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The diagnostic utility of bone marrow biopsies performed for the investigation of fever and/or cytopenias in HIV-infected adults at Groote Schuur Hospital.

by

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DECLARATION

I, Willem Adendorff van Schalkwyk, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

This work has not been published prior to registration for the abovementioned degree.

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LIST OF ABBREVIATIONS

AFB's	Acid-fast bacilli
AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral therapy
AZT	Zidovudine
CNS	Central Nervous System
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
D4T	Stavudine
Fas-L	Fas-ligand
Fas-R	Fas-receptor
GCV	Ganciclovir
HAART	Highly Active Antiretroviral Therapy
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
H-RS	Hodgkin-Reed Sternberg
IF- γ	Interferon gamma
IL-1	Interleukin-1
ITP	Immune Thrombocytopenic Purpura
NHL	Non-Hodgkin lymphoma
NHLS	National Health Laboratory Service
PCP	Pneumocystis pneumonia
PCR	Polymerase chain reaction
PUO	Pyrexia of unknown origin
RNA	Ribonucleic acid
TB	Tuberculosis
TNF- α	Tumour necrosis factor alpha
ZN	Ziehl-Neelsen
3TC	Lamivudine

PART A: RESEARCH PROTOCOL

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TITLE:

The diagnostic utility of bone marrow biopsies performed for the investigation of fever and/or cytopenias in HIV-infected adults at Groote Schuur Hospital.

A.1 Background information

Human Immunodeficiency Virus (HIV) infected patients frequently present with fever and peripheral cytopenias, which are often caused by disseminated infections and malignant disease (Miralles *et al*, 1995; Coyle *et al*, 1997; Benit *et al*, 1997; Armstrong *et al*, 1999). A bone marrow examination may identify underlying disease in some of these patients, with the reported diagnostic yield ranging from 27% to 42% (Bishburg *et al*, 1997; Brook *et al*, 1997; Luther *et al*, 2000; Pacios *et al*, 2004).

There is a high demand for bone marrow biopsies on HIV-patients at Groote Schuur Hospital. A bone marrow biopsy is an invasive and expensive procedure. In the background of limited resources and increasing number of HIV patients, questions are raised regarding the cost-effectiveness of this procedure and the possibility of selecting patients in whom this investigation would be more useful.

The South African context

South African studies investigating the utility of bone marrow biopsies in HIV-patients are sparse. Those performed, were done before the Highly Active Antiretroviral Therapy (HAART) era. The provision of HAART drugs was started in March 2004 in the public sector and is currently the largest Acquired Immune Deficiency Syndrome (AIDS) treatment program in the world. By September 2007, 370 000 individuals in the public sector and 120 000 attending private doctors had been enrolled nationally (UNAIDS/WHO, 2008). Due to severe cellular immunosuppression patients are at risk of concurrent opportunistic infections. Thus, ongoing management of opportunistic and HIV-related illnesses is required in HIV patients; either prior to starting Antiretroviral therapy (ART), during treatment or after treatment failure. A number of infectious and malignant disorders that may be reversed with effective therapies affect the bone marrow and can be elicited through a suitable biopsy. Research is required to develop cost-effective ways of diagnosing and treating opportunistic disease in HIV-infected

individuals in South Africa and to predict the changing requirements for their care as the rollout of HAART becomes more established.

For instance, the Western Cape has the highest worldwide annual tuberculosis (TB) notification rate (1000/100 000 individuals) (Meya *et al*, 2007). Early detection of this disease in our community is a priority and it is important to determine the role of bone marrow biopsies in this regard.

Detection of infectious disease by bone marrow examination

Investigations for infectious diseases include: microbiological cultures of the blood and bone marrow, bone marrow cytological assessment, histological evaluation for the presence of granulomata and the demonstration of micro-organisms by special staining techniques. Because the histological bone marrow examination occurs within days of collection and mycobacterial cultures take 3 to 6 weeks, a considerably quicker diagnosis of disseminated mycobacterial infections may be made on morphological examination prior to microbiology culture results. This allows the rapid institution of antimicrobial therapy which reduces morbidity and mortality (Nichols *et al*, 1991; Ciaudo *et al*, 1994; Benit *et al*, 1997; Hussong *et al*, 1998; Karstaedt *et al*, 2001).

On the other hand, several studies showed bone marrow cultures to be more sensitive than bone marrow microscopy for the diagnosis of disseminated mycobacterial and fungal infections (Ker *et al*, 2002). One example is a study performed by Benit *et al* (1997), who assessed the role of a bone marrow biopsy in 123 HIV-patients who presented with pyrexia of unknown origin (PUO). TB was found in 18 patients and was confirmed by bone marrow culture in 10. Tuberculoid granulomata with acid-fast bacilli (AFB's) were observed in 7 patients. The absence of AFB's or granulomata in the bone marrow trephine biopsy does not exclude the eventual growth of organisms in bone marrow cultures (Prego *et al*, 1990). Cultures are useful for confirmation, identification as well as determination of antibiotic susceptibility of organisms (Ciaudo *et al*, 1994).

Blood cultures

Mycobacterial blood cultures are not taken routinely from patients suspected to have TB in South African hospitals. More emphasis is placed on sputum collection. However, blood cultures have been found to be equivalent to bone marrow cultures for the diagnosis of disseminated mycobacterial and fungal infections in AIDS-patients (Northfelt *et al*, 1991; Ciaudo *et al*, 1994; Ker *et al*, 2002; Pacios *et al*, 2004). Several authors recommend the use of a blood culture as the first step in the routine evaluation of AIDS patients when disseminated mycobacterial or fungal infections are suspected (Engels *et al*, 1995; Keiser *et al*, 1997; Kilby *et al*, 1998; Marques *et al*, 2000). However this practice is generally underutilized in the clinical work-up of these patients. This is likely due to financial constraints as the cost of a mycobacterial blood culture is approximately three hundred and twenty five rand, including sensitivity testing.

Detection of malignant disease by bone marrow examination

Non-Hodgkin lymphoma (NHL) occurs with a 60 – 200 fold increased frequency in HIV-positive compared with HIV-negative patients. Before HAART was available, Primary Central Nervous System (CNS) lymphoma and Burkitt lymphoma were increased approximately 1000 times in comparison with the general population. In 1985 the United States Centre for Disease Control and Prevention included NHL as an AIDS-defining condition. Since the introduction of HAART, the risk of NHL declined substantially and is consistent with improved CD4 counts. Burkitt lymphoma however, showed no evidence of a decline. Because HAART is associated with enhanced survival and a 75% decrease in mortality rate, lymphomas now contribute to a greater percentage of first AIDS-defining illness. (Beral *et al*, 1991; Levine *et al*, 1993; Cote *et al*, 1997; Appleby *et al*, 2000; Swerdlow *et al*, 2008).

Bone marrow biopsies are frequently performed to exclude lymphoma in HIV-patients. These lymphoma patients may also present with fever and cytopenias. High-grade NHL, the most prevalent type of lymphoma in AIDS patients, infrequently present with bone

marrow involvement according to international literature. Thus the diagnostic sensitivity of a bone marrow examination demonstrated in the trephine biopsy by special staining techniques as the primary diagnostic procedure for the diagnosis of lymphoma is thought to be poor (Malyangu *et al*, 2000; Levine *et al*, 2002; Santos *et al*, 2004; Swerdlow *et al*, 2008).

In contrast with the low incidence of bone marrow involvement of Hodgkin Lymphoma (HL) in immune-competent patients, the incidence of bone marrow involvement in patients with HIV is higher, and the bone marrow may also be the site of primary diagnosis (Fend and Kremer, 2007). Before the era of HAART the incidence of HL in HIV-patients was increased by 8 fold (Hussong *et al*, 1998). There has been an unexpected increase in the incidence of this disease since the advent of HAART therapy and this is possibly related to improvements in CD4 counts (Beral *et al*, 1991; Swerdlow *et al*, 2008).

Predictors of a positive bone marrow examination

Several authors have previously compared the clinical and laboratory features of HIV-patients with diagnostic versus non-diagnostic bone marrow biopsies in an attempt to identify predictive features. Some concluded that a bone marrow biopsy is most helpful in patients when fever and cytopenias co-exist rather than fever or cytopenias alone (Claudo *et al*, 1994; Brook *et al*, 1997; Llewelyn *et al*, 2005; Tanaka *et al*, 2006). Keiser *et al* (1997) reported that a CD4 count $< 50 \times 10^6/L$ and a haematocrit $< 25\%$ were predictive of a diagnostic bone marrow biopsy, while others found the bone marrow most likely to yield a diagnosis if the haematocrit is $< 30\%$ (Luther *et al*, 2000). In one of the few South African studies, Karstaedt *et al* (2001) identified wasting, oral thrush, leucopenia and a CD4 count $< 100 \times 10^6/L$ as predictors.

A.2 Aims and objectives

Broad Aim

This study was performed to evaluate the diagnostic yield of bone marrow biopsies performed on HIV-positive patients at Groote Schuur Hospital.

Objectives

The study was structured with the following objectives:

- To determine the diagnostic yield of a bone marrow biopsy to detect neoplastic, infectious and new haematological diseases (excluding non-specific changes found in HIV) by histopathological examination and microbiological investigations.
- To determine the most frequent new diagnoses made.
- To correlate the diagnostic yield of histopathology, bone marrow culture and peripheral blood culture results in the diagnosis of disseminated mycobacterial infections.
- To compare the turnaround times (difference between the date sampled and the date reported positive) between positive mycobacterial bone marrow versus positive blood cultures.
- To determine the correlation between the presence of granulomata in the bone marrow and the detection of AFB's by Ziehl-Neelsen (ZN) staining.
- To evaluate possible predictors of a diagnostic bone marrow biopsy.

A.3 Institutional approval

This proposal was submitted for ethics approval to the University of Cape Town Research Ethics Committee and was approved (Annexure A).

A.4 Methodology

A retrospective review was performed on consecutive patients infected with HIV who had bone marrow biopsies performed at Groote Schuur Hospital between January 2004 and December 2007. Statistical analysis indicated that a sample size of approximately 150 patients would meet our requirements: assuming that the sample of 150 cases was representative of the study population and based on an estimated yield of 33%, there was an acceptable width (approximately 15%) to the 95% confidence interval.

Groote Schuur Hospital serves as a tertiary referral centre in the Cape Town Central Health District of the Western Cape Metro region. HIV prevalence in this region is approximately 15,1% of the total population. As a 936 bed, public sector university hospital, there are approximately 44 000 inpatient admissions and 575 000 outpatient attendances per year (www.capegateway.gov.za). The hospital offers specialized Infectious Disease and Anti-retroviral Clinics. Approximately 1000 bone marrow biopsies are performed in the Haematology Department each year.

Data collection

Cases were selected for this study according to the following inclusion and exclusion criteria:

Inclusion criteria:

- Adults of 18 years and older.

- Patients who were HIV positive, as indicated by the clinician on the bone marrow request form.
- Bone marrow biopsies for which the indications were fever, fever and cytopenias or cytopenias only.
 - Fever was defined as a documented temperature of $\geq 38^{\circ}\text{C}$ measured in the armpit.
 - Anaemia, leucopenia and thrombocytopenia were defined as levels below the National Health Laboratory Service (NHLS) reference ranges at sea level (Annexure B).

Exclusion criteria:

- Patients below 18 years of age.
- Staging bone marrow biopsies on patients with previously diagnosed lymphomas or other malignancies.
- All follow-up bone marrow biopsies for previously diagnosed haematological diseases.
- All bone marrow biopsies referred to Groote Schuur Hospital from other centers.

The laboratory records of the selected cases were retrospectively reviewed. Files were sourced according to the laboratory specimen numbers. Clinical data was obtained from the patient's Groote Schuur Hospital folders which were drawn from archives using the patient hospital numbers. The medical and laboratory information of the selected patients were entered onto a standardized data collection sheet (Annexure C). Only the laboratory number (under which the bone marrow biopsy was performed) was used as identification on the worksheet. The patient's name and personal details were not recorded. Each case was then assigned a random number which was used during statistical analysis. The following information was collected:

- Indication for the bone marrow biopsy.
- Age and gender of the patient.

- Use of anti-retroviral therapy.
- Prior diagnosis of tuberculosis.
- Current tuberculosis treatment.
- Prior diagnosis of lymphoma or another malignancy.
- Laboratory results:
 - Absolute CD4 lymphocyte count result closest to the date of the bone marrow biopsy (performed by CD45-Assisted PanLeucogating methodology on the Epics XL-MCL Flow cytometer in the C17 NHLS Haematology Laboratory, Groote Schuur Hospital).
 - Peripheral blood counts and differential counts (performed on the Bayer Advia 2120 analyzer in the C17 NHLS Haematology Laboratory, Groote Schuur Hospital).
 - Results of bone marrow aspirate and peripheral blood mycobacterial cultures (performed on the BACTEC 9000 blood culture system in the NHLS Microbiology Laboratory, Groote Schuur Hospital).
 - Bone marrow histopathology results (reported in the NHLS Haematology Laboratory, Groote Schuur Hospital).
- Results of ZN and modified ZN stains (retrospectively performed on all the bone marrow trephine biopsies).
- Diagnosis made by bone marrow biopsy. A bone marrow biopsy was considered diagnostic when:
 - There was evidence of a haematologic disease, such as Immune Thrombocytopenic Purpura (ITP), Aplastic Anaemia, Pure Red Cell Aplasia or Megaloblastic Anaemia.
 - There was evidence of previously undiagnosed lymphoma or another malignancy.
 - There was evidence of a disseminated mycobacterial or fungal infection, either by morphologic examination (bone marrow granulomata or identification of micro-organisms by cytochemical staining) or a positive bone marrow or blood culture.

- Changes made to the patient's management based on the bone marrow biopsy result.

Bone marrow histology

Bone marrow trephine biopsies were fixed in Zenker's solution (which contains mercuric chloride, potassium dichromate and sodium sulphate) and decalcified in a weak acid as per standard operating procedures (Annexure D). Three-micrometer sections were cut and stained with hematoxylin and eosin (Annexure E). ZN and modified ZN stains were performed on all the bone marrow trephine biopsies to identify the presence of AFB's (Annexures F & G). In some cases ZN stains were already performed during the initial assessment of the bone marrow, and the rest were performed subsequently as part of the study. Grocott's methenamine silver or periodic acid-schiff stains for fungal elements and other immunohistochemical stains were performed where appropriate.

