apex 1 and Hilus 1 samples of the 5 cadavers of the pilot study were included as control or reference samples

The results of **the first line screening technique** are shown in Figure 3.1 and Figure 3.2. The results from the first sampling round (before embalming) are shown in Figure 3.1 while the second sampling round (3 weeks after embalming) is shown in Figure 3.2. The first round sampling results of the first line screening showed that 15% of the samples tested positive for the presence of TB in the Apex area. In the hilus area, 25% of the samples yielded a positive result as seen in Figure 3.1. The first line screening technique, both the first and second sampling rounds yielded a larger percentage of TB positive samples in the hilus area of the lung.

It is important to note that during the study families of the deceased claimed 2 cadavers before the second sample could be taken. This is the reason for *n=20* for the first sample and *n=18* in the second sample in Figure 3.1 and 3.2

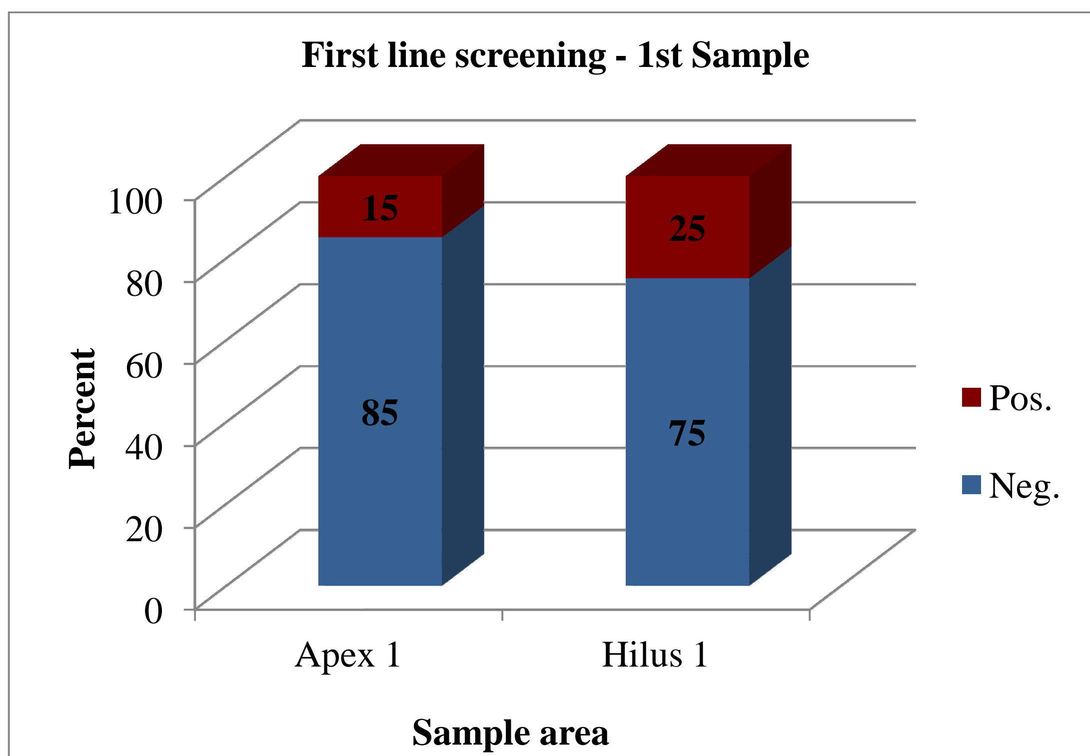

Figure 3.1 Graph depicting the percentage of first samples with a positive and negative TB result, with the first line screening technique (*n=20*).

