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Determining the validity of the mycobacterium polymerase chain reaction assay in histological samples showing granulomatous inflammation with a negative ziehl-neelsen stain

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DETERMINING THE VALIDITY OF THE MYCOBACTERIUM POLYMERASE CHAIN REACTION ASSAY IN HISTOLOGICAL SAMPLES SHOWING GRANULOMATOUS INFLAMMATION WITH A NEGATIVE ZIEHL-NEELSEN STAIN.

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment for the Degree of Master of Medicine In the branch of Anatomical Pathology

Johannesburg 2015

DECLARATION :

I, Deepna Govind Lakhoo (student number 0203829Y), declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Anatomical Pathology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

SIGNATURE : _____

DATE : _____

DEDICATION

In loving memory of my brother, Deepin

ABSTRACT

Background*:* *Mycobacterium tuberculosis* (Mtb) poses a major global health problem. According to the World Health Organization, South Africa is a country with one of the highest reported incidence rates of this disease. Key to overcoming this preventable and treatable disease lies in establishing a reliable and rapid diagnostic approach.

Aims and Objectives: This study aims to investigate the validity of the mycobacterium polymerase chain reaction (PCR) assay applied to formalin-fixed, paraffin-embedded tissue in which the histology showed granulomatous inflammation with no demonstrable acid-fast bacilli.

Methods: A retrospective, cross sectional and non-interventional study was conducted on 121 histopathology cases showing granulomatous inflammation with a negative Ziehl-Neelsen (ZN) stain. The mycobacterium PCR results obtained in these cases were compared against the results of mycobacterium culture obtained from a specimen derived from the same or related site as the biopsy.

Results: The mean age of the study population was 35.3 years and the study cohort included 63 males and 58 females. The sensitivity of nested mycobacterium PCR (detecting the 133 base pair product of the heat shock protein 65 kilo Dalton gene), was 64.1% and the specificity was 68.2%. The positive and negative predictive values were 49% and 80% respectively. Twenty six of the 121 cases studied had a false positive result (21.5%).

CONCLUSION: There are many factors that may influence the result of a PCR assay and the interpretation thereof. Some of these factors include the inability of

the test to distinguish between live and dead bacilli, the high risk of carry over contamination, and the paucibacillary nature of certain samples with an unequal distribution of the few bacilli that may be present. Although the sensitivity and specificity of mycobacterium PCR on paucibacillary, formalin-fixed, paraffin embedded tissue is suboptimal, the interpretation of these results must be performed in conjunction with the overall clinical presentation of the patient.

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TABLE OF CONTENTS

Candidate's declaration ..... **ii**
Dedication ..... **iii**
Abstract ..... **iv**
Acknowledgements ..... **vi**
Table of contents ..... **vii**
List of figures ..... **x**
List of tables ..... **xi**
List of abbreviations ..... **xii**

Chapter 1. Introduction **1**

Chapter 2. Aims and Objectives **3**

2.1 Aims 3
2.2. Objectives 3

Chapter 3. Literature Review **5**

3.1 Introduction 5
3.2. Epidemiology 6
3.2.1 Statistics 6
3.2.2 Burden of disease 6
3.3. Controlling the disease 7
3.4. Diagnosis of tuberculosis 8
3.4.1 Conventional methods used for the diagnosis of tuberculosis 8
3.4.2 Role of cytology and histology in the diagnosis of tuberculosis 10

3.4.2 Nucleic acid amplification techniques 13

3.4.3.1 Xpert MTB/RIF 13

3.4.3.2 Line probe assays 16

3.4.3.3 Nested PCR in formalin-fixed, paraffin embedded tissue 17

3.5. Limitations of the mycobacterium PCR assay 23

3.5.1 False positive and false negative results 23

3.5.2 Advantages and disadvantages 25

3.6. Conclusion 26

Chapter 4. Materials and Methods 27

4.1 Clinical laboratory and hospital settings 27

4.2. Study design 27

4.2.1 Sample selection 27

4.2.2 Mycobacterium polymerase chain reaction 31

4.3. Sample and sample size 35

4.3.1 Inclusion criteria 35

4.3.2 Exclusion criteria 35

4.3.3 Sample size calculation 36

4.3.4 Final sample size 36

4.4. Collection and recording of data 36

4.5. Statistical analysis 37

4.5.1 Reproducibility and validity of results 37

4.5.2 Why specific tests have been used 37

4.6. Ethical considerations 39

Chapter 5. Results 40

5.1 Patient demographics 40

5.2. Mycobacterium polymerase chain reaction results 42

5.3. Results in patients in whom there was no clinical suspicion of tuberculosis . 44

5.4. Results in patients who were on empirical treatment for tuberculosis 45

5.5. PCR results in comparison to culture results, including cases that were culture positive for mycobacterium other than tuberculosis 46

Chapter 6. Discussion 47

6.1 Summary of the main findings 47

6.2. Comparison of results with those from other studies 48

6.3. Clinical importance of specific findings 49

6.4. Shortcomings or limitations of the study 52

6.5. Directions for future research 52

Chapter 7. Conclusion 54

References 55

Annexures 61

Annexure 1 Example of β-globin melt curve graph 61

Annexure 2 Data collection sheet 62

Annexure 3 Protocol approval certificate 63

Annexure 4 Ethics clearance certificate 64

Annexure 5 Turnitin report 65

LIST OF FIGURES

PAGE

Figure 1. Necrotising granulomatous inflammation…….....29

Figure 2: Necrotising granulomatous inflammation with a giant cell……...29

Figure 3: Positive Ziehl-Neelsen stain with acid-fast bacilli……30

Figure 4. Necrotising granulomatous inflammation with a negative ZN stain……30

Figure 5: Agarose gel photograph of mycobacterium PCR products……...33

Figure 6: Percentage of males and females in the study sample……40

Figure 7: Age distribution of the study sample……41

Figure 8: Distribution of the biopsy sites……...42

Figure 9: Graph depicting the overall mycobacterium PCR and culture results ……43

Figure 10: Diagram illustrating the PCR assay results in patients in whom tuberculosis was not clinically suspected …….....45

LIST OF TABLES

PAGE

Table1: Comparison of the findings from similar studies investigating the efficacy of the mycobacterium PCR assay……22

Table 2: Mycobacterium PCR primers and β-globin PCR primers……...34

Table 3: Mycobacterium PCR compared to culture results……...44

LIST OF ABBREVIATIONS

AFB:	Acid-fast bacilli
Bp:	Base pair
β- globin:	Beta globin
DNA:	Deoxyribose nucleic acid
e.g.:	Example
XDR:	Extensively drug resistant
FF-PE:	Formalin-fixed, paraffin embedded
FNAB:	Fine needle aspiration biopsy
H&E:	Haematoxylin and eosin
hsp65:	Heat shock protein 65
HIV:	Human immunodeficiency virus
IS6110:	Insertion sequence 6110
kDa:	Kilo Dalton
T_m:	Melting temperature
µg:	Micrograms
µl:	Microlitre
µm:	Micrometre
ml:	Millilitre
MDR :	Multi-drug resistant
MGIT :	Mycobacteria growth indicator tube
MOTT:	Mycobacterium other than tuberculosis

Mtb	*Mycobacterium tuberculosis*
NAATs:	Nucleic acid amplification techniques
PCR :	Polymerase chain reaction
PTB:	Pulmonary tuberculosis
RFLP:	Restriction fragment length polymerase
RNA:	Ribose nucleic acid
i.e.:	That is
TB:	Tuberculosis
WHO:	World Health Organisation
ZN:	Ziehl-Neelsen

CHAPTER 1

1.0 Introduction

Mycobacterium tuberculosis (Mtb) remains a major global health problem, despite rapid advancements in the medical field.[1] Key to decreasing the morbidity and mortality rate of this disease lies in early detection by rapid and accurate diagnostic testing. Unfortunately, patients that are clinically suspected of having the disease are often sputum smear negative.[2] This is particularly true in the Human Immunodeficiency Virus (HIV) positive population.[3] As such, tissue biopsies are frequently submitted for histopathological evaluation.

Histologically, Mtb displays a pattern of necrotising granulomatous inflammation. As other disease entities may present in this manner, a Ziehl-Neelsen (ZN) stain for acid-fast bacilli (AFB) is also performed, which if present, confirms mycobacterial infection.[4] However, it is not uncommon that even in the presence of high clinical suspicion, the ZN stain is negative, with reported sensitivities of the ZN stain on histological samples ranging from 22% to 80%[5]. Culture, regarded as the gold standard test for detection of Mtb, is accurate but has the pitfall of taking 2-8 weeks to obtain a result[6]. Furthermore, it is not uncommon that clinicians omit submitting a sample for culture at the time of biopsy.

Another diagnostic test is the mycobacterium polymerase chain reaction (PCR), which may be performed on formalin-fixed, paraffin- embedded (FF-PE) tissue. This test is rapid, but its results are dependent on many variables, including primers used,

fixation time, concentration of DNA utilised, length of the PCR product and the PCR protocol itself[7].

This is a retrospective study with the aim of determining the validity of the mycobacterium PCR test utilised in our department. This will be achieved by comparing the PCR results with the culture results from the same or related biopsy site.

CHAPTER 2

2.0 Aims and Objectives

2.1 Aims

The aim of this study is to investigate the validity of the mycobacterium polymerase chain reaction (PCR) assay utilised in the department of Anatomical Pathology in evaluating histological specimens showing granulomatous inflammation without demonstrable acid-fast bacilli.

2.2 Objectives

The objectives of this study are as follows:

- To compare the PCR result obtained from the formalin-fixed, paraffin embedded samples with the culture result from the same or related site, (e.g. bone marrow trephine PCR with bone marrow aspirate culture).
- Describe the age distribution of the study sample.
- Determine the distribution of the biopsy sites.
- Establish in how many cases a clinical suspicion of tuberculosis was raised, based on the clinical history provided.
- Determine the number of patients on antituberculosis therapy at the time of biopsy.
- Establish the number of patients in whom there was no clinical suspicion of tuberculosis, but the PCR results were positive and the culture results were positive.

- To determine in patients who were started on empirical antituberculosis treatment; how many cases yielded positive PCR and culture results respectively.
- Establish the spectrum of mycobacterium species that were cultured and determine if the PCR assay yielded positive results when mycobacterium other than tuberculosis (MOTT) was grown by culture.

CHAPTER 3

3.0 Literature Review

3.1 Introduction

Despite rapid advancements in the medical field, *Mycobacterium tuberculosis* (Mtb) still remains a major global health problem. South Africa is regarded as a "high burden country" by the World Health Organization (WHO), with a reported incidence rate of 860 per 100 000 population in 2013.[1] Key to curbing the scourge of this devastating, yet preventable and treatable disease, lies in establishing a quick diagnosis. Nucleic acid amplification techniques (NAATs) used for the detection of Mtb are rapid. These include assays such as the GeneXpert (Xpert MTB/RIF) which is an automated, real-time nucleic acid amplification assay for the concurrent detection of tuberculosis and rifampicin resistance.[8,9] Line probe assays are able to detect the presence of *M. tuberculosis* complex as well as genetic mutations associated with isoniazid and rifampicin resistance.[8,10,] These tests are rapid and accurate, but are primarily endorsed by the WHO for use on sputum and other fluid samples as well as fine needle aspirate samples.[11] However, the use of these novel tests on histological samples is not yet widely applied. Other PCR based methodologies have been proven useful on histological specimens, but the reliability of PCR is dependent on a number of parameters.

3.2 Epidemiology

3.2.1 Statistics

Tuberculosis (TB) was declared a global public health emergency by the World Health Organization (WHO) in 1993. Today, more than 20 years later, this disease remains a worldwide health challenge.[1]

The statistics are alarming. In 2013, an estimated 9 million people developed tuberculosis and 1.5 million people died from the disease.[1] Thirteen percent of the estimated 9 million people who developed TB in 2013, were HIV positive. The South-East Asian and Western Pacific regions collectively accounted for 56% of the worlds' TB cases in 2013.[1] However, the African region had approximately one quarter of the world's cases and the highest rates of cases and deaths relative to the population size. South Africa, regarded as a high burden country by the World Health Organisation, falls within the top six countries worldwide with the highest incidence rates, with a reported rate of 860 per 100 000 population in 2013.[1] These figures highlight the difficulty in controlling this disease.