Blood and bone marrow mycobacterial cultures

These were performed on request, at the time of the bone marrow biopsy. Peripheral blood or bone marrow aspirate material was inoculated at the bedside into Bactec Myco F/Lytic vials. These contain a Middlebrook liquid medium that supports the growth of mycobacteria, fungi, yeasts and anaerobic organisms. The Bactec vials were incubated at 35 °C under 50% CO₂ for 6 weeks in a Bactec automated blood culture incubator. ZN and Gram stains were performed on those with positive flags and sub-cultures were then performed in a Lowenstein-Jensen medium (Annexure H). Acid-fast organisms were identified by PCR as *Mycobacterium tuberculosis*, *Mycobacterium avium complex* or *Mycobacterium other than tuberculosis*. An in-house polymerase chain reaction (PCR) was used for the identification of *Mycobacterium tuberculosis* (Annexure I) and a Genotype Mycobacterium CM/AS 96 PCR kit for the rest (Mäkinen *et al*, 2006). Drug sensitivities were only performed where requested by the clinician.

Statistical analysis

- Median values and ranges were determined for patient's age, full blood count parameters and CD4 counts. The median CD4 count of patients with bone marrow granulomata was determined.
- The overall diagnostic yield of bone marrow biopsies and cultures were determined and the yield according to indications.
- The diagnostic yields for bone marrow histological examination, bone marrow cultures and blood cultures were determined.
- The mean turnaround times (duration between date sampled and date reported positive) were determined for bone marrow and blood culture results.
- Fisher's exact probability test was used to determine the significance of association between: the presence of poorly-formed granulomata and low CD4 counts ($< 100 \times 10^6/L$); well-formed granulomata and TB diagnoses; caseous necrosis and TB diagnoses; caseous necrosis and CD4 counts $> 200 \times 10^6/L$.
- The analysis to determine the predictive factors for diagnosis was performed using univariate analysis to assess the relationship between each predictor and diagnosis. The Stata 10 statistical software package (StataCorp, College Station, Texas) was used. Adjustments for age and sex were not included in the results because they were not identified as confounding factors (neither age nor sex predicted a new diagnosis on their own).
- A Mann-Whitney U test was performed to determine the statistical significance between the presence and absence of any new diagnosis, new TB diagnosis and the following variables: age, gender, clinical indication, CD4 count, ARV treatment, white cell count, haemoglobin (Hb), platelet count, neutrophil count, lymphocyte count, monocyte count, eosinophil count, presence of granulomata and previous TB diagnosis.
- All probability (p) values reported were two-sided and a value of $< 0,05$ was considered statistically significant throughout the analyses.

A.5 Research plan

The project commenced in January 2007 and ran over 24 months. The first 18 months was dedicated to the retrospective identification of appropriate cases using bone marrow biopsy reports. The relevant information was retrieved from the laboratory records and the medical record department at Groote Schuur Hospital.

During the last 6 months of the project ZN and Modified ZN stains were performed in the Anatomical Pathology Department on all cases included in the study. The stains were reviewed by Dr WA van Schalkwyk.

Data analysis was performed after data collection. Conclusions were then drawn and any additional requirements were attended to. Dr WA van Schalkwyk was responsible for all data collection and performed the data analysis with the help of Dr Jo-Ann McLoughlin, Department of Public Health at the University of Cape Town, and Professor Nicolas Novitzky, Department of Haematology of the University of Cape Town.

A.6 Operational budget

ZN and Modified ZN stains

Unit price: R57.86

Number of samples: 250

Total price: R14 465.00

A.7 Funding

Funding was provided by the NHLS K-project research fund and was obtained early in the description of the methods.

A.8 References

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PART B: LITERATURE REVIEW

B.1 Objectives

The objectives of this literature review were to obtain the following background information:

- The epidemiology of HIV in South Africa.
- The frequency and causes of fever and cytopenias in patients with HIV.
- The role of a bone marrow biopsy in the diagnosis of opportunistic infections and malignancy in HIV positive patients.
- The utility of bone marrow biopsies, bone marrow cultures and blood cultures in the diagnosis of mycobacterial infections in HIV-positive patients in South Africa.

B.2 Search strategy

The literature search was initiated using the Pubmed Central digital archive. Further appropriate papers were identified by searching reference lists. Forty-nine relevant research papers were identified. An author-year referencing method was used.

B.3 Quality criteria

The keywords used for the Pubmed search included HIV and each of the following: South Africa, fever, pancytopenia, thrombocytopenia, anaemia, leucopenia, bone marrow biopsies, opportunistic infections and malignancy. Studies involving HIV positive patients who presented with fever and cytopenias were selected. Particular attention was given to studies performed in Southern Africa. A distinction was made between studies published in the pre- and post-HAART era.

B.4 Summary of the literature

Epidemiology of HIV in South Africa

South Africa is the country with the largest number of HIV infections in the world. 5,7 Million people were living with HIV in South Africa. (UNAIDS/WHO, 2008 [a]). Tuberculosis is the most common medical cause of death in HIV-infected adults. In some areas, two-thirds of patients with active TB are co-infected with HIV (Groenewald *et al*, 2005; Meya *et al*, 2007).

The South African Department of Health has a comprehensive plan for the management, treatment, care and support of patients with HIV. The ARV treatment programme had enrolled approximately 370 000 people in the public sector by September 2007 with and an estimated 120 000 people in the private sector (UNAIDS WHO, 2008 [b]).

Frequency and causes of fever and cytopenias in patients with HIV

HIV-patients frequently present with PUO and peripheral cytopenias. A study performed by Adewuyi *et al* (1999) described the haematological features of HIV infection in adult Zimbabweans. Cytopenias were frequent (47,5% of adults with HIV infection) and the most common abnormalities were lymphopenia (31,5%), anaemia (30,8%), neutropenia (29,6%), thrombocytopenia (24,7%), eosinophilia (23,5%) and leucopenia (11,7%). There was a lack of association between the severity of the haematological abnormalities and the clinical stage of the disease.

Fever and cytopenias are often caused by disseminated opportunistic infections and malignancy (Armstrong *et al*, 1999; Santos *et al*, 2004; Tanaka *et al*, 2006).

Suppression of the bone marrow directly by HIV or by its viral proteins, as well as indirect factors relating to immune and cytokine dysregulation, are also responsible for cytopenias. Most studies show that actual infection of CD34+ stem cells by HIV *in vivo*

is a rare occurrence. CD34⁺ stem cells express low levels of the receptors and co-receptors (CD4, CXCR-4 and CCR-5) necessary to permit HIV infection, making them relatively resistant to direct infection (Volberding *et al*, 2004). It appears more likely that cytokines such as interferon gamma (IFN- γ), tumour necrosis factor alpha (TNF- α) and interleukin-1 (IL-1) are responsible for marrow suppression and cytopenias. These cytokines are released by HIV infected mononuclear-macrophage cells and activated lymphocytes and induce significant bone marrow dysfunction and even progenitor cell death (Levine *et al*, 2001). Haemopoietic progenitor cells derived from bone marrows of HIV-patients have been reported to undergo increased apoptosis. TNF- α increases the expression of the Fas-receptor (Fas-R) on CD34⁺ cells. Increased levels of Fas-ligand (Fas-L), produced by activated T-cells result in increased apoptosis of haematopoietic progenitor cells through the Fas-L/Fas-R pathway. Activation of caspase 1, caspase 3 and caspase 8 appear to be important activators of several effector proteins in this pathway (Sloand *et al*, 2000). Although marrow stromal function has been examined, and susceptibility of stromal cells to HIV infection has been documented, actual infection of the stroma is a relatively rare event. Stromal function appears to play only a minor role in the pathophysiology of marrow dysfunction in HIV (Sloand, 2005).

An association between HIV and thrombocytopenia was first described in 1982. The most common cause of this complication is ITP, which occurs in 30% or more of patient with HIV. While slightly more prevalent in those with advanced disease, ITP typically arises early in the course of HIV infection and can be seen before other manifestations of AIDS (Volberding *et al*, 2003). Defective megakaryopoiesis may also contribute to thrombocytopenia, particularly in patients with advanced disease. Megakaryocytes can be infected with certain strains of HIV, some of which can be cytopathic for the megakaryocytes (Sloand, 2005).

The most frequent cause of anaemia in HIV-infected patients is Anaemia of Chronic Disease (Volberding *et al*, 2003). Cytokines such as IL-1, TNF and interferons play an important role in impairing erythropoietin response by reducing concentrations of marrow progenitors and erythroid colonies, and effecting abnormalities of reticulo-endothelial iron metabolism (Moyle, 2002). Another common cause of anaemia in

patients with HIV is medication (Sloand *et al*, 2005). Zidovudine (AZT) is frequently associated with significant anaemia due to inhibition of thymidine kinase and deoxyribonucleic acid (DNA) chain termination. It inhibits *in vitro* erythroid colony formation in a dose-dependent manner. Severe anaemia can be seen in 24% of those who receive AZT 1500 mg daily, while neutropenia and thrombocytopenia are less common (Volberding *et al*, 2003). Haematologic toxicity is less frequently seen with other nucleoside analogs, such as Lamivudine (3TC) and Stavudine (d4T). The protease inhibitors have little or no effect on haemopoiesis (Sloand *et al*, 2005). Trimethoprim-sulfamethoxazole (Bactrim®), an antibiotic commonly used for the prevention of *Pneumocystis pneumonia* (PCP), is associated with a high rate of side effects, including fever, rash and pancytopenia. In patients with poor nutritional status and in combination with other predisposing factors, the drug can cause megaloblastic anaemia, leucopenia and thrombocytopenia. Bone marrow suppression, seemingly unrelated to folate deficiency, is a frequent side effect of Bactrim® (Watera *et al*, 2007). Treatment of cytomegalovirus (CMV) infection with parenteral ganciclovir (GCV) may result in significant myelosuppression leading to leucopenia and thrombocytopenia after the second week of therapy. Oral GCV appears to have similar haematological effects. The antifungal agent amphotericin B is also frequently associated with myelosuppression. In addition to direct effects on the bone marrow, amphotericin renal toxicity may lead to diminished erythropoietin production (Sloand, 2005).

A significant increase in white cell count, neutrophils and platelets, in addition to CD4+ T-cells, is seen in patients beginning HAART, with a simultaneous decline in plasma HIV ribonucleic acid (RNA) levels. The basis for these improvements is most likely due to the reversal of inhibitory effects of HIV replication. HIV protease inhibitors may have a direct effect on haematopoietic cells by inhibition of caspase 1, resulting in a reduced apoptotic rate and improved colony forming capacity of bone marrow cells (Levine *et al*, 2001).

The role of a bone marrow biopsy in the diagnosis of opportunistic infections and malignancy in HIV positive patients

Bone marrow biopsies are often used to investigate fever and cytopenias in patients with HIV, with reported diagnostic yields ranging from 27% to 42% in different studies. (Luther *et al*, 2000; Pacios *et al*, 2004; Tanaka *et al*, 2006). Karstaedt *et al*, 2001, retrospectively reviewed bone marrow biopsies performed on HIV-patients at Chris Hani Baragwanath Hospital in Johannesburg and also found the yield to be considerable at 38%.

Infectious Disease

One of the most frequent reasons for performing a bone marrow biopsy in these patients is to diagnose opportunistic infections. Microbiologic cultures may be performed on bone marrow aspirate samples and histological examination of the trephine biopsy may identify morphological features of a disseminated infection, such as granuloma formation. Micro-organisms may be demonstrated by special staining techniques or direct microscopy. A variety of opportunistic infections can be identified in this way including *Mycobacterium tuberculosis*, *Mycobacterium avium complex*, *Toxoplasma gondii*, *Histoplasma capsulatum* and *Cryptococcus neoformans* (Jagadha *et al*, 1985; Karcher *et al*, 1990; Nichols *et al*, 1991; Luther *et al*, 2000; Akpek *et al*, 2001). Because the histological bone marrow examination result may be available within days and mycobacterial cultures take 3 to 6 weeks, a considerably quicker diagnosis of disseminated mycobacterial infections can be made on histology. (Bishburg *et al*, 1986; Ciaudo *et al*, 1994; Engels *et al*, 1995; Kilby *et al*, 1998). This allows the rapid institution of antimicrobial therapy which reduces morbidity and mortality (Nichols *et al*, 1991; Benit *et al*, 1997; Hussong *et al*, 1998; Karstaedt *et al*, 2001).

Granuloma formation is the typical inflammatory response seen in tissues infected with mycobacterial or fungal organisms. In the setting of immunodeficiency such as AIDS, conflicting results have been reported regarding the frequency of granuloma formation.

Some authors report granulomata to be less frequent with a low correlation between the presence of granulomata in the bone marrow and the detection of organisms by special stains or culture (Bishburg *et al*, 1986). This was confirmed by Marques *et al* (2000) who evaluated the efficacy of blood cultures, bone marrow cultures and histological examination of the bone marrow for predicting the presence of infection. Bone marrow granulomata were seen in only 9 of 27 patients with mycobacterial infections and AFB's were seen in only 7. In contrast, other groups have found a high percentage of bone marrow granulomata in patients with disseminated mycobacterial infections (Karcher *et al*, 1990; Nichols *et al*, 1991; Ciaudo *et al*, 1994; Luther *et al*, 2000). Karstaedt *et al* (2001) found bone marrow granulomata in 93% of patients with TB. They found, however, that the use of empiric anti-TB therapy on the basis of bone marrow granulomata was microbiologically appropriate in only 41% of patients with non-caseous granulomata. Empirical anti-tuberculosis therapy is still frequently recommended in TB-predominant geographical regions such as South Africa, on the basis of the presence of granulomata in the bone marrow.

Bone marrow cultures are useful to confirm the presence of micro-organisms, as well as for identification purposes and to determine their antibiotic susceptibility (Ciaudo *et al*, 1994). The absence of AFB's or granulomata in the bone marrow trephine biopsy does not exclude the eventual growth of organisms in bone marrow cultures (Prego *et al*, 1990). Several studies have shown that bone marrow cultures are more sensitive than the bone marrow histological examination for the diagnosis of disseminated mycobacterial and fungal infections (Benit *et al*, 1997; Ker *et al*, 2002).

Mycobacterial blood cultures are underutilized in South African hospitals and are not used in the routine investigation of pyrexial patients or in patients suspected of having disseminated mycobacterial infections. Sputum microscopy is preferred. This is likely due to a lack of availability of mycolytic vials and financial constraints as the cost of a mycobacterial blood culture is approximately three hundred and twenty five rand; including sensitivity testing, while sputum microscopy costs thirty rand.

A study performed by Von Gottbert *et al* (2001) in Johannesburg found that bacteraemia with *Mycobacterium tuberculosis* was detected in 28% of HIV-infected patients with suspected TB, including those with localized pulmonary disease on initial clinical assessment. AFB's were detected on sputum microscopy in 42% of these patients and sputum cultures were positive in 57%. They concluded that bacteraemia with *Mycobacterium tuberculosis* was common in HIV-infected patients with suspected TB. Blood cultures were also useful in distinguishing disseminated *Mycobacterium avium* from *Mycobacterium tuberculosis* infections.