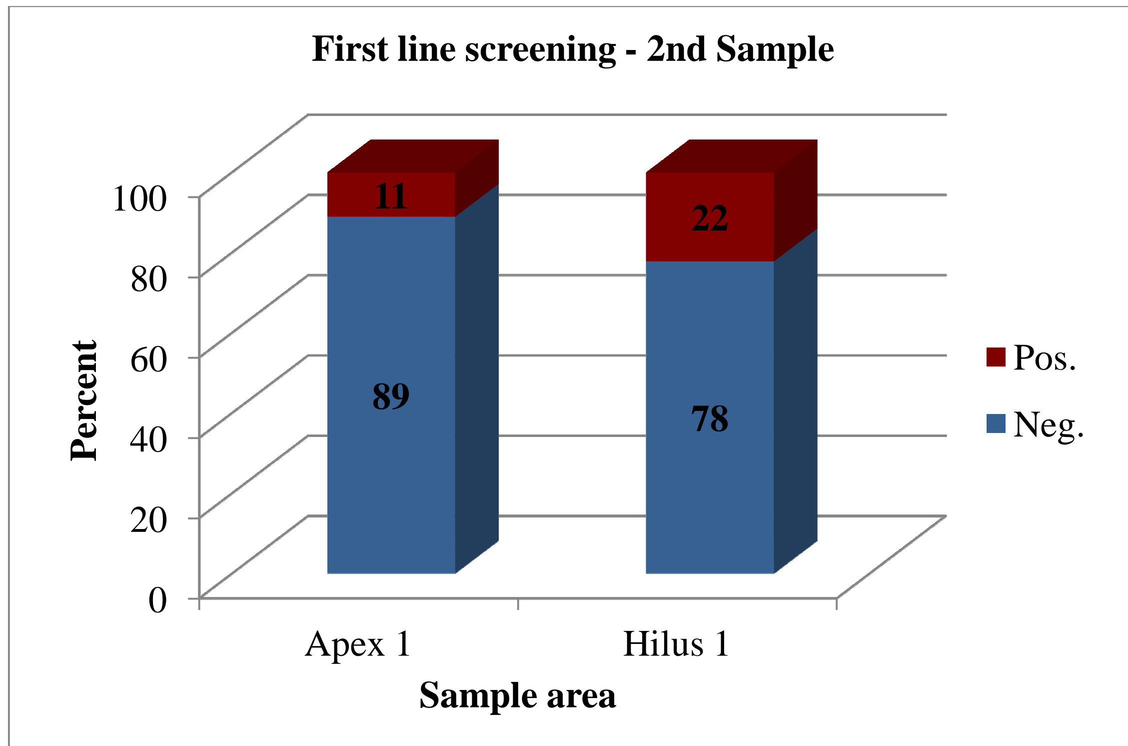

Figure 3.2 Graph depicting the percentage of second samples with a positive and negative TB result, with the first line screening technique (*n=18*).

The first round sampling results of the growth in the main study showed that 50% of the samples tested positive for the presence of TB in the Apex area. In the hilus area, 40% of the samples yielded a positive result as seen in Figure 3.3. With the second sampling round, none of the samples in both the apex and hilus areas tested positive for TB growth (Figure 3.4). When looking at **TB growth**, the first sampling round yielded a 50% and 40% positive TB growth in the apex and hilus areas respectively, yielding a larger percentage of TB positive samples in the apical area of the lung.