3.2.2 Burden of Disease

Tuberculosis places a major burden on the socioeconomic productiveness and viability of a country. This is especially true in high prevalence countries, such as South Africa, where most TB patients are in their twenties to forties, and hence are the most economically productive generation.[12]

3.3 Controlling the disease

The World Health Organization's "Global Plan to Stop TB" strategy has a vision of a TB free world. To accomplish this vision, goals have been set, amongst which are the millennium development goals. One of the millennium development goal targets is to reduce the TB incidence, prevalence and mortality rate by 50% in 2015, compared with their baseline levels in 1990.[1] Whilst progress has been made toward reaching this target, in that the global prevalence has fallen by 41% and the mortality rate has fallen by 45% between 1990 and 2013, the goal is far from being accomplished. [1]

One of the challenges towards achieving this goal is the emergence of multi-drug resistant tuberculosis (MDR TB). This entity has a poor treatment outcome and is an important public health hazard and burden.[12] Globally, approximately 3.5% of new TB cases and 20.5% of previously treated cases had MDR TB in 2013.[1] In line with this issue is the emergence of extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis.* The detection and treatment of this disease is highly dependent on the results of high performance sensitivity tests.[12]

A second challenge facing the health sector in curbing this disease is human immunodeficiency virus (HIV) and Mtb co-infection. In 2013, an estimated 13% of the 9 million people globally who developed TB worldwide were HIV positive.[1] In South Africa alone, a total of 328 896 cases of tuberculosis were notified in 2013. Of these cases, 90% of patients were aware of their HIV status and 62% were HIV positive. These figures highlight the burden of co-infection, particularly within the South African context. [1]

Priority actions are thus needed to accelerate progress towards the millennium development goals. This includes early case detection and diagnosis, rapid testing and detection of MDR TB and HIV testing for TB patients. This is to ensure appropriate treatment and infection control measures are instituted. In May 2014, the World Health Assembly approved a 'post- 2015 global TB strategy'. This strategy aims for a target of a 95% reduction in TB mortality and a 90% reduction in TB incidence by 2035 worldwide (compared with 2015 levels). Accurate and rapid TB diagnostics form a fundamental factor in achieving this goal.[1]

3.4 Diagnosis of Tuberculosis

3.4.1 Conventional methods used for the diagnosis of Tuberculosis

The most widely used, rapid and cost effective method for the diagnosis of pulmonary tuberculosis (PTB) is that of sputum smear microscopy with acid-fast bacilli staining, together with culture for *M. tuberculosis.*[10,13] However, smear and mycobacterium culture can only be performed if a patient is productive of sputum,[8] and these traditional methods have a low sensitivity when sputum samples contain only a small number of organisms.[2]

In order for acid-fast bacilli to be identified on the Ziehl-Neelsen (ZN) stain, 10^4 to 10^6 bacilli per millilitre of tissue or fluid is required.[13] An advancement in conventional tuberculosis microscopy is the development of fluorescent methods (e.g. auramine-rhodamine staining) for the detection of bacilli.[12] This method has increased the sensitivity over that of ordinary light microscopy.[12]

Nevertheless, the gold standard test for the diagnosis of tuberculosis remains culture. [3] This method has a high specificity with a sensitivity of approximately one

hundred fold more than that of smear microscopy.[6] However, mycobacteria are notoriously slow growing, dividing once every 18-21 hours,[14] and require 10^1 to 10^2 bacilli per millilitre of sample for growth to occur.[14,15] Although culture of *M. Tuberculosis* in Lowenstein-Jensen medium is a sensitive assay,[10] it is a protracted process that includes isolation, identification and drug susceptibility testing; thus can take at least 4 to 8 weeks for a result.[6] In recent years, modern liquid media and growth detection systems have been developed, which have been shown to be more rapid and sensitive than the solid medium cultures. [12] The Mycobacteria Growth Indicator Tube (MGIT) is one of the novel culture methods that has an inbuilt fluorescent sensing system which detects even small amounts of bacterial growth based on oxygen concentration changes. [12] A meta-analysis comparing BACTEC MGIT960 and conventional solid media showed that the mean time to detection of bacterial growth (for all mycobacterial species) was 12.9 days as compared to 27.0 days using the Lowenstein-Jensen solid medium. [16] Smear negative specimens took a mean time of 16.5 days as compared to 33.7 days using BACTEC MGIT960 and Lowenstein-Jensen medium respectively.[16] Although the MGIT method of culture shows an improvement in the turn-around time for the culture result, there is still an inherent time limitation of all culture-based diagnostic assays. This potentially has serious implications on patient management, as well as on the implementation of appropriate infection control measures.[5] In resource poor settings, the selective use of mycobacterium culture is recommended, especially in cases with repeated negative smear results.[15] Whilst culture is regarded as the gold standard diagnostic test for Mtb, the specificity of this test may also be diminished due to contamination of specimens in the laboratory. It is reported that despite the use of the best decontamination procedures, a false positive rate of 1 to 4% can be expected.[15]

3.4.2 Role of cytology and histology in the diagnosis of Tuberculosis

In the immunocompromised population, especially in those individuals infected with HIV, the diagnosis of tuberculosis is more challenging. Factors contributing to this include an increase in the frequency of false negative sputa.[3] This increase in sputum smear negativity is contradictory, as patients who are immunosuppressed tend to have higher bacterial loads. The increase in false negative sputa is in part due to the decreased likelihood of bronchial damage and cavitation, thus decreasing the likelihood of bacilli being expelled in the sputum.[3] Furthermore, the greater the degree of immune suppression, the greater the likelihood of extrapulmonary tuberculosis. The frequency of extrapulmonary tuberculosis rises to greater than 50% in those with severe immune deficiency.[3] Therefore, in cases of suspected pulmonary TB (based on clinical features and chest X-Ray findings), with repeated negative sputum smear and culture results; and in cases with extrapulmonary involvement, the cytological as well as the histological evaluation of tissue samples play a key role in confirming the diagnosis.

Tuberculous lymphadenitis is the commonest manifestation of extrapulmonary tuberculosis.[17,18] It accounts for approximately 30-52% of cases of peripheral lymphadenopathy in countries with a high incidence of tuberculosis.[17] In the setting of either peripheral lymphadenopathy or a palpable and accessible mass, fine needle aspiration biopsy (FNAB) has been shown to be the diagnostic procedure of choice.[17,18] This procedure offers a feasible and safe option of specimen collection and can be easily performed in an out-patient setting.[19] Fine needle aspiration biopsy has a diagnostic yield that ranges from 42-83%.[17,18] The procedure involves aspiration of a palpable mass utilising a syringe and needle and preparing at least

one smear for Papaniculaou staining and one air dried smear for Giemsa or Diff-Quick staining and subsequent Ziehl-Neelsen staining.[19] Residual material is subsequently rinsed in mycobacterial culture media.[19] Using the aforementioned method, a local study in HIV positive adults with suspected mycobacterial lymphadenitis showed a diagnostic yield of 80%.[17] Whilst the use of cytology with confirmation of the presence of organisms using acid-fast stains has excellent diagnostic yields, speciation and drug sensitivity testing is only possible through mycobacterial culture or novel molecular techniques.[17,19]

A major challenge in the diagnosis of extrapulmonary tuberculosis lies in the atypical clinical presentation of the disease, given that it often mimics other inflammatory and neoplastic conditions.[9,20] A high degree of clinical suspicion is often required for an early diagnosis. The problem is confounded in cases where the disease presents at sites that are not easily amenable to FNAB, such as the kidney, female genital tract or vertebral spine.[9, 21, 22] In this clinical scenario, histopathological evaluation of tissue specimens play an important role in the diagnosis of tuberculosis.

The key features that are suggestive of mycobacterial infection on histopathological examination include granulomatous inflammation, which may or may not show caseous necrosis, and Langhans-type giant cells.[4] Although tuberculosis is the prototypical granulomatous disease, granulomatous inflammation can be found in a wide variety of clinical entities, amongst which include sarcoidosis, cat scratch disease, lymphogranuloma inguinale, leprosy, brucellosis, syphilis, mycotic infections, berylliosis and some autoimmune diseases. [4, 14, 20, 23]

By applying a Ziehl-Neelsen stain to the formalin-fixed, paraffin-embedded tissue, a diagnosis of tuberculosis can be confidently made if acid-fast bacilli (AFB) are

demonstrated. Factors that influence the detection of AFB visualisation include the type of stain used (Ziehl-Neelsen stain , Kinyoun acid-fast stain, Fite stain, auramine-rhodamine stain, or silver impregnation).[24] The former three stains exploit the ability of the mycobacterium bacillus to retain their staining quality despite decolourisation with acids due to the large amount of lipid in their cell walls. [24] In formalin-fixed, paraffin embedded tissue, acid-fast stains have been shown to have a lower sensitivity, which may be attributable to the formalin and/or xylene treatment required for tissue processing.[25] It is postulated that formalin and /or xylene may alter the property of the bacillus.[25] Demonstration of the mycobacterial antigen by immunohistochemistry is also an option; however, this method has not been widely adopted. [24] Detection of mycobacteria is an often slow and tedious process. Acid-fast bacillus staining has a sensitivity ranging between 22-80%.[5] A study investigating the sensitivity of the ZN stain in lymph node specimens previously diagnosed as tuberculous lymphadenopathy by conventional histopathology, showed that only 3% of cases were positive by ZN staining.[26] Furthermore, another study showed that only 17 of the 53 (32%) of the histological samples showing granulomatous inflammation in patients who were clinically suspected as having TB, were AFB positive.[25] It is therefore evident that in cases with a high clinical suspicion for tuberculosis that demonstrate granulomatous inflammation on histological sections, it is not uncommon for AFB not to be visualised. Thus, mycobacterial infection cannot be conclusively diagnosed if the tissue was not submitted concurrently for mycobacterial culture. The difficulty with extrapulmonary tissue samples is the paucibacillary nature of extrapulmonary disease.[23] Due to the low yield of bacilli, extrapulmonary tuberculosis is associated with low sensitivity of acid-fast bacilli smear and culture evaluation.[23] Nevertheless, it is extrapulmonary

TB, including tuberculous meningitis, pleural effusions and tuberculous osteoarthritis to name a few, that require a prompt, accurate and specific diagnosis.[20] Therefore, developing a means for rapid, specific and sensitive identification of *Mycobacterium tuberculosis* is desirable.[14]

3.4.3 Nucleic Acid Amplification Techniques

The advent of molecular diagnostics has resulted in the development of rapid and sensitive assays which have revolutionised the diagnosis of tuberculosis.[28] Nucleic acid amplification techniques (NAATs) are at the forefront of this technology. There are currently two novel assays that use molecular detection methods that have been endorsed by the WHO for use on patient samples. This includes the Xpert MTB/RIF assay and line probe assays. [8]

3.4.3.1 Xpert MTB/RIF

Xpert MTB/RIF (Cepheid, Sunnyvale, CA, United States) is an automated, real time polymerase chain reaction (PCR) assay that can simultaneously detect *Mycobacterium tuberculosis* DNA and rifampicin resistance in less than two hours.[28] This technology uses a closed cartridge system that contains preloaded liquid buffers and lyophilized reagent beads that are required to process the clinical sample. The assay uses PCR based technology to amplify a 192 base pair (bp) segment of the bacterial ribose nucleic acid (RNA) polymerase rpoB gene. Within this 192bp sequence, lies an 81bp region. Greater than 95% of all rifampicin resistant strains of *Mycobacterium tuberculosis* contain mutations that are localised within this 81bp region. Hence, it is possible to detect the presence of Mtb DNA and rifampicin resistance simultaneously by targeting a single gene target.[8, 9] The assay

uses molecular beacon technology to detect amplicons using real-time PCR. This technology utilises nucleic acid probes that recognise the presence or absence of the rifampicin susceptible (wild type) sequences within the rpoB gene of Mtb. Five different coloured beacons are used, each spanning a different nucleic acid sequence within the rpoB gene. When the beacon binds, it fluoresces, indicating that the rifampicin susceptible gene sequence is present. Failure of fluorescence of a beacon is an indication that the sample is probably resistant to rifampicin.[11]

In December 2010, the WHO endorsed the expansion of the Xpert MTB/RIF test. They recommended its use as the initial diagnostic test in adults and children presumed to have MDR TB or in HIV-associated TB.[11] Since 2010, there has been a vast amount of research regarding the efficacy of the Xpert MTB/RIF test, including its use in the diagnosis of pulmonary, extrapulmonary and paediatric TB. As a result, the WHO published a policy update, in which the contemporary research performed with regard to this assay was systematically reviewed.[11]

The key findings from this policy update were as follows:[11]

- When used as an initial diagnostic test, replacing conventional smear microscopy, Xpert MTB/RIF achieved a pooled sensitivity and specificity of 88% and 99% respectively.
- When used as an add-on test following a negative smear microscopy result, Xpert MTB/RIF yielded a pooled sensitivity and specificity of 68% and 99% respectively.
- Xpert MTB/RIF on fresh or frozen tissue samples from any site excluding lymph nodes demonstrated varied sensitivities, ranging from 42% to 100%.