Blood and bone marrow cultures have been shown to be equivalent testing modalities for the diagnosis of disseminated mycobacterial and fungal infections in AIDS-patients, with a yield of approximately 20% for disseminated mycobacterial infections and 8% for disseminated fungal infections (Marques *et al*, 2000). Their combined use provides a higher diagnostic yield (Northfelt *et al*, 1991; Ker *et al*, 2002; Pacios *et al*, 2004). Several authors have recommended that blood cultures should be the first step in the routine evaluation of AIDS patients when disseminated mycobacterial or fungal infections are suspected. They suggest that an early bone marrow biopsy should be reserved for extremely ill patients in whom a more aggressive approach is needed (Keiser *et al*, 1997; Kilby *et al*, 1998; Marques *et al*, 2000). Other authors feel that, because of the additional diagnostic yield of a biopsy, both peripheral blood and bone marrow cultures should be performed early in the evaluation of this patient group. (Bishburg *et al*, 1986; Fernandez-Aviles *et al*, 1995; Benit *et al*, 1997; Luther *et al*, 2000; Tanaka *et al*, 2006). In the local setting, there are currently no standard protocols.

Malignant Disease

NHL occurs with a 60 – 200 fold increased frequency in HIV-positive compared with HIV-negative patients, particularly in the pre-HAART era. Primary CNS lymphoma and Burkitt lymphoma are increased approximately 1000 times in comparison with the general population. In 1985 the United States Centre for Disease Control and Prevention included NHL as an AIDS-defining condition. Since the introduction of HAART, the

risk of NHL has declined dramatically and is consistent with improved CD4 counts. Burkitt lymphoma however, showed no evidence of decline. Because HAART is associated with enhanced survival and a 75% decrease in mortality, lymphomas now contribute to a greater percentage of first AIDS-defining illness (Beral *et al*, 1991; Levine *et al*, 1993; Cote *et al*, 1997; Swerdlow *et al*, 2008). Before the era of HAART, the incidence of HL in HIV-patients was increased 8 fold (Hussong *et al*, 1998) and there has been an unexpected increase in its incidence since the advent of HAART therapy. This is possibly related to improvements in CD4 counts (Beral *et al*, 1991; Swerdlow *et al*, 2008). An essential feature of HL is the non-neoplastic milieu of reactive cells which surround the Hodgkin-Reed Sternberg (H-RS) cells in the tumour mass. The recruitment of inflammatory cells may provide essential feedback signals that stimulate proliferation or inhibit apoptosis of H-RS cells. HL incidence may decline in severely immunosuppressed HIV-patients because the malignant H-RS cells are not able to recruit lymphocytes and histiocytes required for their survival (Biggar *et al*, 2006). In contrast to the low incidence of bone marrow involvement by HL in immune-competent patients, bone marrow involvement is more frequently seen in HIV patients and the bone marrow may also be the site of primary diagnosis (Fend and Kremer, 2007).

A bone marrow biopsy is often performed to investigate for lymphoma in HIV-positive patients with only fever and cytopenias and no lymphadenopathy nor hepatosplenomegaly. However, high-grade NHL has been reported to less frequently involve the bone marrow which implies a low sensitivity of this investigation as a diagnostic tool (Malyangu *et al*, 2000; Swerdlow *et al*, 2008). Levine *et al* (2002) retrospectively compared staging bone marrow biopsies performed on HIV-patients with indolent B-cell lymphomas to those with high-grade NHL. They found bone marrow involvement to be significantly more common in indolent NHL cases (50%) than high-grade NHL cases (17%). Santos *et al* (2004) found bone marrow biopsies unhelpful in the diagnosis of high-grade NHL in patients with AIDS. Another study in Zimbabwe also concluded that HIV-associated NHL rarely infiltrates the bone marrow (Adewuyi *et al*, 1999).

Predictors of a Positive Bone Marrow Examination

Several authors have previously compared the clinical and laboratory findings of HIV-patients with diagnostic versus non-diagnostic bone marrow biopsies in an attempt to identify predictive features. Some concluded that a bone marrow biopsy is most diagnostic in patients where fever and cytopenias co-exist than fever or cytopenias alone (Ciaudo *et al*, 1994; Brook *et al*, 1997; Karstaedt *et al*, 2001; Llewelyn *et al*, 2005; Tanaka *et al*, 2006). Keiser *et al* (1997) reported that a CD4+ T-lymphocyte count $< 50 \times 10^6/L$ and a haematocrit $< 25\%$ were predictive of a diagnostic bone marrow biopsy, while others found the bone marrow was most likely to yield a diagnosis if the haematocrit was $< 30\%$ (Luther *et al*, 2000).

The utility of bone marrow biopsies, bone marrow cultures and mycobacterial blood cultures in HIV positive patients in South Africa

Karstaedt *et al* (2001) retrospectively reviewed a consecutive case series of 257 adults with HIV infection who had undergone a bone marrow examination at Chris Hani Baragwanath Hospital between January 1996 and December 1997 to assess the diagnostic usefulness of bone marrow biopsy and culture. They concluded that the bone marrow examination gave a unique diagnosis in 24% of patients, which was a high proportion compared to other studies. The most common new diagnosis made was disseminated mycobacterial infections. Predictors identified for a positive bone marrow biopsy were wasting, oral thrush, leucopenia and low CD4 count ($< 100 \times 10^6/L$). A possible explanation for the high yield was that blood cultures for mycobacterial disease were not available for routine use in South African hospitals at that time. They could therefore not be used as an early investigation before a bone marrow biopsy was performed. They concluded that expanding access to mycobacterial blood culture technology at primary care level would limit the number of bone marrow examinations required. They noted that at that stage, most South African HIV-infected patients did not have access to bone marrow biopsies, blood cultures nor CD4 counts. The situation has improved somewhat since then with the introduction of widespread HIV testing, CD4

counts and HAART, however access to bone marrow biopsies and blood cultures for most patients remains poor.

B.5 Identification of needs for further research

The spectrum of opportunistic disease in HIV-patients varies among different regions of the world and it is important to identify local patterns (Kaplan *et al*, 1996). Most published research in this area has been done outside of South Africa. There have been no studies in the Western Cape to examine the utility of bone marrow biopsies in HIV-patients. Published work was done before the rollout of HAART started in the public sector in March 2004. Ongoing management of opportunistic and HIV-related illnesses is required in HIV-patients; either prior to starting HAART, during treatment or after treatment has failed. Research is required for the development of cost-effective ways to diagnose and treat opportunistic disease in HIV-infected individuals in South Africa and to predict the changing requirements for this care as the rollout of HAART becomes more established.

The Western Cape has the highest annual TB notification rate (1000/100 000 individuals) (Meya *et al*, 2007). It is critical to provide adequate and effective care to co-infected individuals. Early detection of multidrug resistant TB in our community is a priority and it is important to determine the role of blood cultures, bone marrow cultures and bone marrow biopsies in this regard.

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The diagnostic utility of bone marrow biopsies performed for the investigation of fever and/or cytopenias in HIV-infected adults at Groote Schuur Hospital, South Africa.

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Key words: Retroviral infection, tuberculosis, bone marrow biopsy, diagnostic utility.

Running title: Diagnostic yield of a bone marrow investigation in patients with HIV infection.

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Abstract

Introduction: A bone marrow biopsy is frequently requested in the work-up of patients with Human Immunodeficiency Virus (HIV) infection who present with fever and/or cytopenias in the search for opportunistic infections and malignancies.

Methods: This is a retrospective review of the results of consecutive bone marrow biopsies performed at our institution over a three year period on HIV positive patients for the investigation of fever and cytopenias. Clinical data, haematological parameters, morphology of bone marrow biopsy, Ziehl-Neelsen staining and microbiological culture results were analyzed. The aim of the study was to determine the diagnostic yield of this investigation.

Results: Sixty three males and 84 female patients were included for the analysis. Indications for bone marrow biopsies were cytopenias in 79% (n= 115), pyrexia of unknown origin (PUO) alone in 1% (n= 2) and both PUO and cytopenias in 20% (n= 30). The bone marrow biopsy gave a high diagnostic yield of 47% (70 patients) and a unique diagnosis in 33% (49 patients). Immune thrombocytopenic purpura and disseminated mycobacterial infections were the most common unique diagnoses made (14% each), followed by malignancies (4%). In this cohort, 4 cases of primary bone marrow involvement by Hodgkin lymphoma and 1 case of involvement by non-Hodgkin lymphoma were diagnosed. Cytopenias and granulomata were significantly associated with lower CD4 counts ($p= 0,01$). Granulomata in the marrow was significantly associated with disseminated tuberculosis ($p= 0,001$), and past diagnosis of tuberculosis.

Conclusions: We conclude that in our study group bone marrow biopsy was a useful investigation with a high diagnostic yield.

Introduction

South Africa is currently experiencing one of the most severe Acquired Immune Deficiency Syndrome (AIDS) epidemics in the world. By the end of 2007 5,7 million people were living with the Human Immunodeficiency Virus (HIV) (UNAIDS/WHO, 2008 [a]). Co-infection with tuberculosis (TB) is the most common cause of death in this population group. In some areas of South Africa, two-thirds of patients with active TB are HIV positive (Groenewald *et al*, 2005; Meya *et al*, 2007). The South African Department of Health currently has a comprehensive HIV treatment plan.

HIV positive patients frequently present with pyrexia of unknown origin (PUO) and peripheral blood cytopenias, which may be caused by dysregulated immune system, co-infection with TB, disseminated opportunistic infections, malignancy, drugs or other haematological conditions. Methods of identifying a disseminated infection with TB include microbiological cultures of the peripheral blood or bone marrow aspirate, Ziehl-Neelsen (ZN) staining of the bone marrow aspirate, histological examination of the trephine biopsy and the demonstration of micro-organisms by special staining techniques. A bone marrow biopsy may be a useful analytical tool in some of these patients, with the reported diagnostic yield ranging from 27% to 42% (Engels *et al*, 1995; Brook *et al*, 1997; Luther *et al*, 2000; Pacios *et al*, 2004; Tanaka *et al*, 2006). Because morphological examination is performed within days while mycobacterial cultures take 3 to 6 weeks to grow, a considerably faster diagnosis of disseminated mycobacterial infections may be made on histological examination (Bishburg *et al*, 1986; Kilby *et al*, 1998; Luther *et al*, 2000). Bone marrow biopsies are often performed for the exclusion of lymphoma in HIV patients.

At our institution we are currently experiencing a high demand for bone marrow biopsies in this patient group. The Western Cape has the highest proportion of HIV-infected individuals taking antiretroviral treatment in the country (UNAIDS/WHO, 2008 [b]). Despite the stabilization of HIV prevalence in the Cape Town Metro region, the 2007 South African HIV and Syphilis Prevalence Survey showed a notable increase in HIV prevalence among antenatal clinic attendees in rural districts of the province (UNAIDS/WHO, 2008 [c]). The Western Cape has the highest annual TB notification

rate (1000/100 000 individuals) and early detection of multidrug resistant TB in our community is a priority (Meya et al, 2007). In the background of limited resources, questions are raised regarding the cost-effectiveness of bone marrow biopsies in HIV patients and the possibility of selecting patients in whom this investigation would be most useful.

The spectrum of opportunistic disease in HIV-patients varies among different regions of the world and it is important to identify local patterns (Kaplan *et al*, 1996). Most published research in this area has been done outside of South Africa. There have been no studies in the Western Cape to examine the utility of bone marrow biopsies in HIV-patients. Published work was done before the rollout of HAART started in the public sector in March 2004. Ongoing management of opportunistic and HIV-related illnesses is required in HIV-patients; either prior to starting HAART, during treatment or after treatment has failed. Research is required for the development of cost-effective ways to diagnose and treat opportunistic disease in HIV-infected individuals in South Africa and to predict the changing requirements for this care as the rollout of HAART becomes more established.

Groote Schuur Hospital serves as a tertiary referral centre in the Western Cape, South Africa. HIV prevalence among antenatal clinic attendees in the region is approximately 15,1% (UNAIDS/WHO, 2008 [d]). As a 936 bed, public sector university hospital, there are approximately 44 000 inpatient admissions and 575 000 outpatient attendances per year, with specialized Infectious Diseases and Anti-Retroviral clinics. This study retrospectively evaluated the diagnostic yield of consecutive bone marrow biopsy results on HIV-positive patients reviewed retrospectively for the period January 2004 to December 2007. The study was approved by the Research Ethics Committee of the University of Cape Town.

Methods

Inclusion criteria were as follows: HIV positive (as indicated by the clinician on the bone marrow request form) adults ≥ 18 years of age who underwent bone marrow biopsies for the investigation of fever, fever and cytopenias or cytopenias alone. Fever was defined as an armpit temperature greater than 38°C . Anaemia was defined as a haemoglobin concentration < 13 g/dL for men and < 12 g/dL for women, leucopenia as a total white cell count $< 4 \times 10^9/\text{L}$ and thrombocytopenia as a platelet count $< 137 \times 10^9/\text{L}$ for men and $< 178 \times 10^9/\text{L}$ for women. The following specimens were excluded: biopsies on patients below 18 years of age, biopsies done solely to stage previously diagnosed lymphoma and follow-up bone marrow biopsies for previously diagnosed haematological diseases. Bone marrow specimens referred to Groote Schuur Hospital from other centres were not included in the study.

A standardized data-collection sheet was used to record the following information: indication for bone marrow biopsy, patient characteristics, prior diagnoses of mycobacterial infection or malignancy, antiretroviral therapy, CD4 lymphocyte count, peripheral blood counts, mycobacterial bone marrow aspirate and blood culture results, final diagnosis made by bone marrow biopsy and resulting changes made to the patient's management. Files were sourced according to the laboratory specimen numbers. Clinical data was obtained from the patient's Groote Schuur Hospital folders which were drawn from archives using the patient hospital numbers. The patient's name and personal details were not recorded. Each case was then assigned a random number which was used during statistical analysis

Posterior iliac crest bone marrow samples were collected and processed according to standard protocols. Samples were fixed in Zenker's solution and decalcified in formic acid. Three-micrometer sections were cut and stained with hematoxylin and eosin. Both ZN and modified ZN stains for acid-fast bacilli (AFB's) were routinely performed in all cases and were microscopically examined throughout the entire area of the sections and not only in areas of granulomatous inflammation. Where appropriate, Grocotts

methenamine silver or periodic acid-schiff stains for fungal elements and other immunohistochemical stains were performed. Peripheral blood and bone marrow aspirate material was inoculated at the bedside into Bactec Myco F/Lytic vials which were incubated at 35°C under 50% CO₂ for 6 weeks. An in-house PCR was used for the identification of *Mycobacterium tuberculosis* while for other mycobacterial organisms a Genotype Mycobacterium CM/AS 96 PCR kit (Mäkinen *et al*, 2006) was employed. Mean turnaround times (duration between date sampled and date reported positive) for bone marrow and blood culture results were determined. Absolute CD4 lymphocyte counts were performed by CD45-Assisted PanLeucogating methodology (Glencross *et al*, 2002).

