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COMPARISON OF MULTIPLE METHODS OF DIAGNOSIS OF MYCOBACTERIAL INFECTION FROM BONE MARROW SAMPLES OF HIV POSITIVE PATIENTS

Benford Ivan Chosamata

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Haematology.

Johannesburg, 2010.

DECLARATION

I, Benford Ivan Chosamata declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Haematology at the Faculty of Health Sciences of the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



26 th Day of July, 2010.

DEDICATION

This dissertation is dedicated to my wife Mary

And daughter Mbumba for their patience

And understanding during the pursuit of this research

PRESENTATION ARISING FROM THIS STUDY

Chosamata BI, Mahlangu JN, Scott L.

Comparison of multiple methods of diagnosis of mycobacterial infection from bone marrow samples of HIV positive patients (poster).

Abstract was presented at the International Society of Laboratory Haematology (ISLH) 2010 23rd International Symposium on Technological Innovations in Laboratory Haematology. Brighton, United Kingdom, 10-12 May 2010.

ABSTRACT.

Background: Mycobacterium tuberculosis (MTB) infection remains a serious public health challenge in sub-Saharan Africa. Rapid and early diagnosis is critical in the successful control of this eminently treatable infection.

This study compared the diagnostic usefulness of culture, bone marrow trephine biopsy granulomata, bone marrow trephine biopsy Ziehl-Neelsen (ZN) stain and bone marrow mycobacterial polymerase chain reaction (PCR) in establishing the diagnosis of mycobacterial infection in HIV infected patients.

Materials and methods: The trephine biopsies of HIV positive patients done for the investigation of suspected tuberculosis were reviewed for granulomata and stained with ZN stain. The corresponding bone marrow aspirates were subjected to DNA real-time PCR analyses using LightCyler TB Kit® (Roche Diagnostic). Culture results were used as diagnostic gold standard.

Results: Of the 60 patients studied, 24 were culture negative. Of the 34 culture positive, 62% were *Mycobacterium tuberculosis* and 38% were *Mycobacterium avium intracellulare.*

Using the culture method as a gold standard, the sensitivities and specificities were 97% and 23% for bone marrow trephine biopsy granulomata, 65% and 58% for bone marrow trephine biopsy ZN staining and 50% and 73% for bone marrow aspirate PCR analysis respectively. Ninety-seven percent of all trephine biopsies with positive ZN stain had granulomata.

Conclusion: The presence of granulomata in bone marrow trephine biopsies of HIV infected patients appear to have a high diagnostic yield whilst mycobacterial PCR has the lowest yield but highest specificity. These results should be confirmed in a prospective case controlled study because the sample size in this study was small, and the study was a retrospective one.

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LIST OF ABBREVIATIONS

AFB	Acid fast-bacilli
AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral
CCD	Charge-coupled device
CD4	Cluster of differentiation number 4
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HE	Haematoxylin and Eosin
HIV	Human immunodeficiency virus
LCTB	LightCycler tuberculosis
MA	Mycobacterium avium intracellulare
MDRTB	Multidrug resistant tuberculosis
MK	Mycobacterium kansasii
MTB	Mycobacterium tuberculosis
ng/ul	Nanogram per microlitre
NHLS	National Health Laboratory Service
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SOP	Standard operating procedure
TB	Tuberculosis
Tm	Melting temperature
WHO	World Health Organisation

XDRTB	Extensively drug resistant tuberculosis
ZN	Ziehl-Neelsen

CHAPTER 1

1.1 LITERATURE REVIEW

1.1.1 Epidemiology of tuberculosis in South Africa

Tuberculosis (TB) is a major public health problem in South Africa, a country which is ranked fifth on the list of 22 high-burden TB countries in the world (World Health Organisation, 2009). There were nearly 460,000 new TB cases in South Africa in 2007, and the TB infection incidence rate was approximately 948 cases per 100,000 populations- a major increase from 338 per 100,000 12 years earlier in 1998 (World Health Organisation, 2009). The reasons for the high burden of TB in South Africa are multi-factorial and some of these are explored in the discussion that follows.

1.1.2 Risk factors for tuberculosis

Globally the incidence of TB is increasing, fuelled in part by the concurrent epidemic of HIV/AIDS currently worst affecting sub-Saharan Africa (Harling *et al.*, 2008). Traditionally TB is regarded as a disease of poverty and many aspects of low socioeconomic status for example overcrowding and malnutrition, are recognised individual and household level risk factors for the disease (Harling *et al.*, 2008). Although for centuries TB has been called the White Plague, in South Africa it is predominantly a disease of black Africans, a by-product of poverty, poor health care and high HIV infection rate (Koening, 2008).

In the old South Africa, people were previously classified as "coloured" (mixed race group), African, white and Asian. TB rates were extremely high in the

coloured and African communities compared to whites and Asians, a reflection of their poor socioeconomic circumstances (Wood *et al.*, 2000).

A South African population study found that TB was associated with lower individual, household and community-level socioeconomic status. Low level of personal education, unemployment and a low level of household wealth were associated with increased odds of TB (Harling *et al.*, 2008). This analysis found alcohol abuse, cigarette smoking and low body mass index (BMI <18.5) each independently to be a risk factor for TB in South Africa.

The hypothesis for alcohol consumption and cigarette smoking to be risk factors is that in addition to their biological effects, they may be proxies for frequenting locations that put one at raised risk of close contact with infectious individuals, such as neighbourhood bars (Harling *et al.*, 2008).

The risk factor of developing TB was reported to increase markedly with advanced HIV disease; WHO clinical stages 3 and 4 and increased erythrocyte sedimentation rate (ESR >75 mm/Hour) were shown to be independently associated with risk of developing TB (Wood *et al.*, 2000).

The multidrug-resistant tuberculosis (MDRTB), largely caused by non-adherence to drug regimens, is further exacerbating the problem. According to the World Health Organisation (WHO) report of 2009, the TB treatment success rate in 2006 was 67%, with 12% defaulting treatment and 9% failing it. The number of laboratory

confirmed cases of MDRTB more than tripled from 2,000 cases in 2005 to 7,350 in 2007. Among all the new TB cases 1.8% was MDR TB, and among the previously treated TB cases MDR TB comprised 6.7%. Since 2007 South Africa has increasingly reported patients with extensively drug-resistant tuberculosis (XDR-TB), and the actual reported cases of XDR-TB had increased from 74 in 2004 to 536 in 2007 (World Health Organisation, 2009). From 2004-2008 more than 24,000 cases of MDR TB were diagnosed, of which 7% were infected with XDR TB (Kapp, 2009). The combination of drug-resistant TB and HIV infection is especially dangerous because the weakened immune systems of HIV-infected persons make them more vulnerable to TB and also more difficult to treat (Koening, 2008).

1.1.3 Tuberculosis treatment approaches.

Many South Africans who test positive for TB are started on first-line drugs while being investigated for drug resistance (Koening, 2008). XDR TB patients are involuntarily isolated or confined to the isolation wards, a move which is controversial, but it has to be done as the disease poses an immediate threat to public health (Koening, 2008).

South Africa has 100% DOTS (directly observed treatment, short course) implementation strategy for TB control. The strategy includes case detection through quality assured bacteriology, standardised short course (6-8 months) treatment with direct observation of doses to ensure adherence, an effective drug supply and management system and monitoring and evaluating, allowing assessment of treatment results (World Health Organisation, 2009).

Before the implementation of DOTS, TB treatment was in many resource poor countries chaotic, non-standardised and poorly monitored and consequently had little epidemiological impact on the incidence of TB. Therefore, the introduction of the DOTS strategy has led to improvements in treatment outcomes for many patients (Cox and Morrow, 2008).

South Africa adopted the Stop TB Strategy launched by WHO in 2006 (Kapp, 2009). The core of this strategy is DOTS. It responds to access, equality and quality constraints, and adopts evidence-based innovations in engaging with private health-care providers, empowering affected people and communities, strengthen health systems and promote research (Raviglione and Upleker, 2006).

1.1.4 Epidemiology of HIV in South Africa

An estimated 5.7 million South Africans are HIV-infected (UNAIDS, 2009) the highest infection being reported in KwaZulu-Natal (Koening, 2008). Tables 1.1 and 1.2 show the prevalence of HIV in South Africa by age, sex, race and province.

Table 1.1 HIV prevalence by sex and age in South Africa in 2008

AGE GROUP (YEARS)	HIV PREVALENCE (%)	
	MALE	FEMALE
2-14	3	2
15-19	2.5	6.7
20-24	5.1	21.1
25-29	15.7	32.7
30-34	25.8	29.1
35-39	18.5	24.8
40-44	19.2	16.3
45-49	8.4	14.1
50-54	10.4	10.2
55-59	6.2	7.7
60+	3.5	1.8

Adapted from Shisana *et al.,* 2009.

Table 1.2 HIV prevalence by sex, age, race and province, South Africa 2008

Variable	HIV+ (%)	95% CI
Sex		
Male	7.9	6.8-9.2
Female	13.6	12.5-14.8
Total	10.9	10.0-11.9
Age group (years)		
2-14	2.5	1.9-3.5
15-24	8.7	7.2-10.4
25+	16.3	15.3-18.4
Population group		
African	13.6	12.6-14.8
White	0.3	0.1-0.9
Coloured	1.7	1.3-2.4
Indian	0.3	0.1-1.2
Province		
Western Cape	3.8	2.7-5.3
Eastern Cape	9.0	7.2-11.2
Northern Cape	5.9	4.5-7.8
Free State	12.6	10.5-15.1
KwaZulu-Natal	15.8	13.4-18.6
North West	11.3	9.1-14.0
Gauteng	10.3	8.3-12.7
Mpumalanga	15.4	11.9-19.7
Limpopo	8.8	6.5-11.9

Adapted from Shisana *et al.,* 2009

1.1.5 HIV risk factors

Multiple sexual partners, early sex debut, unprotected sex, intergenerational sex (particularly younger females having sex with older males) were shown to contribute to the spread of HIV (Katz and Lower-Beer, 2008; Shisana *et al.*, 2009). In South Africa, the most at risk populations were shown to include (Shisana *et al.*, 2009):

- African females aged 20-34;
- African males aged 25-49;
- Males aged 50+
- Men who have sex with men (MSM); and
- People who use drugs for recreational purposes.

1.1.6 Public health programmes

There are several HIV/AIDS communication and awareness programmes including:

- The Khomanani "Coming Together" which is a national government programme intended for all populations in South Africa;
- Soul City, a television drama series aimed at educating the public;
- Soul Buddyz which is intended for children aged 8-12 years; and
- Lovelife, a youth oriented programme (Shisana *et al.*, 2009).

1.1.7 Epidemiology of HIV & TB co-infection

1.1.7.1 The burden of HIV-TB

Mycobacterial tuberculosis and atypical mycobacterial infections are very common in individuals who are immunocompromised as a result of HIV infection (Karstaedt *et al.*, 2001; Sonnenberg *et al.*, 2005). The HIV-TB co-infection rate in South Africa was high, and according to WHO report of 2009, an estimated 73% of new TB patients were co-infected with HIV. An estimated 31% of all HIV-TB cases in Africa were in South Africa. Mortality rates were 38 and 193 deaths per 100,000 populations in HIV negative and HIV positive people respectively (World Health Organisation, 2009). The HIV and TB co-infection at a public hospital in Johannesburg was found to be 95% (John *et al.*, 2007).

1.1.7.2 Clinical presentation of tuberculosis

From the time of HIV infection, the individual's susceptibility to tuberculosis is increased, and as the HIV epidemic in South Africa progresses, the incidence of new cases of TB will continue to increase (Cohen and Maartens, 2004).

Tuberculosis is often an early manifestation of HIV infection and the pattern of clinical presentation depends on the degree of immunosuppression (Charles and Pape, 2006; Mwandumba *et al.*, 2008). In the early stages of HIV infection when the patients' immunity is only partially compromised the clinical features are characteristic of post-primary TB and resemble those seen in HIV-negative individuals such as localised disease with extensive lung destruction and cavitation, upper lobe involvement and positive sputum smears (Harries, 1997). As the CD4 counts decline, HIV-positive patients present with atypical pulmonary

disease (i.e., pulmonary infiltrates with no cavities, lower lobe involvement, intrathoracic lymphadenopathy and negative sputum smear) or extra-pulmonary/ disseminated disease affecting many parts of the body such as the lymph nodes, abdomen, pericardium and haemopoietic system in the form of bone marrow involvement (Harries, 1997; Charles and Pape, 2006; Keshinro and Diul, 2006; Mwandumba *et al.*, 2008).

The classic systemic symptoms include fever, night sweats, anorexia, weight loss and weakness, however these symptoms are non-specific, sometimes resulting in delayed diagnosis or even misdiagnosis (Keshinro and Diul, 2006).

In HIV-positive patients, cough is a symptom reported less frequently than in HIV-negative patients probably because there is less cavitation, inflammation and endotracheal irritation (Harries, 1997). Similarly, haemoptysis, which results from caseous necrosis of the bronchial arteries inside the cavities, is less common in HIV-positive patients (Harries, 1997).