The pooled sensitivity and specificity was 81.2% and 98% respectively. (Twelve studies were included).

- Performance of Xpert MTB/RIF on lymph node biopsy or aspirate samples, revealed a pooled sensitivity and specificity of 84.9% and 92.8% respectively. (Fourteen studies were included).[11]

Given the above findings, the WHO recommendation for the use of Xpert MTB/RIF in the diagnosis of extrapulmonary TB and rifampicin resistance in adults and children is that "it may be used as a replacement test for usual practice (including conventional microscopy, culture or histopathology) for testing specific nonrespiratory specimens (lymph nodes and other tissues) from patients suspected of having extrapulmonary TB". [11]

Three recent studies evaluating the Xpert MTB/RIF assay were conducted in South Africa, a country with one of the highest rates of TB and HIV prevalences.[1] In the first study undertaken by Theron *et al*, [29] sputum samples from 496 patients with suspected TB (at two primary health care clinics in Cape Town), were analysed by Xpert MTB/RIF. In this study, the sensitivity of the assay in smear positive, culture positive cases was 95% and the specificity was 94%. The sensitivity of smear negative cases was 55%.[29]

The second study assessed the use of the Xpert MTB/RIF test in the diagnosis of spinal (extrapulmonary) TB in 71 patients. In this study, the sensitivity and specificity of the assay was 95.6% and 96.2% respectively, as compared to the mycobacterium culture results.[30]

A recent, local study undertaken by Scott *et al*[31] determined the diagnostic accuracy of Xpert MTB/RIF in extrapulmonary specimens.[31] A total of 1175 specimens (including cerebrospinal fluid, fine needle aspirate samples, tissue samples, body cavity fluids, urine, stool and bone) were analysed with the Xpert MTB/RIF assay and compared to the culture results. Only 2% of the study sample were tissue samples, for which an additional step of tissue homogenisation was required. The Xpert MTB/RIF assay showed good performance in pus (sensitivity of 91%) and aspirate samples (sensitivity of 80%). Although the sample size for the tissue specimens was too small to attain statistically significant results, the study showed that the Xpert MTB/RIF assay detected two more positive results as compared to culture. Importantly, this study also illustrated that the Xpert MTB/RIF assay is less affected by contamination from bacteria as compared to culture. Thus, its use in the diagnosis of extrapulmonary TB could reduce labour in the laboratory and subsequently diminish diagnostic delay.[31]

However, to the investigator's knowledge, the diagnostic use of Xpert MTB/RIF on FF-PE tissue has not been sufficiently evaluated. Furthermore, the use of this assay on tissue samples is not part of the South African National Department of Health's Xpert algorithm.[32] Thus, it is clear that although the Xpert MTB/RIF offers a rapid and sensitive method of diagnosing tuberculosis, with an additional advantage of detecting rifampicin resistance, other methods are required for the diagnosis of smear negative pulmonary TB and extrapulmonary TB on FF-PE tissue samples.

3.4.3.2 Line probe assays

Line probe assays were endorsed for use by the WHO in 2008 for the detection of Mtb and drug resistance from smear positive individuals who were at risk for MDR

TB.[8] This technique is based on the principle of reverse hybridisation, in which mycobacterial RNA is amplified by PCR.[10] Specific oligonucleotides are immobilised at known locations on a membrane strip with a biotin labelled PCR product and the hybrids formed are detected colourimetrically.[8] The commercially available MDRTBplus line probe assay (Hain Lifesciences, Germany) allows direct detection of *M. tuberculosis*, isoniazid and rifampicin resistance from smear positive pulmonary specimens.[8] This assay has not been endorsed for used on formalin fixed tissue specimens.

3.4.3.3 Nested Polymerase Chain reaction (PCR) in formalin-fixed, paraffin- embedded tissue

Amplification of *Mycobacterium tuberculosis* DNA (deoxyribose nucleic acid) in formalin-fixed, paraffin-embedded (FF-PE) tissue samples is useful in patients with pulmonary and extrapulmonary TB, where the diagnosis depends on examination of tissue samples.[2] PCR is of value when there is high clinical suspicion for tuberculosis, but clinical samples have not been submitted for culture.[7] PCR performed on FF-PE tissue is of benefit under these circumstances, as this technique can detect the presence of mycobacterium DNA in tissue that has been preserved in formalin or other fixatives that preclude the possibility of culture.[2] The nested technique of PCR consists of two consecutive rounds of PCR. The second round amplifies a DNA sequence within the first amplification product.[33] This amplification assay can produce a result within two to three days, whereas culture may require between two to six weeks.[7] The test can detect a limit of 1-10 bacilli in various clinical samples.[14] In a study by Rish *et al*, as few as 9 organisms could be

detected by nested PCR in a 5 micrometer (µm) section of tissue.[34] Factors that may affect the result of this assay have been extensively researched; including the type of fixatives used, the DNA extraction procedure, the length of the PCR target, the concentration of the target DNA amplified and the PCR target itself.[7]

A good target sequence for the PCR assay is one that will generate a species specific amplicon with a high degree of sensitivity and specificity.[10] The target sequence for analysis should have the following features:

1. A common sequence for all members of the mycobacterium genus, which may function as the primer binding site. [10]
2. Variability within the amplified region between the two primer binding sites which allows for differentiation between species.[10] Thus, a vital aspect of this assay is the choice of a superior target sequence that will generate a species specific amplicon.[10]

Targets for DNA amplification include the insertion sequence 6110 (IS6110), 65 kilo Dalton (kDa) heat shock protein (hsp65), MPB64 protein, 38KDa protein and ribosomal DNA.[14]

Two commonly used targets in clinical practice are IS6110 and hsp65.

IS6110 is usually present in multiple copies within the genome of all members of *Mycobacterium tuberculosis* complex.[7] Species within the Mtb complex include *M. tuberculosis* (Mtb)*, M. bovis, M. bovis BCG, M. africanum, and M. microti*. The IS6110 sequence has been shown to have as many as 24 copies in Mtb, but only a few copies in *M. bovis* and only one copy in *M. bovis BCG*.[14] The target sequence will not amplify in mycobacteria other than tuberculosis (MOTT), namely *M. avium,*

M. intracellulare complex and *M. Kansasii*.[5] Hence, the IS6110 target sequence is specific for *Mycobacterium tuberculosis* complex.

Many studies have assessed the accuracy of PCR using IS6110 as the target sequence. Most studies compared their results against Mtb culture results, which is the gold standard. Other studies, however, compared their PCR results against the clinical suspicion of tuberculosis (based on signs, symptoms and radiological findings). Some authors compared PCR results to histopathological features that were suggestive of tuberculosis. Sensitivities range from 72% to 100%, whilst specificities ranged from 73 to 99%,[2,6,14,21,22,27,33,35,36] **(Table 1)**. However, it should be noted that many of these studies used samples that either had positive smear results or positive culture results for *Mycobacterium tuberculosis,* i.e. included samples with high bacterial loads.

In a study conducted by Salian *et al*, [2] the authors compared the Mtb PCR results on formalin-fixed, paraffin embedded tissue to mycobacterium culture results in 53 of their 60 cases. Their cases included tissue that was AFB positive on histopathological examination. TB PCR (targeting IS6110) yielded a sensitivity of 100% and a specificity of 93%. Of their 3 false positive cases, 2 patients had a prior history of tuberculosis and in the third case, the patient had a good response to antituberculosis therapy despite the negative culture result.[2]

Park and colleagues performed a study on formalin-fixed, paraffin embedded lung tissue.[33] Of their 223 cases, 152 had mycobacterium culture results available. This study also included cases that were AFB positive. Using the IS6110 gene target for PCR, and comparing the results with that of culture; TB PCR yielded a sensitivity of 85% and a specificity of 99%.[33]

Only one study was performed on cases of suspected tuberculosis, where histopathological examination revealed granulomatous inflammation but the Ziehl-Neelsen stain failed to demonstrate acid-fast bacilli.[14] In this study, the PCR result was compared against the clinical diagnosis and the results showed the lowest sensitivity and specificity as compared to other studies. Only a proportion of cases (68 patients in whom there was a clinical suspicion of TB) had culture results available. The authors subsequently compared the PCR results with the culture results and demonstrated a false positive rate of 34% and a false negative rate of 4%.[14] This study showed that in paucibacillary samples, PCR tends to demonstrate inferior results.

Jain *et al*[22] investigated the value of TB PCR in paucibacillary cases of suspected osteoarticular tuberculosis. In this study, TB PCR was compared against the clinicoradiological diagnosis of TB and the 16sr RNA gene was the target for PCR. Notably, the mycobacterium culture result was positive in only 6 cases of clinically suspected TB, whilst the PCR was positive in 49 of the 50 cases. The results of this study suggest that in paucibacillary cases, PCR may perform superiorly compared to culture.[22]

Numerous other studies have evaluated the performance of TB PCR in microbiological specimens (such as sputum, pleural fluid, cerebrospinal fluid etc.).[6, 36, 37] Majority of the studies utilised the IS6110 PCR target. Cheng *et al* [36] included AFB positive specimens; reported a sensitivity of 75.9%, whilst Gholoobi *et al*[6] reported a sensitivity of 58.3%.

The 65KDa heat shock protein (hsp 65) is another commonly used target using nested PCR, followed in some instances by restriction fragment length polymerase

(RFLP) analysis.[10] The hsp 65 gene is highly conserved in all mycobacterium species. Its protein product contains genus specific epitopes.[10] Hence, restriction enzyme digestion of the hsp 65 PCR product can be used in differentiating *M. tuberculosis* from other species. In a study conducted by Cook *et al*, [38] the authors developed a simple nested PCR protocol, in which amplification of a 133bp fragment of the hsp 65 gene, together with RFLP analysis using three restriction enzymes, was successfully able to detect and distinguish between *M. tuberculosis* and atypical species of mycobacteria.[38] A study evaluating PCR using the hsp65 target showed a false positive rate of 27% (3 of 11 cases). However, it was noted that in all three of these false positive cases, there was a culture confirmed diagnosis of *M. avium* complex.[7] Other parameters that may affect the results of PCR include the type of fixatives used, the length of the PCR target and the concentration of the target DNA amplified.[7]

AUTHOR AND DATE	N0. OF SPECIMENS	TISSUE TYPE	PCR TARGET	FINDINGS
Jackson *et al*, 2000[14]	115 specimens, 68 were compared with culture results.	FF-PE tissue. Pulmonary & extrapulmonary	IS6110	Overall sensitivity: 72% Overall specificity: 72% Compared against culture: 2FN and 22 FP
***Jain et al*, 2008**[22]	50 cases from osteo-articular sites	PCR on fresh tissue/ aspirate material	16rs RNA	Comparing PCR with clinicoradiological diagnosis as gold standard: sensitivity: 78.6% specificity: 87.1%
Salian *et al*, 1998[2]	60 cases, culture results available in 53 cases.	FF-PE tissue. Pulmonary & extrapulmonary	IS6110	Sensitivity: 100% Specificity: 93%
Park *et al*, 2003[27]	81 cases, no culture results available	FF-PE tissue. Pulmonary & extrapulmonary	IS6110	Comparing PCR with clinical diagnosis: Sensitivity: 78% Specificity: 88%
Park *et al*, 2010[33]	223 cases, culture results available in 152 cases.	FF-PE lung tissue.	IS6110	Comparing PCR with culture: Sensitivity: 85% Specificity: 99%
Chawla *et al*, 2009[35]	104 cases, no culture results available	PCR on fresh extrapulmonary tissue	IS6110	Comparing PCR with histopathology as gold standard: sensitivity: 74.1% specificity: 96.1%
Cheng *et al*, 2004[36]	155 cases, culture results available in 112 cases	Microbiological specimens (Fluids etc.)	IS6110	Comparing PCR with culture: Sensitivity: 75.9%
Gholoobi *et al*, 2012[6]	30 cases	Microbiological specimens (Fluids etc.)	IS6110	Comparing PCR with culture: Sensitivity: 58.3% Specificity: 77.8%
Thangappah *et al*, 2011[21]	72 cases, 49 cases submitted for PCR	FF-PE tissue from female genital tract	IS6110 & TRC4	Comparing PCR to clinical suspicion of TB: Sensitivity: 57.1%

Table 1: Comparison of the findings from similar studies investigating the efficacy of the mycobacterium PCR assay. *FF-PE = Formalin fixed, paraffin embedded; FN = False negative; FP = False positive.*

In a study conducted by Barcelos *et al*, [39] the authors illustrated that whilst the length of time that a specimen is fixed in formalin does not influence the PCR result; the type of formalin does. Specifically, buffered formalin presented better results as compared to 10% non-buffered formalin.[39] Rish *et al* also showed that up to 7 days of fixation in 10% neutral buffered formalin had a negligible effect on the PCR test.[34]

The longer the fragment length of an amplified target the greater the likelihood of degradation of the amplicon. This is due to the fact that PCR products with a higher molecular weight tend to fragment more easily. This was highlighted by Marchetti *et al*, [7] where the authors showed that PCR target sequences that were longer (223 and 143 base pairs respectively), showed worse results in terms of sensitivity as compared to target sequences that were 106 and 123 base pairs in length.[7] The same authors also showed that the concentration of DNA used may also influence the PCR result. In their study, DNA concentrations of 1microgram (µg) and 3µg yielded the best results in terms of sensitivity, whilst DNA concentrations of 5µg had the highest number of false negative results.[7]

It is thus clear that several factors may influence the results of the PCR assay. In order to optimize results, an individual laboratory should pay attention to all of the above mentioned factors when designing their PCR protocol.