Statistical analysis

Descriptive statistics were employed to define the study population. Overall diagnostic yield, yield according to indication, diagnostic yield for bone marrow histological examination, diagnostic yields for bone marrow and blood cultures were determined. Analysis was performed to establish the predictive factors for a diagnosis. A p (probability) value of < 0,05 was considered statistically significant. Univariate analysis was used to assess the relationship between each predictor and diagnosis. Non parametric statistics were applied to determine possible relationship between unique diagnosis (yes, no), diagnosis of tuberculosis (yes, no) and clinical parameters. Age, gender, fever, CD4 count, ARV therapy, prior diagnosis of TB, current TB treatment, leucopenia, anaemia, thrombocytopenia, neutropenia, lymphopenia and granulomata were analyzed as possible predictors. Analysis was performed using Stata 10 statistical software package (StataCorp, College Station, Texas).

Results

Study population

The review of the records of 147 patients (63 males; 43% and 84 females; 57%) was included in this study. A summary of the clinical and haematological parameters at the time of the bone marrow biopsy are shown in tables I and II. Indications for the bone marrow biopsies are summarized in table III. Most commonly patients presented with anaemia and thrombocytopenia (43%), followed by a pancytopenia (31%), anaemia alone (15%), thrombocytopenia alone (5%) and anaemia with a leucopenia (5%). One hundred and twenty seven patients (86%) had a CD4 count performed and in 115 (91%) the values were $< 200 \times 10^6/L$. The median CD4 count was $73 \times 10^6/L$ (range $1-697 \times 10^6/L$). Thirty eight patients had prior diagnoses of mycobacterial infections and 30 were already on anti-TB treatment. Thirty-two (22%) patients were on antiretroviral therapy, but their adherence to therapy was not well documented. The CD4 count was graded into ≥ 200 , 100-199, 50-99 and $< 50 \times 10^6/L$. Reduced CD4 count grades were significantly related to leucopenia ($p= 0,02$), thrombocytopenia ($p= 0,01$), lymphopenia ($p= 0,001$), monocytopenia ($p= 0,01$) and the finding of granulomata in the marrow ($p= 0,001$). There was also a significant correlation between severity of reduction of CD4 count and the number of haematopoietic lineages reduced (single line, bicytopenia and pancytopenia) ($p= 0,01$). Fever as an indication for the procedure was only significantly associated with lower median lymphocyte count ($p= 0,02$) but did not result in a new diagnosis.

Bone Marrow Morphological Examination

A diagnosis was made by bone marrow biopsy in 44% ($n= 65$) and a new diagnosis was made in 28% ($n= 41$). Granuloma formation was reported in 33% ($n= 49$) and in 37 of these a specific cause for granuloma formation was found. Granulomata in the marrow biopsy were significantly associated with lower median neutrophil, monocyte and CD4

counts (see Table IV). The finding of a granuloma was significantly associated with a new diagnosis of TB ($p=0,0001$).

Two patients were already diagnosed with disseminated cryptococcal infections on cerebrospinal fluid samples. Both had well-defined epithelioid granulomata and fungal stains showed the presence of cryptococci, but bone marrow cultures were negative. Fungal stains were performed on 30 other cases, but none of these were positive. Malignancy was demonstrated in 6 trephine biopsy specimens, and in all of these the diagnoses were confirmed by immunohistochemical stains.

Bone Marrow Microbiological Culture

Microbiological cultures for TB were performed on bone marrow aspirates in 84% ($n=124$) patients and provided a unique diagnosis in 5% ($n=8$). In 15 of the 18 (83%) positive cultures, bone marrow granulomata were seen and in 8 (44%) cases AFB's were demonstrated by ZN stains. *Mycobacterium tuberculosis* was isolated in 13 cases, *Mycobacterium avium complex* in 4 cases and an unidentifiable mycobacterium in 1 culture. The mean time to positive growth for *Mycobacterium tuberculosis* bone marrow cultures was 700 hours (range 385-980 hours) and 582 hours (33-790 hours) for *Mycobacterium avium complex* cultures. No fungi or yeasts were cultured.

Blood Culture

Mycolytic blood cultures were performed on 17 (11%) patients. Mycobacterial organisms were isolated in seven cases: 6 *Mycobacterium tuberculosis* and 1 *Mycobacterium avium complex*. The mean time to positivity for *Mycobacterium tuberculosis* blood cultures was 471 hours (range 375-656 hours) and the one case of *Mycobacterium avium complex* showed a positive result after 265 hours. Two other cases that had cultures performed had no morphological evidence to suggest mycobacterial infection and bone marrow cultures were negative.

Diagnostic Yield

A specific diagnosis was made by bone marrow biopsy or culture in 70 patients, which gave the procedure an overall diagnostic yield of 47%. In 21 of these patients the same diagnoses were made by alternative diagnostic modalities. Following testing of the bone marrow as a diagnostic procedure, unique diagnoses, which resulted in a change of patient management, were thus made in 49 cases (33%). The diagnostic yield according to indication was as follow: PUO and cytopenias led to a new diagnosis in 13 cases (9%), cytopenias alone was responsible for a new diagnosis in 36 cases (25%). Table V summarizes the diagnostic yield of the bone marrow biopsies.

Predictors of a Positive Bone Marrow Examination

The bone marrow examination was helpful (see Table VI) in leading to a new diagnosis in patients with lower (than median) CD4 counts ($p= 0.02$); Hb ($p= 0,01$); platelet counts ($p= 0,01$); neutrophil counts ($p= 0,02$) and if they had had TB in the past ($p= 0,01$). To address the utility of bone marrow biopsy for diagnosis of disseminated mycobacterial infections, analysis was repeated using only tuberculosis as the outcome variable. Man-Whitney U test (see Table VII) indicated that diagnosis of TB was significantly more likely in patients with lower (than median) platelet count ($p= 0,04$), finding bone marrow granulomata ($p= 0,0001$), previous diagnosis of TB ($p= 0,01$) and being on TB therapy ($p= 0,04$).

Discussion

Fever and cytopenias are frequent and significant problems in patients infected with HIV, so it is important to establish as quickly as possible their etiological causes. Consequently, we undertook this retrospective analysis to evaluate the diagnostic utility of the bone marrow biopsy as a diagnostic tool in this patient group. The procedure led to important new diagnoses of auto-immune disease, opportunistic infections, malignancies or other conditions in 33% of the patients.

When comparing these conclusions with earlier similar studies, our diagnostic yield falls within the previously reported ranges of 27% to 42%. (Engels *et al*, 1995; Brook *et al*, 1997; Benit *et al*, 1997; Luther *et al*, 2000; Pacios *et al*, 2004; Tanaka *et al*, 2006). In addition, in the pre-HAART era in South Africa, Karstaedt *et al* (2001) performed a similar review at Chris Hani Baragwanath Hospital in Johannesburg. In this study, bone marrow examination was found to give a unique diagnosis in 24% of patients and the highest yield was obtained in patients with advanced HIV; defined as CD4 count $< 100 \times 10^6/L$. Although our study was performed during the roll-out of HAART in South Africa (initiated March 2004), only 22% (n= 32) of our cohort were on this therapy. Therefore the current study does not reflect the full impact of HAART; whilst the need for HAART treatment in appropriate patients is highlighted.

Twenty one patients (14%) were diagnosed with immune thrombocytopenic purpura (ITP), which added to the high diagnostic yield. The association between HIV and ITP is well described and occurs with an incidence of 5% - 30% (Liebman, 2008). While slightly more prevalent in those with advanced disease, ITP typically arises early in the course of HIV infection and is often seen before other manifestations of AIDS (Volberading *et al*, 2003). In our cohort, the median presentation platelet count of patients with ITP was $15 \times 10^9/L$ (range 6 – $86 \times 10^9/L$) and their median CD4 count was $198 \times 10^6/L$ (range 18 – $561 \times 10^6/L$). The majority (68%; n= 14) presented with anaemia and thrombocytopenia, followed by isolated thrombocytopenia in 32% (n= 7) and none with pancytopenia or PUO. Bone marrow biopsy confirmed the diagnosis of ITP in all these patients and no additional pathology was found. This supports the argument that if the history, physical examination, blood count and blood film examination are consistent with the diagnosis of ITP, the bone marrow biopsy is unnecessary (Provan *et al*, 2003).

The second most common new diagnosis made was disseminated mycobacterial infection found in 20 (14%). In view of the fact that TB is the major cause of death in adults with HIV in South Africa, it is vital to consider this diagnosis in patients who

present with fever and cytopenias. The early detection of multidrug resistant TB in our community is a priority. Another 38 (26%) patients had already been diagnosed with mycobacterial infections before the bone marrow biopsy was performed, and most of them were already on TB treatment. Evidence of granulomatous inflammation was found in 21 (55%) of these patients and 5 (13%) had positive ZN stains. Bone marrow cultures were performed in 30, of which 5 (17%) were positive. Evidence of active TB reflected either that TB therapy was only recently started, non-adherence to treatment or resistant disease, which confirmed the highly relevant diagnostic role of a bone marrow biopsy in patients already on TB treatment who presents with fever and cytopenias.

Of the twenty new cases with a mycobacterial infection, the diagnosis was based on identification of bone marrow AFB's alone in 7 (35%) and on the result of a bone marrow culture alone in another 7 (35%) cases. In another 6 (30%) cases both these investigations were positive. The performance of bone marrow cultures increased the diagnostic yield and confirmed the diagnosis. In two patients AFB's were demonstrated by ZN stains without morphological evidence of granulomatous inflammation in the marrow. The mean time to positivity for mycobacterial cultures were several hundred hours (29 days), while a much quicker diagnosis was made on the trephine biopsy by ZN stains. Thus, both ZN stains and bone marrow aspirate cultures should routinely be performed whenever a mycobacterial infection is suspected. As only 6% of patients had mycobacterial blood cultures performed, an accurate comparison between the diagnostic yield and turnaround times of mycobacterial blood versus bone marrow cultures could not be made. However, blood cultures provided a unique diagnosis in two patients in whom no AFB's could be demonstrated in the trephine biopsy and who had negative bone marrow cultures. Previous studies have shown mycobacterial blood cultures to be an equivalent testing modality to bone marrow cultures for the diagnosis of disseminated tuberculosis and fungal infections in AIDS-patients. Their combined use also led to a higher diagnostic yield (Northfelt *et al*, 1991; Ker *et al*, 2002; Pacios *et al*, 2004).

Evidence of granulomatous inflammation was frequent (33%) and in the majority of cases (69%) was associated with proven disseminated mycobacterial infections either by

identification of AFB's with ZN stains (30%), positive bone marrow/blood cultures (27%) or both (19%). In 8 (24%) patients the diagnosis could not be confirmed, but the diagnosis was considered as highly likely by the reporting pathologist. Since fungal infections are much less frequently seen than mycobacteria in AIDS patients in South Africa, routine cytochemical stains for fungi and yeasts were not performed on the trephine biopsies in this study (Groenewald *et al*, 2005).

Because staging bone marrow biopsies on patients with previously diagnosed lymphomas were excluded from the study, very few malignancies were identified. This confirms the relatively low utility of a bone marrow biopsy as the primary diagnostic procedure for non-Hodgkin lymphoma in HIV patients with fever and/or cytopenias and no other systemic features to suggest lymphoma. Nevertheless, bone marrow involvement is seen with increased frequency in HIV patients with Hodgkin lymphoma (HL) and is confirmed in our study as four (3%) patients who had no other features to suggest malignancy were found to have primary bone marrow HL.

Several authors have previously compared the clinical and laboratory findings of HIV-patients with diagnostic versus non-diagnostic bone marrow biopsies, in an attempt to identify predictive features. Some concluded that a bone marrow biopsy is most diagnostic in patients where fever and cytopenias co-exist than fever or cytopenias alone (Ciaudo *et al*, 1994; Brook *et al*, 1997; Karstaedt *et al*, 2001; Llewelyn *et al*, 2005; Tanaka *et al*, 2006). We could not confirm this observation in our cohort. In our study group a diagnostic marrow was associated with a lower (than median) CD4 count ($p=0,02$), Hb ($p=0,01$), platelet count ($p=0,01$), neutrophil count ($p=0,02$) and if they had had TB in the past ($p=0,01$). Keiser *et al* (1997) reported that a CD4+ T-lymphocyte count $< 50 \times 10^6/L$ and a haematocrit $< 25\%$ were predictive of a diagnostic BM biopsy, while others found the bone marrow most likely to yield a diagnosis if the haematocrit is $< 30\%$ (Luther *et al*, 2000). This is not surprising as most opportunistic infections occur in patients with advanced disease who are severely immunosuppressed (Klimas *et al*, 2008).

Thus we conclude that in patients with pure thrombocytopenia without other cytopenias or fever (0/7), the bone marrow biopsy did not yield a new diagnosis other than ITP. Thus in this group, the procedure could probably be omitted. However for those with other cytopenias and fever, it resulted in a new diagnosis in 28% of patients. Thirdly, it was a useful tool with a quick turnaround time and made a new diagnosis of tuberculosis in 20/147 individuals. In this group, all had anaemia and 17/20 had bi/pancytopenia. Bone marrow cultures for TB are recommended whenever disseminated TB is suspected. Lastly, the bone marrow biopsy also resulted in the new diagnosis of unsuspected malignancy (6/147). A weakness of this study is its retrospective nature and that the biopsies were performed solely at the clinicians request and no selection criteria were used. Another potential bias is the fact that Groote Schuur Hospital is a referral centre for specialist care and its patient population is unlikely to represent the general HIV population.

Table I. Clinical characteristics of the study population on presentation.

Parameter	Value
Age (years); median (range)	34 (18-62)
Gender M/F	63/84
ARV therapy; n (%)	32 (22%)
Previous TB diagnosis; n (%)	38 (26%)
Current TB treatment; n (%)	30 (20%)
Previous diagnosis of malignancy; n (%)	0
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CD4 count ($\times 10^6/L$); median (range)	73 (1-697)
< 50 ($\times 10^6/L$)	54 (43%)
50-99 ($\times 10^6/L$)	15 (15%)
100-199 ($\times 10^6/L$)	18 (14%)
≥ 200 ($\times 10^6/L$)	13 (10%)

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Table II. Haematological parameters of the study population on presentation.

