CHAPTER ONE: LITERATURE REVIEW

1.1 Historical Background

Tuberculosis (TB) is one of the oldest infectious diseases of human kind and still remains one of the biggest killers. The modern members of TB including *Mycobacterium tuberculosis, Mycobacterium cannetti* as well as *Mycobacterium bovis* originated from one progenitor about 15,000 to 35,000 years ago (Gutierrez *et al*., 2005). East Africa has been considered the place were TB started but there is lack of concrete evidence to support this theory (Daniel, 2006). TB may affect bones causing skeletal deformities. These has been found in Egypt and other parts of the world such as Denmark, Italy and Middle East countries which proved that TB is a disease that affect people world-wide (Smith, 2003).

In Greece, TB was described by Hippocrates as Phthisis or consumption, a condition characterized by wasting away with chest pain and coughing with blood in the sputum. The causative organism was not identified until 1882 by Hermann Heinrich Robert Koch who described it as bacillium. The description of the tubercle bacilli as a causative agent was also the beginning of Koch's postulate which still forms the basis for the demonstration of infectious etiology (Daniel, 2005).

Treatment of TB has always been a challenge. A Greek physician Clarissimus Galen recommended fresh air, milk and sea voyages for the treatment of the epidemic, the only form of treatment for a TB patient was care at the sanatorium which included rest, rich diet, well monitored exercise and strict hygiene (Davis, 1996). Streptomycin was the first drug to be discovered in 1943 by Dr Selman Waksman and Albert Schatz, isoniazid was discovered in 1952 and rifampicin followed in 1957. The development of these drugs replaced the sanatorium as a form of treatment (Daniel, 2006).

One of the greatest initiatives in history towards the control of TB was the development of a TB vaccine by Albert Calmette and Camille Gue'rin called Bacille Calmette Gue'rin (BCG) (Hawgood, 2007). Although the vaccine contributed to the decline of TB cases in developed countries, underdeveloped countries are still confounded by the epidemic (WHO, 2009). Despite all efforts done by scientists and medical practitioners in an attempt to control TB infection and complications, TB is still a problem today.

1.2 Epidemiology of Tuberculosis

TB is the most common cause of death world-wide and its incidence has been steadily increasing, which is more evident when comparing the global TB incidence of 9.24 million in 2006 to 9.27 million cases in 2007 (WHO, 2009). These included 4.1 million new smear- positive cases and 14.8% HIV-positive cases. The majority (79%) of the HIV-positive TB patients were in Africa followed by Asia which accounted for 11% of all the HIV-positive TB patients. Approximately 3.1% of new cases were MDR-TB and 19% of the recurring cases were MDR-TB. There was a slight decrease in the prevalent cases from 13.9 prevalent cases in 2006 to 13.7 million in 2007 (WHO, 2008; WHO, 2009). The mortality rate was estimated at 1.32 million in HIV-negative people with an additional of 456 000 among the HIV-positive people and the latter accounted for 23% of the estimated 2 million HIV deaths that occurred in 2007 (WHO, 2009; UNAIDS, 2009).

African countries seem to be the second most affected by the epidemic after Asia with Sub Saharan Africa being the most affected. South Africa is among the 22 countries most affected by TB with a very high number of cases relative to the total population (WHO, 2006). The population of South Africa makes up 0.7% of the world's population and yet contributes 28% of the number of HIV-positive TB cases worldwide and 33% of HIV positive TB cases in Africa (WHO, 2009). The incidence of TB in South Africa is at around 948/100 000 population per year (ppyr); of these 358 /100 000ppyr were smear positive with mortality rate of 38/100 000ppyr and 94/100 000ppyr among HIV-negative and HIV-positive respectively (WHO, 2009). Eighty six percent of the 15 countries with the highest incidence per 100 000 population countries were from the African region

with South Africa ranking second after Swaziland. South Africa alone accounted for 31% of the HIV-positive cases in Africa and ranked fourth among the countries with the highest incidence of MDR-TB (WHO, 2009).

1.3 Characteristics of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (*M. tuberculosis*) belongs to the family Mycobacteriaceae and the genus mycobacteria. These bacteria are non-motile, non-sporulated rods with a lipid cell wall and a high content of guanine and cytosine in their DNA, and for these reasons they are assumed to be more preserved than any other bacteria. *M. tuberculosis* is an obligate aerobe and thus is found in the well aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite that grows inside macrophages with a slow generation time, a characteristic that contributes to its virulence. Under a light microscope the bacilli appear straight or slightly curved with the length of 1-10 µm and 0.2-0.6 µm in width (Draper and Daffe, 2005). The colonies of *M. tuberculosis* on solid media appear rough and crumbly, and on microscopic smear mycobacteria have cording characteristics which are attributed to the trehalose 6.6'-dymycolate, a compound also known as cord factor (Indrigo *et al.,* 2002).

The inner compartments of the wall of mycobacteria consist of three major components; peptidoglycan, arabinogalactam and mycolic acids covalently linked together forming a complex (Brennan, 2003). The peptidoglycan is rigid enough to maintain the shape of mycobacteria. The arabinogalactam is important for cell wall integrity and for anchoring the impermeable mycolic acid layer to the peptidoglycan. Mycolic acids play a role in cell wall impermeability and consist of long chain of fatty acids with 60-90 carbon atoms. About 60% of the mycobacterium cell wall is made out of lipids (Besra and Brennan, 1997). This high lipid content on the cell wall is associated with survival inside macrophages, resistance to lethal oxidation, resistance to antibiotics and acid fastness of the bacilli; the latter is useful in diagnosis particularly in resource poor settings (Murray *et al.,* 2003).

The tubercle bacilli can build its components from basic nitrogen source carbon and from already synthesized organic compounds; thus it is both prototrophic and heterotrophic. During unfavorable conditions mycobacteria can shift from being aerobic to microaerophilic and use lipids in place of carbohydrates as a source of energy (Neyrolles *et al.,* 2006). Mycobacteriae make use of oxygen as a final electron acceptor during aerobic respiration, for this reason tubercle bacilli in hosts have high affinity for tissues with high oxygen tension such as the lungs, particularly well aerated upper lobes. Carbon dioxide is also essential and can be obtained from the environment or from carbonates or bicarbonate. In culture, 5-10% carbon dioxide enhances growth of the bacilli (Palomino *et al.,* 2007).

It takes 12-24 hours for *M. tuberculosis* to divide in culture. This rate is extremely slow compared to other bacteria which take between 15 minutes to an hour to divide. This slow growth rate might be partially due to the cell wall impermeability that interferes with uptake of nutrients which are necessary for growth and multiplication. However, ribonucleic acid (RNA) synthesis has been identified as a major factor that contributes to slow generation time. Harshy and Ramakrishnan (1977) demonstrated that the ratio of RNA to DNA and RNA chain elongation ratio is tenfold lower than that of *Escherichia coli*. The slow growth rate explains the ability of the organism to cause sub-acute to chronic disease and the long time required to observe visible growth in culture (Murray *et al.,* 2003).

1.4 Transmission

TB is mainly transmitted by inhalation of a nuclei droplet containing the bacilli, or by ingestion of contaminated material, usually milk, and by direct inoculation, the latter usually affects health care workers (Dye *et al.,* 2006). The bacterium is highly resistant and can survive long periods in droplet nuclei. Each droplet nuclei contains about 3 bacilli and the minimum infective dose is about 9-10 bacilli. When an infected patient coughs, they release at least 3000 droplet nuclei, and thus TB can spread with relative ease. The chance of a person exposed to TB to be infected depends largely on the

concentration of the infectious droplet nuclei and the length of exposure (CDC, 2005). The risk of becoming infected with TB is even higher in crowded conditions, elderly people and people living with Human Immunodeficiency Virus (HIV) (WHO, 2008).

1.5 Tuberculosis infection

Once the mycobacteriae are inhaled, most of the organisms will be cleared in the upper respiratory epithelium by means of mucociliary escalator. Less than 10% of *M. tuberculosis* enters the alveolar passage through air droplets where it resides inside macrophages. Apart from macrophages, *M. tuberculosis* can also be absorbed by alveolar epithelial pneumocytes II and dendritic cells, the former are found in great numbers in the alveoli (Mehta *et al.,* 1996). Dendritic cells play a role as antigen presenting cells and in the dissemination of the bacilli since these cells are migratory (Smith, 2003).

Once the bacilli are phagotocysed, they reside inside the phagosome. If phagosome lysosome fusion occurs the organism may encounter a harsh environment consisting of acidic pH, reactive oxygen intermediate (RIO) such as H_2O_2 and O_2^- and lysosomal enzymes (Fenton and Vermeulen, 1996). This harsh environment is responsible for the killing of the organism. However, mycobacteriae have the ability to survive intracellular killing by inhibiting the phagosome lysosome fusion (Frehel *et al.,* 1986).

1.5.1 Development of latent tuberculosis

Infected macrophages attract inactivated monocytes, neutrophils and lymphocytes which fail to kill the bacterium efficiently (Fenton and Vermeulen, 1996). Attracted cells then result in the formation of caseous center of the granuloma. The granuloma is composed of fibroblasts, monocytes and lymphocytes which join forces in attempt to kill the Mycobacteria (Dannenberg and Rook, 1994); Mycobacteria fail to multiply in the caseous tissue because of its acidic environment with low availability of oxygen, and the presence of toxic fatty acids. If a sufficient cellular immune response is induced, the

infection may be arrested at this stage. However some organisms may remain dormant for as long as a decade resulting in latent TB (Palomino *et al.,* 2007)

1.5.2 Development of pulmonary tuberculosis

If the infected person cannot control the initial infection in the lung or if a latently infected person's immune system is weakened, the granuloma center can become liquefied and then serves as a rich medium in which the bacilli grow uncontrollably producing more than 10^8 bacilli per cavity (Palomino *et al.,* 2007). The infectious material spread through the bronchi resulting in continuous production of the sputum through persistence cough, this clinical form of TB is known as pulmonary TB (Talaro and Cowan 2006).