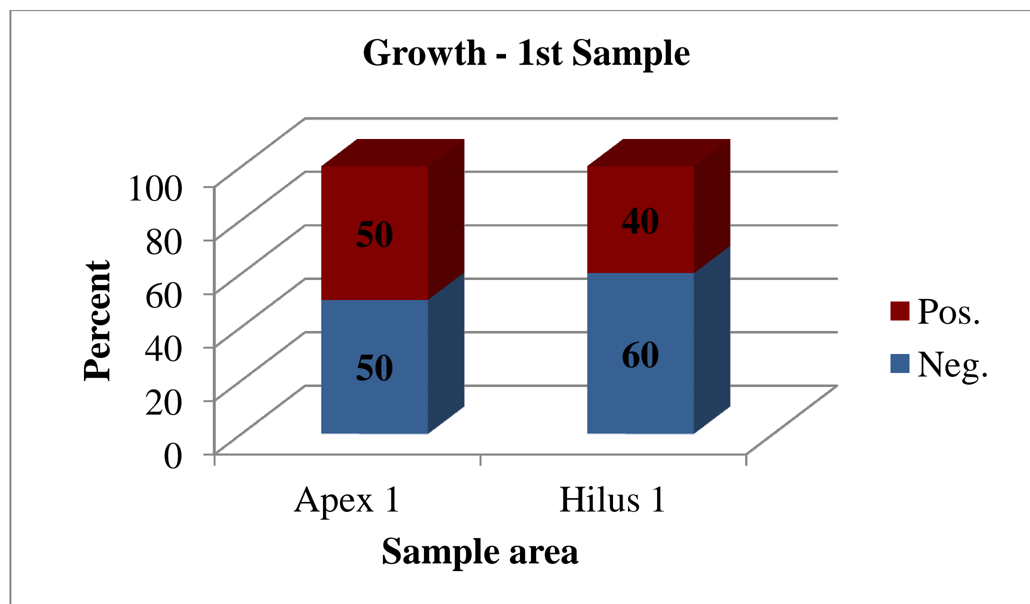

Figure 3.3 Graph depicting the percentage of first samples with a positive and negative TB result, with TB growth (*n=20*).

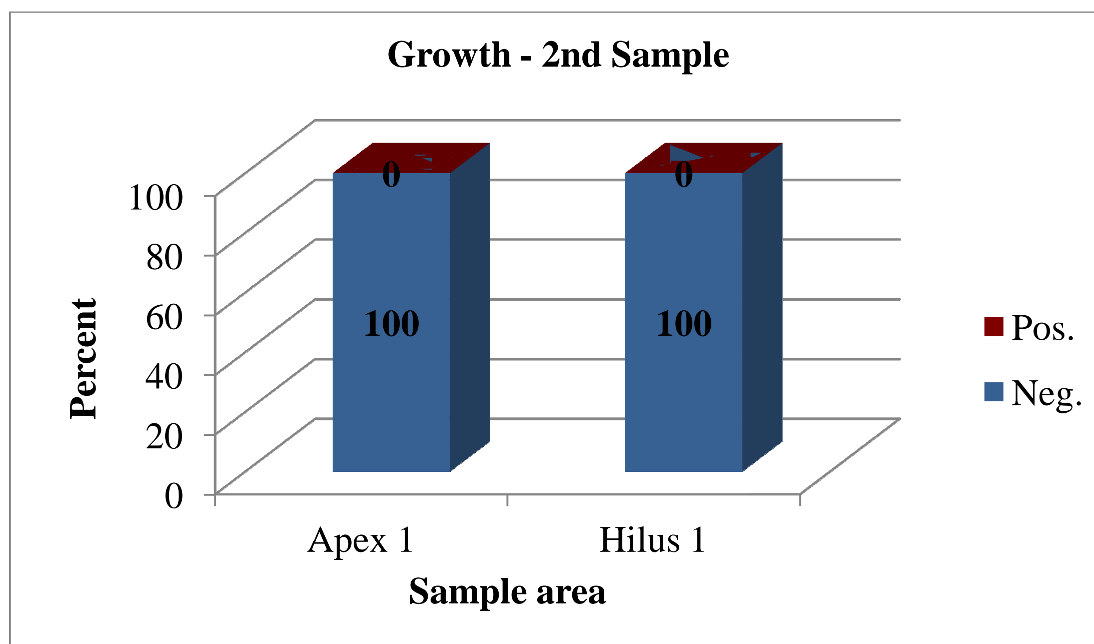

Figure 3.4 Graph depicting the percentage of second samples with a positive and negative TB result, with TB growth (*n=18*).

The results are summarized in **Table 3.3** on the next page. Thirteen of the 20 cadavers (Table 3.3) had a viable strain of MTB before embalming either in the apical or hilus area as tested with TB growth. Fourteen of the 20 cadavers had died of pulmonary TB (PTB), 4 of Miliary TB, 1 of bronchogenic TB spread and 1 of tuberculous meningitis, according to their death certificates. Table 3.3 also indicates the days between date of death and embalming. It is of special interest to mention that cadaver 56-11 still had viable MTB 36 days after death.

Table 3.3 Cause of death according to death certificate and the number of days the MTB is still viable in the cadavers.