Parameter	Value
Leucocyte count (x 10 ⁹ /L); median (range)	4,3 (0,5-22,5)
< 2	28 (19%)
2, 0-2, 9	18 (12%)
3, 0-3, 9	18 (12%)
Haemoglobin (g/dL); median (range)	7,8 (1,9-14,9)
< 6	32 (22%)
6, 0-9, 9	88 (60%)
10-12 (F) / 10-13 (M)	17 (12%)
Platelet count (x 10 ⁹ /L); median (range)	70 (4-712)
< 50	54 (37%)
50-99	43 (29%)
100-178 (F) / 100-137 (M)	11 (8%)
Neutrophil count (x 10 ⁹ /L); median (range)	2,9 (0,2-18,2)
< 0, 5	8 (5%)
0, 5-1, 4	25 (17%)
1, 5-2, 0	7 (5%)
Monocyte count (x 10 ⁹ /L); median (range)	0,2 (0,0-2,3)
Lymphocyte count (x 10 ⁹ /L); median (range)	0,65 (0,0-3,8)
Eosinophil count (x 10 ⁹ /L); median (range)	0,01 (0,0-1,1)

Table III. Indications for bone marrow biopsy.

Indication:	Total n (%)	Unique bone marrow diagnoses n (%)
Anaemia	12 (8%)	3 (6%)
Anaemia and leucopenia	6 (4%)	0
Anaemia and thrombocytopenia	51 (34%)	17 (35%)
Thrombocytopenia	7 (5%)	7 (15%)
Pancytopenia	39 (27%)	9 (18%)
PUO alone	2 (1%)	0
PUO and:		
anaemia	7 (5%)	2 (4%)
anaemia and leucopenia	6 (4%)	4 (8%)
anaemia and thrombocytopenia	7 (5%)	3 (6%)
pancytopenia	9 (6%)	3 (6%)
thrombocytopenia	1 (1%)	1 (2%)

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Table IV. Clinical and haematological parameters associated with bone marrow granulomata (Fisher's Exact Test).

Parameter	No granulomata n=98	Granulomata n=49	p value
White cell count (x 10 ⁹ /L), median	4,9	3,5	0,001
Haemoglobin (g/dL), median	7,7	7,3	0,2
Platelet count (x 10 ⁹ /L), median	72	71	0,3
Neutrophil count (x 10 ⁹ /L), median	3,4	2,3	0,02
Monocyte count (x 10 ⁹ /L), median	0,3	0,2	0,04
Lymphocyte count (x 10 ⁹ /L), median	0,8	0,5	0,1
CD4 count (x 10 ⁶ /L), median	137	40,5	0,001
Previous TB diagnosis	33	22	0,01
Current TB therapy	28	19	0,2
New TB diagnosis	5	16	0,0001

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Table V. Summary of diagnostic bone marrow biopsies.

Diagnoses	Total diagnoses 70 (48%)	Unique diagnoses 49 (33%)
Infectious diseases:		
Disseminated mycobacterial infections	38 (26%)	20 (14%)
Diagnosis based on:		
- Trepine biopsy:	20 (13%)	7 (5%)
- Aspirate culture:	9 (6%)	6 (4%)
- Trepine biopsy and aspirate culture:	9 (6%)	7 (5%)
Disseminated cryptococcal infections	2 (1,4%)	0
Malignant diseases:		
Hodgkin Lymphoma	4 (2,7%)	4 (2,7%)
Non-Hodgkin Lymphoma	1 (0,6%)	1 (0,6%)
Metastatic Malignant Melanoma	1 (0,6%)	1 (0,6%)
Other:		
Immune Thrombocytopenic Purpura	21 (14%)	21 (14%)
Parvovirus-induced pure red cell aplasia	1 (0,6%)	0
Drug-induced pure red cell aplasia	1 (0,6%)	1 (0,6%)
Severe Aplastic Anaemia	1 (0,6%)	1 (0,6%)

Table VI. Predictors of a new diagnosis (Mann-Whitney U test).

Predictor	Rank Sum No	Rank Sum Yes	U*	Z [#]	p-value
Age	7177,000	3701,000	2326,000	-0,30818	0,76
Gender	7472,500	3405,500	2180,500	0,90606	0,37
CD4 count	4876,500	3251,500	1390,500	-2,20652	0,02
ARV treatment	5911,500	2999,500	1918,500	0,39026	0,69
Previous TB diagnosis	7747,000	2984,000	1759,000	2,55909	0,01
Granulomata	7129,500	3748,500	2278,500	-0,50337	0,61
White cell count	6721,000	4010,000	1968,000	-1,69323	0,09
Haemoglobin	6516,500	4214,500	1763,500	-2,54044	0,01
Platelet count	7751,500	2979,500	1754,500	2,57774	0,01
Neutrophil count	5003,500	3252,500	1348,500	-2,41758	0,02
Monocyte count	5387,500	2740,500	1732,500	-0,26904	0,79
Lymphocyte count	5618,500	2637,500	1691,500	0,68611	0,49
Eosinophil count	5515,500	2485,500	1624,500	0,61443	0,54

* U value is the number of times observations in one sample precede observations in the other sample in the ranking.
Z value is a measure of the distance in standard deviations of a sample from the mean.

Table VII. Predictors of a new TB diagnosis (Mann-Whitney U test).

Predictor	Rank Sum No	Rank Sum Yes	U	Z	p-value
Age	9322,500	1555,500	1321,500	-0,00830	0,99
Gender	9471,000	1407,000	1176,000	0,81373	0,42
CD4 count	6977,000	1511,000	941,000	0,85382	0,39
ARV treatment	7630,000	1281,000	1071,000	0,37139	0,71
Previous TB diagnosis	9619,000	1112,000	881,000	2,40629	0,01
TB treatment	7624,000	3107,000	1882,000	2,04934	0,04
Granulomata	8662,500	2215,500	661,500	-3,66180	0,0001
White cell count	9396,500	1334,500	1103,500	1,16550	0,25
Haemoglobin	9055,500	1675,500	1180,500	-0,73611	0,46
Platelet count	8843,000	1888,000	968,000	-1,92113	0,04
Neutrophil count	7006,500	1249,500	1011,500	-0,16085	0,87
Monocyte count	7152,500	975,500	785,500	1,62559	0,10
Lymphocyte count	7260,500	995,500	805,500	1,54146	0,12
Eosinophil count	6999,500	1001,500	830,500	0,98650	0,33

* U value is the number of times observations in one sample precede observations in the other sample in the ranking.
Z value is a measure of the distance in standard deviations of a sample from the mean.

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PART D: SUPPORTING DOCUMENTATION

Annexure A: University of Cape Town ethics approval.

UNIVERSITY OF CAPE TOWN



Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
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24 August 2009

REC REF: 224/2008

Dr WA van Schalkwyk
PO Box 52193
Waterfront
8002

Dear Dr van Schalkwyk

PROTOCOL TITLE: THE DIAGNOSTIC UTILITY OF BONE MARROW EXAMINATIONS PERFORMED FOR THE INVESTIGATION OF FEVER AND/OR CYTOPENIAS IN HIV-INFECTED ADULTS AT GROOTE SCHUUR

Thank you for your letter to the Research Ethics Committee dated 15th August 2009.

It is a pleasure to inform you that the Ethics Committee has **granted annual re-approval** for the above mentioned study.

Approval is granted for one year until 25 August 2010. Progress report is noted with thanks.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

lemjedi

Annexure B: National Health Laboratory Service (NHLS) Full Blood Count reference ranges at sea level.

Parameter	Adult male Reference ranges	Adult female Reference ranges	Units
White cell count	4,0-10,0	4,0-10,0	$\times 10^9/L$
Haemoglobin	13,0-17,0	12,0 -15,0	g/dL
Platelet count	137-373	178-400	$\times 10^9/L$
Neutrophil count	2,0-7,5	2,0-7,5	$\times 10^9/L$
Monocyte count	0,18-0,8	0,18-0,8	$\times 10^9/L$
Lymphocyte count	1,0-4,0	1,0-4,0	$\times 10^9/L$
Eosinophil count	0,00-0,45	0,00-0,45	$\times 10^9/L$
Basophil count	0,00-0,2	0,00-0,2	$\times 10^9/L$

University of Cape Town

Annexure C: Data collection sheet.

Patient number:		
Date of bone marrow biopsy:		Laboratory number:
Indication for bone marrow biopsy:		
Age:		Gender:
ARV therapy:		
Prior TB diagnosis:		
Current TB treatment:		
Prior diagnosis of lymphoma/malignancy:		
Laboratory results		
CD4 count:		Date performed:
White cell count:		
Haemoglobin:		
Platelet count:		
Neutrophil count:		
Monocyte count:		
Lymphocyte count:		
Eosinophil count:		
Mycobacterial blood cultures:		
Date performed:		
Result:		
Time to positivity:		
Mycobacterial bone marrow cultures:		
Result:		
Time to positivity:		
Bone marrow histopathology result:		
Granuloma formation:		
ZN & modified ZN results:		
Diagnosis made by bone marrow biopsy:		
Changes to patient management:		

**Annexure D: Fixation of Trepine Biopsies: NHLS Grootte Schuur Hospital
Standard Operating Procedure.**

STANDARD OPERATING PROCEDURE

Title: FIXATION OF TREPHINE BIOPSIES

Document number: HAE0686

Version number: 3

Written by: Dr Maureen Stein

Checked by: N/A

Approved by: F. Barton

Active date: November 2008

PRINCIPLE

Fixation must preserve the tissue in such a manner that it will retain its original form and yet permit certain investigations. In the fixation of tissues the most important reactions are those, which stabilize the proteins. It seems that fixatives have the property of forming cross-links between proteins thereby forming a gel. Soluble proteins are fixed to structural proteins and thus rendered insoluble. Therefore, the whole structure is given some mechanical strength, which permits the subsequent processes to take place. Fixation normally takes place in aqueous solutions, although occasionally vapor is used. The type of investigation required will govern the choice of a fixative. In our laboratory, Zenker's fluid is the fixative of choice.

SPECIMEN

Bone marrow trephine biopsy.

REAGENTS

ZENKER'S FLUID:

Stock solution

Mercuric chloride	50g
Potassium dichromate	25g

Distilled water 1L

Working Solution

Stock solution 10ml
Acetic acid 0, 5 ml
Formalin 1ml

PROCEDURE

- 1) Place trephine in 10 ml Zenker's working solution.
- 2) Place on roller for 4 hours.
- 3) After 4 hours label cassettes and place trephine in appropriate ones.
- 4) Wash in running tap water for a minimum of 15 minutes to wash out all excess fixative thereby preventing the contamination of the decalcifying agent.

Notes: Zenker's fluid is an excellent fixative for bone marrow biopsies.

ADVANTAGES

- 1) Excellent for staining of nuclei and connective tissue.
- 2) Enhances staining.
- 3) Easy to prepare.

DISADVANTAGES

- 1) After 24 hours the tissue becomes brittle and hardened.
- 2) The solution does not keep after the addition of acetic acid.
- 3) Prolonged fixation also causes shrinkage and damage to or loss of antigens on the cell surface, which will affect immune studies.
- 4) If the incorrect concentration or volume is used, it could alter the staining of the tissue.

FORMAL DECAL for trephines

This must be made up once monthly – in the first week of the month in quantities of 4 litres

For 4 litres

Formaldehyde 400ml
Distilled water 3600ml

Sodium di-hydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) 16g

OR

di-Sodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) 26g

Then discard 340ml of the above solution and add

90% formic acid 175ml
Concentrated hydrochloric Acid 175ml

Precautions

Formaldehyde

Toxic by inhalation, in contact with the skin or if swallowed.

Causes burns.

Possible risks of irreversible effects.

May cause sensitization on skin contact.

Wear suitable protective clothing and gloves.

Use only in well-ventilated areas.

In case of contact with eyes, rinse immediately with plenty of water, report to safety officer and seek medical advice immediately.

In case of accidental spill or if you feel unwell, report to safety officer and seek medical advice immediately.

Formic Acid

Causes severe burns.

Do not breathe vapors.

Wear suitable protective clothing and gloves.

Use only in well-ventilated areas

In case of contact with eyes, rinse immediately with plenty of water, report to safety officer and seek medical advice immediately.

In case of accidental spill or if you feel unwell, report to safety officer and seek medical advice immediately

THE TREPINE PROCESSING PROCEDURE

The first steps are performed in the C17 Haematology laboratory

- The trephine in the cassette is placed in Formal Decal for 5 hours
- It is then placed in water for 30 minutes.

The trephine in the cassette is then referred to the Histology laboratory at Medical School for processing, wax embedding, cutting and H&E staining. The form "bone Marrow Delivery Receipt" is completed with the laboratory label, patient sticker and referring doctor's name.

REFERENCES

Handbook of Histopathological and Histochemical techniques, 3rd Edition. C.F.A. Culling, Butterworth, 1974

**Annexure E: Haematoxylin and Eosin stain: NHLS Groote Schuur Hospital
Standard Operating Procedure.**

STANDARD OPERATING PROCEDURE

Title: HAEMATOXYLIN AND EOSIN STAIN

Version number: 1

Written by: A. VISSER

Checked by: N/A

Active date: November 2008

Objective/Aim

To demonstrate the different tissue structures in sections which forms the basis of histopathologic diagnosis.

Principle

H&E staining usually means staining of nuclei by oxidized haematoxylin (haematein) through mordant bonds of metals such as aluminium, followed by counterstaining by eosin (a xanthene dye), which colours in varying shades the different tissue fibres and cytoplasm. A general tissue demonstration picture is produced.

Haematoxylin itself is not a dye, the major oxidation product is haematein, a natural dye that is responsible for the colour properties. Haematein can be produced from haematoxylin in two ways, either by natural oxidation or by chemical oxidation. Natural oxidation ('ripening') by exposure to light and air can take between 3-4 months. Chemical oxidation uses sodium iodate. The iodate converts the haematoxylin to haematein almost instantaneously so the haematoxylin is available to use immediately. Haematein needs a mordant, ie a tissue-dye link and the most useful are aluminium and iron salts.

Eosin is the most suitable dye to combine with an alum haematoxylin to demonstrate general histological architecture because of its ability to distinguish between the

cytoplasm of different types of cells and between the different types of connective tissue fibres and matrices, by staining different shades of red and pink. In our laboratory we use a combination of Eosin Y and Phloxine B which the pathologists prefer to a straight eosin counterstain.