1.1.7.3 HIV-TB co-infection treatment approaches & challenges

Antiretroviral (ARV) therapy has been shown to reduce the incidence of TB in HIV-infected patients by more than 80% (Badri *et al.*, 2002). Both anti-TB treatment and ARVs are indispensable in the management of patients with HIV-TB co-infection (Sharma *et al.*, 2005). The key therapeutic principles underlying the treatment of HIV-TB are, (i) treatment of TB always takes precedence over treatment of HIV infection, (ii) in patients who are already on ARVs, the same has

to be continued with appropriate modifications both in ARVs and anti-TB treatment, and (iii) in patients not receiving ARVs, the need for and timing of initiation of ARVs have to be decided after assessing the short-term risk of disease progression and death, based on CD4 count and type of TB, on individual basis (Sharma *et al.*, 2005).

In South Africa, the first-line treatment regimen for ARV-naïve patients in the public sector is combination of tenofovir, lamivudine or emtracitabine and efavirenz or nevirapine. Patients who fail first line regimen are commenced on protease inhibitor (PI)-based second line ARV therapy comprising either tenofovir, lamivudine or emtracitabine and lopinavir/ritonavir or zidovudine, lamivudine and lopinavir/ritonavir (South Africa National Department of Health, 2010). The critical component of TB treatment is rifampicin and clinically significant pharmacokinetic drug interactions exist between standard TB treatment and ARV therapy. Rifampicin is a potent inducer of the cytochrome P450 enzyme system, particularly the dominant iso-enzyme CYP3A4; rifampicin therefore increases metabolism and reduces plasma levels of many hepatically metabolised drugs such as non-nucleoside reverse transcriptase inhibitors (NNRTIs) and PIs (Cohen and Maartens, 2004).

There is an additive risk of side-effects and drug toxicity when ARVs and anti-TB drugs are administered together. The common side effects include nausea, hepatitis, peripheral neuropathy and rash and these side effects may make adherence to treatment difficult (Cohen and Maartens, 2004). Patients with

advanced HIV and TB co-infection commonly develop immune reconstitution illness when ARVs are commenced (Cohen and Maartens, 2004).

1.1.8 Approaches in tuberculosis diagnosis

1.1.8.1 Culture of mycobacteria

Blood cultures were not usually processed for the detection of mycobacteria in the past and the processing for mycobacteria began with the emergence of HIV-infected patients (Martinez-Sanchez *et al.*, 2000). The increasing numbers of mycobacterial infections in disseminated forms prompted the searches for rapid, sensitive and safe methods for the recovery of mycobacteria from blood (Askgaard *et al.*, 1992).

Culture methods have evolved since the advent of HIV, for example in Denmark (Askgaard *et al.*, 1992) in early 1980's coagulated blood and bone marrow specimens were inoculated directly on conventional media, that is, Lowenstein-Jensen media (LJ). Later bone marrow or blood was indirectly inoculated onto conventional media or into Bactec 12B media (Becton Dickinson) using the Isolator® lysis centrifugation system. This system was based on centrifugal concentration of the mycobacteria in a lysing solution containing sodium polyanethol sulfonate (SPS) which counteracted phagocytic, lysozymal and complement activities in blood. The concentrate was inoculated on LJ media or into a 12B vial. The Bactec 12B bottles contained a Middlebrook 7H12 broth supplemented with ^{14}C-labelled palmetic acid. The microbial metabolism led to release of $^{14}CO_2$ into the atmosphere above the broth, which was measured by an

automated ionisation detector Bactec 460 TB Instrument (Becton Dickinson). The amount of $^{14}CO_2$ was arbitrarily represented as a growth index (GI).
From 1988 direct inoculation of bone marrow or blood was introduced using 13A Bactec vials (Becton Dickinson). These vials were inoculated at the bedside with approximately 5 ml of blood or 2 ml of bone marrow. These 13A medium, formulated specifically for culturing mycobacterium from blood, was a Middlebrook 7H9 broth supplemented with casein hydrolysate, Tween 80, SPS and ^{14}C-labeled palmitic acid. Upon receipt at the laboratory the 13A vials were supplemented with 0.5 ml of OADC Enrichment (oleic acid, albumin, dextrose, catalase and sodium chloride). The released $^{14}CO_2$ was measured in the automated Bactec 460 TB Instrument. Currently in South Africa the National Health Laboratory Service is using Bactec Myco/F lytic blood culture bottles (Becton Dickinson), the Bactec 9240 Blood Culturing System (Becton Dickinson) and Accuprobe DNA probes (Geneprobe) as described in section 2.2.4 in the materials and methods below.

The blood or bone marrow aspirate culture methods are regarded as the gold standard for the diagnosis of mycobacterial infection. Blood and bone marrow aspirates have been reported to have similar mycobacterial yields (Keiser *et al.*, 1997; Kilby *et al.*, 1998; Marques *et al.*, 2000; Llewellyn *et al.*, 2005)

Sputum cultures are traditionally carried out in resource-poor countries (Siddiqi *et al.*, 2003; Keshinro and Diul, 2006). The sputum culture is much more sensitive than routine sputum microscopy as it is able to detect 10-100 AFB per µl (Keshinro and Diul, 2006). In high prevalence countries sputum culture can detect

pulmonary TB in around 80% of true cases (Siddiqi *et al.*, 2003; Parry, 1993). However the sensitivity for sputum cultures seems to be lower in AIDS patients with advanced immune deficiency, due to paucibacillary nature of pulmonary TB in these patients (Parry, 1993; Siddiqi *et al.*, 2003; Charles and Pape, 2006).

In terms of specificity, culture methods have the big advantage by allowing differentiation between *Mycobacterium tuberculosis* and other mycobacteria. For reference laboratories in high-burden countries, culture methods mainly serve as a first step in drug susceptibility testing (DST). DST itself will be reserved to track drug resistance. In these laboratories, culture may also enhance research.

The main disadvantage of culture methods is not the need for more sophisticated resources and more qualified technicians or higher cost, but its slowness. This is certainly so with the classical egg- or agar-based media (Löwenstein-Jensen, Ogawa, Middlebrook), for which a positive result requires 3-6 weeks, limiting culture methods as the first-line diagnostic tool (Siddiqi *et al.*, 2003; Keshinro and Diul, 2006). The delays in the diagnosis lead to delays in treatment initiation; a greater number of patients may potentially become infected through contact with an infected person (Cobbert *et al.*, 2006). The diagnostic delay of culture, together with the presence of advanced disease in many cases at presentation and the high index of suspicion among clinicians in countries with a high prevalence of TB, often obliges the clinicians to commence anti-mycobacterial therapy based solely on clinical suspicion (Gazzola *et al.*, 2008).

The commercial liquid systems (Bactec) may reduce this time by 50% or more, but they are more costly and place higher demands on equipment, so that they cannot be routinely used in resource-poor countries (Foulds and O'Brien, 1998).

1.1.8.2 Sputum smear microscopy

The conventional diagnosis of TB infection relies on sputum smear microscopy for acid-fast bacilli (AFB) (Parry, 1993). It is the simplest available diagnostic method and has been the mainstay of TB diagnosis for the past 100 years and a key technique in the DOTS strategy (Reid and Shah, 2009). Traditionally using direct ZN staining of sputum samples, sputum smear microscopy is highly specific in areas where there is high prevalence of TB, suitable for application in developing countries, relatively inexpensive and can be accomplished under field conditions (Foulds and O'Brien, 1998; Steingart *et al.*, 2006).

The AFB sputum smear microscopy has a sensitivity of only 50-60% in well-equipped laboratories staffed with skilled technicians, partly because a positive smear requires 5,000-10,000 AFB per µl of sputum sample (Parry, 1993; Siddiqi *et al.*, 2003; Charles and Pape, 2006). AFB microscopy detects those cases that are epidemiologically most important, i.e., those that are most likely to transmit the infection to those in close contacts (Foulds and O'Brien, 1998). Nonetheless, in this era of increasing HIV prevalence, the sensitivity of sputum smear microscopy is diminished as people who are co-infected with HIV are likely to have smear-negative pulmonary or extrapulmonary disease (Reid and Shah, 2009). HIV-

infected smear-positive patients tend to excrete significantly fewer organisms per ml of sputum than HIV-negative patients, which can lead to AFB being missed (Harries, 1997; Siddiqi *et al.*, 2003).

Smear-negative pulmonary TB in HIV/AIDS patients, particularly in low-income countries, is a substantial epidemiological problem, accounting for 24-61% of TB (Getahun *et al.*, 2007). In areas with high HIV prevalence, smear-negative TB has a much worse prognosis (Hargreaves *et al.*, 2001) due in part to delays in diagnosis and treatment initiation (Pablos-Mendez *et al.*, 1996).

An alternative to ZN staining of sputum is fluorescence microscopy. It is about 10% more sensitive than the conventional microscopy and has similar specificity, however it is technically complex and costly (Steingart *et al.*, 2006). However a Kenyan study found that fluorescence microscopy was more cost effective than conventional sputum microscopy and that it shortened the diagnostic process (Kivihya-Ndugga, L *et al.*, 2003). Fluorescence microscopy increases the number of sputum samples that can be read in a given time, because fewer fields must be examined under the microscope, and therefore cost effective than ZN staining (Reid and Shah, 2009).

Another method of improving sputum microscopy is the bleach and smear method. Sputum is chemically liquefied then concentrated by centrifugation or sedimentation. The best established method uses sodium hypochloride (NaOCl), the household bleach. This method has shown greater sensitivity than with ZN stain even among HIV positive patients. The main disadvantages of the technique

are long processing time and lack of a standardised method (Reid and Shah, 2009).

AFB sputum microscopy has limited value for diagnosis of TB in children, and does not, by definition, identify smear-negative TB, which is more likely in HIV-positive patients than HIV-negative individuals (Shingadia and Novelli, 2003; Siddiqi *et al.*, 2003).

The collection and testing of three consecutive smears (spot-morning-spot) over 2 days for TB screening is a matter of contention, especially where prevalence of TB is high (Reid and Shah, 2009). Reducing the number of smears- with patient giving 2 samples on 1 day rather than three samples over 2 days- could decrease the burden on patients and laboratories, and thus improve the sensitivity and specificity of direct microscopy (Reid and Shah, 2009).

Although the chances of finding AFB are greater with three sputum samples than with two samples or one (Harries *et al.*, 1996), the incremental yield and increased sensitivity gained by examining a third sputum specimen is small (about 2-5%) (Mase *et al.*, 2007).

World Health Organisation recently recommended that "the number of specimens to be examined to be reduced from 3 to 2 in places where a well-functioning external quality assurance (EQA) system exists, where work load is very high and human resources are limited" (World Health Organisation, 2007).

1.1.8.3 Chest X-ray

X-Ray diagnosis of pulmonary TB is popular with physicians everywhere in the world, thanks to its speed, simplicity and ease of use when equipment is available, and possibly also because the physician sees it as his tool which he understands better (Foulds and O'Brien, 1998). X-Ray has a high sensitivity, and it can detect TB in patients who do not excrete any bacilli, however it lacks specificity (Foulds and O'Brien, 1998). Other chest conditions may be accompanied by radiological abnormalities misread as TB, especially for AIDS patients, who may present with a variety of upper respiratory illnesses and may be (improperly) treated for TB (Foulds and O'Brien, 1998). These conditions include pneumocystis jiroveci pneumonia (PCP), pulmonary kaposi's sarcoma, cryptococcosis and nocadiosis (Harries, 1997). In areas with poor diagnostic facilities, it has been suggested that more stringent working case definitions be applied to presume pulmonary TB in order to assist in differentiating TB from these other conditions (Harries, 1997). No radiographic pattern is absolutely diagnostic of TB, although the classical hallmarks of the disease are cavitation, apical distribution, bilateral distribution, pulmonary fibrosis, shrinkage and calcification (Harries, 1997). Atypical findings such as infiltrations without cavitation involving lower lobes and hilar lymphadenopathy may also be seen, and are more common in patients with HIV infection (Harries, 1997). When the HIV testing was not available or when patients refused HIV testing atypical abnormalities on chest x-ray in patients with pulmonary TB alerted clinicians to the possibility of associated HIV infection (Mlika-Cabanne *et al.*, 1995a; Mlika-Cabanne *et al.*, 1995b). Chest radiographic manifestations correlate with the degree of immunosuppression (Daley, 1995;

Post *et al.*, 1995). Patients with relatively well-preserved immune function will show cavitation and upper lobe infiltrates typical of post-primary reactivation in immunocompetent patients. Those with severe immunosuppression will show changes atypical of primary TB, such as intrathoracic adenopathy and a miliary pattern (Daley, 1995). In HIV-positive TB patients in South Africa, the mean CD4 count was $389x10^6$/l in patients with upper zone infiltration compared with a mean CD4 count of $105x10^6$/l in patients with lower or mid-zone infiltrates, adenopathy, interstitial pattern or normal radiograph (Post *et al.*, 1995).