Reference	Cause of death (according to death certificate)	Number of days between date of death and embalming	Viable MTB before embalming (TB growth)	Viable MTB 3 weeks after embalming (TB growth)
22-09	PTB*	25	Yes	No
24-09	Miliary TB	29	Yes	No
34-09	PTB*	17	No	No
38-09	Bronchogenic TB	18	Yes	No
40-09	PTB*	22	Yes	No
42-09	PTB*	23	Yes	No
44-09	Miliary TB	23	Yes	No
55-09	PTB*	19	Yes	No
56-09	PTB*	29	No	No
75-09	PTB*	25	Yes	No
78-09	Miliary TB	19	Yes	Claimed
80-09	Miliary TB	27	Yes	No
10-10	PTB*	11	No	Claimed
13-10	PTB*	12	No	No
14-10	PTB*	14	Yes	No
25-10	PTB*	40	No	No
29-10	PTB*	29	Yes	No
38-11	*TB meningitis*	21	No	No
53-11	PTB*	10	No	No
56-11	PTB*	36	Yes	No

***PTB = Pulmonary TB**

Table 3.4 shows the number of male and female cadavers tested for the study. Samples were taken from 16 male and 4 female cadavers. From the 16 male cadavers, 11 samples were taken from the left lung and 5 from the right lung. From the 4 female cadavers, 2 samples were taken from the left lung and 2 samples from the right lung. The positive and negative indicate if the sample tested was TB growth positive or negative thus containing viable MTB (Table 3.4) before embalming.

Table 3.4 Comparison between the number of right and left lungs and male and female cadavers tested.

	Left lung			**Right lung**			**Total**
	Total	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	
Male	**11**	**8**	**3**	**5**	**2**	**3**	**16**
Female	**2**	**2**	**0**	**2**	**1**	**1**	**4**
Total	**13**	**10**	**3**	**7**	**3**	**4**	***n = 20***

In Table 3.4 for the male cadavers tested, 10 of the specimens out of the 16 (62.5%) tested contained viable MTB in either the apical or hilus area. Three of the 4 female cadavers tested contained viable strains of MTB (75%) in either the apical or the hilus area. Thirteen samples were taken from the left lung and 10 of those samples tested positive for MTB (77%). Seven samples

were taken from the right lung and 3 of those samples tested positive for MTB (43%).

4. DISCUSSION

4.1 PILOT STUDY

Only 2 of the cadavers (in pilot study included in main study) were tested after 6 months. The reason, after six months the cadavers are released to the medical students for dissection purposes. The reason why only 2 cadavers (22-09 and 24-09 in Table 3.3) were tested is that none of the samples tested contained viable MTB 3 weeks after embalming and thus it was not necessary to test them again after 6 months. The financial implication of the test also played a role in only testing the 2 cadavers. The 2 cadavers tested after 6 months tested negative with the ZN stain as well as no growth was detected after 6 weeks. This indicates that the medical students and embalmers can handle the cadavers after 6 months of embalming with minimum safety precautions concerning TB.

The results from the pilot showed that three weeks after embalming none of the tested cadaver’s lungs contained viable MTB. Although some of the samples had positive results after 3 weeks with the ZN staining, these samples

contained non-viable MTB as no growth was detected after 6 weeks. ZN staining only tests the presence of MTB and not the viability.

4.2 MAIN STUDY

From the results, it is clear that the embalming fluid is effective in rendering the bacilli non-infectious because no growth was detected 3 weeks after embalming with the MGIT culture method. Before embalming, 40% and 50% of the apical and hilus samples tested positive with the TB growth respectively and no growth was detected 3 weeks after embalming. The culture method (MGIT) can differentiate between dead and live bacilli. This indicates that the embalming fluid effectively rendered the bacilli non-viable.

When using the ZN stain, both the first and second sampling rounds yielded a larger percentage of TB positive samples in the hilus area of the lung. Although positive samples were observed with the ZN stain 3 weeks after embalming, this technique only tests for the presence of acid-fast bacilli, not the viability of the bacilli. The sensitivity is directly proportional to the number of acid-fast bacilli present (Mackie *et al.* 1978), and can be a reason for the higher concentration of bacilli present in the hilus area.

TB growth in the first sampling round yielded a 50% and 40% positivity in the apex and hilus areas respectively, yielding a larger percentage of TB positive

samples in the apical area of the lung. This indicates that more viable MTB is found in the apical area of the superior lobe than in the hilus region. Experiments conducted with macrophages demonstrated that a high oxygen pressure promote growth of the MTB bacilli (Cardona and Ruiz-Manzano 2004). Thus the apical area with higher concentration of oxygen is an optimal environment for the aerobic MTB bacillus (Martin, Cline and Marshall 1953). Another reason for the higher concentration of MTB in the apical area may be the impaired lymphatic drainage in the apical area according to Leung (1999).