3.5 Limitations of the mycobacterium polymerase chain reaction assay

3.5.1 False positive and false negative results

Although TB PCR has been shown to be a rapid, sensitive and specific diagnostic test, it has some limitations. The major limitation of PCR is that it cannot distinguish

live from dead bacilli as the test is not dependent on bacterial replication.[22, 35] As such, PCR results should be interpreted with caution in patients with reactivation TB, asymptomatic infection and in those who have recently received antituberculosis treatment.[14, 35] In cases where the PCR test is positive but the subsequent culture result is negative, there is a major clinical dilemma as to whether antituberculosis treatment should be maintained or discontinued. Many authors suggest that under these circumstances, if all precautions have been taken to avoid contamination of the PCR procedure, then the results of the positive PCR should be judged in light of the clinical picture and histopathological features. [14, 35, 36]

Due to the extremely sensitive nature of the PCR test, false positive results are mainly due to carry-over contamination. [14, 35, 21] Therefore, meticulous specimen handling, including DNA extraction and PCR setup should be undertaken for reliability of results.[14]

False negative PCR results are mainly a consequence of the paucibacillary nature of extrapulmonary TB. Other contributory factors for false negative PCR results include loss of DNA during extraction; uneven distribution of mycobacteria in tissue samples; inadequate samples submitted for PCR; extensive necrosis within the tissue and inhibitors to PCR that may be present in the sample. [14, 33, 35] In order to circumvent these problems, appropriate controls (internal control such as amplification of the beta-globin (β-globin) gene, as well as positive and negative controls) need to be incorporated to the PCR protocol.[14]

3.5.2 Advantages and disadvantages

In summary, the advantages of PCR carried out on formalin-fixed, paraffin embedded tissue are as follows:

1. It is a sensitive and rapid diagnostic assay, particularly useful if tissue has not been concurrently submitted for culture.[22]
2. The test is of great value in circumstances where disease progression is rapid and destructive, and diagnosis by culture confirmation would delay treatment with undesirable and unfavourable consequences.[22]
3. As PCR is a very sensitive test that can detect the presence of only a few bacilli, a very small quantity of specimen is required for a diagnosis to be made.[26]
4. PCR can differentiate between *Mycobacterium tuberculosis* complex and atypical mycobacterial species.[22]

The disadvantages of PCR carried out on formalin-fixed, paraffin embedded tissue are as follows:

1. PCR cannot differentiate between dead and live bacterial organisms and as such, cannot differentiate between active, latent and recently treated disease.[26]
2. The diagnostic accuracy of 'in-house' methods may be variable.[12]
3. PCR has suboptimal sensitivity in paucibacillary cases. [14,33,35]
4. High initial capital costs and maintenance costs may limit is use in resource-poor settings.[10]

3.6 Conclusion:

PCR is a rapid diagnostic test that may be very valuable especially in circumstances where tissue has not been concurrently submitted for TB culture. However, due to the many factors that can alter the reliability and reproducibility of this molecular test, an individual laboratory should validate its use.

CHAPTER 4

4. 0 Materials and Methods

4.1 Clinical laboratory and hospital setting

The department of Anatomical Pathology, University of Witwatersrand and the National Health Laboratory Services (NHLS) affords its services to patients within the greater Johannesburg region. This includes patients attending Charlotte Maxeke Johannesburg Academic Hospital, Chris Hani Baragwanath Hospital, Helen Joseph Hospital and others. The histopathological reporting of all tissue samples from patients attending these hospitals is carried out by this department.

The records of all cases of mycobacterium PCR that were performed on formalin-fixed, paraffin embedded tissue samples in the department of Anatomical Pathology, University of Witwatersrand from the period 2008 to 2012 were retrieved.

4.2 Study design

This is a retrospective cross sectional, non-interventional laboratory based study.

4.2.1 Sample selection

Using the 'DISA' laboratory database system, the histological reports for all cases showing granulomatous inflammation with subsequent negative Ziehl-Neelsen (ZN) stains for acid-fast bacilli were retrieved. Using 'DISA', cases for which corresponding mycobacterium culture results were available, were then identified. The mycobacterium culture test must have been performed on a specimen retrieved

from the same site as the biopsy sample submitted for histology, or from a closely related site, e.g. bone marrow trephine submitted for histology and bone marrow aspirate submitted for mycobacterium culture evaluation.

The histological haematoxylin and eosin (H&E) slides as well as the corresponding Ziehl-Neelsen (ZN) stains for each of these cases were reviewed by the author as well as a pathologist. This was to ensure that the histological pattern of inflammation was indeed granulomatous and that the ZN stain was negative for acid-fast bacilli, **(Figures 1- 4).**

The mycobacterium polymerase chain reaction results for all these selected cases were then reviewed.

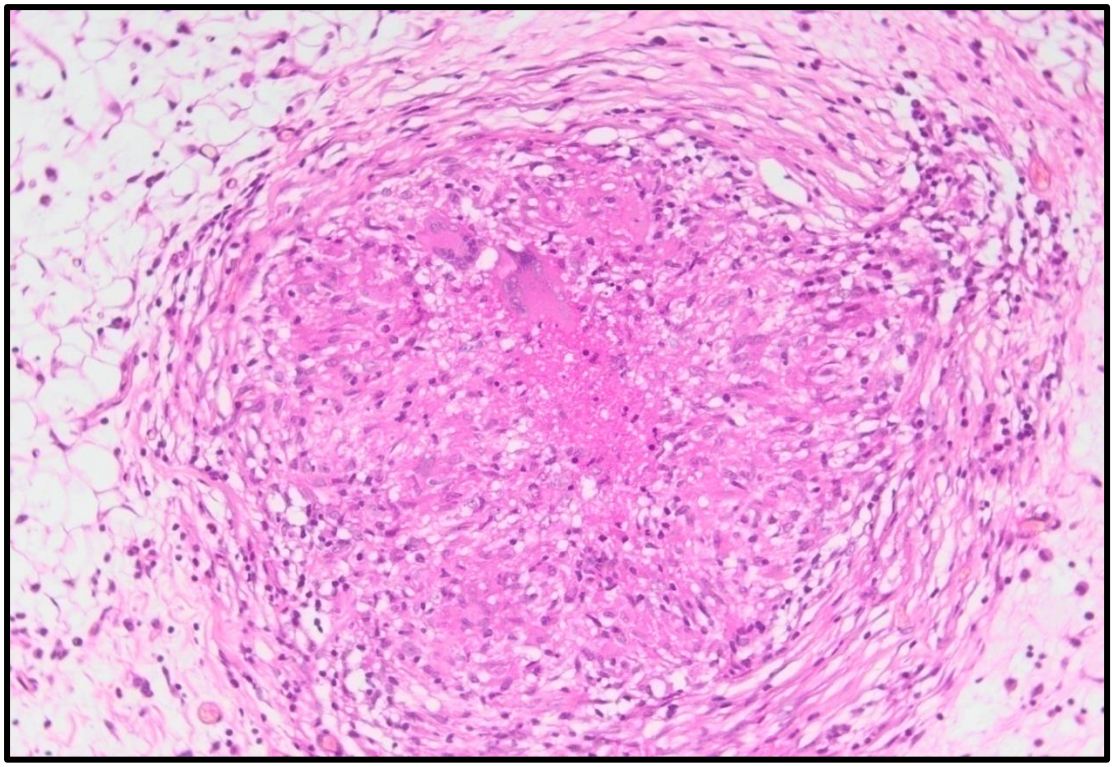


Figure 1: Necrotising granulomatous inflammation. *This photomicrograph illustrates a necrotising granuloma comprising an aggregate of epithelioid histiocytes and Langhans-type giant cells; surrounded by a chronic inflammatory cell infiltrate. (H&E, 4 µm; original magnification 200x).*

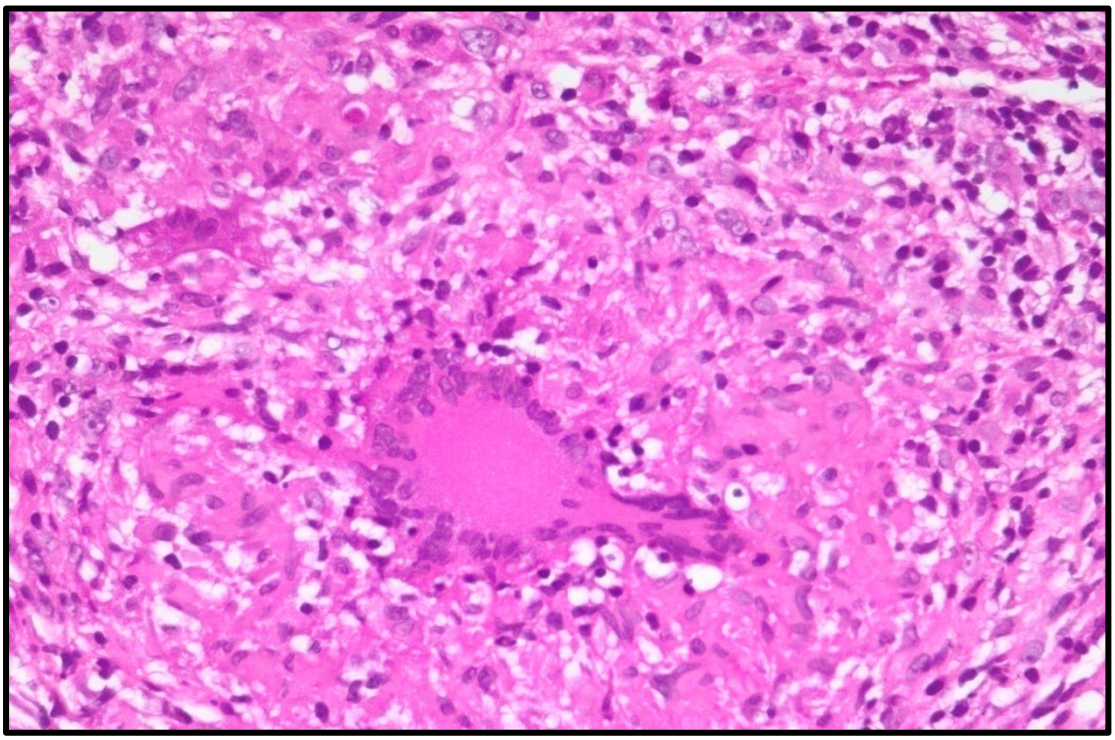


Figure 2: Necrotising granulomatous inflammation with a giant cell at the centre of the field. *(H&E, 4 µm; original magnification 400x).*