1.1.8.4 Extra pulmonary TB (EPTB)

The common forms seen in sub-Saharan Africa, and associated with HIV infection, are lymphadenopathy, serous effusions (particularly affecting pleura and pericardium) and miliary disease (Harries, 1997). Patients usually present with constitutional symptoms: fever, night sweats and weight loss and local features related to the site of the disease (Harries, 1997; Keshinro and Diul, 2006). Definitive diagnosis of extra pulmonary disease is often difficult in district hospitals in Africa, and the degree of diagnostic certainty requires specialised services such as histology (Harries, 1997). Bone marrow specimens have been used in the diagnosis of extra pulmonary tuberculosis (Bishburg *et al.*, 1986; Askgaard *et al.*, 1992; Riley *et al.*, 1995; Keiser *et al.*, 1997; Akpek *et al.*, 2001). When the sample is a bone marrow trephine biopsy the standard histopathologic approach is to do levels through the block to look for histological patterns suggestive of mycobacterial infection such as granulomata and to stain the trephine biopsy with the Ziehl-Neelsen stain for AFB once granulomata have been identified. This

approach may be justified as one study reported seeing AFB only in conjunction with granulomata, and suggested that it might be unnecessary to use ZN stains if no granulomata were seen (Riley *et al.*, 1995). Granulomata are the histopathologic hallmark of tissue mycobacterial infection; they represent an inflammatory response of an intact immune system to control mycobacterial infection (Marques *et al.*, 2000). In the setting of immunodeficiency the presence of granulomata has been noted to be less frequent, most likely as a direct consequence of impairment of the immune system in this condition (Marques *et al.*, 2000). Some studies however found no correlation between the presence of granulomata and the detection of mycobacteria, and that stainable organisms were found in the absence of granulomata (Bishburg *et al.*, 1986; Marques *et al.*, 2000).

1.1.8.5 PCR-based genetic tests

Detection is based on multiplication not of whole bacilli, as in culture, but of their genetic material, chromosomal DNA or ribosomal RNA. The main advantage of PCR-based techniques is their speed of analysis which improves diagnostic turnaround time. In principle, only 1-2 days are needed. This is true for diagnosis of TB, and even more so for applications such as diagnosis of drug resistance (mainly rifampicin) and species identification using probes. This early diagnosis will result in early treatment initiation, therefore reducing the potential for the disease transmission. PCR may forego the use of trial of anti-tubercular treatment for confirmation of diagnoses. The involvement of the reticuloendothelial system may constitute a critical clinical sign of disseminated disease with bacillary load too low

to be isolated by conventional culture procedures but detectable by PCR (Singh *et al.*, 2006). Conditions such as systemic candidiasis, nocardiosis, cryptococcosis that can easily simulate clinical presentation of disseminated tuberculosis can be quickly excluded by PCR (Singh *et al.*, 2006).

The main disadvantage of PCR-based tests is their extremely high cost, especially when more convenient and more sensitive commercial test kits are available (van Cleeff *et al.*, 2005). For resource-poor countries, detection of TB using PCR seems unaffordable and perhaps inaccessible. Even if TB was confirmed in a patient, dissemination to the bone marrow may not have occurred at the stage in which the sample was collected from the patient (Singh *et al.*, 2006).

A number of different nucleic acid amplification tests for mycobacteria have been shown to offer more rapid and potentially highly specific diagnosis. The first PCR study was conducted based on DNA coding for 16S rRNA for detection and identification of mycobacteria which included *M.tuberculosis* complex, *M.avium, M.intracellulare, M.kansasii* complex, *M.sacrofulaceum, M.xenopi, M.fortuitum and M.smegmatis* (Kox *et al.*, 1995). Gamboa *et al.,* (1998) using a ligase chain reaction technology, the LCx M.*tuberculosis* Assay (Abbott Laboratories, Diagnostics Division) amplified the chromosomal gene of MTB which codes for antigen b which is specific to MTB.

The PCR amplification of a 316-bp fragment of IS6110 insertion element of MTB complex has been reported (Kolk *et al.*, 1998; Ritis *et al.*, 2005). Kivihya-Ndugga *et al.,* (2004) used sputum sample and the Amplicor test system (Roche

Diagnostics Systems) to amplify a 584-bp ribosomal DNA sequence specific to the MTB complex. Using samples such as sputum, skin biopsies, synovial fluid, lymph node aspirates, ascitic fluid and bone marrow aspirate the investigators amplified the 62kDa gene (165-bp) of MTB with the use of PCR system 2700 thermal cycler (Negi *et al.*, 2005). Using bone marrow aspirate samples other investigators amplified 240-bp long region of the MPT64 gene for MTB using a thermocycler (Singh *et al.*, 2006).

The polymerase chain reaction has been shown to be useful in amplifying mycobacterial DNA in pulmonary and extra pulmonary samples. It has been used in clinical samples such as sputum, cerebral spinal fluid, pleural effusion, peripheral blood and bone marrow aspirates. Bone marrow aspirate has also been shown to be a valuable sample and PCR may be useful to identify the mycobacterial infection.

South Africa is a middle income nation with a nationally coordinated laboratory services that perform among others, culture methods, PCR analysis and histological examination of samples. The comparison of these three methods of TB diagnosis has not been reported in the setting of high prevalence of HIV-TB co-infection. We hypothesised that PCR analysis would be highly sensitive and specific in the diagnosis of mycobacterial infection from bone marrow samples and that the findings would stimulate the roll out of mycobacteria molecular diagnostics in South Africa. This might have an impact on costs of TB diagnosis and the management of TB in HIV-positive patients in South Africa. Other sub-

Saharan African countries, unlike South Africa, are resource-poor; the culture methods are not readily or routinely available and detection of TB using PCR is very unrealistic. The comparison of bone marrow trephine biopsy granulomata and bone marrow trephine biopsy Ziehl-Neelsen stain would help assess their diagnostic utility, and if highly diagnostic, could be promoted as alternatives in these resource-poor countries.

1.1.9 Aim of the current study

The aim of this study was to compare the diagnostic usefulness of TB culture, bone marrow trephine biopsy granulomata, bone marrow trephine biopsy Ziehl-Neelsen stain and bone marrow aspirate mycobacterial DNA PCR analyses in the diagnosis of mycobacterial infection in HIV-positive patients.

CHAPTER 2

2.1 MATERIALS AND METHODS.

2.1.1 Study design

This was a retrospective, uncontrolled study whereby the haematology laboratory bone marrow trephine biopsy registers were reviewed for those trephine biopsies which had ZN stain for acid-fast bacilli. These bone marrow trephine biopsies had ZN stain if mycobacterial infection was suspected or if bone marrow trephine biopsy histological findings were suggestive of mycobacterial infection. Once the samples were identified, laboratory records were reviewed on the laboratory information system for mycobacterial culture results and CD4 count results of samples drawn at around the same time the bone marrow aspirate and trephine biopsy were done. The patients who had mycobacterial culture done had their CD4 count result, bone marrow trephine biopsy reports and archived unstained air-dried bone marrow aspirate smears retrieved from the laboratory records and archive.

The bone marrow trephine biopsy histology reports were reviewed for reported presence or absence of granulomata and the results of ZN stain for acid-fast bacilli. The bone marrow trephine biopsy's corresponding unstained bone marrow aspirates were retrieved from the archive and were used for deoxyribonucleic acid (DNA) extraction for PCR analysis for *Mycobacterium tuberculosis*, *Mycobacterium avium intracellulare* and *Mycobacterium kansasii*.

2.1.2 Ethical clearance

Permission to use patient materials and laboratory medical records for this study was sought from the institutional review committee prior to study initiation.

Ethics approval was granted by the University of Witwatersrand Human Research Ethics Committee. The ethics clearance certificate number is M080618, and it is included in appendix A of this research report.

2.1.3 Study population selection

The subjects for this study were patients who presented to the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and Helen Joseph Hospital, which are the University of the Witwatersrand Medical Faculty teaching hospitals in Johannesburg, between January 2007 and December 2008.

2.1.4 Inclusion criteria.

- Patients who had bone marrow aspiration and trephine biopsy done to investigate clinically suspected tuberculosis.
- HIV positive by any HIV test method.
- Males and females > 18 years old.
- Patients who had blood and/or bone marrow aspirate mycobacteria culture samples taken around the time bone marrow aspirates and trephine biopsies were taken.

2.1.5 Exclusion criteria

- Patients who never had blood or bone marrow mycobacteria culture done.
- Patients with negative or unknown HIV test results.
- Patients without bone marrow trephine biopsy results.

The study design is shown in figure 2.1 below.

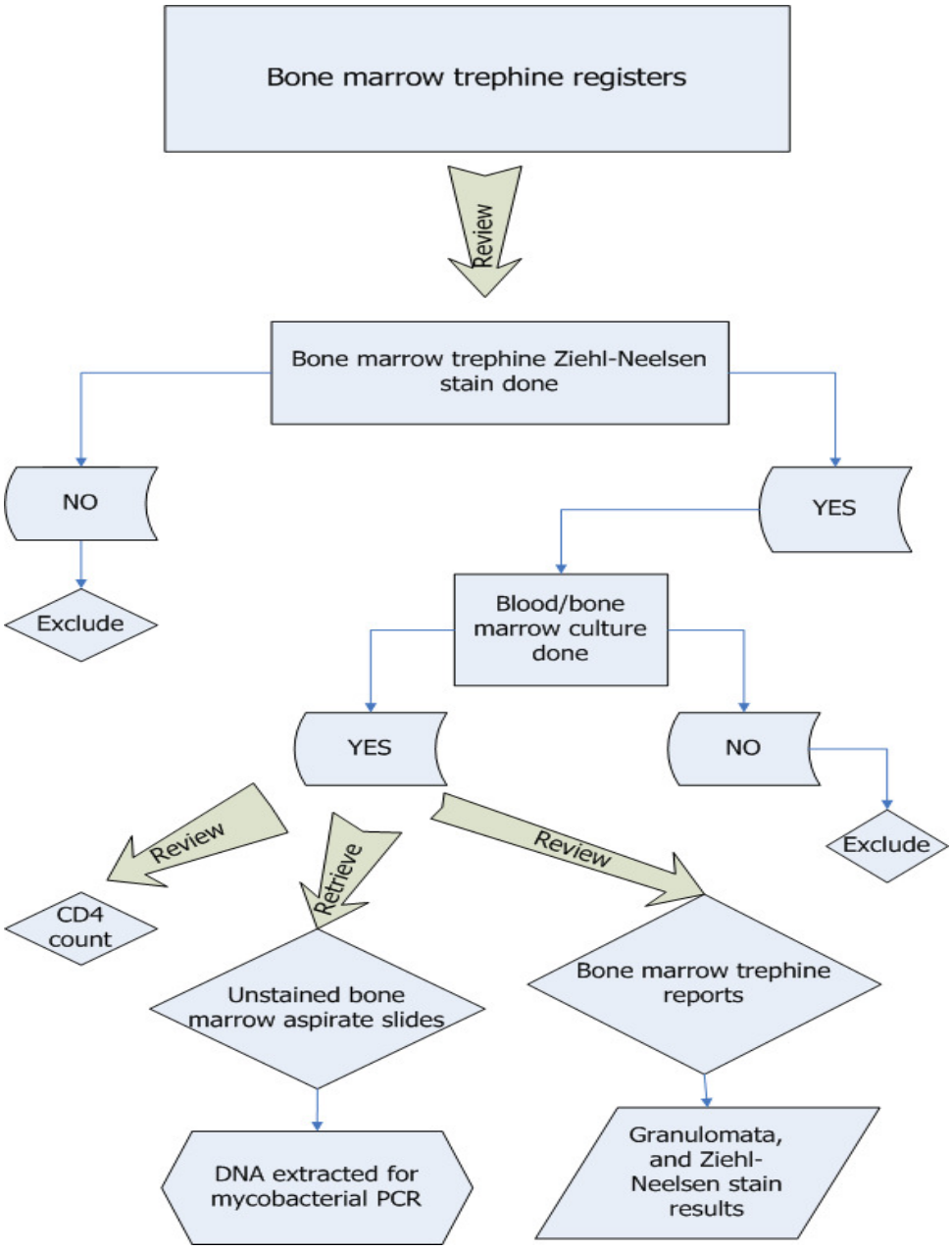


Figure 2.1 Flowchart of the study design

2.2.1 DNA extraction

High Pure PCR Template DNA Preparation Kit instructions manual and the National Health Laboratory Service DNA extraction standard operation procedure were used for the extraction of the DNA (Roche Applied Science, 2008a; Moodly, 2009) as shown in figure 2.2. In detail, the unstained air-dried bone marrow aspirate smears were washed off the slides using 500µl of the Blood Washer (Roche Applied Science, Germany).

Two hundred microlitres was transferred to 1ml Eppendorf tube (Merck Chemicals, South Africa) and the following were added:

- 200µl of the Binding Buffer (Roche Applied Science, Germany) [6M guanidine-HCl, 10mM urea, 10mM Tris-HCl, 20% Triton X-100(v/v), pH 4.4].
- 40µl of reconstituted Proteinase K (Roche Applied Science, Germany) which had to be thawed after being stored at -20^{o}C. Proteinase K was for sample lysis and inactivation of endogenous DNase.
- The mixture was vortexed and then incubated at 70^{o}C in a pre-warmed heating block for 10 minutes.
- 100µl of 99% isopropanol (Merck Chemicals, South Africa) was added to precipitate the DNA.
- The sample was pipetted into the upper reservoir of a combined Filter Tube-Collection Tube (Roche Applied Science, Germany) assembly.
- The sample was centrifuged for one minute at 8000g using Eppendorf centrifuge 5424 (Merck Chemicals, South Africa).
- The flowthrough liquid and the collection tube were discarded.