Thirteen of the 20 cadavers (Table 3.3) had a viable strain of MTB before embalming either in the apical or hilus area as tested with TB growth. Although all 20 cadavers' death certificates indicated that the cause of death was PTB, miliary TB or tuberculous meningitis, 7 of the samples still tested negative for viable MTB as no growth was detected on the MGIT culture 6 weeks after embalming. A reason for this might be that they were on TB treatment before death, which rendered the bacilli non-viable, thus resulting in the negative results for MTB.

As seen in the results (Table 3.3), 14 of the 20 cadavers had died of pulmonary TB (PTB), 4 of miliary TB, 1 of bronchogenic TB and 1 of

tuberculous meningitis according to the death certificates. Miliary TB, as discussed in Chapter 1, is the spread of MTB to the rest of the body. The name comes from the pattern that one can see on a chest X-ray, the tubercle looks like tiny spots on the lung, resembling a millet seed. Miliary TB can occur after the primary infection or during reactivation of a latent infection in about 15% of TB patients (Bloom and Murray 1992).

Bronchogenic is defined as originating in the bronchi, thus bronchogenic TB spread is the spread of TB through the bronchi. Endobronchial TB is MTB infection in the tracheobronchial tree. Endobronchial TB may be a result of the direct implantation of the MTB bacilli in the bronchus, another cause may be from rupture of caseous material from lymph nodes (Ip, So, Lam and Mok 1986). Lymphatic spread along the bronchial tree and haematogenous spread is less common (Ip *et al.* 1986). It clinically resembles bronchogenic carcinoma.

Tuberculous meningitis is a form of disseminated TB and the most devastating type of meningitis according to Haldar, Sharma, Gupta, and Tyagi (2009). If left untreated, the case fatality rate is 100%. In tuberculous meningitis, the bacilli travel in the blood to the meninges where they form small subpial foci,

called Rich foci (Thwaites, Chau, Mai, Drobniewski, McAdam, and Farrar 2000). If these foci rupture, the bacilli are discharged into the subarachnoid space.

Sixteen male cadavers and 4 female cadavers were included in the sample. Eighty percent of the cadavers tested were male, and died of PTB or a TB related infection. Adult men are diagnosed more often with TB, according to the WHO (2002). Diwan and Thorson (1999) also stated that more males than females are infected with TB. They attribute this difference to immune response differences between the two genders. Difference in roles in society may also influence the risk of exposure to MTB (WHO 2002).

4.2.1 Resistance of *Mycobacterium* tuberculosis

According to previous studies, after death, MTB can remain infectious for about 8 days in unembalmed lung tissue (Nolte 2005), up to eight days at 20 - 25°C and up to 14 days if stored between 2 - 4° C in sputum (Collins and Grange 1999). From the results in this study, it is clear that MTB can survive in dead bodies with significant post-mortem intervals. The MTB remained viable for 14 – 36 days before embalming took place as seen in Table 3.3. As

mentioned previously, after death, the dead bodies are stored in the mortuary. If not claimed, the department of Basic Medical Sciences is notified. These bodies are kept in a fridge at -5 – -9°C.

MTB has very simple growth requirements, multiply slowly (every 20 hours), grows optimally in about 38°C (Devi, Shaila, Ramakrishnan and Gopinathan 1975), and is able to grow in harsh conditions. MTB is also highly resistant to extreme temperatures, as it remained viable for 36 days in cadaver 56-11 (Table 3.3) in cold temperatures ranging between -5 – -9°C. MTB can be preserved by freezing on glass beads at -70°C. A study by Giampaglia, de Brito, Martins, Ueki, Latrilha, de Oliveira, Yamauchi and da Silva Telles (2009) showed that 94% of the strains preserved at –70°C on glass beads could be recovered within 30 days.