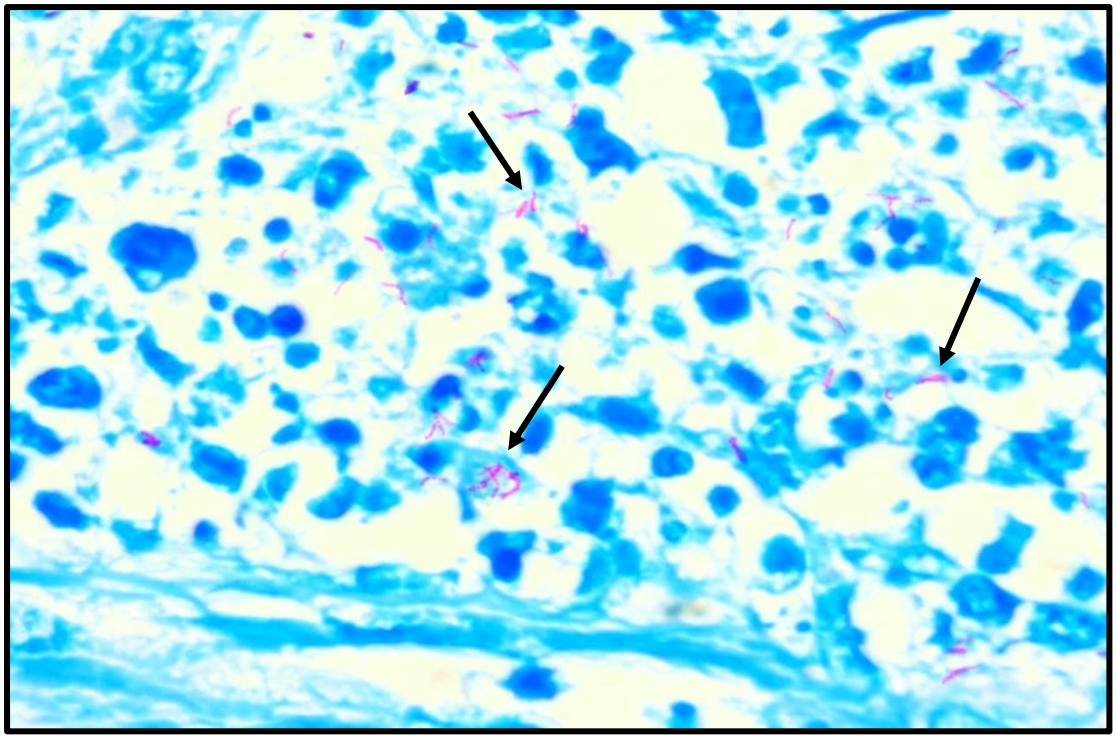


***Figure 3**: **Positive Ziehl-Neelsen stain with acid-fast bacilli indicated by arrows.** (ZN, 4 µm; original magnification 1000x).*

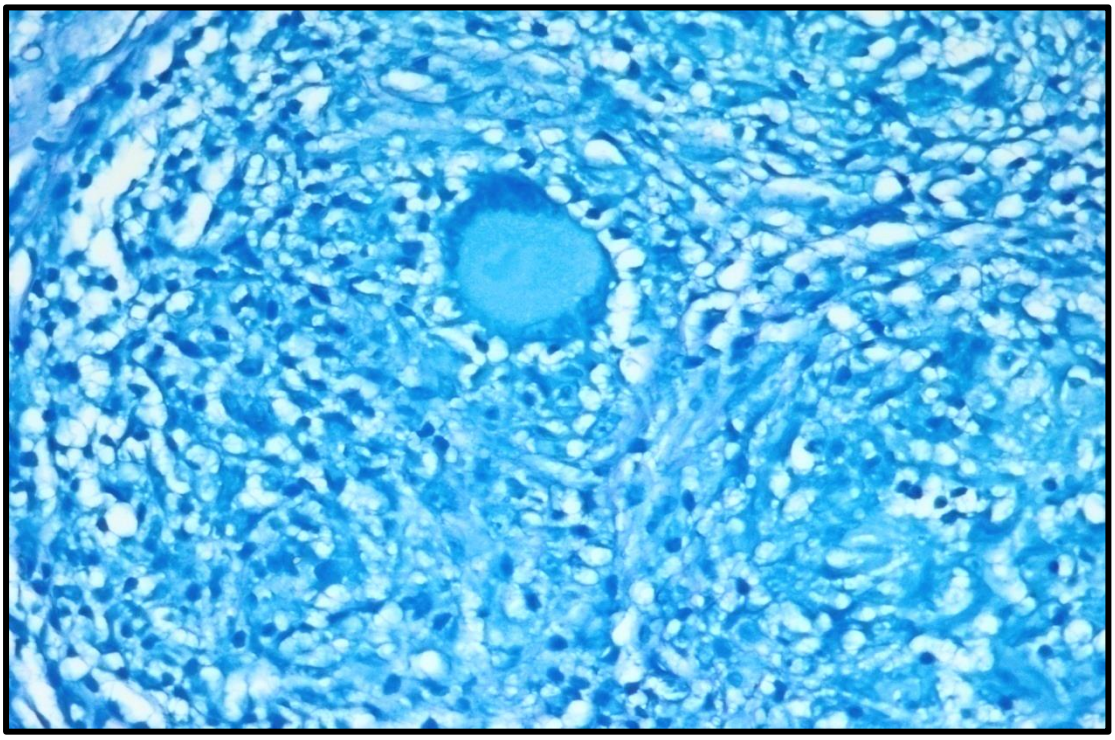


***Figure 4: Necrotising granulomatous inflammation with a negative Ziehl-Neelsen stain.** (ZN, 4 µm; original magnification 400x).*

4.2.2 Mycobacterium polymerase chain reaction

A. DNA extraction/ isolation:

Ten micrometre sections are prepared from the formalin fixed, paraffin embedded samples. The blocks are sectioned using new blades for each sample to prevent cross contamination and the work area as well as work tools are cleaned with 3% Virkon between each block handled. Work areas are also decontaminated with ultra violet light between subsequent procedures. These processes are in place to control cross contamination.

The paraffin is dissolved using 1ml xylene, followed by treatment with 1ml ethanol. Following centrifugation, DNA is extracted from the samples using the DNA Micro QIA amp kit (Qiagen, Whitehead Scientific), according to manufacturer's instructions. DNA is quantified using the Nanodrop 1000 Spectrophotometer.

B. Mycobacterium Nested PCR[38]

Nested PCR is performed using primers designed to amplify a region of the gene encoding the 65-kDa mycobacterial heat shock protein.

The first round of nested PCR produces amplicons that are of 310 and 231 base pairs in length. The following components are added to obtain a 50 µl standard reaction: "5µl of template DNA, 200µM dNTP's (Roche), 0.38 µM of each primer viz. T1U1, T1U2 and T1D (Whitehead Scientific), **(Table 2)**. 1.0U Taq DNA polymerase (Roche), 10x Reaction Buffer (with $MgCl_2$, 15mM) in a total volume of 50µl".

The samples are placed in the 9700 Gene Amp PCR System (Life Technologies), under the following thermal conditions: "Initial denaturation to 94ºC for 4 minutes;

subsequent 35 cycles consistent of 94 ºC for 1 minute (denaturation), 57 ºC for 2 minutes (annealing) and 72 ºC for 2 minutes (extension). A final extension at 72 ºC for 7 minutes completes the PCR run."

The second round of nested PCR results in a 133 bp product. For nested reamplification 5 µl of first round PCR product is transferred into a 45 µl of master mix solution containing second primers T2U and T2D, **(Table 2)**. dNTP's, primers and Taq DNA polymerase are maintained at the concentrations of the first round master mix. PCR is repeated as above, except that an annealing temperature of 52 ºC is used.

C. Agarose gel electrophoresis:

The amplified second round PCR products are then visualised by agarose gel electrophoresis. The PCR products are electrophoresed at 100 volts utilising a 3% agarose gel(Celtic Diagnostics). After completion of electrophoresis the amplified fragments are visualised with ethidium bromide (Merk) staining on a UV-transilluminator and photographed. Positive samples appear as a visible band with a molecular size of 150bp, **(Figure 5)**.

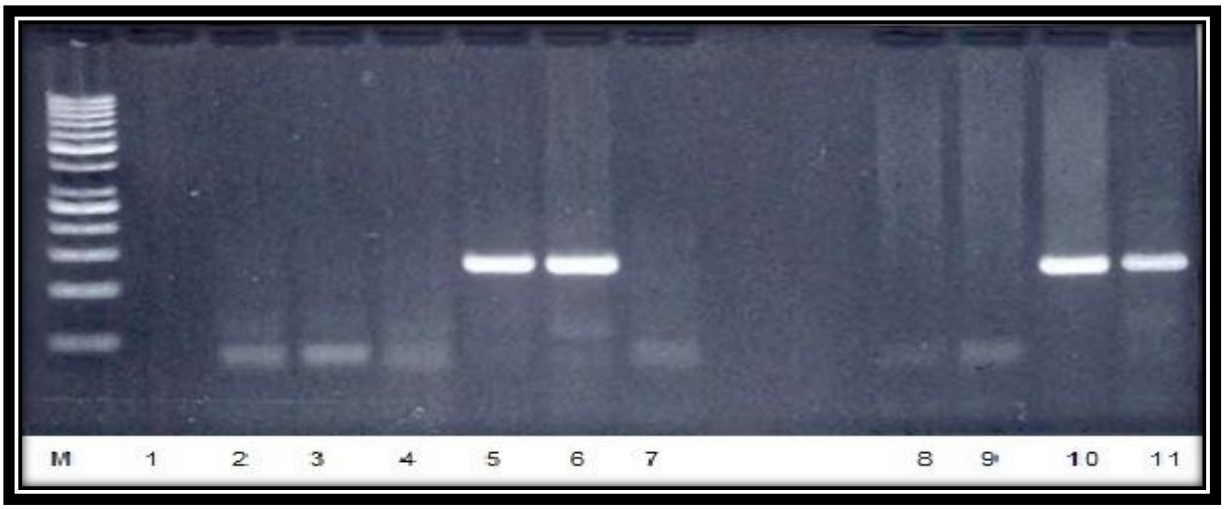


Figure 5: Agarose gel photograph of mycobacterium PCR products. *A positive result is seen on lanes 5, 6 and 10. M= Molecular weight ladder at 50 bp gradations. 11= Positive control.*

D. Controls

Both positive and negative controls are utilised. The positive control includes paraffin embedded samples that had previously tested positive with the Ziehl-Neelsen stain for acid-fast bacilli. The negative control used includes a no-template control in which nuclease-free water is substituted as a template.

Primers PC04/ GH20 (Whitehead Scientific), **(Table 2)**, targeting the β-globin housekeeping gene serves as a control for efficacy of extraction and amplification of DNA from paraffin embedded tissue material.

E. Real-time amplification of the β-globin gene[40]

This assay is performed using the Corbett Research RotorGene 6000 (Whitehead Scientific) RT-PCR machine using the Bioline SensiMix™SYBR No-Rox kit (Celtic Diagnostics). A final volume of 20 µl reaction mix is made using "0.2 mM of each

primer, 10 µl 2x SensiMix™SYBR No-Rox Master Mix (with $MgCl_2$, 50mM) and 2µl of template DNA". The thermal cycling profile of this assay consists of an initial denaturation step at 95ºC for 10 minutes, followed by 50 cycles consistent of 95ºC for 10 seconds (denaturation), 55ºC for 10 seconds (annealing) and 72 ºC for 15 seconds (elongation). After amplification, melt curve analysis is carried out at 95ºC with a ramp rate 1ºC/5seconds. The average melting temperature (T_m) of the β-globin amplicon is 85.5 ±1.0°C. **(see Annexure 1)**

Primer Name	Sequence
T1U1	5'-AAG GAG ATC GAG CTG GAG GA –3'
T1U2	5'-AGG CGT TGG TTC GCG AGG G –3'
T1D	5'-TGA TGA CGC CCT CGT TGC C –3'
T2U	5'-GTC TCA AAC GCG GCA TCG –3'
T2D	5'-GTC ACC GAT GGA CTG GTC –3'
PCO4	5'-CAA CTT CAT CCA CGT TCA CC-3'
GH20	5'-GAA GAG CCA AGG ACA GGT AC-3'

Table2: Mycobacterium PCR primers (T1U1, T1U2, T1D, T2U and T2D) and β-globin PCR primers (PCO4 and GH20).

F. Interpretation of the PCR results:

Given that the controls produce the expected results, the clinical samples are interpreted as follows:

1. A sample in which a 133 bp fragment is amplified, is reported as positive, regardless of the outcome of the internal control.
2. A sample in which a 133 bp fragment is not amplified, is reported as negative.
3. A sample that is negative, in which the internal control is negative, is reported as invalid.

4.3 Sample and sample size

4.3.1 Inclusion criteria

- Included in the study sample were histological cases from 2008 to 2012, where the histology showed granulomatous inflammation with a negative Ziehl-Neelsen stain.
- All of these cases had to have been submitted for TB PCR and must have had a corresponding culture result.