Prerequisite

Thickness of sections: \pm 2-3 μ m

Solutions

Mayer's acid alum haematoxylin (add in the following order):

- | | |
|---|----------|
| 1. Hot distilled water (\pm 70°C) | - 2000ml |
| 2. Haematoxylin (dissolve) – CI 75290 | - 3g |
| 3. Potassium alum/Aluminium potassium sulphate (dissolve) | - 100g |
| 4. Sodium iodate | - 0.4g |
| 5. Citric acid | - 2g |
| 6. Chloral hydrate (when cold) | - 100g |

Garvey's modified Mayer's haematoxylin:

1. Dissolve 45g ammonium or potassium alum (*aluminium ammonium- or aluminium potassium sulphate*) in 900ml distilled water with the aid of heat.
2. Dissolve 2.5g haematoxylin in 100ml absolute ethanol (alcohol).
3. Combine above solutions and add 0.2g sodium iodate and 1g citric acid.

Mix well. The solution is stable for several months.

Eosin:

- Solution 1: 1% Eosin Y (CI 45380)
- Solution 2: 1% Phloxine B (CI 45410)
- Solution 3: Mix 2 parts of 1% eosin and 1 part of 1% phloxine. The mixture must ripen for \pm 2 weeks!
- Solution 4: sol.3 is diluted with equal parts of distilled water. The mixture must ripen for \pm 2 weeks. This is the working solution – 'Ready-for-use'.

Scott's tap water substitute:

- | | |
|--|----------|
| Tap water | - 2000ml |
| Sodium bicarbonate (sodium hydrogen carbonate) | - 7g |

Magnesium sulphate	- 40g
40% formaldehyde	- 20ml

CARE: Chloral hydrate is toxic if swallowed; eosin is an irritant; formaldehyde is toxic.

Method for routine H+E staining

1. Bring sections to water.
2. Haematoxylin – ½ to 2min. depending on freshness of solution.
3. Rinse well in water.
4. Blue in Scott's – 30sec.
5. Wash in running water – 2min.
6. Counterstain in eosin – 2min.
7. Rinse, dehydrate, clear and mount.

Results

- Nuclei - blue
- Cytoplasm - pink-red
- Other tissue components - varying shades of pink.

Notes

- H+E staining: eosin is easily removed in water and 96% alcohol.
- Haematoxylin: chloral hydrate is an antioxidant/preservative and prevent bacterial growth; due to its toxicity, it is replaced with ethanol in Garvey's haematoxylin. Ethanol is a more effective penetrator and bacteriostat.
- Eosin: when fresh, the stain has a tendency to wash out easily.
- Scott's: 1% formalin is added to prevent the growth of mould.

Safety considerations

Refer to '*Appendix: Safety Considerations for Special Stains*' at back of file or *Material Safety Data Sheets*–files (kept in Main Laboratory) for more comprehensive information.

References

- ❖ NHLS Histology Laboratory Special Stains Manual, Division of Anatomical Pathology, UCT Medical School, Groote Schuur Hospital, Cape Town.
- ❖ Garvie, W; (1991) J. Histotechn 14(3): 164-165.
- ❖ Manual of Histological Techniques and their Diagnostic Application; Bancroft J.D. and Cook, H.C.; Churchill Livingstone; 1994.
- ❖ Cellular Pathology Technique – 4th Edition; Culling C.F.A., Allison R.T. and Barr W.T.; Butterworths; 1985.
- ❖ Red Cross Hospital Haematology Laboratory, Standard Operating Procedures.

University of Cape Town

Annexure F: Ziehl-Neelsen stain: NHLS Groote Schuur Hospital Standard Operating Procedure.

STANDARD OPERATING PROCEDURE

Title: ZIEHL-NEELEN STAIN

Version number: 1

Written by: A. Visser

Checked by: N/A

Active date: June 2008

Objective/Aim

To demonstrate Mycobacteria, especially Mycobacteria tuberculosis.

Principle

Mycobacteria (eg. tubercle bacilli) have a lipid rich cell wall capable of taking up strong phenol-dye solutions so that they retain the dye upon subsequent differentiation in acid or alcohol (i.e. acid- and alcohol-fast).

The phenol in the staining solution probably acts as a surface tension depressant to allow the dye ions to enter the lipid envelope of the tubercle bacilli more easily.

Prerequisite

Thickness of sections: \pm 2-3 μ m

Control: ZN.

Solutions

Carbol Fuchsin:

Supplied from Microbiology at C17.

OR:

Carbol Fuchsin:

Dissolve 1g Basic Fuchsin (CI 42510) in 10ml ethanol.

Dissolve 5g phenol (carbolic acid) in 100ml distilled water.

Mix the 2 solutions together.

Filter.

(Basic Fuchsin: use the coarse granule, not the more purified type specified for Schiff's reagent – CI 42510).

Loeffler's Methylene Blue:

Stock solution:

Absolute alcohol	- 100ml
Methylene Blue (CI 52015)	- 0.8g

Working solution:

Distilled water	- 99ml
1% Potassium hydroxide	- 1ml
Stock solution	- 30ml

Filter.

*CARE: Carbol Fuchsin solution includes reagents which are **toxic**; potassium hydroxide causes severe burns.*

Method

1. Take sections to water.
2. Filter on Carbol Fuchsin.
3. Flame slide until steam rises – leave for 5min.
4. Flame slide again – leave for 5min.
5. Rinse in water.
6. Rinse in 1% acid alcohol until excess colour is removed.
7. Rinse in water.
8. Place slide in 25% sulphuric acid – 5-20min.
9. Wash in running tap water to remove acid – 10min.

10. Counterstain with Loeffler's Methylene Blue – a few seconds. to 1min ('sky' blue).
11. Rinse in water.
12. Dehydrate, clear and mount.

Results

- Acid fast bacilli, etc. - red
- Nuclei - blue

Notes

- Avoid decalcification in strong acids as acid-fastness may be lost.
- It is important to take through a control as the Carbol Fuchsin solutions deteriorate with age (and newly prepared solutions may not work).
- Do not overstain with the counterstain.
- In a crisis, the times can be halved.

Safety considerations

Refer to '*Appendix: Safety Considerations for Special Stains*' at back of file or *Material Safety Data Sheets*–files (kept in Main Laboratory) for more comprehensive information.

References

- ❖ NHLS Histology Laboratory Special Stains Manual, Division of Anatomical Pathology, UCT Medical School, Groote Schuur Hospital, Cape Town.
- ❖ Manual of Histological Techniques and their Diagnostic Application; Bancroft J.D. and Cook, H.C.; Churchill Livingstone; 1994.

**Annexure G: Modified Ziehl-Neelsen stain: NHLS Groote Schuur Hospital
Standard Operating Procedure.**

STANDARD OPERATING PROCEDURE

Title: MODIFIED ZIEHL-NEELEN STAIN

Version number: 1

Written by: A. VISSER

Checked by: N/A

Active date: May 2008

Objective/Aim

For the demonstration of especially leprosy bacilli.

Principle

Leprosy bacilli are much less acid- and alcohol-fast than tubercle bacilli and their lipid envelope is more easily affected by fat solvents, diminishing the staining reaction. Therefore, in this modification, differentiation with acid and alcohol is minimal and dewaxing is done in a mixture of a vegetable oil and xylene, or turpentine.

Also see SOP for the standard Ziehl-Neelsen.

Prerequisite

Thickness of sections: \pm 2-3 μ m

Control: LEP.

Solutions

Please see ZN SOP.

CARE: Carbol fuchsin solution includes reagents which are toxic, acids cause severe burns.

Method

1. DO NOT TAKE TO WATER. Dewax in turpentine – 10min.
2. Blot and rinse in water.
3. Filter on Carbol-fuchsin solution (do not heat) – 20min.
4. Rinse in water.
5. Decolourise in 5% H₂SO₄ – 10-15min.
6. Wash in running water – 5-10min.
7. Counterstain lightly with Loeffler's Methylene Blue.
8. Rinse, blot, air dry, place in xylol and mount.

Results

- Bacilli of M.Lepae and other mycobacteria, Nocardia asteroides - red
- Background - blue

Safety considerations

Refer to 'Appendix: Safety Considerations for Special Stains' at back of file or *Material Safety Data Sheets*–files (kept in Main Laboratory) for more comprehensive information.

References

- ❖ NHLS Histology Laboratory Special Stains Manual, Division of Anatomical Pathology, UCT Medical School, Groote Schuur Hospital, Cape Town.
- ❖ Manual of Histological Techniques and their Diagnostic Application; Bancroft J.D. and Cook, H.C.; Churchill Livingstone; 1994.

Annexure H: Processing of MGIT (Mycobacterial Growth Indication Tubes), Lowenstein-Jensen slopes and MYCO/F Lytic bottles: NHLS Groote Schuur Hospital Standard Operating Procedure.

STANDARD OPERATING PROCEDURE

Title: PROCESSING OF MGIT TUBES, LJ SLOPES and MYCO/F LYTIC BOTTLES

Document number: MIC0700

Version number: 2

Written by: C. Visser

Checked by: N/A

Approved by: K. Mentoor

Active date: March 2009

PURPOSE

This protocol describes the steps to be taken when placing MGIT tubes / LJ slopes into the incubator and when to remove the tubes / slopes. It also describes how to follow-up positive MGIT tubes / LJ slopes and Myco/F Lytic bottles.

PRINCIPLE

- The MGIT (mycobacterial growth identification) system is an automated, liquid medium based culture system for detecting mycobacterial growth. Once specimens have been inoculated, MGIT tubes are placed into a MGIT incubator unit. Growth is automatically evaluated by the machine by measuring changes in the fluorescence of the tube, which is influenced by a change in the oxygen tension in the medium. Once a change in fluorescence is measured, the tube is “flagged positive” by the incubator.
- LJ (Lowenstein-Jensen) medium is a solid, egg based medium for culture of mycobacteria. It is often used as an adjunct to liquid based media, although there is some evidence that the MGIT media alone is sufficiently sensitive. Certain specimens (detailed in the specimen processing SOP) are inoculated onto LJ media as well. Growth is measured visually.
- The Myco/F Lytic bottle is liquid culture used with the BACTEC 9000 blood culture system to allow the detection of mycobacteria. Growth is automatically evaluated by the machine by measuring changes in the fluorescence sensor at the bottom of the

tube, which is influenced by a change in the oxygen tension in the medium. Once a change in fluorescence is measured, the bottle is “flagged positive” by the incubator.

LIMITATIONS

Refer to Isenberg, H.D; Clinical Microbiology Procedures Handbook, Volume 2, Bactec MGIT 960 Automated System section 7.4.2.

SCOPE

This SOP applies to all MGIT, LJ cultures and MycoF/ Lytic bottles which have been inoculated with either clinical or quality control samples.

This SOP must be followed by any medical technologist / student medical technologist / technician / medical scientist / pathologist / pathology registrar who may be working in the laboratory and following up cultures.

ABBREVIATIONS

The following abbreviations are used in this SOP:

MGIT	Mycobacterial Growth Indicator Tube
LJ	Lowenstein-Jensen medium
AUR	Auramine stain
ZN	Ziehl-Neelsen stain
LED	Light emitting diode
2% BA	2% Blood agar
LIS	Laboratory Information System

EQUIPMENT AND SUPPLIES

Biosafety cabinet

Glass slides

MGIT tubes (purchased from Becton Dickinson)

LJ slopes (supplied by media laboratory)

MycoF/ Lytic BACTEC culture bottles

2% Blood agar plates (supplied by media laboratory)

Bactec 960 MGIT incubator unit

37 degree incubator (aerobic)

35 degree incubator (CO₂)

REAGENTS

Precipitating fluid

SAFETY PRECAUTIONS

M. tuberculosis is spread by the airborne route. Many laboratory procedures are accompanied by the formation of aerosols containing bacteria but most importantly, mycobacteria. All workers in the TB laboratory must be aware of aerosol formation and the dangers thereof. **Any work that involves opening either a MGIT tube or an LJ slope MUST be performed in biosafety cabinet.**

Refer to the NHLS Safety Manual for additional information regarding Health and Safety.

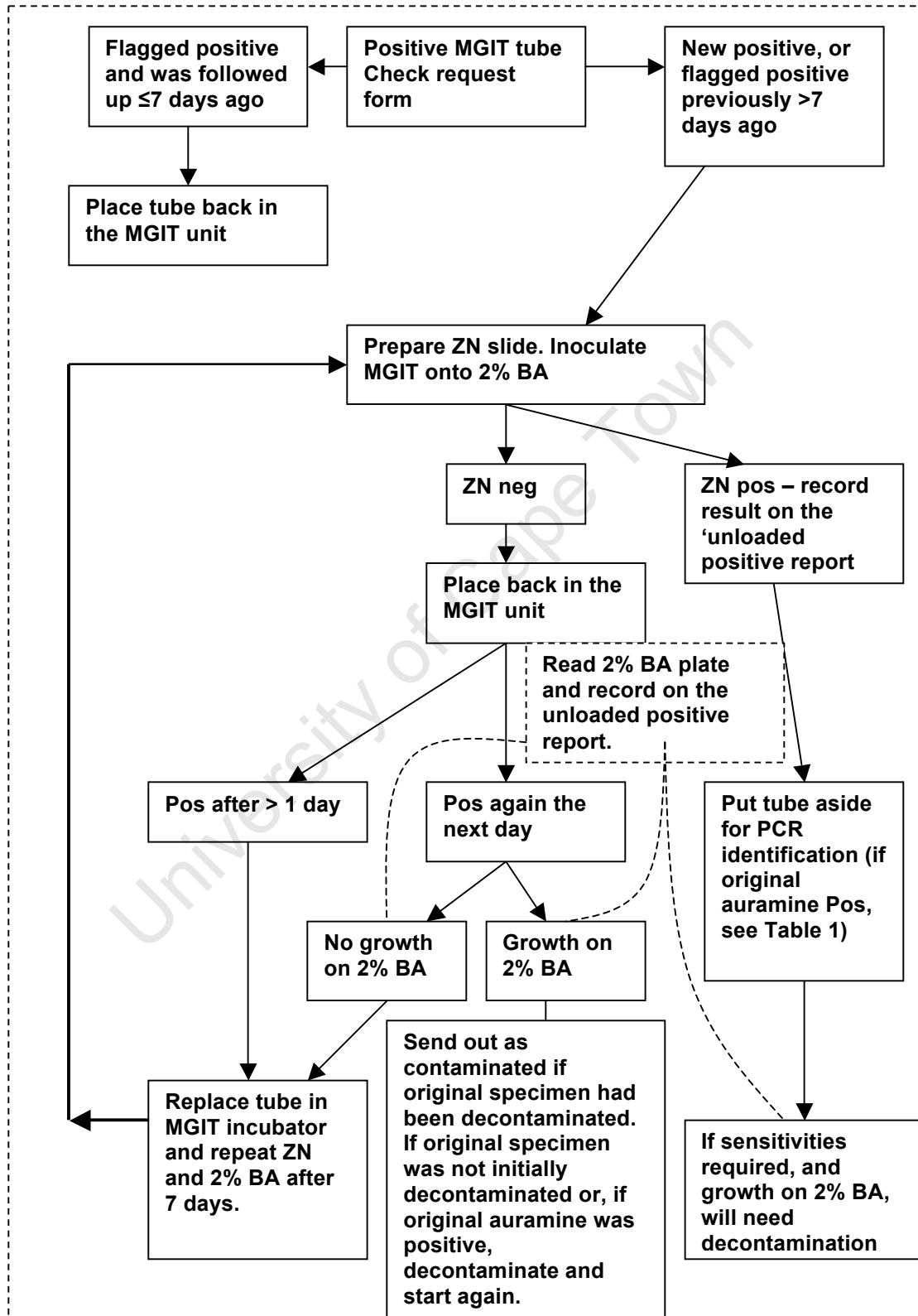
INCUBATION OF MGIT TUBES

- i. Open the drawer of the MGIT incubator unit.
- ii. Select the 'insert tube' icon.
- iii. The MGIT tube has two barcodes on it – one printed on the tube by the manufacturer and the other the barcode on the laboratory generated label (this barcode is associated with the laboratory number). First scan the MGIT tube's barcode into the MGIT incubator, and then scan the laboratory generated barcode. The barcode scanner is located on the front of the incubator
- iv. Insert the MGIT tube into the spot indicated in the MGIT drawer by the red/green LED.
- v. If a specimen is in a container other than a MGIT tube (such as a large bone specimen) incubate it in the freestanding aerobic (37°C) incubator. This culture will be inspected manually (see later)