Approximately 10% of all people who are infected with TB will develop the disease at some point in their lives. However, HIV infected individuals have a 10% probability of developing the disease every year. The risk of developing active TB disease is increased more than a hundred times in HIV infected persons than in the uninfected. An Infected individual may suffer repeated episodes of the disease, which may occur due to reactivation of dormant bacilli in primary lesions (CDC, 2005).

1.5.3 Extra pulmonary Tuberculosis

Tuberculous bacilli may escape from the granuloma and spread from the lungs to other tissues through the lymphatic system and blood resulting in different forms of extra pulmonary TB such as miliary TB, TB meningitis, lymph node TB, renal TB and pleural TB (Palomino *et al.,* 2007).

1.5.4 Pleural tuberculosis

Pleural TB is the most common form of extrapulmonary TB after lymph node TB (Sharma and Mohan, 2004). Pleural involvement may be primary or secondary to pulmonary TB. The incidence of pleural tuberculosis may vary with geographical distribution. In the United States pleural involvement has been seen in 4% of all TB

cases while in Spain it has been seen in up to 23 % of all TB cases (Reider *et al.,* 1990; Vidal *et al.,* 1986). Similarly Valdes *et al*., 1996 found that 25% of pleural effusion cases were attributed to tuberculosis. The increase in HIV pandemic has contributed to the escalation of pleural involvement in HIV patients ranging from 15% to 90% (Batungwanayo *et al.,* 1993). HIV patients develop pleural tuberculosis in the early stage of immunosuppression. In South Africa a high frequency of pleural TB effusion in HIV-positive patients have been reported (Saks and Posner, 1992).

1.5.4.1 Pathogenesis of pleural tuberculosis

Pleural TB occurs 6 to 12 weeks following primary infection when a small subpleural focus releases its contents into the pleural space. In the early stage of the infection, neutrophils may predominate. However, in the later stage of infection the release of mycobacterial antigens into the pleural space induces a predominantly CD4+ T cell mediated delayed type hypersensitivity reaction. Briefly, macrophages together with major histocompatability complex (MHC) class I, or II present mycobacterial antigen to the T Cell such as CD4+and CD8+, the T cell then produces Th1 type cytokines INF-Ý and IL-2 which in turn activate macrophages for the intracellular killing of mycobacterium (Light, 2007).

This inflammatory response results in increased capillary permeability and subsequent impairment of the lymphatic clearance of proteins and pleural fluid due to damage of the stomata, hence accumulation of fluid in the pleural space (Barnes *et al.,* 1989). Thus pleural tuberculosis is not caused by the mycobacterial infection but rather by this delayed hypersensitivity reaction to mycobacterial antigens (Light, 2007). T lymphocytes immunity is demonstrated by higher levels of INF-Ý in the pleural fluid compared to the peripheral blood of the same patient (Mitra *et al.,* 2005).

1.5.4.2 Clinical presentation of pleural tuberculosis

Pleural TB is the third leading cause of effusions after malignancy and pneumonia (Porcel and Vives, 2003). TB Pleural effusion may present as an abrupt illness that is characterized by fever, pleuritic chest pain, non-productive cough and dyspnea. Pleuritic chest pains and non-productive cough are the most common symptoms and present in more than 70% of cases (Sharma and Mohan, 2004). TB pleural effusions are typically unilateral, small to moderate in size. The effusion seldom occupies more than two thirds of the hemithorax (Cases *et al.,* 2006).

Fever, dyspnea, night sweats, fatigue, hepatosplenomegally and lymphadenopathy are more common in HIV infected patients (Richter *et al.,* 1994). HIV patients with pleural TB tend to be older and are more likely to be smear and culture positive for tuberculosis; this is more likely because of a Th2-type response which is more prevalent in HIV co-infected. Th2 induce a response that is both immune suppressive and also stimulate the growth of mycobacterium (Martin *et al.,* 2004).

1.6 Influence of HIV on TB

Being the most common opportunistic infection among HIV positive patients, TB has re-emerged as a global emergency. HIV has substantial influence on the incidence, clinical manifestation, treatment, diagnosis and disease outcome of TB. HIV mediated immune suppression impairs granuloma formation, resulting in ineffective containment of the bacilli and diminished formation of pulmonary cavities. These in turn result in frequent intrapulmonary disease and lower concentration of bacteria in sputum (Klautau and Kuschnaroff, 2005; Murray, 2005).

HIV is the single most common factor activating latent TB and for progression in adults (Frieden *et al.,* 2003). A person with latent TB who becomes HIV positive develops active tuberculosis at a higher rate of 7 to 10 % per year rather than 10% per lifetime. Persons with HIV who are newly infected with *M. tuberculosis* progresses to active

tuberculosis at a rate as high as 37% in the first 6 months rather than 2 to 5% in the first 2 years (Frieden *et al.,* 2003).

TB has become one of the most common causes of morbidity and mortality in HIV positive living in less developed countries, even though it is a preventable and treatable disease (WHO, 2008). TB/HIV co-infection also increases the rate of transmission of TB causing TB outbreaks at community level which also threatens the health of HIV negative persons. Most of the outbreaks reported involve multi drug resistant strains (Corbett *et al.,* 2003; WHO, 2008).

1.7 Laboratory Diagnosis of TB

The diagnosis of tuberculosis and screening of contacts is the foundation key for controlling spread of active TB infection. This is because successful treatment of TB largely depends on early diagnosis. TB diagnosis becomes even more challenging in patients with immunosuppression (for example in HIV infected), in the case of latent infection, extrapulmonary TB and in children (Perkins and Cunningham, 2007).

1.7.1 Specimens

It is important that the right type of specimen is collected, properly transported and carefully processed for effective diagnosis. The definitive diagnosis of pleural TB depends on the demonstration of *M. tuberculosis* in sputum, pleural fluid and pleural biopsy (Gopi *et al.,* 2007). Specimens should be collected in sterile leak-proof containers and transported rapidly to avoid overgrowth of other organisms (Palomino *et al.,* 2007).

1.7.2 Specimen Decontamination

Specimen decontamination is an important part of preparation for culture where samples from non-sterile sites are involved. This step is done to eliminate normal flora and maximize mycobacterial isolation. The decontamination methods include sodium hydroxide method, which utilizes 2-4% sodium hydroxide; N-acetylcysteine-sodium

hydroxide, sodium chloride and sodium hydroxide method; oxalic acid method; Cethyl pyridinium and sodium chloride and Ogawa Kudoh method (Della Latta, 2004).

1.7.3 Microscopy

Active tuberculosis is diagnosed by acid fast bacilli (AFB) smear microscopy, which detects the presence of AFB in clinical samples. There are three main methods used in AFB staining namely; Zeihl-Neelsen (ZN) and Kinyoun staining methods, which utilize the use of carbol fuschin and the ability of mycobacteria to resist decolorization. ZN staining method requires a heating step. The third method is termed Auramine O method, which utilizes a flourochrome, Auramine O, as the primary stain and is read at a lower magnification than the former two stains and it make use of a fluorescence microscope (Murray *et al.,* 2003).

The demonstration of AFB in pleural fluid is positive in only 5% of the cases (Valdes *et al., 1998*), with the exception of those co- infected with HIV and empyema cases (Gopi et al., 2007). The use of induced sputum for the diagnosis of pleural TB has increased detection of AFB microscopy to about 12% (Conde *et al.,* 2003). The sensitivity of AFB microscopy is even higher by Ziehl-Neelsen staining of pleural biopsy (Valdes *et al.,* 1998).

The main advantages of AFB microscopy are that it is a very rapid and inexpensive method for use in identifying highly infectious patients. However, this method has a relatively low sensitivity for AFB detection especially in children and HIV patients and in case of extra pulmonary TB such as pleural tuberculosis, as it requires bacterial loads of at least 10^4/ml. Moreover this method cannot distinguish between viable and non viable bacteria which may not be well suited for monitoring treatment success (Palomino *et al.,* 2007).

1.7.4 Culture

Culture is still the gold standard for diagnosing pleural TB because it is the most sensitive method and it also allows for subsequent strain characterization including drug susceptibility testing (DST) (Pfyffer *et al.,* 1997). The liquid media include Middlebrook 7H9 and Bactec radiometric 12B broth. The solid culture medium includes Lowenstein-Jensen agar and Middle brook 7H10 and 7H11 agar. Mycobacterium grows slowly and as result culture on solid medium takes 2– 8 weeks for visible growth on agar, and often treatment gets to be initiated before the laboratory results are available (Talaro and Cowan, 2006)

The sputum culture has 30 to 50% yields in patients with both pulmonary TB and pleural TB. However in patients with pleural TB alone, only 4% of the cases can be detected. Pleural TB culture has a diagnostic yield in 10-35% of the cases, and an even higher yield can be attained with pleural biopsy culture between 39-65% (Sharma and Mohan, 2004).

Different automated systems exist and this includes BACTEC TB 460 and BACTEC MIGIT960 system (Becton Dickinson, Sparks MD). These methods detect bacterial CO_2 production and O_2 consumption making use of radiometric, fluorescent, colometric or pressure sensors that allows continuous monitoring and eliminate the need for mature colony formation. The implementation of automated culture methods have increased the sensitivity of culture since these methods employs liquid medium and in the sense that it takes about 18 days instead of 33 days for a positive culture to be noted (WHO, 2008).