- The filter tube was combined with a new collection tube.
- 500µl of the Inhibitor Removal Buffer (Roche Applied Science, Germany) [5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6] was added to the upper reservoir of the sample, followed by centrifugation at 8000g for one minute.
- The flowthrough liquid and the collection tube were discarded, and the filter tube was combined with a new collection tube.
- 500µl of the Wash Buffer (Roche Applied Science, Germany) [20 mM NaCl, 2 mM Tris-HCl, pH 7.5] was added to the upper reservoir of the sample, followed by centrifugation for one minute at 8000g.This step was performed two times.
- The flowthrough liquid was discarded and the filter tube was combined with the same collection tube, and centrifuged at 14000g for 10 seconds to remove the Wash Buffer.
- The collection tube was discarded and the filter tube was inserted into a sterile 1.5 ml microfuge tube (Roche Applied Science, Germany).
- 100µl of pre-warmed (70^{o}C) Elution Buffer [10 mM Tris-HCl, pH 8.5] (Roche Applied Science, Germany) was added to the tube, followed by centrifugation at 8000g for one minute.
- The microfuge tube with the eluted DNA was stored at -20^{o}C until PCR analysis was performed.

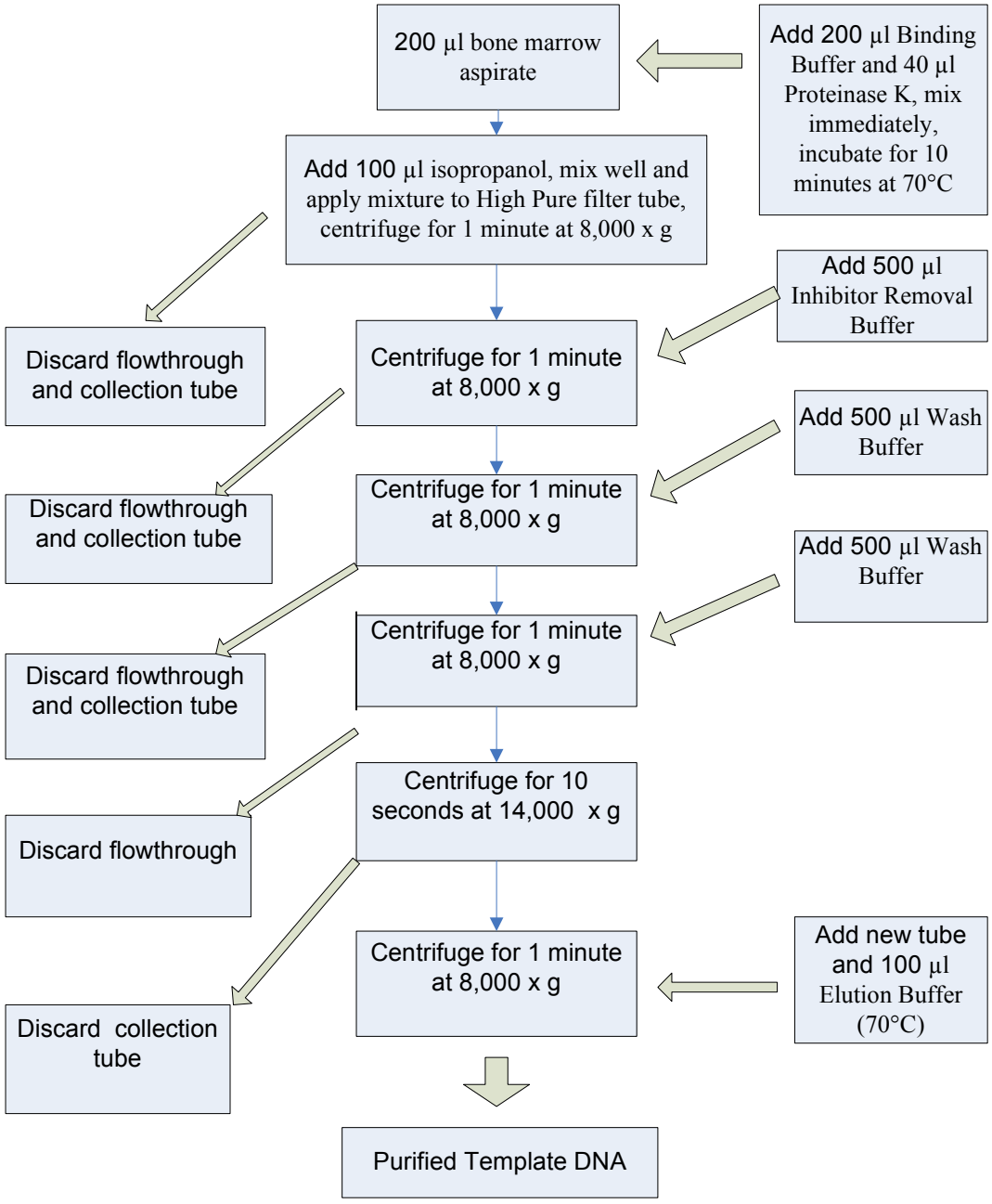


Figure 2. 2 Flowchart of manual DNA extraction

2.2.2 DNA quantification

DNA concentration was measured using NanoDrop® ND-1000 Spectrophotometer according to the manufacturer's manual (NanoDrop Technologies, 2006) and the National Health Laboratory Service standard operating procedure (Instrument Working Group, 2009). This instrument is a full-spectrum (220-750nm) spectrophotometer that utilised patented sample retention technology that employed surface tension alone to hold the sample in place. No cuvettes and other sample containment devices were required. It measured the concentration in nanogram per microlitre (ng/µl) based on absorbance at 260 nm and the selected analysis constant. The concentration was calculated using Beer's Law. The instrument has the capability to measure highly concentrated samples without dilution. Its detection limit is 2 ng/µl and has upper limit of detection of 3700 ng/µl of double stranded DNA. No reagents were required.

A 1µl DNA solution from stored sample was pipetted onto the end of a fibre optic cable (the receiving fibre). A second fibre optic cable (the source fibre) was brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends. A pulsed xenon flash lamp provided the light source and a spectrometer utilising a linear charge-coupled device (CCD) array was used to analyse the light after passing through the sample. When analysis was complete, the DNA concentration was displayed on the instrument's screen.
Before the samples were loaded onto the instrument, a blanking cycle using distilled water was performed. This was to ensure that the instrument was working well and that there was no non-specific fluorescence evidenced by a

spectrum with a relatively flat baseline. Upon completion of each sample measurement, both the receiving fibre and the source fibre were wiped using soft laboratory wipes to prevent sample carryover. The instrument required no calibration as the wavelength was auto calibrated based on known peaks in the xenon lamp spectra each time the software was started.

2.2.3 Amplification of mycobacterial DNA by PCR.

PCR detection was based on multiplication not of whole bacilli, as in culture, but of their genetic material, namely, chromosomal DNA. LightCycler TB Kit® for the detection of *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium kansasii* and LightCycler® Mycobacterium Detection Kit (Roche Applied Science, 2008b; Roche Applied Science, 2008c) were used for mycobacterial DNA polymerase chain reaction.

An approximately 200 base pair fragment was amplified from isolated mycobacterium DNA using specific primers. The oligonucleotide primers bound a portion of the 16S rRNA gene that included the hyper variable region A of the mycobacterium. The amplicon was detected by fluorescence using a specific pair of HybProbe probes. These probes consisted of two different oligonucleotide sequences that hybridised to an internal sequence of the amplified fragment during the annealing phase of the PCR cycle. One probe was labelled at the 5'-end with LightCycler® Red 640 fluorophore and was phosphorylated at the 3'-end to avoid extension. The other probe was labelled at the 3'-end with flourescein. After hybridisation to the template DNA, the two probes came in close proximity,

resulting in fluorescence resonance energy transfer (FRET) between the fluorophores. The emitted fluorescence for the amplicon was detected using LightCycler® Red 640. A synthetic internal control was detected using HybProbe probes labelled with LightCycler® Red 705.

Real Time Polymerase Chain Reaction LightCycler® 1.5 (Roche Applied Science, Germany) instrument was used for the PCR. The PCR kits, that is, the LightCycler TB Kit® and the LightCycler® Mycobacterium Detection Kit comprised the following: enzyme, reaction mix, PCR grade H_20, detection mix, positive control, negative control, internal control and uracil-DNA glycosylate. LightCycler TB Kit® did not contain uracil-DNA glycosylate.

Additional equipment and reagents required included:

- Ultra violet dead-air box cabinet (locally made by the NHLS maintenance department).
- LightCycler capillaries (Roche Applied Science, Germany).
- LightCycler capping tool (Roche Applied Science, Germany).
- Eppendorf (Merck Chemicals, South Africa) bench top micro centrifuge.
- LightCycler carousel (Roche Applied Science, Germany) which provided centrifuge adapters that enabled the LightCycler capillaries to be centrifuged in a standard micro centrifuge rotor.
- 10, 100, 1,000 µl Finnitip® pipettes (AEC Amersham, South Africa).
- Nuclease-free, aerosol-resistant pipette tips (10 µl tips were supplied by AEC Amersham, South Africa; and the 50, 200, 1000 µl tips were supplied by Whitehead Scientific, South Africa).

- Sterile reaction (Eppendorf) tubes for preparing master mixes and dilutions.
- Gloves (Kendon Medical Supplies, South Africa).
- Marker pen for labelling tubes.
- Bleach (NHLS Diagnostic Media Products, Johannesburg) and ethanol (Merck Chemicals, South Africa) in spray bottles for cleaning the UV cabinet.
- Standard fridge with freezer.
- LightCycler capillary releaser (Roche Applied Science, Germany)
- Sharps bin, for used LightCycler capillaries.

Unfortunately Roche, South Africa, due to patency secrecy, could not disclose the sequences of the primers, the detection mix nor the compositions of the internal controls, negative controls, positive controls, the enzymes and the reaction mix.

2.2.3.1 PCR set up.

The PCR preparation mixes are shown in tables 2.1 and 2.2.

Table 2.1 PCR mix preparation for LightCycler TB Kit®

Component	Volume for one reaction
LCTB MMX (pink)	11 µl
Master Mix (vial 1)	4 µl
Internal Control	1 µl
Total reagent mix	**16 µl**

LCTB MMX = LightCycler TB detection mix
Master Mix = Enzyme + Reaction mix

Table 2.2 PCR mix preparation for LightCycler® Mycobacterium Detection Kit

Component	**Volume for one reaction**
Detection Mix (vial 3)	11 µl
Master Mix (vial 1)	4 µl
Internal Control (vial 6)	0.75 µl
Uracil-DNA Glycosylase (vial 7)	0.25 µl
Total reagent mix	**16 µl**

LightCycler® capillaries were placed in pre-cooled centrifuge adapters. The reagent mix was carefully mixed by pipetting up and down, and then 16 µl of reagent mix was pipetted into each capillary.

Four micro litres of each patient sample was added to its respective capillary tube. Four microlitres each of a positive and a negative control were added to separate capillary tubes. The capillary tubes were sealed with stoppers then centrifuged at 3000 revolutions per minute for five seconds. The PCR was run under the following conditions:

- Denaturation at 95^{o}C for 600 seconds.
- Amplification: 45 cycles at 95^{o}C for 10 seconds, 50^{o}C for 10 seconds and 72^{o}C for 20 seconds.
- Melting curve: 1 cycle at 95^{o}C for 60 seconds, 40^{o}C for 120 seconds and 68^{o}C for zero seconds.
- Cooling: 1 cycle at 40^{o}C for 30 seconds.

2.2.3.2 PCR Data interpretation

Colour compensation file was required for analysis of data. Manual melting temperature (Tm) was used to analyse the results. *Mycobacteria* species were viewed in channel 640, and the Internal Control was viewed in channel 705.
If the positive control and/or the negative control failed, the entire run was considered invalid and had to be repeated. For the valid run the control results were interpreted according to the table 2.3 below:

Table 2.3 Interpretation of a valid run

Mycobacterium (channel 640)	**Internal Control (channel 705)**	**Result interpretation**
Positive	Positive	Positive
Negative	Positive	Negative
Positive	Negative	Positive
Negative	Negative	Invalid result

Positive control PCR (channel 640) showed a single melting peak with a Tm value of 58.1 ± 1.5^{o}C (figure 3.2). The negative control always showed no signal in channel 640, but it showed a Tm of 63.1 ± 1.5^{o}C in channel 705 (figure 3.4). The *Mycobacteria* species were viewed in channel 640 and their melting temperature ranges are shown in table 2.4

Table 2.4 Result interpretation

Organism	**Mean Tm (^{o}C)**
Mycobacterium tuberculosis	55-57 ± 1.5
Mycobacterium kansasii	59-62 ± 1.5
Mycobacterium avium	50-53 ± 1.5

2.2.4 Mycobacterial culture

The cultures were done at NHLS Mycobacterial Referral Laboratory, Johannesburg as per standard operating procedures (Rautenbach, 2008; TB Laboratory Working Group, 2008; Dolby, 2009).