The high lipid content of the MTB bacilli is responsible for the resistance against acid and alkali. Thus, the high lipid content also makes it more resistant to disinfection. A previous study by Best, Sattar, Springthorpe, and Kennedy (1990) indicated that phenol (5%) inactivated MTB, and ethanol (70%) was only effective against MTB in suspension. According to Kappel,

Reinartz, Schmid, Holter, and Azar (1996) the antibacterial action of alcohol is increased when alcohol is added to formalin.

A study done by Fukunaga, Murakami, Gondo, Sugi, and Ishihara (2002) found that treatment with 10% formalin lowered the sensitivity of bacilli in ZN staining. Gerston *et al.* (2004) attempted to test the viability of MTB in formalin fixed lungs. The lungs were removed during autopsy and placed in 2 - 3 L of 10% buffered formalin for a minimum of 2 weeks. Twelve of the 138 cases still had viable MTB; 8 of the 12 viable cases were fixed in formalin from 20 - 49 days. Gerston *et al.* (2004) concluded that MTB can survive in lungs fixed in 10% formalin.

4.2.2 Risk of transmission of *Mycobacterium* tuberculosis

The transmission of MTB during embalming is due to the inhalation of infectious aerosols that may escape through the mouth of the cadaver. This risk may be reduced by a proper ventilation system in the mortuary (or embalming room) or placing a cloth over the cadaver's mouth (Morgan 2004). Other measures, such as wearing a mask, can also be taken to reduce the risk of infection. Only high filtration masks are capable of preventing the

inhalation of the bacilli in the infectious aerosol. Ultraviolet germicidal irradiation (UVGI) can also be used as this highly effective method uses ultraviolet light to kill or inactivate the MTB in the air (Riley 1994).

According to Healing, Hoffman and Young (1995), TB is classified as a medium risk to healthy people and stated that, "The embalming of people who have died of tuberculosis is unlikely to be hazardous because there is little aerosol formation but, because air may be expelled from the lungs of a body when it is lifted, it is recommended that the face of the corpse is covered temporarily with a disposable cloth." From the results obtained in this study it is clear that MTB can remain viable after death for up to 36 days and precautions should be taken during embalming. The high pressure used to disperse the embalming fluid through the arteries is responsible for the aerosol formation expelled through the mouth of the cadaver.

This study also fulfilled its initial aim: "To investigate if MTB is still viable in human cadaver lung tissue and especially in granulomatous tissue after embalming has taken place". The results clearly demonstrated that the embalming fluid used at the department of Basic Medical Sciences renders the MTB not viable. Has the following objective been achieved: "The aim was to

test the efficacy of the embalming fluid used at the department of Basic Medical Sciences (University of the Free State) on eliminating *Mycobacterium tuberculosis* in human cadaver lung tissue." The study concluded that the embalming fluid is very efficient concerning eliminating MTB.

5. CONCLUSIONS

5.1 LIMITATIONS

A possible limitation of the study is the sample size that consisted of 20 cadavers. The small sample size was due to financial restraints. The laboratory tests done were very expensive amounting to the amount of R35 000. The results were consistent and no growth was detected 3 weeks after embalming in any of the apical or hilar samples of the 20 cadavers tested, the small sample size may be acceptable for the purpose of this study.

Another limitation could possibly be that 13 samples were obtained from the left lung and only seven samples from the right lung. As describe in Chapter 2, this was because the accessibility of the sample from the apex was restricted if the head was either flexed to the left or to the right side. According to Leung (1999), the right lung is affected more often. A further study might be necessary to confirm this finding.

5.2 VALUE OF STUDY

The results indicate that the embalming fluid used at the department of Basic Medical Sciences (UFS) is therefore effective in rendering the cadaver sterile and non-infectious as far as MTB is concerned. The results clearly demonstrated no growth was detected 3 weeks after embalming. The composition of the ingredients of the embalming fluid needs no modification as it renders the bacilli non-infectious.

The results obtained will be used to ensure that the correct procedures and safety precautions are taken by the personnel of the department to prevent transmission of MTB to the embalmer and the students. The findings will thus be used to launch a standard operating procedure to ensure that the embalmer will be at a minimum risk of contracting MTB from a cadaver during the embalming process.

The results also indicate that minimum safety precautions need to be taken by embalmers and students 3 weeks after embalming as far as MTB is concerned. The six-month period, which is currently the time between embalmment and

dissection by students, also do not need to be changed, as none of the cadavers had viable MTB bacilli after 3 weeks.