HIV positive patients with low CD4+ cells who are co-infected with pleural tuberculosis have a higher yield of pleural fluid culture as opposed to HIV negative patients since the former has difficulty in controlling the mycobacteria therefore high levels of the mycobacteria will be found in the pleural fluid of these patients (Luzze *et al.,* 2001).

1.7.5 Molecular methods

Molecular methods are based on the principle that complementary nucleic acids strands bind to form double stranded (hybrid) complexes. These methods make use of oligonucleotide primers and enzymes that catalyze reaction which amplify target, probe or a signal. These methods include the Accuprobe system (Genprobe, Sandiego California), Polymerase chain reaction (PCR) and real-time PCR. The advantage of molecular methods is their excellent specificity and sensitivity as compared to conventional methods and the results are available within a short period of time (Nagesh *et al.,* 2001).

1.7.5.1 The Accuprobe system

This method uses a DNA probe labeled with an Acridinium ester directed at the rRNA of the mycobacterium. The unhybridized probe is chemically degraded and the esterified acridinium on the hybridized probe is hydrolyzed by the addition of alkaline hydrogen peroxide solution and emits visible light which can be measured with luminometer. Although the probes employed in the Accuprobe have a detection limit of 10^6 CFU/ml and requires actively growing cultures, its sensitivity is 100% specific for the *M. tuberculosis* complex (Goto *et al.,* 1991).

1.7.5.2 Polymerase chain reaction (PCR)

PCR uses primers that are either species specific or genus specific. In PCR the mycobacterial DNA in clinical samples is extracted, amplified and then identified. The double stranded target DNA is first denatured by heating, and then a pair of oligonucleotide primers is annealed to the complementary strands of each of the target strands. Primers have been designed to target specific proteins such as the conserved 65-KDa heat shock protein, or repetitive insertion sequences such as IS6*110* which are specific to and repeated 1 to 20 times in the chromosome of the members of the mycobacterial tuberculosis complex. The enzyme polymerase then carries the extension of the new DNA strand, the cycle is repeated 20 to 40 cycles, the amplified

product is then identified using agarose gel electrophoresis and stained with ethidium bromide stain which is visualized by UV transillumination (Palomino *et al.,* 2007).

The DNA can also be detected by binding to a nylon or nitrocellucellose membrane matrix where in the amplified product is hybridized with a probe that is complementary to an internal region of the amplified product. The sensitivity of PCR varies from 50 to 100%, however the specificity is high ranging from 95- 100%. Several factors may affect the sensitivity of a PCR reaction for the detection of Mycobacteria species and these may include the extraction procedure, amplification procedure and number of bacilli present in a specimen. PCR has been used to detect mycobacterial DNA in pleural fluid, with sensitivities ranging from 20 to 80% and specificities of 78 to 100% (De Wit *et al*., 1992; Danielle *et al*., 2003).

1.7.5.3 Real-time PCR

Real-time PCR is also known as quantitative real-time PCR or kinetic PCR. This is a PCR technique that is used to amplify and simultaneously quantify a target DNA molecule and moreover it can detect one or more specific sequences in a DNA sample within an hour or less (Bankowski and Anderson, 2004). The main advantage of real-time PCR is its excellent sensitivity and specificity, low contamination risk, ease of performance and speed (Heginbothom *et al*., 2003).

Different detection techniques are employed with real-time PCR; a less specific method make use of SYBR green to detect any double stranded DNA. A more sensitive and specific real-time PCR makes use of flourecent probes technology, and these include the Taqman probes, molecular beacons and FRET hybridization probes. These detection methods rely on the transfer of light energy between two adjacent dye molecules, a process called fluorescence resonance energy transfer (Espy *et al.,* 2006).

Real-time PCR assay have been used for the diagnosis of many clinically important mycobacterium species in clinical specimens (Lim *et al*., 2007). Low organism in a sample and the presence of inhibitors may result in false negative results (Takahashin and Nakayama, 2006). Real-time PCR have been shown to have a sensitivity of 95.8%

and 100% specificity for the diagnosis of extra pulmonary tuberculosis (Takahashin *et al.,* 2008).

1.7.6 Tuberculin Skin test (TST)

TST has been in use to for more than 100 years. It is mainly used to identify patients actively infected with TB, measure the prevalence of infection in a community and to select susceptible patients or high risk patients for BCG vaccination. With TST, 0.1ml of 5 TU purified protein derivative (PPD) is injected on the forearm and the patients are examined after 48-72hours. A positive reaction is indicated by erythema and induration of > 10mm in size in immune-competent individuals; however, in immune compromised patients the induration of > 5mm is indicative of a positive test (Palomino *et al.,* 2007).

The positive TST test in areas of low TB prevalence and no vaccination is strongly suggestive of pleural tuberculosis. However one third of patients presenting with pleural tuberculosis may have a negative TST test. The negativity of the TST has been reported in 30% of the immune competent and in more than 50% of HIV infected patients (Relkin *et al.,* 1994). A negative TST test in pleural tuberculosis may be due to recent infection, circulating mononuclear cells suppressing the specifically sensitized circulating T-lymphocytes in blood and sequestration of PPD derivative- reactive T lymphocytes in the pleural space (Rossi *et al.,* 1987).

TST test contain proteins which are common with the BCG and other mycobacterium species found in the environment, as a result, TST would often give false positive results, and the sensitivity of the skin test in known TB cases is estimated to be 70%. In immunocompromised patients the sensitivity decreases to as low as 30% (Palomino *et al.,* 2007).

1.7.7 Interferon gamma release assay

Interferon-gamma release assay is based on the principle that T lymphocytes of the TB infected individual produce INF-γ when exposed to TB antigens (Tufariello *et al.,* 2003). INF-γ when released activates macrophages, increasing their bactericidal activity against the phagocytosed mycobacteria and also plays a role in the formation of the granuloma. These assays make use of more specific antigens which are early secretory antigen (ESAT6) and culture filtrate protein (CFP10) and therefore have advantage over the skin test for the detection of latent tuberculosis (Hooper *et al.,* 2009).

The commercially available kits include the enzyme –linked immunospot (ELISPOT) T SPOT-TB assay (Oxford Immunotech, Oxford, United Kingdom) and the Quantiferon – TB and its modified version QuantiFERON- TB Gold assay (Cellestis International, Carnegie, Australia). The T SPOT-TB assay is more sensitive but less specific compared to the QuantiFERON- TB Gold assay (Kim *et al.,* 2009).

High levels of INF-γ have been found in pleural fluid with sensitivity ranging from 78 to 100% and specificity ranging from 95 to 100% for the diagnosis of pleural TB (Jiang *et al.,* 2007). Due to a high number of inconclusive results with the QuantiFERON TB Gold assay, it was concluded that the test cannot be recommended for routine use in the diagnosis of pleural tuberculosis in TB/HIV endemic resource limited setting (Baba *et al.,* 2008). Although these tests have been shown to be useful in the diagnosis of pleural tuberculosis, for various reasons such as limited evidence and its applicability in a high HIV/TB setting, they are not routinely used (Joshi *et al.,* 2008).

1.7.8 Adenosine Deaminase (ADA)

Adenosine deaminase (ADA) is an enzyme that catalyses the hydrolysis of adenosine to inosine and deoxyadenosine to deoxyinosine in the metabolism of purines, with the subsequent release of ammonia. ADA plays a role in the proliferation and differentiation

of lymphocytes such as T lymphocytes. It also plays a role in the transformation of monocytes to macrophages. The levels of ADA increases in diseases such as TB where cell mediated immunity is stimulated (Sharma and Mohan, 1996).

Testing for ADA activity in pleural fluid is an inexpensive method and relatively simple technique. High levels of ADA in pleural tuberculosis were reported for the first time in 1978 (Piras *et al.,*1978). Other than TB, High levels of ADA also occur in empyemas, some lymphomas, brucellosis, histoplasmosis and rheumatoid arthritis. However these conditions can easily be differentiated from pleural tuberculosis in terms of clinical picture and high levels of polymorphonuclear leukocytes instead of lymphocytes seen with TB effusion (Gopi et al.,2007).

Two isoenzymes of ADA exist, ADA1 and ADA2 of which ADA1 have high activity in lymphocytes and monocytes and ADA2 is more common in monocytes and macrophages. ADA2 is more predominant in pleural tuberculosis due to increased release by monocytes and macrophages following phagocytosis of mycobacteria. ADA1 is more predominant with empyemas and other infections (Zemlin *et al.,* 2009).

Pleural fluid ADA activity has been shown to be a valuable biochemical marker that has a high sensitivity and specificity for TB diagnosis (Mo-Lung *et al*., 2004).The sensitivity and specificity of ADA in the diagnosis of pleural tuberculosis differ from population to population based on the prevalence of tuberculosis. In high prevalence of TB the sensitivity of ADA is quite high, in contrast, low ADA sensitivity and specificity are seen in countries were TB is less prevalent (Valdes *et al.,* 1993). The sensitivity of ADA ranges from 47.1 to 100% and its specificity ranges from 0% to 100% in diagnosing pleural tuberculosis (Goto *et al.,* 2003).

The ADA levels in HIV positive patients and in immunocompromised patients are comparable; Baba *et al.,* 2008 showed that ADA activity is a sensitive marker even in HIV positive patients with low CD4+count. The levels of ADA are not correlated to the absolute values of lymphocyte cells but rather to the level of maturation of these cells (Ferrer, 1997).