4.3.2 Exclusion criteria

- Excluded from the study sample, were all cases wherein the histology slides or the original paraffin-embedded tissue blocks could not be retrieved from filing for review.
- Cases that upon review were positive for acid-fast bacilli with the ZN stain, were excluded.
- Excluded were cases where the culture result was reported as “contaminated specimen”.

4.3.3 Sample size calculation

In a study carried out by the investigator in 2012, titled “A study to ascertain the number of cases with negative Ziehl-Neelsen granulomatous inflammation with a positive mycobacterium PCR result”, a total of 269 cases of mycobacterium PCR were performed in a period spanning one year. Extrapolating from this figure, in a five year period, the expected total number of mycobacterium PCR tests performed would be 1345.

It is further estimated that only one third of the above cases would have a corresponding mycobacterium culture result, leaving 444 cases. An additional 10% of cases were excluded due to exclusion criteria.

Therefore, for a population size of 400 with a confidence level of 95%; a precision level (margin of error) of 5% and a variability of 30%, the sample size is calculated at 120.

4.3.4 Final sample size

After reviewing the histology slides and retrieval of the PCR results with the corresponding culture results, a sample size of 121 cases was attained.

4.4 Collection and recording of data

The information collected on each case was entered onto a data capture sheet **(see Annexure 2)**. This data was subsequently transferred into a Microsoft Office Excel (2007) spread sheet for data analysis.

4.5 Statistical analysis

4.5.1 Reproducibility and validity of results

The reliability or reproducibility of a test is the ability of that test to produce comparable results each time that it is performed.[41] The validity of a test is the likelihood that the results of a study are true and free of bias. This is dependent on the integrity of the study design.[41]

The following steps were taken to ensure that this study would produce valid and reliable results:

- An appropriate study design was chosen.
- The test was conducted in a population where the prevalence of disease (i.e. tuberculosis) is high.
- An appropriate sample size was met.
- The test (i.e. TB PCR) was conducted using a standard, reproducible protocol with appropriate controls.

4.5.2 Why specific tests have been used

Descriptive statistics were used to describe the demographical information of the study population, i.e., age and gender distributions.

As this study compares a diagnostic test against a gold standard test, the following statistical tests were performed:

- Sensitivity – This is defined as the proportion of subjects with a particular disease who will have a positive result.[42,43]

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives} + \text{False negatives}}$$

- Specificity – This is defined as the proportion of subjects without a particular disease who will have a negative result.[42,43]

  - $$\text{Specificity} = \frac{\text{True negatives}}{\text{False positives+True negatives}}$$

- A major limitation of both sensitivity and specificity is that they are of no value in estimating the probability of disease in an individual patient. It is of more practical use, when faced with an individual patient, to know the probability that a test result will give the correct diagnosis. For this reason, positive and negative predictive values were also calculated. [42,43]

- Positive predictive value – is defined as the proportion of people with a positive test result who will truly have the disease. [42,43]

$$\text{Positive predictive value} = \frac{\text{True positives}}{\text{True positives} + \text{False positives}}$$

- Negative predictive value – is defined as the proportion of people with a negative test result who do not have the disease [42,43]

$$\text{Negative predictive value} = \frac{\text{True negatives}}{\text{False negatives} + \text{True negatives}}$$

Both positive and negative predictive values are dependent on the prevalence of disease.

Lastly, the Chi-square test was utilised in this study to determine the association between a positive TB PCR result and a TB culture result. This test measures the probability of association between two discrete non-parametric variables. A ‘p value’ of <0.05 was selected for this test. A limitation of this test is that although it gives an

association between two variables, it does not measure the strength of the association.[44]

4.6 Ethical considerations

This was a retrospective study carried out on archived slides and paraffin wax embedded blocks. The patients' names were not recorded on the data capture sheet. Each case was identified with consecutive numbers and laboratory numbers only. The identity of the patient is known only to the principle investigator and was not divulged to anyone else. The Department of Anatomical Pathology has been granted blanket approval by the committee for Research on Human Subjects (medical) of the University of the Witwatersrand to conduct such retrospective studies.

However, a separate application was submitted to the Human Research Ethics Committee, University of the Witwatersrand, which has been approved, (clearance number: M140274), **(see Annexure 4).**

CHAPTER 5

5.0 Results

5.1 Patient demographics

A total of 121 cases of Ziehl-Neelsen negative granulomatous inflammation with TB PCR and TB culture results were identified.

Fifty two percent of the cohort (63/121) were male and 48% (58/121) were female **(Figure 6).**

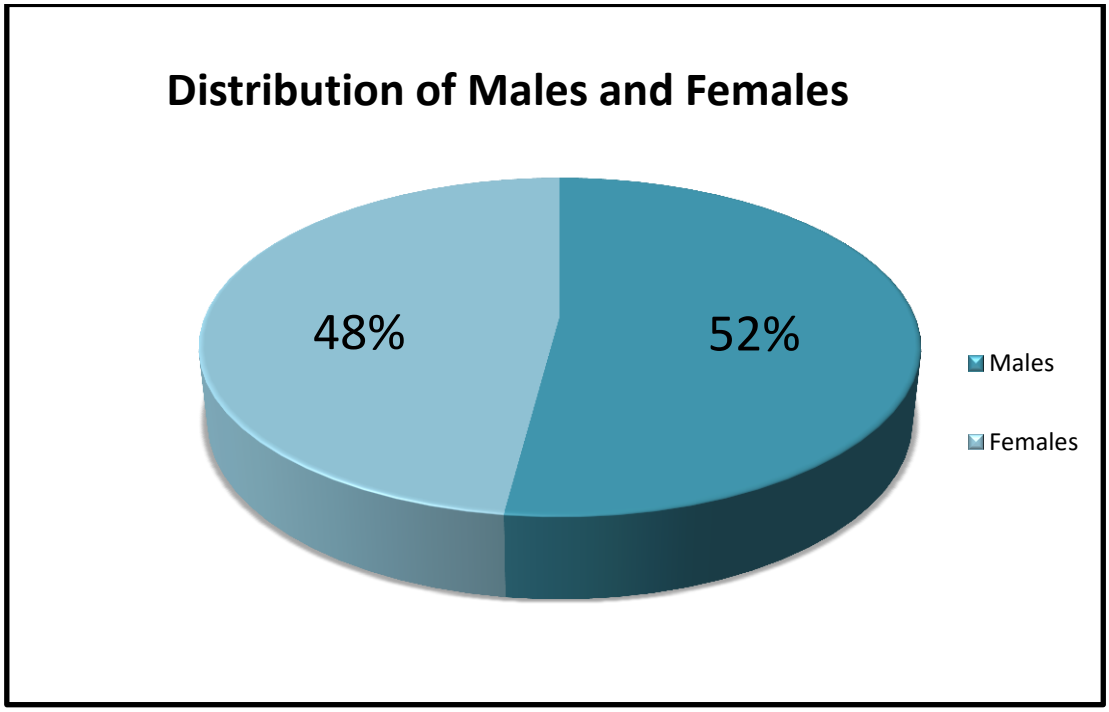


Figure 6: Percentage of males and females in the study sample.

The mean age of the study population was 35.3 years; with majority of patients (58.7%) falling within the 21-30 and 31-40 age categories **(Figure 7).**

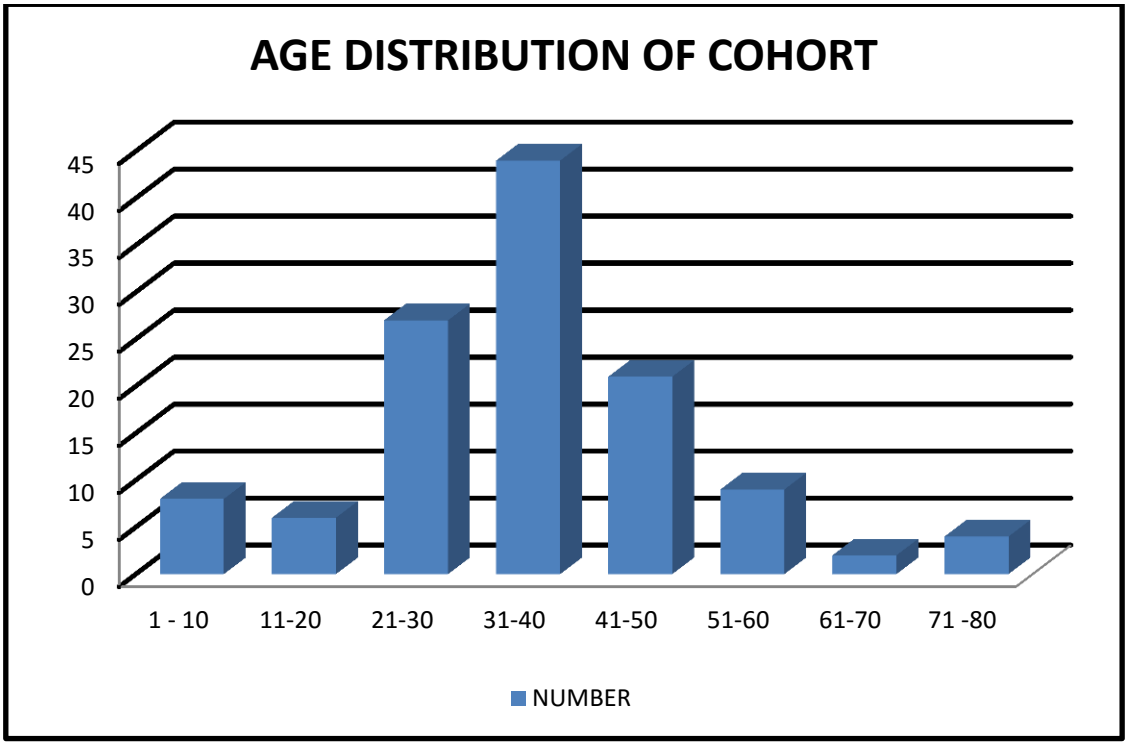


Figure 7: **Age distribution of the study sample.**

Majority of cases were biopsies from extrapulmonary sites, whilst only 5% of samples were pulmonary biopsies. A large proportion of cases (46%) were bone marrow trephine biopsies, with corresponding culture results obtained from the bone marrow aspirate. Other biopsy sites included those from the vertebra, soft tissue, skin, lymph nodes, liver, neck region, synovium and bone **(Figure 8).**

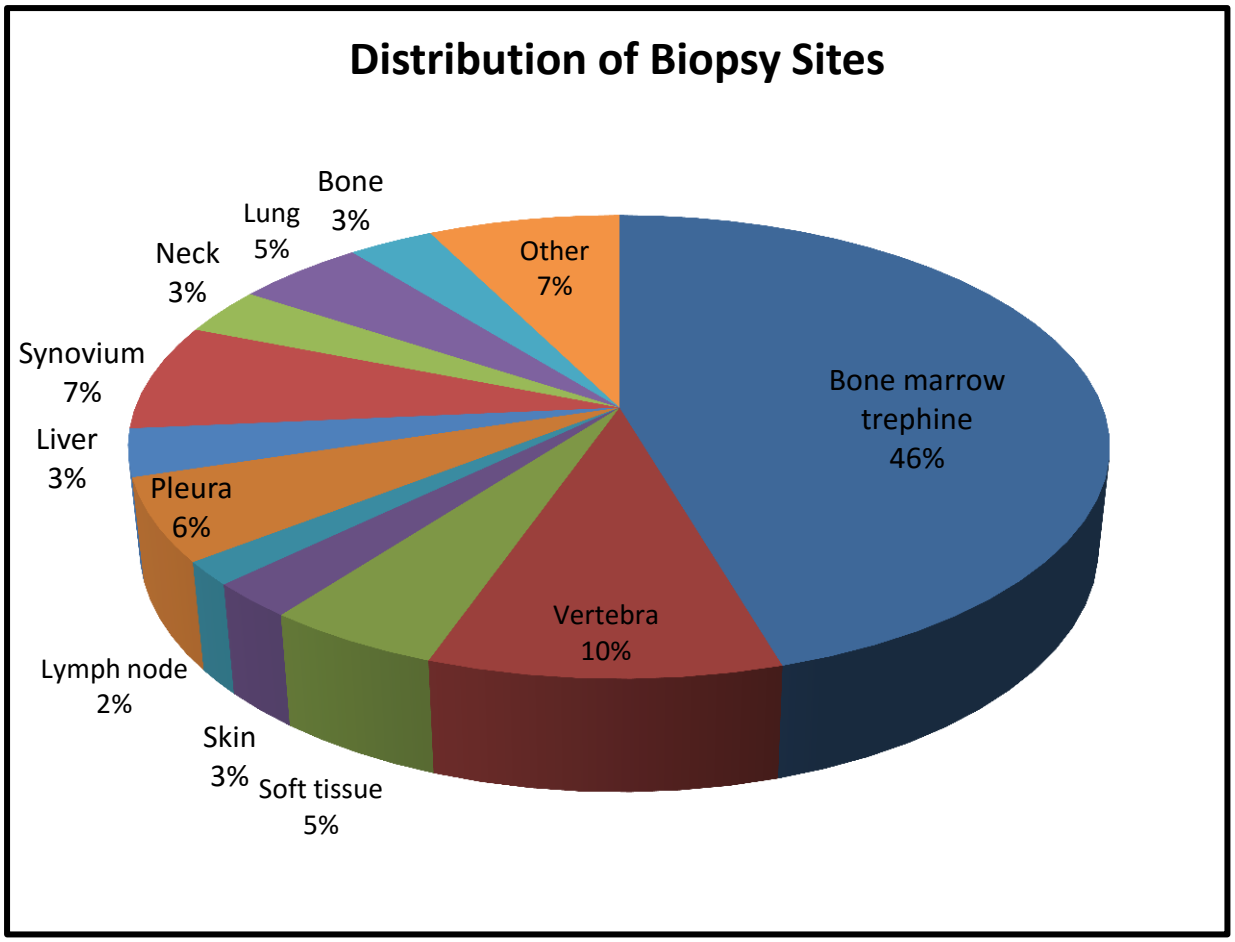


Figure 8: **Distribution of the biopsy sites.** *Majority of samples (46%) were derived from the bone marrow, followed by the vertebra.*