PROCESSING OF POSITIVE MGIT TUBES

- i. Every morning remove all MGIT tubes that have flagged positive. This is performed **daily** from Monday to Friday, apart from public holidays.
- ii. Open the drawer of the MGIT incubator unit and press the positive icon on the screen (picture of a tube with a "+" in it) and remove the MGIT tubes indicated by the LED. Keep MGIT's from each machine separate.
- iii. Print an "unloaded positives" report for each unit. Press the printer icon (picture of a printer), and then the positive vial icon. See appendix A for example of an unloaded positives report.
- iv. All tubes that have already been processed in the preceding week should be returned to their corresponding MGIT units. Only tubes that have not flagged positive before and those that last flagged positive more than a week ago are to be processed further.

Flowchart 1: Summary of what to do with unloaded positive tubes.



A: TUBES PREVIOUSLY REMOVED < 7 DAYS AGO

- i. Return all these MGIT tubes to the appropriate MGIT unit within 5 hours of removal. Barcode the MGIT tube back into the MGIT unit (only the MGIT tube's barcode needs to be scanned).

B: NEW POSITIVES AND TUBES PREVIOUSLY REMOVED > 7 DAYS AGO

- i. For each MGIT, label a slide for ZN staining and a quadrant on 2% BA plate to check for contamination.
- ii. **Work under the hood when working with the MGIT tubes.**
- iii. Place a drop of precipitating fluid on the slide to ensure adherence of the smear.
- iv. Aspirate (fish) approximately 0,1ml from the bottom of the MGIT tube, inoculate the BA quadrant and prepare a slide for a ZN.
- v. Dry the slides under the hood and stain according to the ZN stain technique.
- vi. Incubate the 2% BA plates overnight in the CO₂ incubator.
- vii. Once the ZN has been read, record results on the unloaded positive report. Slides can be discarded.
- viii. If the ZN is negative, date the MGIT tube and return to the appropriate MGIT unit within 5 hours of removal. Only the MGIT tube's barcode needs to be scanned.
- ix. If the ZN is positive, refer to the 'Positive MGIT's / LJ's for Identification' flow diagram UNLESS:
 - the ZN appearance is typical of *M. tuberculosis* (with cording and beaded appearance) **and**
 - the initial auramine performed on the specimen was positive **and**
 - sensitivity testing has **not** been requested **and** the specimen was a sputum, a tracheal aspirate or induced sputum

In the above situation the specimen can be sent out with the comment "MTBID" resulted on the LIS. If in doubt, consult a senior technologist or a pathologist.
- x. Place MGIT tubes or LJ's in the 'awaiting TBPCR' box or the 'awaiting HAIN' box.
- xi. If ZN appears atypical, inoculate on LJ slope for a possible MOTT ID.
- xii. Read the 2% BA plates and record the results on the unloaded positive report.
- xiii. Growth on 2% BA from MGIT's that are ZN positive must be entered on the LIS.
- xiv. Refer to the Flow Chart or Table 1 for the appropriate course of action depending on the results of the 2% BA culture and the ZN.
- xv. If the culture is contaminated with an organism that may be a *Nocardia*, consult with a pathologist or registrar to determine whether the organism should be identified and reported.
- xvi. ALWAYS remember to check whether an LJ slope was inoculated as well. If a specimen is being discarded and an LJ was inoculated, the LJ can be incubated for the remainder of the 42 days unless it also becomes contaminated

Table 1: Summary of action to be taken for positive MGIT tubes

<u>Original auramine</u>	<u>2% blood agar</u>	<u>Specimen type</u>	<u>ZN performed on MGIT</u>	<u>Action</u>
Nag	Growth	Any specimen that has been decontaminated	Negative	Send out as contaminated
Nag	Growth	Any specimen that was NOT decontaminated	Negative	Decontaminate
Pos	Growth	Any	Negative	Decontaminate
Nag or Pos	No growth	Any	Negative	Re-incubate in MGIT machine for a further 7 days and re-assess with ZN and 2% BA at that stage
Nag	No growth	Any	Pos	Check flow diagram (page 8)
Nag	Growth	Any	Pos	Check flow diagram (page 8)
Pos	No growth or growth	Sputum	Pos	Not for PCR unless sensitivity testing required, or unless ZN on MGIT doesn't resemble <i>M. tuberculosis</i> . Result as "MTBID"
Pos	No growth or growth	Any, other than sputum	Pos	Into PCR box

PROCESSING OF NEGATIVE MGIT TUBES

This is performed on Monday to Friday, except for public holidays.

With the MGIT 960 instrument tubes are held for 42 days before they are removed as negative. Those tubes ready for removal from the system will be indicated on the display and on the appropriate drawer.

- i. Open the drawer of the instrument, press the negative icon (bottle with a “-“on it), and remove the tubes indicated by the LED's.
- ii. Print an “unloaded negatives” report by pressing the printer icon, and then the icon of a bottle with a “-“on it. See appendix B for an example of an unloaded negatives report.
- iii. Correlate the MGIT accession number to the accession number on the unloaded negative report and tick each one and sign the report,
- iv. Visually inspect all negative MGIT tubes. If there appears to be granules, perform a ZN. If the ZN is positive, subculture to a LJ slope to confirm viability and complete a worksheet.

- v. If ZN is negative send out as negative.
- vi. Using the MGIT, enter results on the LIS.
- vii. If any specimens have had LJ slopes inoculated, remove the corresponding LJ slopes from the incubator and enter the result of the LIS.
- viii. The negative MGIT tubes and LJ's are sent for autoclaving before being discarded.

PROCESSING OF MGIT CULTURES NOT IN THE MGIT INCUBATOR

These are specimens such as bone that were too large to fit into a normal MGIT tube.

- i. Examine the culture medium weekly (usually a Monday) for any sign of visible turbidity
- ii. If the bottle is turbid, proceed as for a positive MGIT culture (sections A or B depending on the age of the culture)
- iii. If there is no visible growth after 6 weeks, perform a ZN stain on the liquid in the bottle.
 - If the ZN is negative, send out as No Growth
 - If the ZN is positive, put aside for PCR.

INCUBATION OF LJ SLOPES

Slant the LJ slopes overnight with the caps loose in the slanting rack in the CO₂ incubator. The following morning tighten the caps and store on a labeled (“1st week”, “2nd week” etc) tray in the 37°C incubator. A **“TB week” stretches from Monday to Friday.**

READING OF LJ SLOPES

A: ROUTINE READING OF SLOPES

Usually on Mondays (if there is a long weekend –the first working day of the week)

- i. Rotate week indicators on the LJ trays
- ii. Examine the LJ slopes for visible growth.
- iii. Any slopes with growth should be put aside.
- iv. Slopes at the end of week 6 must be discarded if no growth. Record the result on the LIS.
- v. Aerate LJ slopes in week 3 and the “awaiting ID” racks as follows:
 - Loosen caps – **DO NOT REMOVE** – and replace bottle in rack.
 - Allow to stand on the bench until bottles have cooled to room temperature.
 - Re-incubate overnight with the caps loose.
 - Remove rack the following day. Allow to cool again and then tighten caps before replacing the rack in the incubator.

B: LJ SLOPES WITH VISIBLE GROWTH:

- i. Find out if the corresponding MGIT has flagged positive yet. If so, work from the MGIT tube, and use the LJ as a backup if necessary. Enter the result of the LJ on the LIS.
- ii. If the MGIT has not flagged yet, proceed by preparing a ZN slide.
- iii. Using a cotton swab, remove a portion of the growth from the LJ slope, prepare a slide for ZN.
- iv. If the ZN is positive, place the LJ in the “PCR” box
- v. If the ZN is negative, and the slope appears contaminated, enter the result on the LIS as contaminated and discard the LJ slope.
- vi. If the culture is contaminated with an organism that may be a Nocardia, consult with a pathologist or registrar to determine whether the organism should be identified and reported.

MYCO/F LYTIC BOTTLES

When bottle flags positive it will be sent from the blood culture lab to the TB laboratory with its corresponding form.

- i. Prepare a slide for a Gram and ZN stain.
- ii. Sub an aliquot from the bottle onto a 2% BA and incubate under CO₂ conditions.
- iii. Read the ZN and Gram stain. If the ZN and Gram stain reveal no organisms return the Myco/F Lytic bottle to the blood culture machine within 3 hours.
- iv. If the bottle repeatedly flags positive in the blood culture machine (this usually happens with bone marrow specimens), subculture the bottle onto a LJ slope. Place this LJ slope together with its corresponding Myco/F Lytic bottle in the sloping tray and the following day place into the ‘Awaiting further identification’ box and incubate off-line for 6 weeks. Read the LJ weekly as indicated above.
- v. If the ZN is positive, subculture the Myco/F Lytic bottle onto an LJ slope. Enter a provisional culture result onto the LIS as follows:
 - Result: RES (result to follow)
 - Gram: NBO or gram result
 - Organism: MYCOB (if TBZ positive)
- vi. Order a TBZ and result as “pos” or “neg”.
- vii. If the ZN is negative and the Gram stain shows the presence of organisms return the Myco/F Lytic bottle with the corresponding form to the blood culture laboratory for follow up. Before returning the bottle to the Blood Culture Lab, remove about 10ml fluid from the Myco/F Lytic bottle into a Sterilin® tube. Decontaminate the fluid as per protocol. Inoculate a MGIT tube and incubate for 42 days.

CLINICAL APPLICATION

Although microscopy is the primary means of diagnosing TB in South Africa, culture is sometimes indicated to make a diagnosis of TB. Detection of mycobacterial growth from a clinical specimen is one way of confirming a diagnosis of tuberculosis.

REFERENCES

1. Bactec MGIT 960 Users Manual. Becton Dickinson and Company. Sparks, Maryland, USA. 1999
2. Kent PT, Kubica GP. Public Health Mycobacteriology - A Guide for the Level III Laboratory. Centres for Disease Control. Atlanta Georgia. 1985

University of Cape Town

Annexure I: Mycobacterium Tuberculosis PCR identification: NHLS Grootte Schuur Hospital Standard Operating Procedure.

STANDARD OPERATING PROCEDURE

Title: MTB PCR IDENTIFICATION

Document number: MIC0709

Version number: 2

Written by: Mrs K Mentoor

Checked by: N/A

Approved by: Dr A Whitelaw

Active date: March 2009

MTB PCR IDENTIFICATION

PURPOSE

The purpose of this SOP is to describe how acid fast organisms that are growing in either a MGIT or LJ culture are identified as *M. tuberculosis complex* by means of a specific PCR (polymerase chain reaction) assay.

PRINCIPLE

A PCR assay was developed for the amplification of a 336 – bp repetitive fragment in the chromosome of *Mycobacterium tuberculosis*. Briefly, organisms are boiled to kill them and release their DNA, the DNA is used as a template in a PCR assay where the specific fragment of mycobacterial DNA, if present, is amplified by means of a thermostable polymerase enzyme. The presence of the specific amplification product is detected by electrophoresing the sample on an agarose gel, and visualising the DNA under ultraviolet light. The assay is specific for *Mycobacterium tuberculosis complex* and can be used to detect the amount of DNA present in less than 10 organisms.

LIMITATIONS

- Use of incorrect primers or expired primers.
- Incorrect reagents and inaccurate volumes used in the mastermix.
- Incorrect connection of electrodes on the gel tank.
- Insufficient DNA

- Cross contamination while loading gels.
- A negative test result does not exclude the possibility of infection.

SCOPE

This SOP applies to all MGIT tubes or LJ slopes which have cultured acid fast bacilli.

RESPONSIBILITY

This SOP must be followed by any medical technologist / student medical technologist / medical scientists / pathologist / pathology registrar who may be working in the laboratory and processing specimens for mycobacterial culture.

ABBREVIATIONS

The following abbreviations are used in this SOP:

TB	Tuberculosis
MOTT	Mycobacterium other than tuberculosis
MGIT	Mycobacterial Growth Indicator Tube
LJ	Lowenstein-Jensen medium
AUR	Auramine stain
ZN	Ziehl-Neelsen stain
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
DMSO	Di-Methyl Sulfoxide
dNTPs	Deoxy-nucleotide triphosphate mixture
MgCl ₂	Magnesium chloride
TE	Tris-EDTA
TAE	Tris-acetate-EDTA
LIS	Laboratory Information System
2% BA	2 % Blood Agar

EQUIPMENT AND SUPPLIES

- Biosafety cabinet
- 1,5ml Eppendorf tubes
- 0,5ml Eppendorf tubes
- Air displacement pipettes: 1000ul, 200ul, 20ul.
- Filtered pipette tips (aerosol resistant tips)
- PCR thermocycler
- Polypropylene tubes 15ml capacity (NOT polystyrene tubes)
- Heating Block
- Microwave oven
- Electrophoresis tanks
- Electrophoresis trays
- Gel combs (18-20 teeth ideally)
- Power-pack for electrophoresis
- UV transilluminator
- Digital camera linked to PC.