1.8 Treatment of pleural tuberculosis

Drug treatment is an important aspect in the control of the TB epidemic and also contributes in breaking up the chain of transmission if treatment regimens are completed successfully. The main aim of TB treatment is to kill those bacilli which are metabolically active, and also to eliminate or sterilise bacilli which are replicating less actively in granuloma lesions (Palomino *et al.,* 2007).

Drugs for treating TB are usually classified as first- and second-line drugs. The first-line drugs include isoniazid (INH), rifampicin(RIF), pyrazinamide(PZA), ethambutol (EMB) and streptomycin (SM). Second-line drugs include the amino glycosides such as kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, thioamides, prothionamide and several fluoroquinolones (Onyebujoh *et al.,* 2005).

Pleural tuberculosis may resolve spontaneously within 4 to 16 weeks, however if it is not treated, there may be subsequent development of pulmonary or other forms of extrapulmonary TB in up to 65% of cases (Light, 2007). The treatment for pleural tuberculosis is similar to that of pulmonary tuberculosis, it comprises of two phases, a first phase of isoniazid (INH), rifampicin, and pyrazinamide for the first 2 months; second phase of isoniazid and rifampicin for the next 4 months (2HRZ/4HR). Ethambutol should be added if one or more of the following criteria are met: more than 4% primary resistance to isoniazid; previous treatment with antituberculosis medication; the patient is from a country with a high prevalence of drug resistance (Bass *et al.,* 1994).

HIV positive patients are treated with the same treatment as for HIV negative patients; however it is essential for the clinician to consider drug interactions between highly active antiretroviral therapy and antituberculosis treatment. Rifampin is a potent inducer of the cytochrome P-450 enzyme and therefore this drug should not be used with protease inhibitors and non-nucleoside reverse transcriptase inhibitors, alternatively adjusted doses of rifabutin can be used (Onyebujoh *et al.,* 2007). Moreover, delaying antiretroviral treatment until at least 2 months after the initiation of anti -TB treatment is beneficial as concurrent treatment of HIV and TB tend to cause clinical deterioration in the condition of patients as a result of the immune reconstitution inflammatory syndrome (IRIS) taking effect, causing immune-mediated injury on recovery of T cells following antiretroviral treatment (Lawn *et al.,* 2007).

1.9 Study problem

The diagnosis of tuberculous pleuritis remains a challenge because the most widely used conventional diagnostic tools, such as culture and microscopic observation of acid fast bacilli of pleural fluid are unable to rapidly detect *M. tuberculosis* with sufficient sensitivity and specificity. Pleural fluid adenosine deaminase (ADA) assay activity has been shown to be a valuable biochemical marker that has a high sensitivity and specificity for tuberculosis (TB) diagnosis (Mo-Lung *et al*., 2004). The use of ADA as a diagnostic marker has additional merits since results can be produced rapidly. However, pleural effusions are caused by other diseases such as malignancies, lymphoma and collagen vascular diseases (Light, 2002).

More than 90% of TB effusions show a lymphocytic predominance, and hence TB requires exclusion in any lymphocytic exudative effusion where no alternative cause is found. The diagnosis of TB pleural effusion remains a common clinical challenge (Light, 2002). It is important to find a rapid and reliable test for the diagnosis of pleural TB

particularly in developing countries such as South Africa where there is high TB incidence and HIV infection, the standardization of ADA values is of importance in high HIV prevalence settings.

1.10 Aim

The aim of the study was to evaluate the diagnostic value of the ADA assay for the diagnosis of tuberculous pleuritis at Dr. George Mukhari (DGM) tertiary laboratory.

1.11 Objectives

a) To identify *M. tuberculosis* in pleural fluid using microscopy, culture, ADA and real-time PCR

b) To determine the sensitivity and specificity of ADA against culture

c) To determine the sensitivity and specificity of real-time PCR against culture

d**)** To compare the sensitivity and specificity of ADA assay and real-time PCR for the diagnosis of pleural tuberculosis

d) To determine the correlation between ADA values and CD4+ counts

CHAPTER TWO: MATERIALS AND METHODS

2.1 Study design

The study was a case control study evaluating the ADA assay for diagnosis of TB pleuritis in comparison with non-TB patients with clinically significant pleural effusions at the Dr. George Mukhari (DGM) Tertiary Laboratory. The patients in the study group were divided into two groups (Group A and B): Group A comprised of patients suspected of having TB (cases) and Group B were non-TB patients with other clinical conditions such as cancer and heart failure (controls).

2.2 Specimen collection

Hundred and seven consecutive pleural fluid specimens submitted to the Dr George Mukhari Tertiary Chemical Pathology laboratory were included in the study. These were from 71 suspected TB cases (patients with suggestive symptoms of tuberculosis, the symptoms included coughing for more than two weeks, night sweats, fever and loss of weight) and 36 controls (patients with other clinical cause of pleural effusion and these were used as controls), and the study was conducted over a 5 months period**,** from January 2009 to May 2009. Patient demographic data which included, age, sex, clinical history and CD4 counts were collected from National Health Laboratory Services (NHLS) DISA system. The study was approved by the Medunsa Research Ethics Committee.

2.3 Microbiological Methods

Specimens from both the control group and the suspected cases were processed for AFB microscopy, culture and real-time PCR. The control group were analyzed by

microbiological methods in order to eliminate TB, and the suspected cases were analyzed to determine the definitive cases of TB.

2.3.1 Culture on blood agar

Pleural fluid samples were cultured on blood agar at 37^{o}C for 48 hours to check for contamination. There was no growth observed on blood agar for all samples after incubation. None of the samples showed contamination, therefore they were not decontaminated. None of the samples included in the study had blood clots.

2.3.2 Concentration of samples

All pleural fluid samples were concentrated by centrifugation at 3000 x g for 15 minutes, supernatant discarded and the pellet resuspended in 2ml of PBS buffer pH 6.8. The samples were mixed by vortexing for 5 seconds. One millilitre of the sample was used for AFB smear microscopy and culture and 1 ml was used for real-time PCR.

2.4 Microscopy

2.4.1 Smear Preparation

A drop of smear precipitating fluid was placed onto a clean microscope slide and a drop of concentrated pleural fluid was added. The mixture was then spread evenly to make a smear. The smears were air dried and placed on an electric slide warmer at 65 to 75^{o}C for 2hours for the fixation of the smear. The slides were allowed to cool prior to staining.

2.4.2 Auramine O Staining

Microscopy was carried out using auramine O staining. Briefly, the smears were flooded with flourochome stain for 15 minutes, rinsed with water and excess water drained. The smears were decolorized by flooding with 0.5% acid alcohol for 30 to 60 seconds, rinsed with water and excess water drained. The smears were counterstained with potassium permanganate for 2 minutes, rinsed with water and excess water

drained. The smears were allowed to air dry, and then examined with a fluorescent microscope (K530 excitation filter and BD 12 barrier filter), using the high power objective (40X, total magnification, X 400), and verified using a high power objective (100X, total magnification X1000). A positive result appeared as fast acid bacilli with green fluorescence.

2.5 Culture

About 0.2 ml of the sample was inoculated onto Lowenstein-Jensen (L-J) slants. The LJ bottles were placed in slanting position and incubated at 37^{o}C for 24hours to ensure that the sample was in contact with the culture medium. The L-J bottles were then placed upright taking in consideration that the slants were completely covered by the sample. The cultures were aerated weekly. Excess fluid was poured off when visible growth was observed and any subsequent fluid was poured off when aerating. Cultures were incubated for up to 8 weeks.

2.6 Ziehl-Neelsen (ZN) staining

All the specimens which showed visible growth on L-J were stained with ZN. The smears were prepared from cultures as described above. The heat-fixed smears were flooded with carbol fuchsin and heated to steaming with a Bunsen burner, avoiding boiling. The slides were allowed to stand for 5 minutes, rinsed with tap water and drained. The smears were decolorized with 3% acid-alcohol for 3 minutes, rinsed with tap water and drained. The smears were counterstained with methylene blue for 30 seconds, rinsed with tap water, drained and air dried. Slides were examined under oil immersion objective, 100 X magnifications. Acid fast bacilli (AFB) appeared as red bacilli against a blue background. Acid-fastness was graded as follows: Negative=No AFB seen; Scanty=1-9 AFB seen/100 fields; 1+=10-99 AFB/100 fields; 2+=1-10 AFB/field; 3+= >10 AFB/field.

2.7 Real-time PCR

2.7.1 DNA extraction

The extraction of DNA was done on concentrated pleural fluid specimens using the Amplicor respiratory sample preparation kit according to the manufacturer's instructions (Roche Diagnostics, Germany). Briefly, 500µl Wash solution (RW) was added into 1.5ml tube together with 100µl of concentrated pleural fluid sample. The mixture was vortexed for 5 seconds, centrifuged at 12500g for 10 minutes, and the supernatant was discarded. About 100µl of Lysis buffer (RL) was added into the pellet and the tubes were vortexed for 5 seconds to re-suspend the pellet. The samples were then incubated at 60^{o}C for 45 minutes. Following incubation, the samples were spun down for 5 seconds, 100µl of neutralizing buffer (RN) added and the mixture vortexed for 5 seconds. The lysate was stored at 2-8^{o}c until use.