Bactec Myco/F lytic blood culture bottles (Becton Dickinson, USA), the Bactec 9240 Blood Culture System (Becton Dickinson, USA) and Accuprobe DNA probes (GeneProbe, USA) for mycobacteria identification were used. Other equipment and materials required were:

- Lowenstein Jensen Media (LJ) (NHLS Diagnostic Media Product, Johannesburg)
- Syringes and needles,
- Light microscope,
- Microscope slides,
- Stains, and
- Aerobic incubator.

Five millilitres of blood or bone marrow specimen were collected by hospital staff and inoculated into the Bactec Myco/F lytic blood culture bottles (Becton Dickinson, USA). These bottles contained Middlebrook 7H9 and brain heart infusion broth formulation for the recovery of mycobacteria. Specific modifications were made to enhance the growth and recovery of mycobacteria. These modifications included ferric ammonium citrate to provide iron source for specific strands of mycobacteria. Other supplements included saponines (blood lysing agent), anticoagulants and specific proteins and sugars to provide nutritional supplements.

The culture bottles were placed in the Bactec 9240 Blood Culture System (Becton Dickinson, USA) a continuous-monitoring blood culture system. Each culture bottle contained a sensor which could detect decreases in oxygen concentration resulting from micro organism metabolism and growth. The sensor was monitored by the Bactec Blood Culture System for increasing fluorescence which was proportional to the decrease in oxygen. A positive determination indicated the presumptive presence of micro organisms in the bottle.

The culture bottles that were positive were processed as follows:

- A smear was prepared and a Ziehl-Neelsen stain (NHLS Diagnostic Media Products, Johannesburg) was performed to establish the presence and morphology of the acid-fast bacilli.
- Depending on the morphology of the micro organisms, the following detection kits were used for species identification: Accuprobe Mycobacterium Tuberculosis Complex Culture Identification test (GeneProbe, USA), Accuprobe Mycobacterium Avium Complex Identification test (GeneProbe, USA) and Accuprobe Mycobacterium Kansasii Identification test (GeneProbe, USA).

Positive and negative controls were used routinely in the Accuprobe tests. The choice of Accuprobes used depended on the morphology of the organism seen on Ziehl-Neelsen stain. If, for example, the morphology suggested mycobacterium tuberculosis, mycobacterium tuberculosis was used as a positive control and mycobacterium kansasii was used as a negative control.

The Accuprobe mycobacterial identification test was a rapid DNA probes that utilised the technique of nucleic acid hybridisation for the identification of

mycobacterium. A single strand DNA probe with a chemiluminescent label that was complimentary to the ribosomal RNA of the target organism, combined to form a stable DNA: RNA hybrid, which was measured using a luminometer. The luminometer was interpreted as follows:

Cut-off range: 30,000 RLU.

Repeat range: 20,000-29,999 RLU

Negative < 10,000 RLU

Positive > 30,000 RLU.

If the culture bottles were positive but no mycobacteria were observed on stain, a subculture was made onto LJ medium for 6 weeks, and read every week. If positive mycobacteria identification was done, the species were identified using the Accuprobes. If negative after 6 weeks, the sample was reported as no growth.

2.2.5 Bone marrow trephine biopsy histological preparation

Bone marrow trephine biopsy histological preparation was done in the Histopathology Department of the National Health Laboratory Service Charlotte Maxeke Johannesburg Academic Hospital as per tissue processing standard operating procedure (Anatomical Pathology Working Group, 2007). The trephine biopsies were fixed in a neutral buffered formalin (Barr Pharmaceuticals) once obtained from the patients. When the trephine biopsies were received in the Histopathology department they were further fixed for 24 hours in 10% formalin (Merck Chemicals, South Africa) and then placed in cassettes. They were then

softened in ethylenediaminetetraacetic acid (EDTA) for 24-48 hours. The following steps were then done:

2.2.5.1 Processing

Equipment Tissue-Tek® VIP™ (Siemens, Southern Africa) was used to process the trephine biopsies. The whole process included immersion of trephine biopsies into different concentrations of formalin, then alcohol followed by the clearing agent and wax impregnation.

In detail there were 14 stages. Stages 1 and 2 involved the immersion of the trephine biopsy in 10% formalin for 2.5 hours per each stage. Stage 3 involved the immersion of the trephine biopsy in 80% alcohol (SMM Instruments, South Africa) for 30 minutes. In stages 4 and 5 the trephine biopsy was immersed in 95% alcohol (SMM instruments, South Africa) for 30 minutes per stage. In stages 6, 7, and 8 the trephine biopsy was immersed in absolute alcohol (SMM Instruments, South Africa) for 30 minutes, 30 minutes and 40 minutes respectively. In stages 9 and 10 the samples were immersed in xylene (Merck Chemicals, South Africa) for 30 and 40 minutes respectively. Stages 1 to 10 were performed at 40^0C. Stages 11 to 14 were performed at 60^0C and all involved immersion of the trephine biopsy in wax (Siemens, Southern Africa) for 30 minutes per stage.

2.2.5.2 Embedding

Tissue-Tek® TEC™ (Siemens, Southern Africa) was used. Hot wax was put onto the sample cassettes followed by cooling.

2.2.5.3 Cutting

Microtome was used to cut the embedded trephine biopsy. The cut sections were placed onto the glass slides and then floated in 40-50^{o}C water bath for the sections to stretch. The slides were then put on a 50^{o}C hot plate for at least 15 minutes to allow the sectioned trephine biopsies to adhere to the glass slides.

2.2.5.4 Final step

The trephine biopsies were immersed in xylene, then 100% alcohol followed by 95% alcohol to dehydrate the trephine biopsies. They were then washed in tap water.

The trephine biopsies were then ready for haematoxylin and eosin (HE) stain and the Ziehl-Neelsen stain.

2.2.6 Haematoxylin and eosin stain

The bone marrow trephine biopsies were stained with haematoxylin stain (Merck Chemicals, South Africa) for 10 minutes and then rinsed with tap water to remove excess haematoxylin. The trephine biopsies were then immersed in Scott's water (Merck Chemicals, South Africa) for 1 minute to make the nuclei blue, and then they were washed in tap water. They were stained with eosin (Merck Chemicals, South Africa) for 5 minutes and then washed in tap water to remove excess eosin. They were then immersed in 3 basins of 100% alcohol each to dehydrate the samples, followed by 2 basins of xylene to clear the sections. The cover slips were put onto the slides to protect the trephine biopsies from scratches and drying out.

2.2.7 Ziehl-Neelsen stain.

Ziehl-Neelsen stain (NHLS Diagnostic Media Products, Johannesburg) for demonstration of acid fast bacilli was done in the NHLS Histopathology Department at the CMJAH as per currently used standard operating procedure (Anatomical Pathology Working Group, 2009). The trephine biopsies were stained with filtered carbol fuschin for 5 minutes and then rinsed in tap water. Ninety-five percent alcohol was used to rinse the trephine biopsies to remove the excess carbol fuschin followed by tap water rinsing. Seventy percent acid alcohol was used for differentiation until the sections were pale blue followed by rinsing in tap water. Methylene blue was added to the trephine biopsies for counter staining for 1-2 minutes followed by rinsing in tap water. The trephine biopsies were then dried by air or by blotting. Cover slips were mounted onto the slides. Ziehl-Neelsen stain was always done with a known ZN positive section as a control to control for staining and procedural artefacts.

2.3 STATISTICAL ANALYSIS.

All results were tabulated in Microsoft Excel® spread sheets. Mycobacterial culture was considered the gold standard for the diagnosis of mycobacterial infection in this study. A 2 X 2 contingency table was constructed to calculate sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each of the three diagnostic methods.

CHAPTER 3

3.1 RESULTS

3.1.1 Study population

A total of seventy patients were reviewed and assigned study numbers 1 to 70. Ten patients (study numbers 25, 26, 27, 37, 53, 54, 55, 61, 68 and 69) were excluded from the study due to lack of complete data such as HIV status and /or incomplete culture results. Sixty patients met the inclusion criteria and were therefore included in the study. These patients presented to Charlotte Maxeke Johannesburg Academic Hospital and Helen Joseph Hospital with clinical suspicion of mycobacterial infection.

In the eligible study population of 60 patients, females comprised 31 subjects (51%) and males comprised 29 subjects (49%). The mean age was 35.5 years (range 21-55 years) as shown in table 3.1. The study population laboratory findings, that is, the culture results, bone marrow trephine biopsy ZN stain, CD4 count, mycobacterial DNA PCR result and bone marrow trephine biopsy histological findings, are shown in the table 3.2

Table 3. 1 Demographics of the study population

Study Number	Age (years)	Sex	Study Number	Age (years)	Sex
1	47	F	34	35	F
2	40	F	35	24	F
3	32	F	36	36	F
4	39	M	38	36	F
5	25	M	39	32	M
6	33	F	40	37	F
7	49	M	41	31	M
8	38	F	42	44	M
9	29	F	43	34	F
10	39	F	44	35	F
11	42	M	45	36	F
12	32	M	46	34	M
13	27	F	47	39	M
14	29	M	48	29	F
15	47	M	49	37	F
16	40	M	50	41	M
17	40	M	51	25	M
18	30	F	52	37	M
19	40	F	56	29	F
20	40	F	57	55	M
21	49	M	58	45	M
22	30	M	59	30	F
23	44	F	60	23	M
24	31	M	62	33	M
28	31	F	63	45	M
29	40	M	64	24	M
30	54	F	65	25	F
31	30	M	66	51	M
32	28	F	67	21	F
33	22	F	70	35	F

M = Male; F = Female
Number of patients screened= 70
Number of patients eligible for study = 60

Table 3. 2 Study population culture, trephine, CD4 and PCR results

Study number	Culture	ZN stain	Granu loma	CD4 Count ($X10^6$/l)	PCR	Study number	Culture	ZN stain	Granu loma	CD4 Count ($X10^6$/l)	PCR
1	-	-	-	127	-	34	MTB	+	+	9	-
2	-	-	+	7	MTB	35	MTB	+	+	0	MTB
3	-	-	-	ND	-	36	MA	+	+	40	-
4	-	-	+	ND	-	38	MTB	+	+	ND	MTB
5	-	+	+	ND	-	39	MTB	+	+	31	-
6	-	+	-	6	-	40	MTB	-	+	4	-
7	-	-	-	42	-	41	MA	-	+	8	MA
8	-	-	+	375	-	42	MTB	+	+	18	-
9	-	-	+	70	-	43	MA	+	+	73	MA
10	-	-	+	194	-	44	MTB	+	+	20	MTB
11	-	+	+	33	MTB	45	MTB	-	+	848	-
12	-	+	+	57	MTB	46	MTB	-	+	9	-
13	-	-	+	146	MK	47	MA	+	+	0	MA
14	-	-	+	98	-	48	MA	+	+	0	MA
15	-	-	+	196	-	49	MA	+	+	31	MA
16	-	+	+	22	MTB	50	MA	-	+	34	MA
17	-	+	+	62	-	51	MTB	-	+	ND	-
18	-	+	+	21	-	52	MA	+	+	1	MA
19	-	-	-	21	-	56	MTB	+	+	24	MTB
20	-	+	+	14	-	57	MTB	-	+	18	-
21	MTB	-	+	65	-	58	MA	+	+	ND	-
22	MTB	+	+	89	-	59	MA	+	+	2	MA
23	MTB	+	+	2	-	60	MTB	+	+	2	MTB
24	MA	+	+	7	MK	62	MTB	-	+	23	-
28	MA	-	-	8	-	63	-	-	+	3	-
29	MTB	-	+	32	-	64	-	+	+	67	-
30	MTB	+	+	14	MTB	65	-	+	+	136	MTB
31	MTB	-	+	40	-	66	-	-	+	207	-
32	MA	+	+	32	MTB	67	-	-	-	13	-
33	MTB	+	+	37	MTB	70	-	+	+	14	MTB

MA = Mycobacterium avium
MTB = Mycobacterium tuberculosis
MK = Mycobacterium kansasii

- = Negative
+ = Positive
ND = Not done

3.1.2 Mycobacterial culture

In the presence of adequate controls, 34 out of 60 patients analysed had positive mycobacterial culture results (study numbers 21-24, 28-36, 38-52, 56-60 and 62) and 26 out of 60 were mycobacterial culture negative (study numbers 1-20, 63-67 and 70). The following culture negative patients: study numbers 1, 14, 15, 63, 64, 65 and 66 were on anti-tuberculosis therapy for unspecified duration at the time the samples were collected for analysis.

Of the 34 positive culture results, 21 (62%) were *Mycobacterium tuberculosis* and 13 (38%) were *mycobacterium avium*. There was no *mycobacterium kansasii* detected on culture methods in this study population.

Of the 26 culture negative patients, 5 patients (patient study numbers 11, 12, 16, 63 and 70) showed positivity for granulomata, ZN staining and PCR for MTB. Further 4 patients (patient study number 5, 17, 18 and 64) showed positivity for granulomata and ZN staining.

3.1.3 Bone marrow trephine biopsy granulomata

Out of the 34 patients with positive mycobacterial culture, 33 patients (97%) had granulomata present in their bone marrow trephine biopsies and only 1 (3%) had no granulomata present.