5.2 Mycobacterium polymerase chain reaction results

Of the 121 cases submitted for TB PCR, 51/121(42%) were positive whilst 70/121(58%) were negative. The cases that were submitted for TB culture showed a positive result in 39/121(32%) and a negative result in 82/121(68%) of cases **(Figure 9)**.

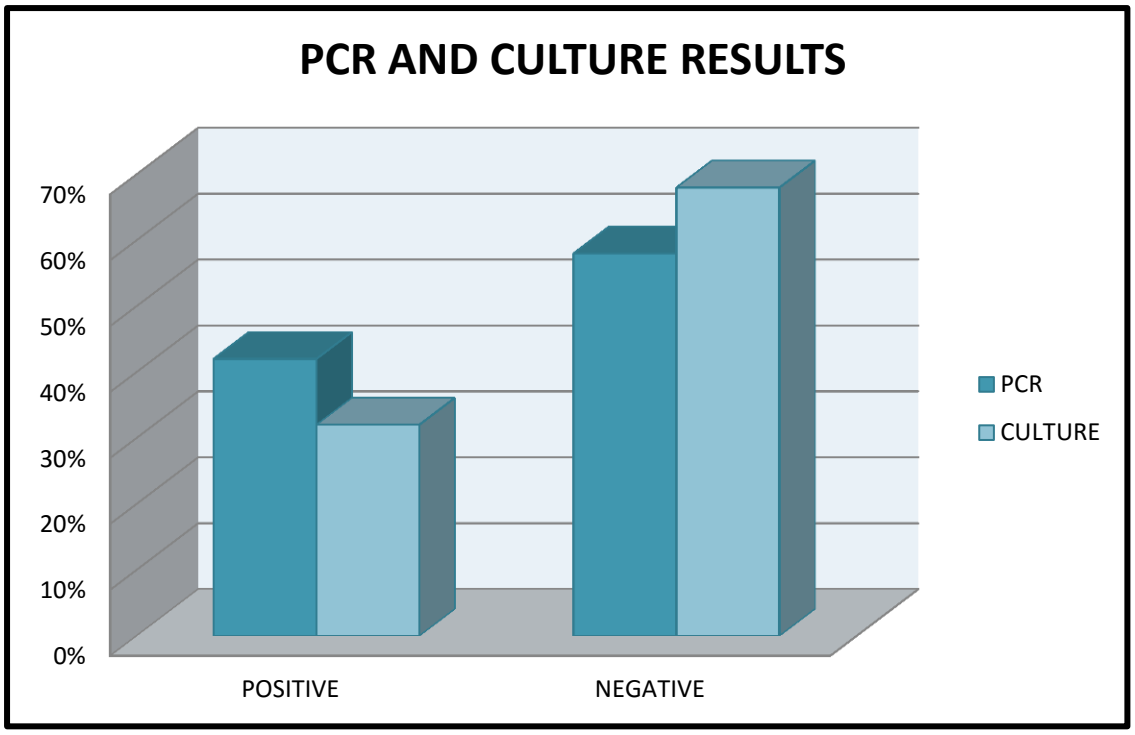


Figure 9: Graph depicting the overall mycobacterium PCR and culture results. *A higher percentage of cases were positive for PCR as compared to culture.*

Twenty five cases (20.7%) had positive TB PCR and positive TB culture results; whilst 46.3% (56/121) had negative TB PCR and TB culture results. There were 14 (11.6%) false negative cases and 26 (21.5%) false positive cases **(Table 3).**

The sensitivity of nested mycobacterium PCR targeting hsp65 is 64.1%, whilst the specificity is 68.2%. The positive and negative predictive values are 49% and 80% respectively. The Chi-square statistic comparing the results of TB PCR results and TB culture results is 11.4 (p value = 0.000744). These results are statistically significant, and highlight an association between PCR and culture.

	CULTURE POSITIVE	CULTURE NEGATIVE
PCR POSITIVE	25 (20.7%)	26(21.5%)
PCR NEGATIVE	14(11.6%)	56(46.3%)

Table 3: **Mycobacterium PCR compared to culture results.** Twenty six cases had false positive results whilst 14 cases had false negative results.

5.3 Results in patients in whom there was no clinical suspicion of tuberculosis

Of the 121 patients, there was a clinical suspicion of tuberculosis in 73 (60.3%) of cases. In 11 patients, there was no clinical suspicion and in 37 (30.6%) of cases, this information was not available.

Mycobacterium PCR was however performed in these 11 cases as the histology showed granulomatous inflammation and considering the high prevalence of this disease in South Africa, it is not a disease to be missed.

Of the 11 patients in whom there was no clinical suspicion of TB, 7 patients had negative TB PCR and culture results and 2 patients were positive by both PCR and culture. There were 2 false positive and no false negative results **(Figure 10)**.

Amongst these 11 cases where tuberculosis was not suspected, 4 patients had malignant diagnoses. In 2 of the 11 cases, the clinical suspicion was that of sarcoidosis (which also produces a histological pattern of granulomatous inflammation). Both these cases had a negative TB PCR and culture result. One

patient had a diagnosis of rheumatoid arthritis, and both the TB PCR and culture results were positive. One patient had a clinical diagnosis of gout. This patient had a positive TB PCR result with a negative culture result. In the remaining 3 cases, further clinical information was not available.

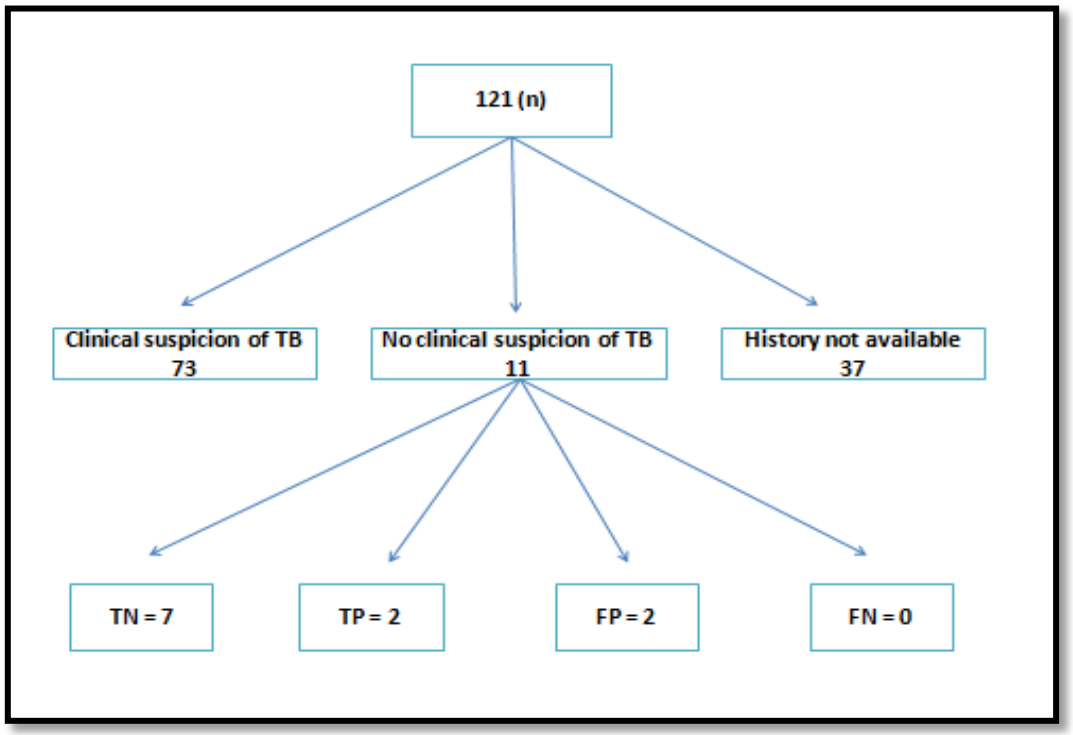


Figure 10: Diagram illustrating the PCR assay results in patients in whom tuberculosis was not clinically suspected. *TN= True negative; TP= True positive; FP= False positive; FN= False negative; n= number.*

5.4 Results in patients who were on empirical treatment for tuberculosis.

Twelve of the 121 patients (9.9%) were on empirical TB treatment, for variable durations, at the time of biopsy. The information regarding the treatment status for the remaining patients is unknown. Of these 12 patients, 7 tested negative for both TB PCR and culture. Five of the 12 patients had a positive PCR test but were culture

negative. None of the patients who were on empirical TB treatment tested PCR negative but culture positive.

5.5 PCR results in comparison to culture results, including cases that were culture positive for mycobacterium other than tuberculosis (MOTT).

There were 25 true positive cases. Of these, 23/25 (92%), grew *Mycobacterium tuberculosis* by culture. One case that was positive for mycobacterium PCR, grew *Mycobacterium mucogenicum* on culture. This is a rapidly growing non tuberculous mycobacterium that is a well recognised cause of skin and soft tissue infections. This biopsy was indeed taken from a skin lesion from a patient's nose. One additional case that was positive for mycobacterium PCR and culture, unfortunately had insufficient material for species identification.

There were 14 false negative PCR results. Eleven of the 14 cases negative for PCR, grew *M. tuberculosis* complex by culture. In 1 case, the culture result was positive for *M. avium/ intracellulare* and in another the culture was positive for *Mycobacterium gordonae*. In the remaining cases there was insufficient material for species identification.

CHAPTER 6

6.0 Discussion

6.1 Summary of the main findings

A retrospective study including 121 cases showing Ziehl-Neelsen negative, granulomatous inflammation, with corresponding mycobacterium culture and PCR results were included.

The sites of tissue biopsy were predominantly extrapulmonary, with only 5% of biopsies originating from the lung. The majority of the cases were derived from the marrow (46%), followed by the vertebrae (10%). This highlights the observation that the majority of the cases that are submitted for histological evaluation are cases that are not amenable to FNAB.

Of the 121 cases, nested mycobacterium PCR (targeting the 65kDa hsp65 gene) was positive in 51 cases (42%), whilst mycobacterium culture was positive in 39 cases (32%). Using the culture result (from the same or related site as the tissue submitted for histology) as the gold standard for comparison, PCR yielded a sensitivity of 64.1% and a specificity of 68.2%. Positive and negative predictive values of 49% and 80% respectively were attained. The Chi square test showed good agreement ($p < 0.05$) between PCR and culture results.

There was no significant difference in the number of males affected (52%), compared to females (48%). However, the age distribution of the study sample revealed that majority of the patients (58.7%) fell into the 21-30 and 31-40 year categories. This finding is concordant with the observation that in high prevalence

countries such as South Africa, most cases of tuberculosis occur in patients in their twenties to forties; that is, the most economically productive generation.[12]

Most of the biopsies were derived from extrapulmonary sites (95%). A large proportion of the cases were bone marrow trephine biopsies, with the corresponding bone marrow aspirate samples that were submitted for mycobacterium culture. The tissue evaluated for mycobacterium PCR was therefore not precisely the same tissue that was submitted for culture, which ultimately may have influenced the results. However, this could not have been circumvented, as in practice; most clinicians fail to concurrently submit tissue in normal saline for culture evaluation.