2.7.2 LightCycler Run

Real-time PCR was performed using the LightCycler mycobacterium detection kit according to the manufacturer's instructions (Roche Diagnostics-Germany). A total of 20µl PCR mixture containing 0.25µl of 8.5 uracil DNA glycosylase, 0.75µl internal control, 4µl master mix, 11µl mix and 4µl DNA sample was prepared in a capillary tube. The master mix contained the enzyme hot start Taq polymerase, $MgCl_2$, deoxynucleoside triphosphate (dTTP, dCTP, dATP, dGTP) and PCR buffer. The detection mix contained the primers, and hybridising probes; flourescein and the acceptor probe LC Red. The Light cycler run was conducted according to the following protocol: Incubation at 40^{o}C for 10minutes, denaturation at 95^{o}C for 10 minutes, amplification consisted of 45 cycles of : 95^{o}C for 10 seconds, 50^{o}C for 10 seconds with a single acquisition mode and 72^{o}C for 20 seconds. This was followed by melting curve which also comprised of three stages which are: 95^{o}C for 1 minute, 40^{o}C for 2 minutes and 70^{o}C for 1 minute, ramp rate 0.1 and a continuous acquisition mode. This was followed by cooling at 40^{o}C for 30 sec. A melting temperature (Tm) ranging from 55-57^{o}C indicated *M. tuberculosis*; 59-62^{o}C *M. kansasii* and 50-53 ^{o}C *M. avium*.

2.8 ADA analysis

The ADA was analysed using a commercial colorimetric assay kit according to manufacturer's instructions (Diazyme General Atomics, CA., USA). Briefly, 25µl of specimen was mixed with 500µl of 21 mM adenosine in 50 mM phosphate buffer, pH 7.0, and incubated for 60 min at 37°C. The reaction was interrupted by incubation with 1.5 ml of phenol nitroprusside at 37°C for 30 min (106 mM phenol, 0.17 mM sodium nitroprusside) in the presence of 1.5 ml of sodium hypochlorite (11 mM NaOCl plus 125 mM NaOH). The amount of ammonium ion released by the ADA reaction was determined as absorbance (optical density, OD) at 628 nm wavelength. To control for the ammonium present in the patient's specimen before addition of exogenous adenosine, specimens without substrate were run in parallel (specimen blank). A standard (15 mM ammonium sulfate stock solution) and a reagent blank (50 mM phosphate buffer pH 7.0) were also included in the assay. The activity in the patient's specimen was calculated with the formula: Activity in specimen = (OD specimen – OD specimen blank) / (OD standard – OD reagent blank) x 50 with the result expressed in Units/L. The ADA value of ≥30 Units/L was considered to be positive. The assay was done on a Beckman DXC Synchon analyzer using user defined methods

2.9 Data Analysis

The results of the ADA assay and real-time PCR against culture, the gold standard for diagnosis of TB were entered into 2X2 tables, where in the sensitivity was calculated by using the formula: TP/ (TP+FN), specificity by using the formula: TN/ (FP+TN), positive predictive value (PPV) by using formula: TP/ (TP+FP), and negative predictive value (NPV), by using the formula TN/(TN+FN); where TP = the true positives; FN= false negatives; FP= false positives and TN= true negatives. Correlation coefficient between ADA and CD4 counts was done using Excel Analysis toolkit. The mean and standard deviation of ADA and the mean age were calculated using epi info version 3.3. Comparison between two groups were done using epi info version 3.3 where in a p value of ≤0.05 was considered significant, and a p value of ≥0.05 was considered insignificant.

CHAPTER THREE: RESULTS

3.1 Study population

A total of 107 pleural fluid specimens were collected, and 71 of these were suspected pleural TB cases, 36 samples were controls (non tuberculosis pleural effusion). Only 34 of the controls were used in the study as 2 of the samples were insufficient for other tests.

3.2 Microbiological Methods

3.2.1 Microscopy

All of the 34 (100%) controls were AFB negative, and 3 of the 71 (4%) suspected pleural TB cases were AFB positive and 68 (96%) were negative. These results are shown in Figure 3.1.

3.2.2 Culture

None of the specimens from the control group showed visible growth on LJ culture medium. Of the 71 suspected pleural TB cases, 50 (70%) were culture negative and 21(30%) were culture positive. These results are shown in Figure 3.1. All the culture positive samples were confirmed by Ziehl-Neelsen staining as acid fast bacilli.

3.3 Molecular Methods

3.3.1 Real-time PCR melting curve analysis

None of the specimens from the control group were positive by real-time PCR. Of the 71 suspected pleural TB cases, 14 (20%) were real-time PCR positive and 57 (80%) were real-time PCR negative. These results are shown in Figure 3.1. All specimens positive by real-time PCR were identified as *M. tuberculosis*, the melting curve analysis peak for *M. tuberculosis* positive specimen is shown in Figure 3.2.

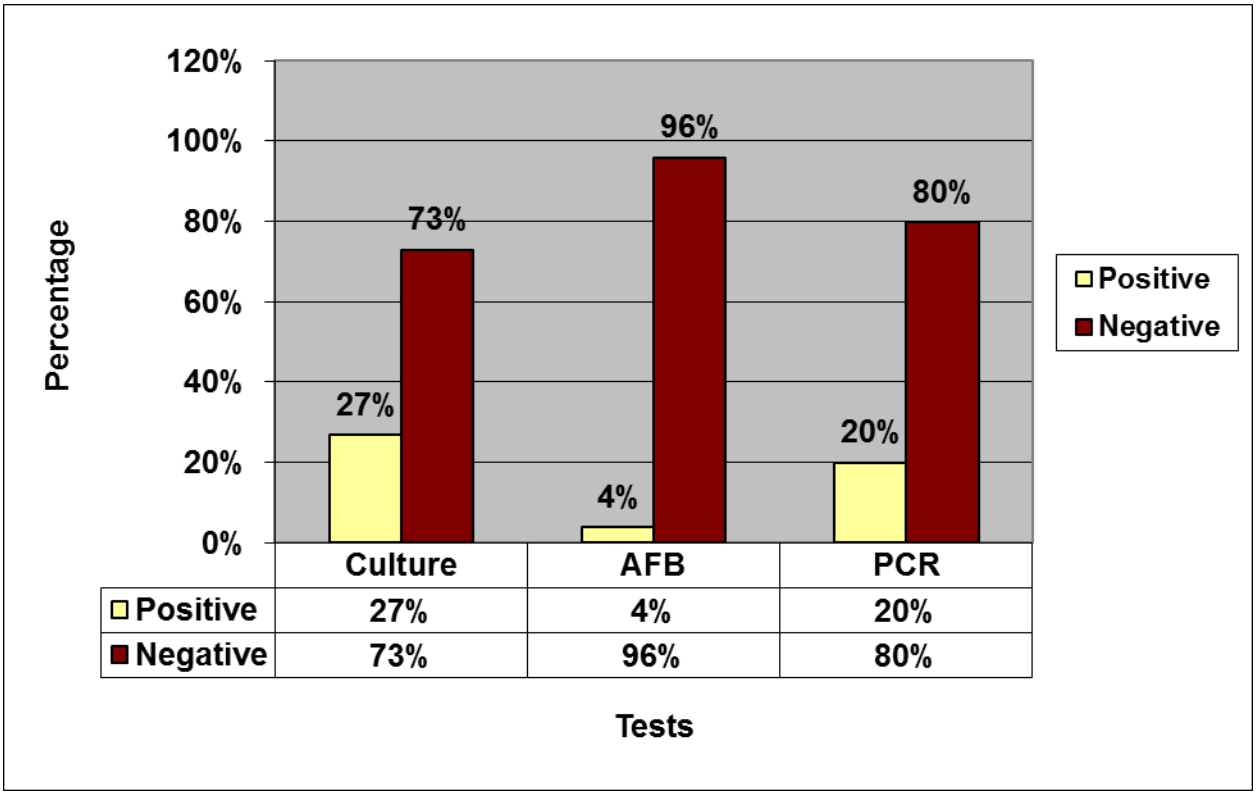

Figure 3.1 Results of AFB, culture and real-time PCR among suspected cases (n= 71)