Out of 26 mycobacterial culture negative patients, 20 (77%) had granulomata in their bone marrow trephine biopsies, and 6 (23%) did not have granulomata.

3.1.4 Bone marrow trephine biopsy Ziehl-Neelsen (ZN) stain

In the presence of adequate ZN positive controls, 22 of 34 (65%) culture positive patients had positive ZN stain on their bone marrow trephine biopsies while 12 out of 34 (35%) were negative for ZN stain. Fifteen of 26 (58%) culture negative patients had negative ZN stain on their bone marrow trephine biopsies. Five of these 15 patients were on anti-tuberculosis therapy at the time of specimen collection. Eleven of 26 (42%) culture negative patients stained positive for ZN stain on bone marrow trephine biopsies. Two of these 11 patients were on anti-tuberculosis therapy.

3.1.5 Bone marrow trephine biopsy Ziehl-Neelsen stain and bone marrow trephine biopsy granulomata

Thirty-three patients had positive bone marrow trephine biopsy Ziehl-Neelsen stain; 32 (97%) of which had granulomata in their bone marrow trephine biopsies. Using ZN stain as standard, granulomata showed a sensitivity and a specificity of 97% and 22% respectively.

3.1.6 CD4 count

Six patients did not have their CD4 count done. The mean CD4 count for 54 patients was $67.7x10^6$/l (range 0-$848x10^6$/l).

Out of 34 culture positive patients 31 had CD4 count done, and their mean CD4 count was $49x10^6$/l (range 0-$848x10^6$/l). The patients diagnosed with *Mycobacterium avium intracellulare* on culture had a mean CD4 count of

19.7x10^6/l (range 0-73x10^6/l). The patients who were diagnosed with *Mycobacterium tuberculosis* had a mean CD4 count of 67.6x10^6/l (range 0-848x10^6/l).

Out of 26 culture negative patients 23 had CD4 count done and their mean CD4 count was 84x10^6/l (range 3-375x10^6/l.

3.1.7 DNA extraction

DNA was extracted from 70 patients ten of which (study numbers 25, 26, 27, 37, 53, 54, 55, 61, 68 and 69) were excluded. Sixty met inclusion criteria and their DNA concentration is shown in table 3.3.

Table 3. 3 Patient DNA concentration

Study number	DNA concentration (ng/µl)	Study number	DNA concentration (ng/µl)
1	43.1	34	7.2
2	242.5	35	3.0
3	19.5	36	190.6
4	49.5	38	43.8
5	8.4	39	29.7
6	7.3	40	12.7
7	10.2	41	140.9
8	14.5	42	15.0
9	201.6	43	128.8
10	40.5	44	26.8
11	13.3	45	188.0
12	38.8	46	22.2
13	20.8	47	2.7
14	91.5	48	4.6
15	23.2	49	3.1
16	115.0	50	2.5
17	210.0	51	2.7
18	18.3	52	73.0
19	24.0	56	16.5
20	8.1	57	27.7
21	175.1	58	22.3
22	46.9	59	106.4
23	5.8	60	141.8
24	15.7	62	201.5
28	12.7	63	7.3
29	176.0	64	6.7
30	26.3	65	42.8
31	28.3	66	10.1
32	136.6	67	41.4
33	203.3	70	12.0

ng/µl = nanogram per microlitre.

3.1.8 Mycobacterial DNA PCR

Mycobacterial DNA PCR analysis was performed on bone marrow aspirates from all 60 patients as described above. For all PCR runs the positive controls and negative

controls were successful as shown in figures 3.1 and 3.2. The Internal Control was added to positive control, negative control and the study samples, and co-amplification of the internal control is shown in figures 3.3 and 3.4.

In the presence of adequate positive and negative controls 17 of the 34 (50%) mycobacterial culture positive patients yielded positive mycobacterial DNA on PCR. A typical positive PCR is shown as a green curve in figures 3.1 and 3.2.

The mycobacterial culture result of patient study number 24 yielded *Mycobacterium avium*, but the mycobacterial DNA PCR analysis yielded *Mycobacterium kansasii*. Mycobacterial culture for patient number 32 yielded *Mycobacterium avium* but the DNA PCR yielded *Mycobacterium tuberculosis* (table 3.2)

Out of 26 mycobacterial culture negative patients, mycobacterial DNA was amplified in 7 samples (23%); and 19 samples (73%) were negative on PCR. A typical negative PCR result is shown as a pink flat curve/ line in figures 3.1 and 3.2.

Out of 24 mycobacterial DNA PCR positives 14 (58.3%) were *Mycobacterium tuberculosis*, 8 (33.3%) were *Mycobacterium avium intracellulare* and 2 (8.3%) were *Mycobacterium kansasii*. The mycobacteria subtypes were differentiated by their melting temperatures (Tm) as shown in figure 3.2 (red for MA with Tm of 49.1^0C and green for MTB with Tm of 55.3^0C).

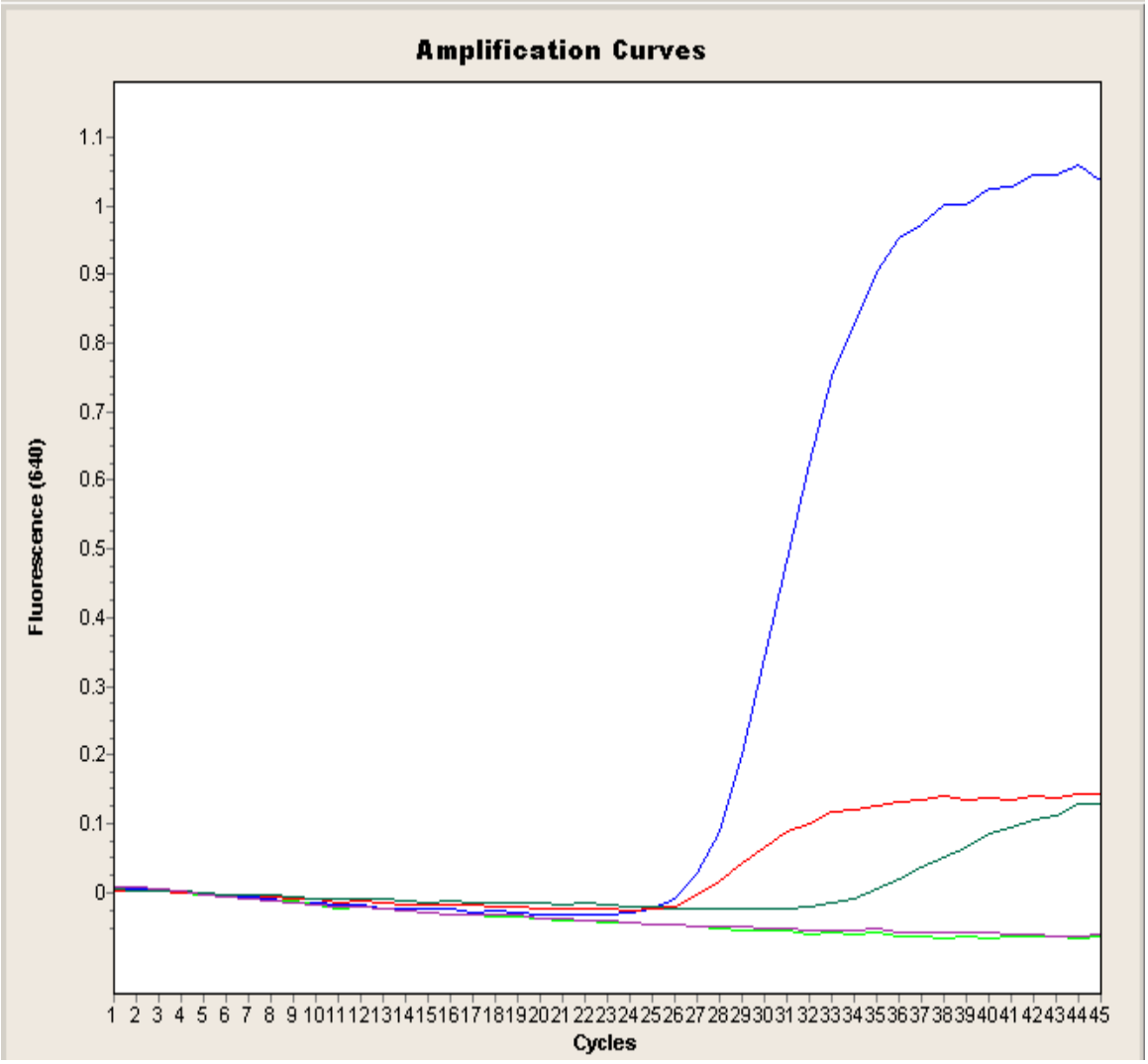


Figure 3.1 Target amplification curves for controls and representative patients

- Positive Control
- Study number 48: *Mycobacterium avium intracellulare*
- Study number 56: *Mycobacterium tuberculosis*
- Negative Control
- Study number 62: No amplification

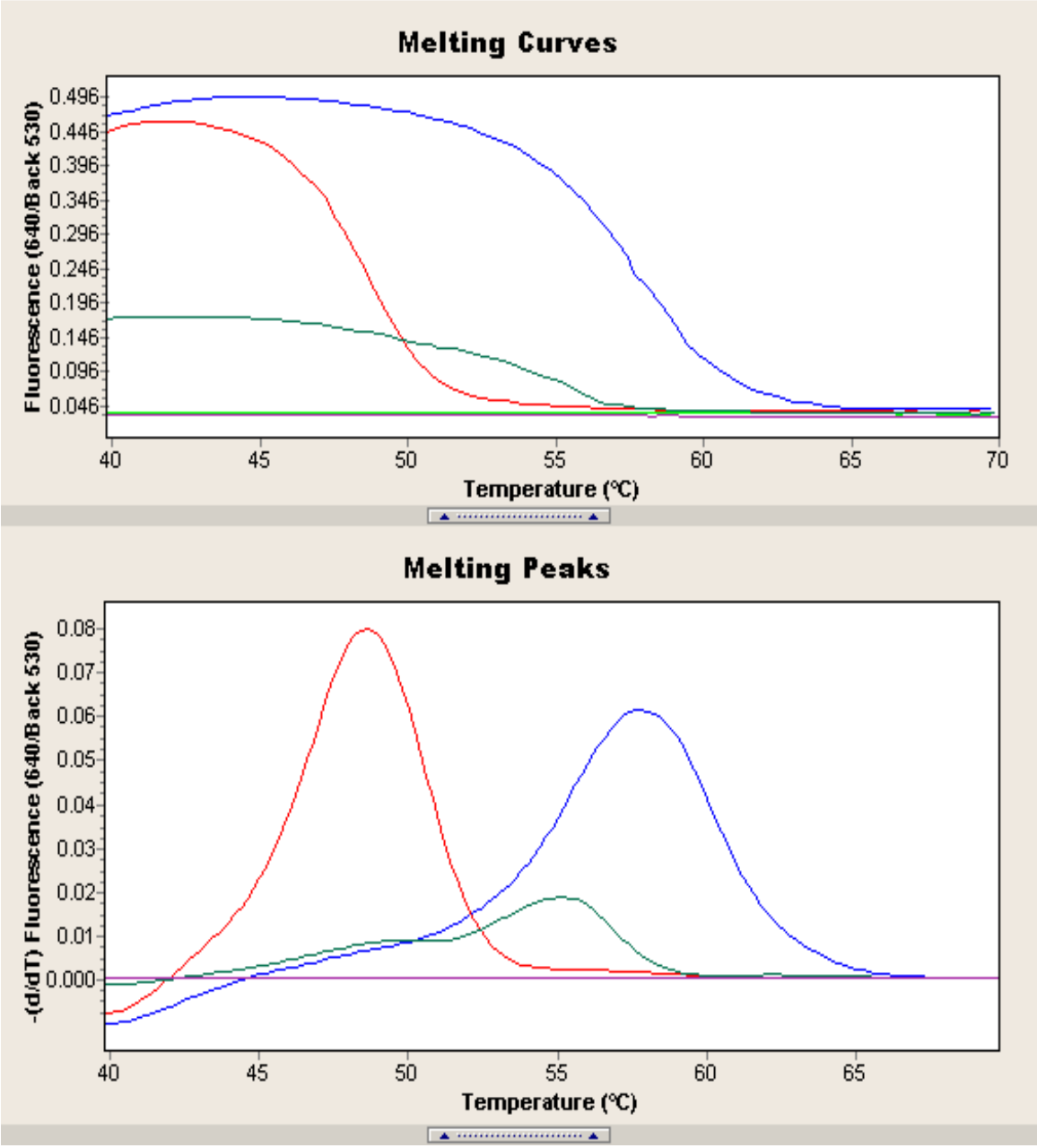


Figure 3.2 Target melting curves and melting peaks for controls and representative patients

- Positive Control
- Study number 48: *Mycobacterium avium intracellulare*
- Study number 56: *Mycobacterium tuberculosis*
- Negative Control
- Study number 62: No amplification