5.3 FURTHER RECOMMENDATIONS

These findings however, did not test for the viability of other organisms such as HIV after embalming has taken place on the cadaver. Although the results obtained indicate that the embalming fluid is an effective bactericide concerning MTB, the question arises whether it is an effective virucide. A further study could therefore be conducted to investigate the efficacy of embalming fluid as a virucide.

6. SUMMARY

THE EFFECT OF THE EMBALMING FLUID, USED BY THE DEPARTMENT OF BASIC MEDICAL SCIENCES (UFS), ON THE VIABILITY OF *MYCOBACTERIUM TB* IN HUMAN CADAVER LUNG TISSUE.

Embalming fluid contains substances such as formalin, ethanol, phenol, and other solvents to prevent decomposition temporarily. These agents disinfect, preserve, and/or sanitize. The risk of contracting a disease such as tuberculosis (TB) among persons, who are in close contact with recently deceased people, is high and the risk varies according to occupation. Workers at Anatomy Departments and embalmers are some of those people who are at a greater risk of contracting tuberculosis carried by cadavers.

The question thus arises whether the penetration of formalin and other embalming agents into the tissue infected with *Mycobacterium tuberculosis* (MTB) is sufficient to render the bacilli non-infectious. The aim is to test the efficacy of the embalming fluid used at the department of Basic Medical

Sciences (UFS) on eliminating *Mycobacterium tuberculosis* in human cadaver lung tissue.

The cadavers were accompanied by their death certificates indicating the cause of death. Only cadavers whose death certificate indicated that the cause of death was TB, was selected to be included in the study. Closed needle biopsies were performed on 20 cadavers to obtain lung tissue from the apical and hilar areas. With the use of a pro-cut biopsy needle, a sample of lung tissue was obtained by inserting the needle through the 3rd intercostal (hilar sample) and the supraclavicular space (apical sample). The first sample was taken **before embalming**. The second sample **3 weeks after embalming**. Tissue was then retrieved and deposited into a sterile specimen container, with saline as transport medium, and transported to Pathcare Laboratory (Drs Dietrich, Voigt, Mia, and partners) in Bloemfontein. The following diagnostic tools were used by Pathcare: direct microscopy from aspirates (lungs in the case of Pulmonary TB or from granulomatous lesions), MGIT culture, identification using PCR techniques, if positive.

Before embalming 50% of the apical samples tested positive for MTB and 3 weeks after embalming none tested positive for MTB. Before embalming

40% of the samples taken from the area close to the hilus (perihilar), tested positive for MTB, 3 weeks after embalming none tested positive. The results show that 3 weeks after embalming none of the tested lung samples contained viable MTB. Thirteen of the 20 cadavers tested did have a viable strain of MTB before embalming occurred. It is of special interest to mention that one cadaver still had viable MTB 36 days after death. According to previous studies, after death, MTB can remain infectious for about 8 days in unembalmed lung tissue and up to 14 days if stored between 2 - 4°C. From this result, it is clear that MTB can survive in dead bodies with significant post-mortem intervals.

It is evident from the results that the embalming fluid used at the department of Basic Medical Sciences (UFS) renders the bacilli non-infectious, because no growth was indicated 3 weeks after embalming

Keywords: Embalming fluid, viability, *Mycobacterium tuberculosis*, cadaver, tuberculosis, UFS, formalin, lungs.

OPSOMMING

DIE EFFEK VAN DIE BALSEMVLOEISTOF, GEBRUIK DEUR DIE DEPARTEMENT VAN BASIESE MEDIESE WETENSKAPPE (UV), OP DIE LEWENSVATBAARHEID VAN *MYCOBACTERIUM TB* IN MENSLIKE KADAWER LONGWEEFSEL.

Die balsemvloeistof bevat stowwe soos formalien, etanol, fenol en ander oplosmiddels om ontbinding tydelik te voorkom. Hierdie agente ontsmet, bewaar en/of reinig. Die risiko van die opdoen van 'n siekte soos tuberkulose (TB) is hoër onder persone wat in noue kontak met lyke is en die risiko wissel ook volgens beroep. Werkers by Anatomie Departemente is van die persone wat 'n groter risiko beloop om tuberkulose vanaf kadawers te kry.