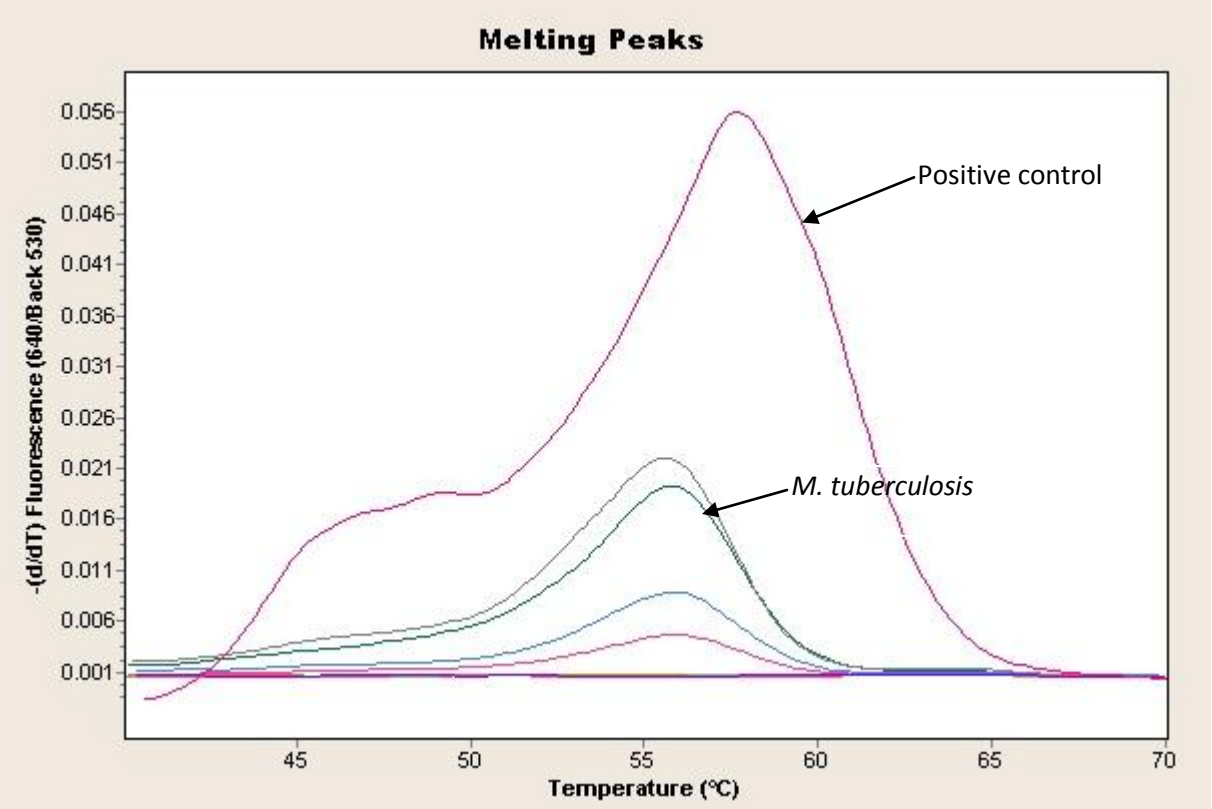

Figure 3.2 Real-time PCR melting curve analysis peaks for *M. tuberculosis* specimens.

3.4 The clinical diagnosis of the control group

Of the 34 control group, 13 had malignancy (38%), 9 had congestive cardiac failure (26%), 5 had chronic renal failure (15%), 2 were diabetic (6%), 2 had pneumonia (6%), 2 had poly arthritis (6%) and 1 had anaemia (3%). None of the controls were positive for TB by any microbiological methods performed. The clinical diagnosis of the control group is indicated in Table 3.1

Table 3.1 Clinical diagnosis of the control group (n=34)

Clinical diagnosis	Number(n)
Malignancy	13(38%)
Congestive cardiac failure	9(26%)
Chronic renal failure	5(15%)
Pneumonia	2(6%)
Poly arthritis	2(6%)
Diabetic	2(6%)
Anemia	1(3%)

3.5 Demographic data

3.5.1 Demographic data of the confirmed pleuritis

Among the 21 confirmed TB pleuritis, 10(48%) were female and 11(52%) were male. Age ranged 20yrs-65yrs with a mean age of 38.5yrs.These results are shown in Table 3.2.

3.5.2 Demographic data of the clinical TB pleuritis

Among the 50 clinical TB pleuritis, 25(50%) were female and 25(50%) were male. Age ranged 19yrs-85yrs with a mean age of 38.9yrs.These results are shown in Table 3.2.

3.5.3 Demographic data of control group

Among the 34 controls patients, 19(56%) were females and 15(44%) were males. This group consisted of adults with age ranging from 23 -78yrs with a mean age of 49yrs. These results are shown in Table 3.2.

Table 3.2 Demographic data of the patients (n=105)

Sex	Confirmed TB pleuritis (n=21)	Clinical TB pleuritis(n=50)	Control group (n=34)
Female	10(48%)	25(50%)	19(56%)
Male	11(52%)	25(50%)	15(44%)
Mean Age	38.5yrs	38.9yrs	49yrs

3.6 Chemistry

3.6.1 Adenosine deaminase assay

3.6.1.1 Confirmed TB pleuritis

The ADA levels among the confirmed pleural TB cases ranged from 8-134.3 U/L with a mean ADA of value of 52.2 U/L. The range and mean ADA results are shown in Table 3.3. Of the 21 confirmed TB pleuritis cases, 17 (81%) were ADA positive and 4(19%) were negative. These results are shown in figure 3.3.

3.6.1.2 Clinical TB pleuritis

The ADA levels among the clinical TB cases, ranged from 5.6U/L-200U/L with a mean ADA of value of 50.1 U/L. The range and mean ADA results are shown in Table 3.3. Of

the 50 clinical TB pleuritis, 39(78%) were ADA positive and 11(22%) were ADA negative. These results are shown in figure 3.3.

3.6.1.3 Control group

The ADA levels among the control group ranged between 2.4U/L and 98U/L, with a mean ADA value of 12.7U/L. The range and mean ADA results are shown in Table 3.3. Two (6%) of the 34 controls were ADA positive with the value of 39U/L and 98U/L respectively, and 32 (94%) were negative. Both patients with elevated levels of ADA had pneumonia. These results are shown in figure 3.3.

Table 3.3 The range of and mean ADA values in different diagnostic categories

Cases	Range (U/L)	Mean ADA(U/L) ±SD
Confirmed TB cases (n=21)	8-134.3	52.22 ±21.66
Clinical TB (n=50)	5.6-200	50.12 ±21.77
Controls (n=34)	2.4 -98	12.70 ± 8.64

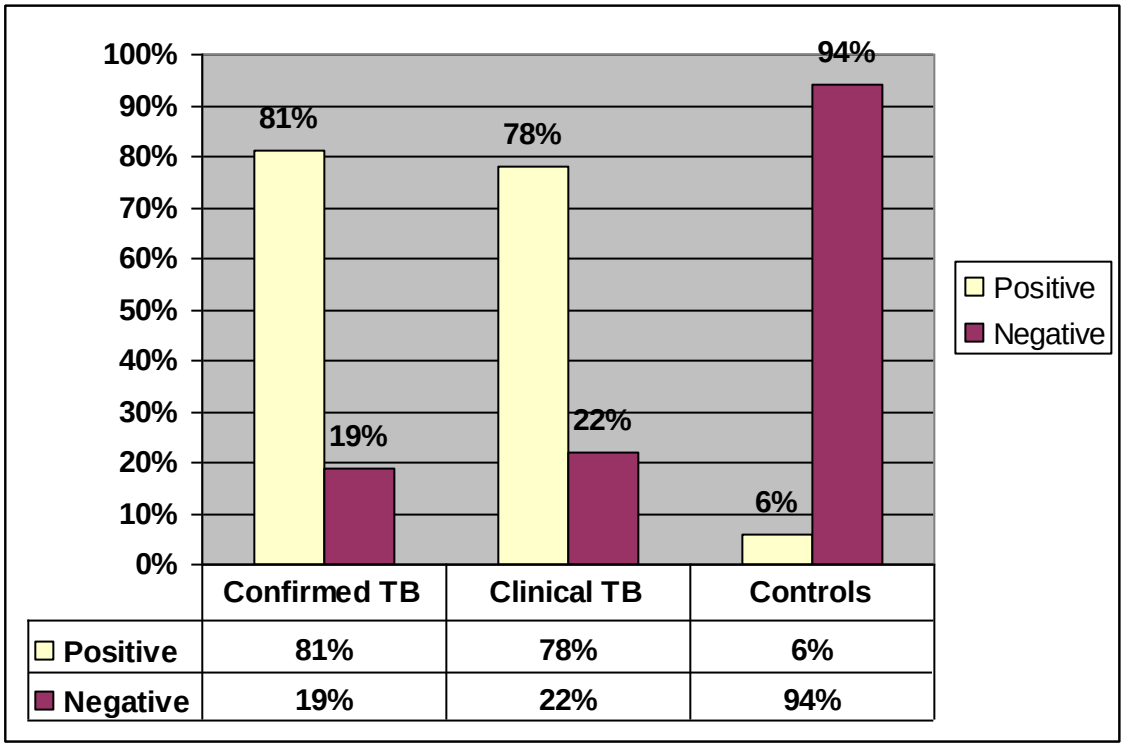

Figure 3.3 Results of ADA among confirmed, clinical TB and controls

3.7 Data analysis

3.7.1 Real-time PCR

The sensitivity and specificity of real-time PCR among the confirmed TB pleuritis were 67% and 100% respectively. The positive predictive and negative predictive values were 78% and 86% respectively. These results are shown in Appendix A.

3.7.2 ADA

The sensitivity and specificity of ADA among the confirmed TB pleuritis were 81% and 94% respectively; among the clinical TB cases were 78% and 94% respectively, and among the total TB cases were 77% and 94% respectively. The positive and negative predictive values among the confirmed TB pleuritis were both 89%; among the clinical TB cases were 95% and 74% respectively, and among the total TB cases were 96% and 67% respectively. These results are shown in Tables 3.4 and Appendix B, C, and D.

Table 3.4 The sensitivity, specificity, positive predictive value and negative predictive value of ADA among different study groups

Study group	Sensitivity	Specificity	PPV	NPV
Confirmed TB pleuritis	80%	94%	89%	88%
Clinical TB pleuritis	78%	94%	95%	74%
Total TB pleuritis	77%	94%	96%	67%

3.8 Correlation between ADA values and CD4 counts

Of the 21 confirmed cases of TB, 12 had known CD4 counts, and all were below 200. Of the 50 clinical TB, 20 had known CD4 counts, the scatter graph and correlation coefficient of ADA and CD4 counts of the confirmed cases is shown in Figure 3.4. The scatter graph and correlation coefficient of ADA and CD4 counts of the clinical TB cases is shown in Figure 3.5. There was no correlation between ADA values and CD4 counts in both the confirmed TB and clinical TB cases as shown in Fig 3.4 and Fig 3.5 respectively.