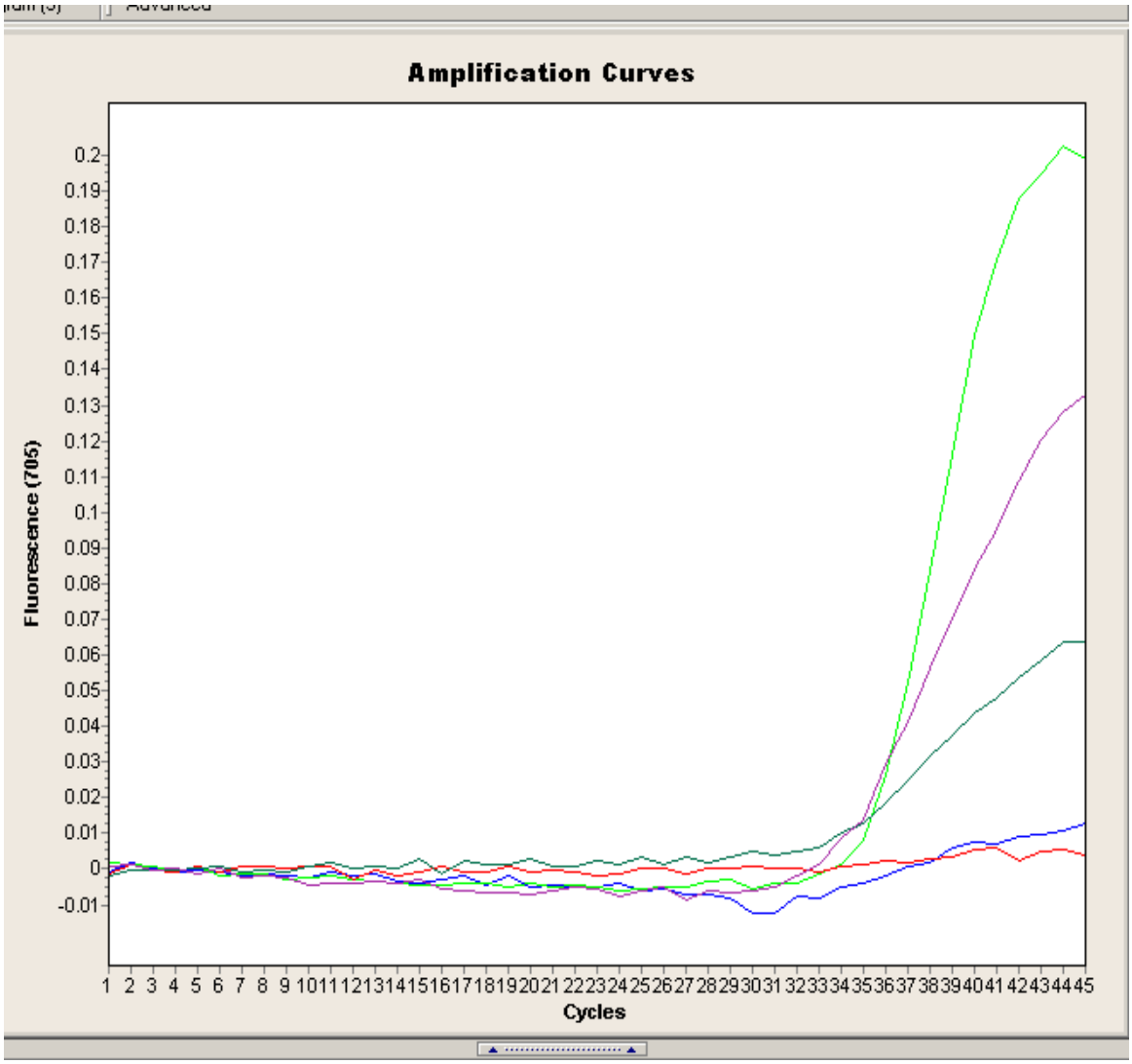


Figure 3.3 Internal Control amplification curves

- Positive Control
- Study number 48
- Study number 56
- Negative Control
- Study number 62

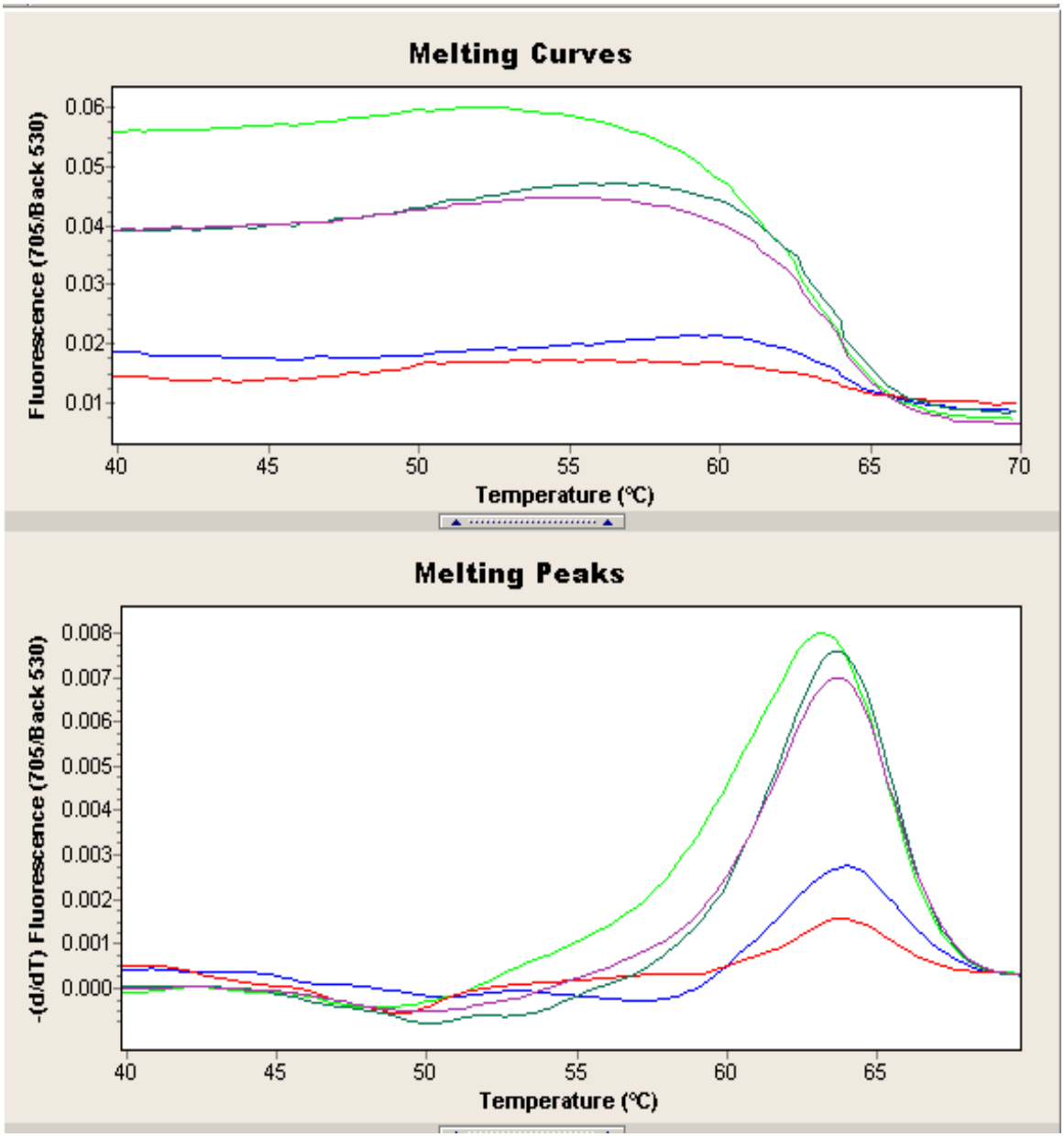


Figure 3.4 Internal Control melting curves and melting peaks

- Positive Control
- Study number 48
- Study number 56
- Negative Control
- Study number 62

3.1.9 Statistical comparison of different methods

The sensitivity, specificity, positive predictive value and negative predictive value of each diagnostic method were calculated using culture method as gold standard and the results are shown in table 3.4.

Table 3. 4 Comparison of the three diagnostic method results using mycobacterial culture as gold standard

Diagnostic method	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %
Mycobacterial DNA PCR	50	73	71	53
Bone marrow trephine biopsy granulomata	97	23	62	86
Bone marrow trephine biopsy Ziehl-Neelsen stain	65	58	67	56

Sensitivity refers to how good a test is at correctly identifying people who have the disease. It is calculated by dividing the number of true positives (TP) by the sum of true positives (TP) and false negatives (FN) (Loong, 2003).

[Sensitivity= TP/TP+FN].

True positives are defined as sick people correctly identified as sick, and false negatives as sick people incorrectly identified as healthy (Loong, 2003).

Specificity refers to how good the test is at correctly identifying people who are well. It is calculated by dividing true negatives (TN) by sum of true negatives (TN) and false positives (FP) (Loong, 2003). [Specificity= TN/TN+FP].

True negatives are defined as healthy people correctly identified as healthy and false positives as healthy people incorrectly identified as sick (Loong, 2003).

Positive predictive value (PPV) is the proportion of patients with positive test results who are correctly diagnosed. It is calculated as follows: (Altman and Bland, 1994; Loong, 2003)

PPV= TP/ TP + FP, or

(Sensitivity)(Prevalence)/(sensitivity) (prevalence) + (1-specificity) (1-prevalence).

Negative predictive value (NPV) is the proportion of patients with negative results who are correctly diagnosed. It is calculated as follows: (Altman and Bland, 1994; Loong, 2003)

NPV= TN/ TN+FN, or

(Specificity)(1-prevalence)/(specificity)(1-prevalence)+(1-sensitivity) (prevalence).

CHAPTER 4

4.1 DISCUSSION

4.1.1 Mycobacterial Culture

No test is considered the gold standard for the diagnosis of mycobacterial infection (Escobedo-Jaimes *et al.*, 2003). This study however regarded the culture method as a gold standard against which other methods were compared. No distinction was made between bone marrow aspirate cultures and blood cultures as these two methods have been shown previously to have similar mycobacterial diagnostic yields (Riley *et al.*, 1995; Kilby *et al.*, 1998; Akpek *et al.*, 2001; Pacios *et al.*, 2004).

In this study, mycobacterial infections by culture methods comprised 62% *Mycobacterium tuberculosis* (MTB) and 38% *Myobacterium avium intracellulare* (MA). In a study of patients from Chris Hanni Baragwanath Hospital, 29 positive blood cultures comprised 86% MTB and 14% MA (Karstaedt *et al.*, 2001). In another South African study of blood culture, 23 out of 71 patients were found to be culture positive; 16 out of 23 (70%) had MTB and 7 out of 23 (30%) had MA (von Gottberg *et al.*, 2001).

In a study conducted in Kenya using sputum samples, of the 490 positive cultures, 486 (99%) were identified as MTB (Kivihya-Ndugga, L. *et al.*, 2004). In another Kenyan study (Gilks *et al.*, 1995) mycobacterial blood culture yielded 23% MTB and 6% MA.

In Malawi in a study of 173 patients presenting with HIV infection and fever 20/173 (12%) were diagnosed with MTB and only one (0.5%) was diagnosed with MA on blood culture (Archibald *et al.*, 2000).

In a Mexican study using sputum, urine, bone marrow aspirates, blood, biopsies of lymph nodes, pleura, larynx, bone or skin all the culture results yielded MTB (Escobedo-Jaimes *et al.*, 2003).

In a Spanish study blood and bone marrow aspirate cultures revealed 39% MTB and 61% MA (Pacios *et al.*, 2004). A study in Boston (Akpek *et al.*, 2001) a culture positivity of bone marrow, blood, sputum or bronchoaveolar lavage revealed 25/56 (57%) MA and 5/56 (9%) MTB.

In a study to detect mycobacteria in bone marrow during the investigation of pyrexia of unknown origin at St Mary's Hospital in London (Riley *et al.*, 1995) bone marrow cultures of HIV positive patients revealed 82.3% MA, 9.8% MTB. The rest was made up of MK and *Mycobacterium xenopi*.

In a study by Marques *et al.,* (2000) in USA 27 patients had mycobacterial infection detected by blood and/or bone marrow culture. Twenty-five of 27 (94%) were MA and 2 of 27 (7%) were MTB. In another study in USA by Keiser *et al.,* (1997) 30 cultures of bone marrow aspirates revealed 22 (73%) MA, 1(3%) MTB and the remaining 24% comprised histoplasma and aspergillus.

In this study MTB was the predominant mycobacterial species. The prevalence of MA in this study is lower than the prevalence reported in UK , USA and Europe (Riley *et al.*, 1995; Keiser *et al.*, 1997; Marques *et al.*, 2000; Akpek *et al.*, 2001). However, the prevalence is higher than the prevalence reported from other African

countries (Morrissey *et al.*, 1992; Gilks *et al.*, 1995; Archibald *et al.*, 2000; Kivihya-Ndugga, L. *et al.*, 2004).