6.2 Comparison of results with those from other studies.

In this study, nested PCR had sensitivity and specificity of 64.1% and 68.2% respectively. The test had a higher false positive rate (21.5%) as compared to the false negative rate (11,6%). However, consideration needs to be given to the fact that this study was performed in a study sample where the Ziehl-Neelsen (ZN) stain was negative for acid-fast bacilli (AFB). Hence, all samples were paucibacillary in nature. Many of the prior studies performed on formalin-fixed, paraffin embedded tissue included samples that were positive for acid-fast bacilli. Acknowledging the fact that in order for AFB to be identified with the ZN stain, 10^4 to 10^6 bacilli per millilitre of tissue or fluid is required, [13] thus by including samples that are AFB positive, PCR is likely to achieve a higher sensitivity.

A similar study to the current one was performed by Jackson *et al*. [14] The authors also investigated formalin-fixed, paraffin embedded tissue showing granulomatous

inflammation without demonstrable acid-fast bacilli. The PCR target used in this study was IS6110. Although their initial sample size was 115 cases, a culture result was only available in 68 cases. When compared to the clinical diagnosis of tuberculosis, the sensitivity and specificity for Mtb PCR was 72% and 73% respectively. When using the gold standard culture for comparison, this study demonstrated a false negative rate of 2.9% (2 of 68 cases) and a false positive rate of 32.3% (22 of 68 cases).[14] Congruent to the current study, (which demonstrated a false positive rate of 21.5%) Jackson *et al* also illustrated the finding of a higher false positive rate in paucibacillary tissue samples.[14]

To the investigator's knowledge, this is the only local study carried out on formalin-fixed, paraffin embedded tissue without demonstrable AFB; using the hsp65 gene target for PCR. Although this study had inferior results with comparable studies; it must be noted that hsp65 is a pan-mycobacterial gene target; hence a higher false positive rate can be expected due to contaminants from other mycobacterial species. Cook *et al*[38] have described false positive results when using the hsp65 gene target due to saprophytic "tap-water" species of mycobacteria that may have been introduced during tissue processing.[38] In addition, a higher false negative rate can also be expected as only tissue without detectable acid-fast bacilli by the Ziehl-Neelsen stain were included.

6.3 Clinical importance of specific findings.

In the current study, there were 11 patients in whom the clinical differential diagnosis of mycobacterial infection was not considered. However, as the histological pattern showed granulomatous inflammation, and granting consideration to the fact that

tuberculosis is so prevalent in South Africa; all the specimens were submitted for mycobacterium PCR. In four of the eleven patients, a malignant diagnosis was queried (carcinoma in 2 cases, multiple myeloma in 1 case and Hodgkin lymphoma in 1 case). In three of the cases, both the PCR and culture results were negative. One of these patients had a previous diagnosis of breast cancer and presented with a vertebral lesion that was subsequently biopsied. Although the clinical suspicion was that of metastatic breast carcinoma to the vertebra, both the mycobacterium PCR and the culture results were positive. This highlights the value of PCR, as a delay in waiting for the culture result may have had undesirable and devastating consequences for the patient. In two patients, the clinical diagnosis was that of sarcoidosis. Tuberculosis and sarcoidosis are clinical and histopathological mimickers of each other as they are both granulomatous diseases.[45] However, the treatment of these two disease entities significantly differ and it thus crucial to make this distinction. In the two patients in whom sarcoidosis was queried; both had negative mycobacterium PCR and culture results, thus verifying a diagnosis of sarcoidosis. It is thus clear that in many different clinical scenarios, and when used in the correct clinical setting, mycobacterium PCR proves to be of significant value.

Furthermore, within the present study, there were 12 patients who were on empirical antituberculosis treatment for an unspecified duration; prior to biopsy. Five of these patients still had a positive PCR result despite a negative culture result. Taking into consideration that these are patients in whom the clinical suspicion was so high as to warrant empirical treatment, one has to question whether the PCR results are indeed "false positive" results. PCR has a major limitation in that it cannot differentiate between dead and live bacilli.[35,36] In addition, the mycobacterial load may remain substantially high despite treatment, even though the organisms are not

capable of generating infection.[37] It is also possible that even with the institution of treatment, as PCR is theoretically capable of detecting a single organism, the sample will have a positive result well into the course of treatment. In a study by Yuen *et al*, [46] the authors showed that the TB PCR remained positive after four weeks of antituberculosis treatment in 29 patients, 16 of whom who had become culture negative.[46] PCR is therefore not able to differentiate between active; and prior or recently treated disease. Thus, a positive result under this circumstance has to be considered in the light of the clinical scenario, rather than assuming that it is false positive result. It is also noteworthy that none of the patients in the current study who were on antituberculosis therapy, had a negative PCR result with a positive culture result. A negative PCR result is thus useful in excluding tuberculosis as a diagnostic consideration.[37] This finding is further substantiated by the higher negative predictive value (of 80%) that was achieved in this study.

As the hsp 65 gene is highly conserved in all species of mycobacterium, the use of this gene target in the PCR assay cannot differentiate *M. tuberculosis* from other mycobacterium species. The advantage of using this target is that PCR may aid in the diagnosis of atypical mycobacterial infections. However, due to the nature of this being a pan-mycobacterial target, contamination from other mycobacterial species may result in a lower specificity of the test. In the current study, one patient had a positive mycobacterium PCR result which grew *M. mucogenicum* on culture. This case would have been negative and potentially missed if a *M. tuberculosis* species specific probe had been used.

6.4 Shortcomings or limitations of the study.

A major limitation of this study is that it was retrospectively conducted; hence not all of the specimens submitted for histopathological examination and mycobacterium culture were derived from the exact same site. Although the biopsy sites were either the same or closely related, this may have produced a sampling error, which could have potentially affected either the PCR or culture results. A second limitation is that the microbiological culture was not always performed on a tissue specimen. For example, a pleural biopsy specimen may have been submitted for histopathology, whilst the pleural fluid may have been submitted for culture. Although the sites are closely related, the bacillary load present in each type of specimen may have differed, which may have impacted the results.

A more accurate and reliable method would be conduction of a prospective study, wherein the investigator would receive a tissue sample in normal saline, and divided the same tissue for histopathological evaluation and culture. However, as many clinicians submit tissue immediately in formalin to maintain tissue preservation, this may be difficult to achieve in clinical practice.

6.5 Directions for future research.

As shown in this study, mycobacterium PCR is a useful tool for the diagnosis of Mtb. Although the sensitivity and specificity was lower compared to other similar studies, this was the only study that was conducted on paucibacillary tissue using the hsp65 gene target. Additional research may be of value in determining the performance of a species specific probe, such as IS6110, on paucibacillary tissue. However, with

major technological advancements being made in the field of molecular pathology to improve our diagnostic abilities and hence our patients' lives, it would be prudent to direct further research towards more recent technologies. For example, there would be great value in determining the use and efficacy of Xpert MTB/RIF technology in formalin fixed, paraffin embedded tissue samples. Additional research with larger sample sizes on the use of Xpert MTB/RIF on fresh or frozen tissue is also essential, in order to include this as part of the national policy guidelines. If this were a feasible option, the diagnosis of extrapulmonary and smear negative pulmonary tuberculosis would be expedited.

CHAPTER 7

7.0 Conclusion

Despite the development of novel diagnostic assays; the diagnosis of tuberculosis, particularly extrapulmonary tuberculosis and smear negative pulmonary tuberculosis, remains challenging. Major progress in the field of diagnostics has been made with the development of novel nucleic acid amplification techniques, which have a major advantage of delivering rapid results.

The aim of this study was to investigate the validity of the mycobacterium PCR assay in formalin-fixed, paraffin embedded tissue showing granulomatous inflammation without demonstrable acid-fast bacilli. Although the study revealed suboptimal sensitivity and specificity results, these figures cannot be interpreted at face value. The results of the mycobacterium polymerase chain reaction assay are of most value when used in conjunction with the patient's clinical history, signs and symptoms, and other ancillary investigations.

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ANNEXURES

ANNEXURE 1: Example of β-globin melt curve graph

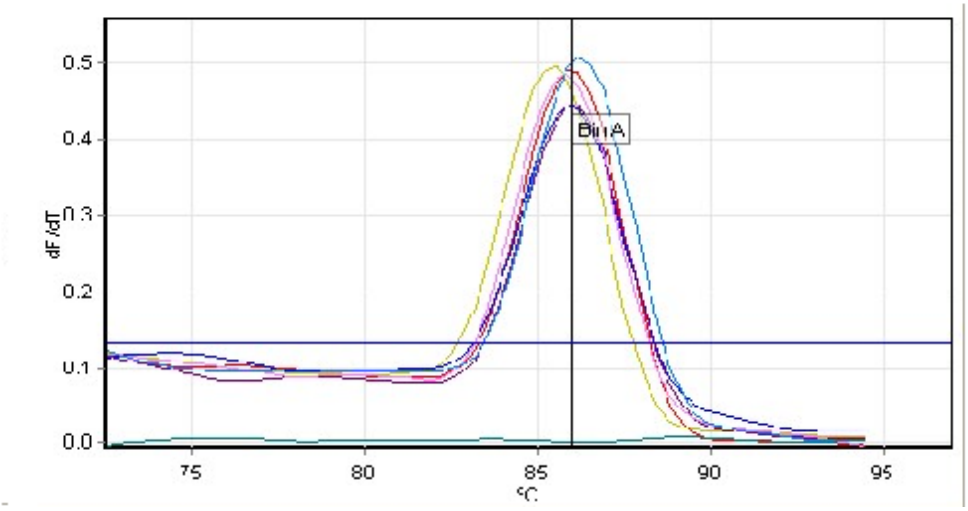
www.qiagen.com

Melt Report

Cycle	Cycle Point
Hold	Hold @ 95°C, 10min 0s
Cycling (50 repeats)	Step 1: Hold @ 95°C, 10s
	Step 2: Hold @ 60°C, 10s
	Step 3: Hold @ 72°C, 15s, acquiring to Cycling A([Green][1][1])
Melt	Ramp from 72°C to 95°C
	Hold for 90s on the 1st step
	Hold for 5s on next steps,Melt A([Green][1][1])

Bin Name	Temperature	Sample No.	Sample Name	Peak
Bin A	86.01	1	T5783	86.0
		2	T5784	85.5
		3	T5785	86.0
		4	T5786	86.0
		5	T5787	85.8
		6	MSI_62	86.2
			Mean	85.92
			Std. Dev.	0.22

No.	Color	Name	Genotype	Peak 1
1		T5783	PASSED	86.0 (Bin A)
2		T5784	PASSED	85.5 (Bin A)
3		T5785	PASSED	86.0 (Bin A)
4		T5786	PASSED	86.0 (Bin A)
5		T5787	PASSED	85.8 (Bin A)
6		MSI_62	PASSED	86.2 (Bin A)
7		NEGATIVE		



ANNEXURE 2: Data collection sheet

NO	LAB NO	AGE	SEX	SUS OF TB	CURR ON TB TX	BX SITE	PCR	CULT SITE	CULT R	ORG CULTURED

ANNEXURE 3: Protocol approval certificate

Faculty of Health Sciences
Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172040

Reference: Ms Thokozile Nhlapo
E-mail: thokozile.nhlapo@wits.ac.za

14 May 2014
Person No: 0203829Y
PAG

Dr D Lakhoo
P O Box 803
Lenasia
1820
South Africa

Dear Dr Lakhoo

Master of Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *Determining the validity of the Mycobacterium polymerase chain reaction assay in histological samples showing granulomatous inflammation with a negative Ziehl-Neelsen stain.* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

ANNEXURE 4: Ethics clearance certificate

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140274

NAME: (Principal Investigator)	Dr Deepna Lakhoo
DEPARTMENT:	School of Pathology/Anatomical Pathology CM Johannesburg Academic Hospital
PROJECT TITLE:	Determining the Validity of the Mycobacterium Polymerase Chain Reaction Assay in Histological Samples Showing Granulomatous Inflammation with a Negative Ziehl-Neelsen Stain
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Reubina Wadee

APPROVED BY: Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 24/03/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

yearly progress report.

Principal Investigator Signature

31/03/2015

M140274Date

ANNEXURE 5: Turnitin Report