- Rubber gloves
- Bench top centrifuge

REAGENTS

Taq polymerase	purchased commercially
MgCl ₂ (25mM)	purchased commercially
PCR buffer	purchased commercially
dNTPs	purchased commercially
Ultra pure water	purchased commercially
Agarose	purchased commercially
DMSO	purchased commercially
TE buffer (10mM Tris, 1mM EDTA pH 7.6)	supplied by media lab
Loading buffer (bromophenol blue and sucrose)	supplied by media lab
Ethidium bromide (5mg / ml stock solution)	made up in-house

TAE buffer supplied by media lab as 50xTAE

Make 1X TAE with either of the following volumes:

<u>50X TAE</u>	<u>Distilled water</u>
20ml	980ml
40ml	1960ml

Primers K1 and S2 synthesised by UCT dept cell biology

The primers are diluted to a working stock of 20pmol/ul

K1: 5' - GCC AGA ACC GAC CAA CCC GCC GAT A - 3'

S2: 5' - GCG GCT CGG GCG GCG TCG GTG GCT T - 3'

SAFETY PRECAUTIONS

M. tuberculosis is spread by the airborne route. Many laboratory procedures are accompanied by the formation of aerosols containing bacteria but most importantly, mycobacteria. The technologist must be aware of aerosol formation and the dangers thereof. Therefore all procedures using live cultures, as far as possible, should be performed in the laminar flow cabinet. Once the samples have been boiled, the organisms are dead and do not pose an infection risk.

Ethidium bromide is potentially carcinogenic and teratogenic. Nitrile gloves must be worn whenever working with the solution. Hands must be washed after any use of ethidium bromide – even if gloves were worn as well. For more details on the handling and disposal of ethidium bromide and ethidium bromide containing gels, refer to the appropriate SOP (Molecular: Handling of Ethidium Bromide).

SPECIMENS FROM OTHER LABORATORIES

In addition to the MGIT or LJ cultures from the TB laboratory at Groote Schuur Hospital, cultures from both Tygerberg Hospital and 2 Military Hospital are sent for PCR identification of mycobacteria

Specimens from Tygerberg Hospital usually arrive once a week.

- Date stamp the referral worksheet

- ii. Sort MGIT's according to the referral sheet provided by TBH.
- iii. If all the specimens that are listed on the referral sheet have arrived, sign the sheet, photocopy it, and send the copy back to the Tygerberg Hospital TB laboratory. If any specimens are missing, contact the Tygerberg TB laboratory to inform them.
- iv. Register each of the specimens with a GSH laboratory number, and label the forms and MGIT tubes with a Groote Schuur lab number (SCH prefix).
- v. Place a red sticker on the MGIT tubes / LJ slopes for HAIN PCR and separate the MGIT tubes and LJ slopes into awaiting HAIN and TBPCR boxes.

SORTING OF POSITIVE CULTURES

All positive cultures where TBCUL has been requested and certain trials will be included in TBPCR's.

If a patient has more than one positive culture, only one of those need to be identified by PCR, unless samples are taken >1 month apart. If a specimen is positive in both the MGIT and on the LJ slope, it is only necessary to confirm one of these cultures as *Mycobacterium tuberculosis*. If the growth on the LJ slope is heavy use this for PCR instead of the MGIT culture.

- i. Print a TBPCR worksheet from the LIS:
Select worksheet
Area: MTB F5
Grp: * F7
Code: MTB Close – choose Y
- ii. Arrange the MGIT tubes / LJ slopes / BacT– ALERT bottles according to the PCR worksheet.
- iii. Label one Eppendorf tube with the laboratory number for every culture to be analysed.

CONTROLS

The following controls are included in every PCR run:

- i. A negative control (TE buffer as described below) every 10th specimen
- ii. Negative control – water (set up at time of preparing mastermix)
- iii. *Mycobacterium tuberculosis* (H37Rv)

All the controls must be included on the PCR worksheet

PCR ASSAY

The PCR assay is carried out in 4 different areas of the laboratory in order to minimise the chance of cross contamination. It is vital that the different steps of the PCR procedure are carried out in the appropriate area of the laboratory.

- a) Preparation of culture suspensions for PCR (area 1)
- b) Preparation of PCR amplification mixtures (area 2)
- c) Addition of template (area 3)
- d) Amplification (area 4)

Extraction of DNA – (TB laboratory):

For MGIT tubes and BacT-Alert bottles:

Aliquot approximately 1 – 1.5ml of the culture fluid into the correctly labelled tube.

For LJ slopes:

Aliquot 1 – 1.5ml of TE buffer into an Eppendorf tube.

Using a sterile cotton swab, make a heavy suspension of the organism from the LJ slope in the TE buffer.

For TE control:

Aliquot 1 -1.5ml TE buffer into an Eppendorf tube

- i. Check that the number on the tube and on the specimen correspond.
- ii. Tighten the caps of the tubes securely to prevent the release of the contents during the heating step.
- iii. Switch on the heating block and allow too reach temperature of 95°C.
- iv. Place the tubes in the heating block and incubate for 30 minutes.
- v. After boiling, place the tubes at 4°C for at least 20 minutes to allow cooling and condensation to take place.
- vi. Centrifuge for 3 - 5 minutes at 3200 rpm in a bench top centrifuge.
- vii. Return tubes to racks. The cooling and centrifugation steps ensure that all products of the boiling procedure, including released DNA, are confined to the bottom of the tube. Thus, the possibility of contamination of the environment with target DNA when the tubes are opened, is reduced.
- viii. The boiled DNA suspensions are stored at 4°C if not used immediately.

Preparation of reagents – area 2:

PCR reagent mixtures are made up in the PCR ‘clean’ room. PCR cleanliness is very important. Hands must be washed and laboratory coats removed before entering this area. Pens, paper and all PCR ‘clean’ reagents are available so it is not necessary to bring anything additional into this room. Nothing leaves this area except you and the newly aliquoted PCR master mixes. For more detail on the use of the PCR clean room, refer to the SOP Molecular: General techniques.

Make up a master-mix using the following formula. All the reagents except water and DMSO are stored at -20°C ($\pm 2^\circ$) in the clean room. DMSO and water are kept at room temperature in the clean room.

- Multiply the volume of each component by the number of tests being done plus 1 (e.g. 31 tests plus 1 = 32). Don’t forget to include the appropriate number of controls. This allows for the inevitable loss of minute volumes while pipetting.

Formula for TB PCR assay

Ultra pure water	19,7 μ l
10X buffer	5 μ l

MgCl ₂ (25mM)	3µl
DMSO	5µl
dNTP's	2µl
Primer S2 (20pmol/ul)	2,5ul
Primer K1 (20pmol/ul)	2,5µl
Taq polymerase (only added later – see below)	0,3ul

- i. Add all the components to a polypropylene tube.
- ii. Mix well by pipetting up and down repeatedly 5-10 times.
- iii. Add Taq polymerase enzyme: 0.3 µl per reaction (the concentration is 5 units / 1µl). Mix well once more. It is very important the two mixing steps be performed.
- iv. Aliquot 40ul of the mastermix into 0,5ml eppendorf PCR tubes
- v. To one tube of mastermix add 10ul of sterile water from the PCR clean room. This serves as the negative control with water.
- vi. Discard any remaining mastermix
- vii. Place mastermix tubes into ice blocks and store at -20°C.

Addition of DNA (area 3):

- i. Place the mastermix tubes inside the laminar flow hood, and the tubes with extracted DNA template outside the hood. There should only be one tube with template DNA in the hood at any one time. Ensure that all mastermix tubes are closed before bringing a new tube of DNA template into the hood.
- ii. Aspirate 10ul of the DNA mixture from the tube by tilting the tube and allowing only the pipette tip to enter the tube opening
- iii. Load the appropriate PCR tube with 10µl of DNA preparation, using filter tipped aerosol resistant tips. The 10µl volume is added to and mixed with the PCR mixture by ticturating 2-3 times. One tube should have no DNA added – the tube with mastermix and water that was added in the PCR clean room (negative control).

Amplification – area 4:

The amplification steps are performed in the DNA amplification laboratory.

Thermocycler 6 has been programmed with the appropriate conditions.

- Initial denaturation step: 1 cycle for 8 minutes @ 95°C
- 40 cycles of 95°C (denaturation) for 25 seconds alternating with 69°C (annealing and extension) for 60 seconds.
- Indefinite hold at 4°C.
 - i. Turn on the thermocycler
 - ii. Transfer the tubes into the thermocycler and close the lid
 - iii. Press “Run” (the F1 key) – a list of programmes appears on the display
 - iv. Use the down arrow to highlight the “tb-pcr” programme
 - v. Press “Start” (the F1 key)

- vi. Once the run has finished (the samples are in the indefinite 4°C hold cycle), turn off the thermocycler (or quit the programme) and remove the tubes.

Alternatively, the cycle can be set up manually on one of the other thermocyclers. Refer to the manufacturer's instructions for details of how to do this, or ask a senior member of staff. If you are unsure, please ask rather than fiddle!

DETECTION OF PCR PRODUCT

Amplification of the specific PCR product is determined by agarose gel electrophoresis. For details on the running of agarose gels, refer to the Molecular: General techniques SOP

The following is a brief description of the agarose gel electrophoresis methodology.

- i. Prepare a 3% agarose gel by adding 3g agarose to 100 ml TAE buffer, and heating in the microwave until completely dissolved
- ii. Allow the agarose to cool to about 50-60°C (the bottle can be held comfortably in your hand without burning)
- iii. While waiting for the agarose to cool, prepare a gel tray by taping the open ends closed with masking tape.
- iv. Add ethidium bromide to the gel – 2ul of stock (5mg/ml) to 100 ml agarose. The final ethidium bromide concentration is 0,1ug/ml. Remember to wear gloves when working with ethidium bromide, and to discard pipette tips into the sharps container.
- v. Pour the cooled agarose into the tray to a depth of about 7mm
- vi. If there are slots in the gel tray for the combs, these should be used. If there are no slots, place a comb about 5mm from the end of the tray, and another comb about halfway down the gel. The second comb should be placed such that it is approximately 4 cm from the first comb, and there is at least 4 cm between the second comb and the end of the gel. Allow the gel to cool and set completely before gently removing the combs.
- vii. Place the gels into running tanks containing just enough 1XTAE running buffer to cover them.
- viii. If there is any excess gel in the bottle, store it in the dark (ethidium bromide is light sensitive). The gel can be reused by melting it in the microwave.
- ix. Add 10ul loading buffer to the amplification mixtures and mix well.
- x. Using a pipette, aspirate 10ul of the amplification product and dye, place the tip of the pipette at the corner of an empty well in the gel, and slowly inject the mixture into the well. The dye allows one to visualise the mixture settling in the bottom of the well.
- xi. Load the samples sequentially into the wells.
- xii. Molecular weight marker VIII (10ul) is loaded into the first and last well of every line of wells.
- xiii. To prevent cross contamination it is advisable to leave a blank space between the negative control and the patient sample.
- xiv. Once all the samples have been loaded, place the lid on the tank, and DNA will migrate from the negative (black) electrode to the positive (red) electrode..
- xv. Turn on the powerpack and electrophorese the gel at 100V. During loading (and the first five minutes of electrophoresis), ensure that the tank and the gel remain

stable (no knocking or bumping) so that PCR mixture load from one well is not carried over to other wells.

- xvi. The dye allows one to visualise how far the samples have migrated. The power can be turned off when either:
- The dye has migrated about 4,5cm (ie virtually touching the next set of wells if two rows of wells were used)

OR

- If one set of wells were used, the dye should have migrated at least 4,5cm and at most to within 0,5cm of the end of the gel.
- xvii. Remove gels carefully and view on the UV transilluminator.
- xviii. Photograph using the digital camera (see PCR-Digital Camera).
- xix. Name the file with the current month, date and year and save in the TB PCR folder. Print a copy of the picture.

RESULTS

The controls must be checked first. The expected results are:

H37 RV	336 bp band
TE control	no amplification
Mastermix / water control	no amplification

If the controls have not worked as expected, discuss it with the technologist in charge of the TB lab or one of the pathologists. The following is a general description of the approach to failed controls.

Positive H37RV has not amplified:

- If the H37RV controls has not amplified, the batch must be repeated (the positives are also repeated in case the positive control was mistakenly loaded into the wrong lane).
- The problem must always be discussed with the technologist in charge of the TB lab, or one of the pathologists to discuss further troubleshooting options

The negative control (TE or water) is positive:

- Repeat entire batch

READING PCR RESULTS

- M. tuberculosis* PCR product is indicated by the presence of a 336bp fragment. The result is reported as *Mycobacterium tuberculosis complex*.
- M. bovis* and *M. bovis* BCG gives a 260bp fragment.
- Other mycobacterial species give a negative PCR result (no amplification).
- Label the lanes on the photograph to correspond with the number in the PCR worksheet.
- Transcribe the results onto the PCR worksheet and the LIS.
- If PCR is negative and there is no record of growth on 2% BA:
 - subculture onto an LJ slope and incubate at 37°C and move to the 'Awaiting Identification' box the following day
 - complete an 'Awaiting Identification' formIf PCR is negative and there is a record of growth on 2% BA:

- Incubate the MGIT tube in the 'Awaiting Identification' box
- Complete an 'Awaiting Identification' form.

Discuss with the technologist in charge of the TB lab, or with one of the pathologists regarding further identification procedures. Record all decisions in this regard on the request form.

- vii. If more than one specimen comes up positive in the MGIT culture, from the same patient, it is only necessary to confirm ID by PCR on one specimen. Send out each report as *M. tuberculosis complex*.
- viii. All isolates that appear to be *M. bovis* BCG (i.e. 260 bp fragment) should be confirmed by the specific multiplex PCR for *M. bovis* BCG. Repeat the electrophoresis of any specimen where a positive result is based on the presence of a weak 336bp band adjacent to a bright 336bp band. Always be aware that a positive result in such cases might be due to the carry-over of positive product from one well into an adjacent well.

CLINICAL APPLICATION

It is important to positively identify mycobacteria as *M. tuberculosis* for the following reasons:

- Treatment of tuberculosis differs significantly from treatment of other mycobacterial infections
- Non-tuberculous mycobacteria may be non-pathogenic in some instances, whereas *M. tuberculosis* is always regarded as a pathogen.
- Infection with *M. tuberculosis* has public health implications

Use of PCR provides a quick and reliable way of positively identifying cultures of acid fast bacilli as *M. tuberculosis*, shortening the time to a final report being issued and thus hopefully allowing for more rapid initiation of therapy and contact tracing.

NOTE:

Positive TB culture results from patients at Red Cross Children's Hospital are notified via email to Infectious Disease clinicians at Red Cross Children's Hospital.

ATTACHMENTS:

- Master Mix Record Sheet
- TB Primer Reconstitution Record Sheet
- In-house PCR Worklist

REFERENCES

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Annexure J: British Journal of Haematology Author Guidelines:

<http://www.wiley.com/bw/submit.asp?ref=0007-1048&site=1>

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