Die vraag ontstaan dus of die penetrasie van formalien en ander balseming agente in weefsel wat met *Mycobacterium tuberculosis (*MTB) besmet is, voldoende is om die basille nie-aansteeklik te maak. Die doel is om die doeltreffendheid van die balsemvloeistof wat gebruik word by die Departement van Basiese Mediese Wetenskappe (UV) met betrekking tot *Mycobacterium tuberculosis* te toets.

Die kadawers is vergesel deur hulle doodsertifikate wat die oorsaak van dood aangedui het. Slegs kadawers wie se doodsertifikaat aangedui het dat die oorsaak van dood TB was, is gekies om ingesluit te word in die studie. Geslote naald biopsies is uitgevoer op 20 kadawers om longweefsel te verkry van die apikale en hilus areas. Met die behulp van 'n "pro-cut" biopsie naald is longweefsel deur die 3de interkostale (hilus) en die supraklavikulêre spasie (apikale) verkry. Die eerste monster is geneem voor balseming. Die tweede monster is geneem 3 weke na die balsem proses. Weefsel is onttrek en in 'n steriele monster houer gedeponeer, met soutoplossing as vervoer middel, en vervoer na Pathcare laboratorium (Drs Dietrich, Voigt, Mia, en vennote) in Bloemfontein. Die volgende diagnostiese instrumente is gebruik deur Pathcare: direkte mikroskopie aspirates (longe in die geval van pulmonêre TB of van granulomateuse letsels), MGIT kultuur metode, identifiseer met PKR tegnieke, indien die uitslag positief is.

Voor balseming het 50% van die apikale monsters positief getoets vir MTB en 3 weke na die balseming het geen monster positief getoets vir MTB met die MGIT kultuur metode nie. Voor balseming het 40% van die monsters wat geneem is uit die area naby die hilus, positief getoets vir MTB en 3 weke na die balseming het geen positief getoets. Die resultate toon dat 3 weke na

balseming geeneen van die long monsters lewensvatbare MTB bevat het nie. Dertien van die 20 kadawers wat getoets is, het 'n lewensvatbare stam van MTB bevat voor balseming. Dit is van besondere belang om te noem dat een kadawer nog lewensvatbare MTB 36 dae na dood gehad het. Volgens vorige studies, na dood, kan MTB aansteeklik bly vir ongeveer 8 dae in longweefsel wat nie gebalsem is en tot 14 dae indien gestoor tussen 2 - 4°C. Vanuit hierdie resultate is dit duidelik dat MTB kan oorleef in lyke met beduidende post-mortem-intervalle.

Dit is duidelik uit die resultate dat die balsemvloeistof wat gebruik word by die Departement van Basiese Mediese Wetenskappe (UV) die basille onaansteeklik maak, as gevolg van die feit dat daar geen groei 3 weke na balseming is nie.

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APPENDIX A

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P.O. Box 4266 Bloemfontein 9300
Tel: +27 (0) 51 4014600 Fax / Faks: +27 (0) 51 4014685
Practice No. 5200539

Client name :	Ms Correia	Mnemonic:	CP CORJB
		Copy doctor:	Ms Correia Dept Anatomy Medical faculty
Account to	Dr SS Weyers , Pathcare, Bloemfontein		
Corpse number:		SEX: M / F	LAB NO:
Sampling date (DD / MM / YYYY)	2 0		
Study reference number			

LABORATORY TESTS	VISIT	
Sample type		
Lung biopsy: R ☐ L ☐		
☐ Hilus	**Base sample**	☐
☐ Apikaal		
Micro analysis: **M TBR** **M TAFB** (Radiometric TB culture + Ziehl Nielsen)	**3 weeks**	☐
	6 month	☐
Please tick ✓ appropriate boxes		

SPECIAL INSTRUCTIONS	TUBES REQUIRED
Please send all samples with copy of requisition to TB Lab N1 city, attention Fadheela Patel **Please sent results to Dr Fanie Weyers, at Haematology**	Tissue samples Received:

Rec by	Initial; Date;	Logged by	Initial Date	Checked by Name:	Date:

BCT/ Requisition form:

Version	1	Date	09.12/2009

Date printed:09/12/09