The relative rarity of disseminated MA in developing countries despite its high frequency of isolation from soil and water (Morrissey *et al.*, 1992) was attributed to several factors. Exposures to MA in the environment or water supply might be less common than in developed countries due to differences in behaviour or in water supply systems. For example, potable hot water was identified as one source of MA infection in USA, and that hot water was not widely available in developing countries (von Reyn *et al.*, 1994). Poor patients in developing countries might be dying from more virulent infections before they were immmunosuppressed enough for MA to develop (Gilks *et al.*, 1995). Patients with latent tuberculosis for example, were likely to reactivate this mycobacterial infection before they reached the low CD4 count associated with susceptibility to MA. Alternatively, prior or continued exposure to MTB or non tuberculous mycobacteria might be eliciting or boosting a broad antimycobacterial immunity, which remained effective against MA even in advanced HIV infection (Gilks *et al.*, 1995).

In contrast with the studies from sub-Saharan countries such as Kenya and Malawi showing MTB as common and MA as relatively uncommon this study suggested that MTB and MA are relatively common in HIV-infected patients in South Africa. These findings were similar to those found in a study of febrile adult (≥15 years old) HIV-infected patients in Bangkok (Archibald *et al.*, 1999).

The possible explanation for relatively common MA infection in South Africa may be that South Africa being a middle income nation with unusually high level of income inequality (Harling *et al.*, 2008), has better health services than other resource-poor sub-Saharan countries. Also exposures to MA in the environment or water supply might be more common than in other sub-Saharan countries as potable hot water was identified as one source of MA infection in USA (von Reyn *et al.*, 1994), and hot water was likely more widely available in South Africa than in other sub-Saharan countries.

Disseminated mycobacterial infections caused by *Mycobacterium tuberculosis* and *Mycobacterium avium* are common complications of late stage human immunodeficiency virus infection, and the disease diagnosis remains a critical problem for clinicians (Gazzola *et al.*, 2008). Multiple methods of diagnosing disseminated bacterial infections are crucial. The use of culture alone could have missed 9 out of 26 (35%) culture negative patients who were positive for granulomata, ZN staining and /or PCR.

4.1.2 Bone marrow trephine biopsy granulomata

In this study the presence of granulomata had a high sensitivity (97.1%) of detecting patients who had mycobacterial infection by culture method. This finding differed greatly with two studies which reported a 24.4% granulomata in culture positive patients (Riley *et al.*, 1995) and 9 (33.3%) granulomata in 27 patients with mycobacterial infection detected by culture (Marques *et al.*, 2000). This study

however concurred with the Japanese study which demonstrated granulomata in all the cases of miliary tuberculosis (Kinoshita *et al.*, 1994).

The results of this study suggest that the presence of granulomata can be used as a screening test, however the specificity is low (23.1%). Keiser *et al.,* (1997) reported that pathologic findings of the bone marrow trephine biopsies were predictive of disseminated infections. Patients with granulomata were 4.1 times more likely to have a positive culture than those without granulomata.

Despite granulomata showing high sensitivity for mycobacterial infection many disorders have been implicated in the pathogenesis of bone marrow granulomata; but they are not specific and do not usually show characteristic features that typify a specific diagnosis (Eid *et al.*, 1996). However some specific pathologic findings can point toward certain diagnoses, for instance, caseation in tuberculosis, Reed-Sternberg cells in Hodgkin's lymphoma and poorly organised granulomata in HIV infection (Eid *et al.*, 1996). In this study no caseations were present in the trephine biopsies and many trephine biopsies had poorly formed or loosely organised granulomata. The differential diagnoses of bone marrow granulomata include:

- Malignant neoplasms – most being non-Hodgkin's and Hodgkin's lymphoma. The granulomata in patients with lymphoma can be due to the invasion of the bone marrow or, more frequently, a non-specific immunologic change due to cancer;
- Infectious causes such as bacterial, viral, fungal and parasitic infections. Viral causes include Epstein-Barr virus (EBV), cytomegalovirus (CMV) and

HIV. Disseminated fungal infections of histoplasmosis are common in immunocompromised patients;

- Sarcoidosis;
- Drugs such as sulphonamide and procainamide
- Connective tissue diseases (Eid *et al.*, 1996).

In this study 20 out of 26 mycobacterial culture negative patients had granulomata in their bone marrow trephine biopsies. The granulomata might be due to other infectious causes other than mycobacteria. Most of these study subjects were severely immunocompromised and therefore quite susceptible to infections such as CMV, EBV and histoplasmosis. None of the subjects were diagnosed with malignant neoplasms. One out of 34 mycobacterial culture positive patients and 6 out of 26 mycobacterial culture negative patients did not have granulomata in their bone marrow trephine biopsy. These granulomata might be truly absent or might have been missed as in HIV infection granulomata are poorly organised (Eid *et al.*, 1996). The examination of several cores of bone marrow trephine biopsy is sometimes required to detect granulomata.

4.1.3 Bone marrow trephine biopsy Ziehl-Neelsen stain

Bone marrow trephine biopsy ZN stain gave a sensitivity of 64.7% and a specificity of 57.7%. This sensitivity was higher than that reported by Marques *et al.,* (2000) who demonstrated acid-fast bacilli in only 7 of 25 patients (28%) infected with MA and in 1 patient (50%) infected with MTB. This result suggests that ZN staining of

bone marrow trephine biopsy can be of high clinical utility when disseminated mycobacterial infection is suspected.

4.1.4 Bone marrow trephine biopsy Ziehl-Neelsen stain vs. Bone marrow trephine biopsy granulomata

The relationship between bone marrow trephine biopsy ZN acid-fast bacilli positivity and bone marrow trephine biopsy granulomata was explored. This study found that 97% of all trephine biopsies that stained positive for acid-fast bacilli had granulomata. This study finding concurred with Riley *et al.,* (1995), who reported seeing acid-fast bacilli only in the presence of granulomata. This finding, however, differed from that of Bishburg *et al.,* (1986) who observed that granulomata were absent in any of the infected bone marrow specimens despite the abundance of mycobacteria on histological examination.

In the light of this study, doing ZN stain without seeing granulomata on bone marrow trephine biopsy is wasteful. Ziehl-Neelsen stain for mycobacteria should be used only if granulomata are seen on the trephine biopsy.

4.1.5 CD4 Count

The patients in this study had a low mean CD4 count of $67.7x10^6$/l. The patients with *Mycobacterium avium intracellulare* had a much lower mean CD4 count than patients with *Mycobacterium tuberculosis* ($19.7X10^6$/l vs. $67.6x10^6$/l). These findings are similar to those reported by Marques *et al.,* (2000) in USA who observed that the patients with MA infection had a much lower mean CD4 count than those with MTB.

4.1.6 Mycobacterial DNA PCR

The real-Time PCR of mycobacterial DNA in this study when compared to mycobacterial culture as gold standard showed a low sensitivity of 50%.

This sensitivity is comparable to those published in literature, for example in the Italian study of TB PCR (Gazzola *et al.*, 2008), the sensitivities of blood and bone marrow aspirate samples were 53% and 60% respectively.

A study in Zambia using sputum specimens, an 'in house' TB PCR and a gold standard incorporating both microbiological and clinical data showed 55% sensitivity (Kambashi *et al.*, 2001). These investigators concluded that the low PCR sensitivity precluded its use as a tool in the routine diagnosis of pulmonary tuberculosis. The above two studies are different from the two Kenyan studies. One of them using sputum samples and culture as a gold standard, the sensitivity of TB PCR with at least one of the 3 samples was 93% (van Cleeff *et al.*, 2005). In another study using sputum samples and culture as a gold standard a sensitivity and specificity of 93% and 84% respectively were reported (Kivihya-Ndugga, L. *et al.*, 2004).

In an Indian study using clinical samples, clinical, radiological and histopathological evidence of TB including clinical response to antitubercular treatment as a gold standard PCR was reported to have the sensitivity and specificity of 74.4% and 97.3% respectively (Negi *et al.*, 2005).

In another Indian study, using bone marrow aspirates for mycobacterial DNA PCR and clinical categories: definite tuberculosis, probable tuberculosis and possible tuberculosis as a gold standard obtained the following PCR sensitivities: 62.5% in

definite diagnosis, 40% in probable diagnosis of tuberculosis and 12% in possible diagnosis (Singh *et al.*, 2006).

Using acid fast smear and culture as a gold standard, the PCR of extra pulmonary samples showed a sensitivity of 90% (Sekar *et al.*, 2008). Using non respiratory clinical samples, and culture and final clinical diagnosis as a gold standard the PCR sensitivity and specificity of 77.7% and 98.7% respectively were reported (Gamboa *et al.*, 1998).

These studies have reported different ranges of sensitivities and specificities depending on the gold standard and other methodological factors used. Although investigators had previously reported that the choice of PCR kit was not important, but attention to detail with which the whole method was conducted (Noordhoek *et al.*, 1996), there is need for standardisation of reagents and methodology, and particularly standardisation of the gold standard (Kivihya-Ndugga, L. *et al.*, 2004).

A recent study looked at the performance characteristics of the Cobas Amplicor MTB test and RT-PCR and with culture as a gold standard different sensitivities were obtained. The Cobas Amplicor had a sensitivity of 88.9% and the RT-PCR had a sensitivity of 66.7% (Desikan *et al.*, 2009). The investigators concluded that the observed variation might be due to genotypic differences, and that a change in geographical region might alter the reported performance characteristics of some commercially available PCR tests.

The 50% sensitivity obtained in this study although in agreement with other studies is low for effective use as a tool in the diagnosis of mycobacterial infections. This low sensitivity may be due to several factors. The PCR

amplification kit as per manufacturer's instructions was for use on sputum samples. This study however adapted the use of the kit from sputum samples to bone marrow aspirates, and this might have contributed to the low sensitivity. The low sensitivity of PCR could also be due to the absence of organisms in the small amount of bone marrow aspirates smeared on a slide due to non uniform distribution of micro organism in the clinical samples.

The presence of PCR reaction inhibitors contributing to low sensitivity was unlikely because each sample was run with an internal control which had to be co amplified for the run to be deemed successful. Internal controls for each run for this study were co amplified as shown in figures 3.3 and 3.4.

Mycobacterial DNA was amplified in 7 of 26 (23%) of culture negative samples. Cross-contamination was not a problem, since all samples were run with a negative control which remained negative during each run. The PCR mix for LightCycler Mycobacterium Detection Kit contained Uracil-DNA Glycosylase which minimised the risks of contamination from previous PCR runs.

Two out of 7 patients who had a positive PCR (study numbers 2 and 13) had negative ZN stain on bone marrow trephine biopsy, while 5 out of 7 patients (patient number 11, 12, 16, 65 and 70) all had disseminated acid-fast bacilli on bone marrow trephine biopsy. Patient 65 was also documented to have a positive sputum smear for AFB. The positive PCR might indicate the presence of non-viable AFB, particularly in patients with a history of mycobacterial infection in the past. However a negative AFB culture could not entirely exclude a clinical diagnosis of mycobacterial infection particularly in patients with clinical and radiological

features suggestive of mycobacterial infection. Although mycobacterial DNA PCR has been shown to be rapid, sensitive and specific diagnostic assay, the results should be interpreted with care in the clinical setting (Cheng *et al.*, 2004).

4.2 Study limitations

There are a number of limitations to this study. Firstly the number of study patients was small (n=60). Secondly the PCR amplification kit as per manufacturer's instructions was for use on sputum samples. This study however adapted the use of the kit from sputum samples to bone marrow aspirates.

This study would have been strengthened by the addition of clinical presentation of patients. This was not done because most of the bone marrow aspirates/ trephine biopsy request forms did not contain full clinical information on the patients. Obtaining full clinical information might have allowed for clearer definitions of indications for bone marrow aspirate/ trephine in HIV positive patients in our environment.

The study did not evaluate whether tuberculosis was diagnosed by any other means such as sputum smears or sputum cultures.

The study did not assess the cost of culture, ZN staining, histological examination of trephine biopsy and the PCR. The assessment of the cost could provide some insight into the most cost-effective means of investigating this group of patients, as the costs of tests are a major issue in South Africa.

4.3 CONCLUDING REMARKS

In this study *Mycobacterium tuberculosis* was a predominant species. The prevalence of *Mycobacterium avium* was higher than that reported in other African countries but still lower than that reported in the United States and Europe.

Granulomata had a highest sensitivity of detecting patients who had mycobacterial infection. Granulomata could be used as a screening test for mycobacterial infections, and when present majority of them stained positive for acid-fast bacilli. It might be unnecessary to use ZN stains for mycobacteria if no granulomata were seen on the trephine biopsy. This study showed also that Ziehl-Neelsen stain of bone marrow trephine biopsy was quite sensitive in detecting those with mycobacterial infection.

In resource-poor countries where culture methods are not readily available, histological examination and Ziehl-Neelsen staining of bone marrow trephine biopsy may offer a highly diagnostic tool in the diagnosis of disseminated tuberculosis.

The sensitivity of mycobacterial DNA PCR was low for adoption of PCR as a sole tool in the diagnosis of mycobacterial infections.

APPENDIX A: ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Chosamata

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080618**
PROJECT	Comparison of multiple methods of diagnosis of mycobacterial infection from bone marrow samples of HIV positive patients
INVESTIGATORS	Dr B Chosamata
DEPARTMENT	Molecular Med & Haematology
DATE CONSIDERED	08.06.27
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.07.29 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr J Mahlangu

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Dr B CHOSAMATA

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