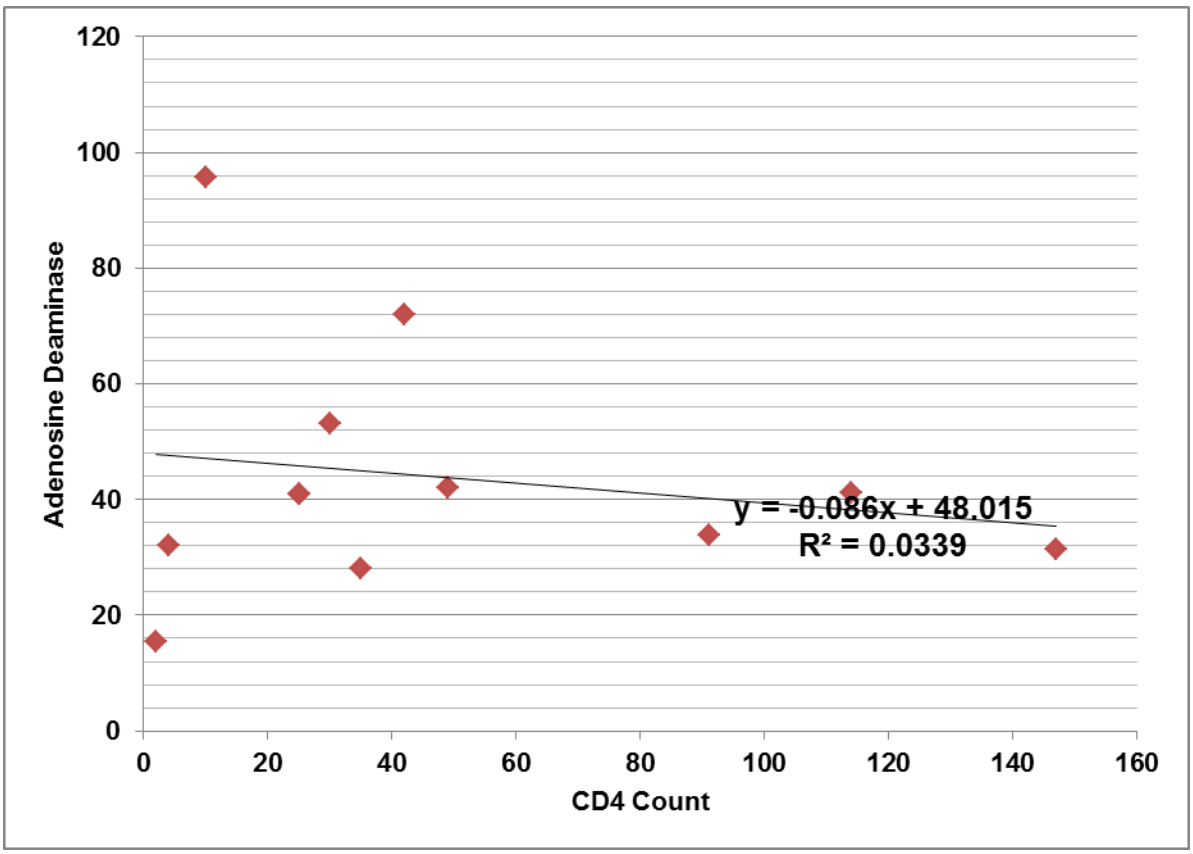

Figure 3.4: Correlation plot of ADA and CD4 counts in confirmed TB pleuritis patients (n=12).

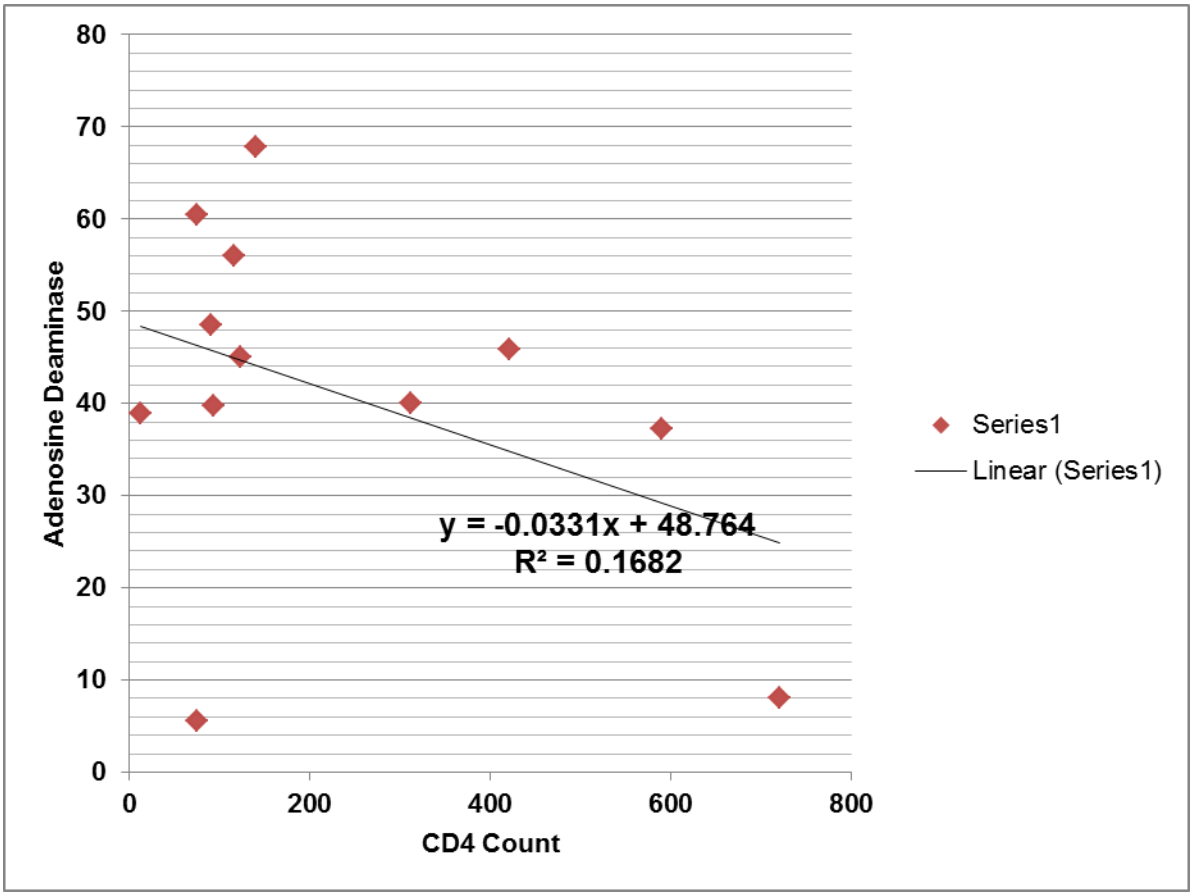

Figure 3.5: Correlation plot of ADA and CD4 counts in clinical TB pleuritis patients (n =20).

CHAPTER FOUR: DISCUSSION

The diagnosis of pleural tuberculosis remains a challenge because of low number of bacilli in the pleural fluid. The diagnosis of TB pleural effusion depends on the demonstration of *M. tuberculosis* in the sputum, pleural fluid, or pleural biopsy specimen (Gopi *et al.,* 2007). Although AFB microscopy is a rapid, inexpensive and relatively simple method, it has low sensitivity. Culture method is more sensitive than AFB microscopy, detecting 25-37% of all pleural tuberculosis cases (Valdes *et al.,* 1998), however it takes 4 to 8 weeks for a visible growth on solid medium. Therefore it is important to find a rapid and reliable test for the diagnosis of pleural TB particularly in high developing countries such as South Africa where there is high TB incidence and HIV infection rate.

ADA has been considered as a marker for cell-mediated immunity and its activity has been observed in various infections including pleural TB. ADA activity may help to differentiate pleural disease due to TB from pleural disease due to other non-infectious disorders such as malignancy. Pleural fluid ADA activity has been shown to be a valuable biochemical marker that has a high sensitivity and specificity for pleural TB diagnosis (Mo-Lung *et al*., 2004). Different cut off values have been used ranging from 30U/L to 50U/L.

The aim of the study was to evaluate the diagnostic value of the ADA assay for the diagnosis of tuberculous pleuritis at Dr. George Mukhari (DGM) tertiary laboratory. In this study, a cut off value of 30U/L was used, which is expected to give a specificity of 98% for TB diagnosis (Blake and Berman, 1982).

The mean ADA among the confirmed TB cases was 52.22±21.66 U/L and this was significantly higher than that of the control group (12.70 ± 8.64 U/L), with a p value of <0.0001. In this study the mean ADA of the clinical TB was 50.12 ±21.77 and this was also significantly higher than that of the controls (P <0.0001). These results are in

agreement with several other studies: In a study by Baganha *et al.,* 1990, high levels of ADA activity were observed in pleural fluid and serum activity of patients with pleural TB effusion as compared to neoplastic effusions. Lamsal *et al.,* 2007 studied 29 patients with TB pleural effusion and 13 patients with pleural effusion due to other non-TB respiratory disease. In that study the mean ADA in the pleural fluid of tubercular pleural effusion patients was significantly higher (90.29 ± 54.80 IU/l) compared to pleural effusion patients with non-TB respiratory disease (24.43 ± 9.28 IU/l) (p=0.0001). In their study, Gaga *et al.,* 2005 also showed that ADA values were significantly higher in tuberculosis patients as compared to patients with malignant effusions (p<0.001). In our study, there was no statistical difference between mean ADA of the clinical TB and that of the confirmed TB, suggesting that ADA is a useful tool in diagnosis TB pleuritis where microbiological methods cannot confirm the diagnosis.

ADA levels in non-tuberculous lymphocytic effusions seldom exceed the diagnostic cut off for TB. In our study, the ADA was above the cut off of 30U/L value in two (5.8%) patients of whom the diagnosis of pleural tuberculosis was ruled out; both the patients had pneumonia with ADA levels of 98U/L and 39.8 respectively. This is in agreement with one study where high levels of ADA have been reported in some cases of parapneumonic effusions and adenocarcinoma (Dikensoy *et al.,* 2002). Lee *et al*., 2001 measured ADA levels in non tuberculous lymphocytic effusion and found that ADA values were above the cut off of 40U/l in one complicated parapneumonic effusion and two cases of lymphoma. A study by Porcel and Vives, 2002 also showed that it is rare that ADA in non-tuberculous lymphocytic effusions can be above the cut off value. In that study, only 2 of 8 patients with high ADA level had uncomplicated parapneumonic effusion with ADA levels of 58.9 and 40.5 U/L respectively. Jime´nez *et al.,* 2003 studied 410 patients with nontuberculous lymphocytic effusions and the ADA level reached the diagnostic cut-off for tuberculosis (40 IU/L) in seven of the 410 cases. Two patients had bronchogenic carcinomas, two had complicated parapneumonic effusions, one had a diagnosis of lymphoma, one had a mesothelioma and one case was idiopathic. Roughly one-third of parapneumonic effusions had ADA levels above 40 U/L (Porcel *et al*., 2006). Differences in ADA activity between tuberculosis and malignancy may be due to

differences in TH phenotypes or the presence of memory CD4+ cells in tuberculosis (Gaga *et al.,* 2005).

In this study the ADA sensitivity and specificity among the confirmed TB pleuritis were 80.95% and 94% respectively. The positive predictive and negative predictive values were 89% and 89% respectively. The ADA sensitivity and specificity among the clinical TB pleuritis were 78% and 94% respectively. The positive predictive and negative predictive values were found to be 95% and 74% respectively. These results are in contrast to those of Baba *et al.,* 2008, where the specificity and sensitivity of ADA were found to be the same in both the confirmed TB cases and the clinical TB cases. In our study, only the specificities were similar in both the groups, while the sensitivity was 78% in the clinical group which is lower than 80% observed with the confirmed TB cases.

This study has shown that ADA activity has high sensitivity and specificity for the diagnosis of pleural TB. This is in agreement with previous studies where high sensitivities and specificities were observed: In a study by Mo-Lung *et al*., 2003, 210 patients with pleural effusion were studied, and high ADA sensitivity and specificity of 87.3% and 91.8% respectively were found. A study by Kashyapi *et al*., 2006 evaluated cerebrospinal fluid ADA activity for the diagnosis of TB meningitis, and ADA yielded a sensitivity of 82% and specificity of 83%. A study by Zaric *et al*., 2008 evaluated the diagnostic value of ADA assay in 121 patients and found a high sensitivity of 89.2% which was higher than that of our study and a low specificity compared to that of our study (70.4%). A study by Porcel *et al*., 2010 evaluated the ADA assay in more than 2000 patients using a cut off value of 35U/L and found that the ADA assay had a sensitivity of 93%, which was higher than that of our study, and specificity of 90%, which was lower than our study.

In our study, the mean ADA value of TB pleuritis patients with high CD4 count above 200 was 42.51±17.51 U/L and the mean ADA among TB pleuritis patients with low CD4

count below 200 was 44.25±14.36 U/L. This study found that there was no statistical difference between the mean ADA values of patients with high CD4 count above 200 and those with low CD4 count below 200 which shows that ADA is still a valuable marker even in immunocompromised patients. Several studies were in accordance with these results of our study, and have shown that ADA activity is still a sensitive marker even in immunocompromised patients (Gaga *et al.,* 2005; Baba *et al*., 2008). In contrast, a study by Hsu *et al.,* 1993 showed a low sensitivity of ADA activity in 10 immunocompromised chronic disease patients. A study by Corral *et al.,* 2004 also showed that ADA has low sensitivities for the diagnosis of tuberculosis meningitis. Reuter *et al.,* 2005 reported low sensitivity of ADA in tuberculous pericarditis patients with advanced HIV disease compared to cases with higher CD4 cell count.

Furthermore, our study showed that there was no correlation between the ADA and CD4 count. A study by Ocana *et al.,* 1983 is in agreement with our study and no correlation was found between ADA and CD4 counts. In a study by Baganha *et al.,* 1990, no correlation was noted between ADA and CD4 counts. A previous South African study is in accordance with this study and has shown no correlation between ADA and CD4 counts (Baba *et al.,* 2008). In contrast to our study, a study by Gaga *et al.,* 2005 showed a good correlation between ADA and T cells.

In this study, the sensitivity of real-time PCR against culture was found to be 66% and specificity was 100%. This is in agreement with a study by Bonington *et al.,* 1998, where sensitivity of 60% and 100% specificity were obtained using the Roche amplicor *Mycobacterium tuberculosis* PCR for the diagnosis of tuberculous meningitis. A study by Nagesh *et al.,* 2001 evaluated PCR for the diagnosis of pleural TB, and showed a sensitivity of 70%, which was similar to our study, with 100% specificity.

The results of our study are in contrast with those of previous studies where the sensitivity and specificity of PCR were found to be lower: In a study done by Daniel *et al.,* 2003, which used a PCR based on the amplification of the portion of the *M. tuberculosis* genome located in the *IS6110* insertion sequence in pleural fluid specimen, the sensitivity and specificity were 31.3% and 96.6% which were lower than that of our

study. Moon *et al.,* 2005 studied 111 patients for whom the exclusion of tuberculous pleural effusion was necessary using the Cobas Ampicor MTB test (Roche Diagnostic System), the sensitivity and specificity were 17.1% and 98.1% respectively which were lower than that of our study. However, another study by Hasaneen *et al.,* 2003 found that the PCR of pleural biopsy specimens had 90% sensitivity and 100% specificity, which were higher than those of our study.

In our study, all (100%) of the pleural fluid specimens from the control group were AFB negative, and of the 71 suspected cases only three (4.2%) were AFB positive confirming the low diagnostic yield of microscopy. This is in agreement with a study done by Valdes *et al.,* 1998, which demonstrated that AFB is positive in 5% of pleural fluid specimens. Direct examination of pleural fluid using the Ziehl-Neelsen staining method was found to have a sensitivity between 0-1percent (Yew *et al.,* 1991).

None of the pleural fluid specimens from the control group were culture positive on LJ, and 19 of 71(27%) of the suspected cases were positive on LJ. Culture detects only about 25-37% of all pleural tuberculosis cases (Valdes *et al.,* 1998), and the low positivity of culture has also been demonstrated by Danielle *et al.,* 2003. The low positivity of culture may be due to difficulties of culturing *M. tuberculosis*, since this technique is only capable of detecting *M. tuberculosis* in samples containing 50 to 1,000 bacilli per milliliter (Wolinsky *et al.,* 1994).

CHAPTER FIVE: CONCLUSION

This study has shown that the ADA assay has a high sensitivity and specificity and hence it is still a useful tool for the diagnosis of pleural tuberculosis. The study also showed that the ADA activity in pleural fluid can differentiate between pleural disease due to TB and effusion due to non tuberculous lymphocytic effusions with only 2 of 34 (5.8%) of the controls having ADA levels above the cut off for TB. Furthermore there was no statistical difference between the mean ADA values of patients with high CD4 count above 200 and those with low CD4 count below 200. No correlation was observed between ADA activity and CD4 counts which show that ADA is still a valuable marker even in immune-compromised patients. The sensitivity and specificity of the ADA assay were high even in immune-compromised patients with low CD4 counts below 200. The ADA assay performed better than all the other microbiological methods such as AFB microscopy and culture, and also real-time PCR. Therefore the ADA is useful for the diagnosis of TB pleuritis, and in addition, the result is available on the same day compared to culture which takes about 2 weeks and real-time PCR which is less sensitive.

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APPENDICES

Appendix A: 2x2 Table showing comparison of real-time PCR against culture, the gold standard

Real-time PCR		**TB confirmed**	**None TB**	
	+	**14**	**0**	**14**
	-	**7**	**34**	**41**
	Total	**21**	**34**	
Sensitivity	**14/21x 100=67%**			
Specificity	**34/34 x 100=100%**			
PPV	**14/18 x 100=78%**			
NPV	**32/37 x 100=86%**			

Appendix B: 2x2 Table showing comparison of ADA against culture, the gold standard in TB confirmed patients

ADA		**TB confirmed**	**None TB**	
	+	**17**	**2**	**19**
	-	**4**	**32**	**36**
	Total	**21**	**34**	
Sensitivity	**17/21x 100= 81%**			
Specificity	**32/34 x 100=94%**			
PPV	**17/19 x 100=89%**			
NPV	**32/36 x 100=89%**			

Appendix C: 2x2 Table showing comparison of ADA against culture, the gold standard in TB clinical TB patients

ADA		**Clinical TB**	**None TB**	
	+	**39**	**2**	**41**
	-	**11**	**32**	**43**
	Total	**50**	**34**	
Sensitivity	**39/50 x 100=78%**			
Specificity	**32/34 x 100=94%**			
PPV	**39/41 x 100=95%**			
NPV	**32/43 x 100=74%**			

Appendix D: 2x2 Table showing comparison of ADA against culture, the gold standard in TB total TB patients

ADA		**Total TB**	**None TB**	
	+	**55**	**2**	**57**
	-	**16**	**32**	**48**
	Total	**71**	**34**	
Sensitivity	**55/71x 100=77%**			
Specificity	**32/34 x 100=94%**			
PPV	**55/57 x 100=96%**			
NPV	**32/48 x 100=67%**			