

**THE CONTRIBUTION OF FINE NEEDLE ASPIRATION BIOPSY IN
THE DIAGNOSIS OF MYCOBACTERIAL LYMPHADENOPATHY
WITH PARTICULAR REFERENCE TO CHILDREN**

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

A handwritten signature in brown ink, appearing to read 'C. Welch', is positioned above the 'Signature:' label.

Signature:

Date: 5 August 2009

In all first author papers the candidate obtained ethics consent, assisted with the sample collection, collected the data, drew up the analysis plan and wrote the first draft of the manuscript.

In addition the candidate was involved in all the microscopic evaluations and learnt the laboratory techniques used in the molecular analyses.

In all the other papers the candidate helped conceptualise the study, assisted with sample collection and evaluation of microscopic slides and made a substantial contribution to the manuscript.

SUMMARY

Expediting a diagnosis of tuberculosis in children, particularly those who are immunocompromised due to HIV/AIDS, is essential, as they are vulnerable to develop severe forms of disease due to their immature or compromised immune systems. A significant percentage of children (8 to 10%) with TB have TB lymphadenitis, in isolation, or in combination with other disease manifestations.

Fine needle aspiration biopsy (FNAB) is a simple and minimally invasive procedure well tolerated by children. It may be performed as an outpatient procedure by clinicians as well as nurses, and excellent results can be achieved with training in the correct procedure.

The aim of this dissertation was to demonstrate that FNAB may contribute significantly to the diagnosis of mycobacterial lymphadenitis, with particular reference to children TB suspects. We first established that TB lymphadenitis is a common clinical problem in children in TB endemic areas and that FNAB is an efficient simple and effective diagnostic modality in children with peripheral lymphadenopathy.

We then proceeded to document the diagnostic yield and time to diagnosis of FNAB compared to conventional laboratory specimens collected in children.

We investigated the value of additional diagnostic modalities such as autofluorescence in improving the ability of cytology to make a definitive diagnosis of mycobacterial infection based on cytomorphology and identification of the organism.

In countries where organisms such as *Mycobacterium bovis BCG* and non-tuberculous mycobacteria are prevalent, culture with subsequent speciation is essential. The amount of material harvested during FNAB is minuscule, and requires immediate bedside inoculation for optimal yields.

We developed an inexpensive and effective transport medium to facilitate mycobacterial culture from FNAB, even if this is collected at an outside facility. It is ideally

suited for use in clinics and rural hospitals as it is stable at room temperature, maintains viability of the organism for seven days, and the closed lid format reduces contamination.

Mycobacterial culture even using liquid-based media, takes up to 6 weeks, and this delay is unacceptable particularly in children. We developed a Nucleic Acid Amplification Technique (NAAT) using High Resolution Melt Analysis and applied this novel technique to FNAB specimens submitted in transport medium. Although sensitivity remained suboptimal, the technique is highly specific, simple and rapid. Its use could be incorporated into routine microbiology laboratories, to assist with rapid diagnosis while cultures are pending.

We collected a solid body of evidence, which will promote the use of FNAB in suspected mycobacterial lymphadenopathy, particularly in children in resource-limited countries. The utilisation of the diagnostic methods identified will expedite speciation and allow early and appropriate initiation of therapy. This is in keeping with Millennium Development Goal 6: to combat TB by early detection of new cases and effective treatment.

OPSOMMING

Kinders met tuberkulose (TB), en veral diegene met gekompromiteerde immuniteit as gevolg van MIV/VIGS, het 'n verhoogde neiging om ernstige siektebeelde te ontwikkel vanweë hul onvolwasse of gekompromiteerde immuunsisteme. 'n Spoedige diagnose van TB in kinders is dus noodsaaklik. 'n Betekenisvolle persentasie van kinders (8 tot 10%) met TB het TB limfadenitis met of sonder meegaande ander siekteverskynsels.

Fynnaaldaspirasiebiopsie (FNAB) is 'n eenvoudige en minimale indringende prosedure wat gereedelik deur kinders aanvaar word. Geneeshere en verpleegkundiges wie toepaslike opleiding in die uitvoering van FNAB ontvang het, kan die prosedure op buitepatiënte uitvoer en uitstekende resultate behaal.

Die doel van hierdie studie was om aan te toon dat FNAB betekenisvol kan bydra tot die diagnose van mikobakteriële limfadenitis in veral kinders met vermoedelike TB. Daar was eerstens bevestig dat TB limfadenitis 'n algemene kliniese probleem is in kinders in TB endemiese areas en dat FNAB 'n doeltreffende, eenvoudige en effektiewe diagnostiese modaliteit is in kinders met perifere limfadenopatie.

Vervolgens was FNAB se diagnostiese opbrengs en die tydsverloop tot diagnose vergelyk met dié van konvensionele laboratoriummonsters wat in kinders verkry word.

Die bydrae van verdere diagnostiese modaliteite soos outofluoressensie tot 'n verbetering in sitologie se rol in die diagnose van mikobakteriële infeksie, soos gebaseer op sitomorfolgie en identifisering van organismes, is ondersoek.

In lande waar organismes soos *Mycobacterium bovis* BCG en nie-tuberkulose mikobakterië heersend is, is kultuur en spesiebepaling noodsaaklik. Die hoeveelheid materiaal wat met FNAB verkry word is baie min en vereis onmiddellike okulasie vir die beste resultate.

Tydens hierdie studie is 'n goedkoop en effektiewe vervoermedium ontwikkel om mikobakteriële kultuur van FNAB verkreeë monsters te fasiliteer, selfs al is die monster vanaf 'n buite fasiliteit bekom. Die vervoermedium is baie geskik vir gebruik in klinieke en plattelandse hospitale. Dit is stabiel by kamertemperatuur, handhaaf lewensvatbaarheid van organismes vir sewe dae, en die geslote dekselmaat verminder kontaminasie.

Mikobakteriële kultuur neem tot ses weke, selfs met die gebruik van vloeistofgebaseerde mediums. Sodanige vertraging in die diagnose is veral in kinders onaanvaarbaar. Tydens hierdie studie is 'n Nukleïnsuur Amplifikasietegniek ontwikkel deur die aanwending van Hoë Resolusie Smeltanalise en is hierdie nuwe tegniek toegepas op FNAB verkreeë monsters wat in die vermelde vervoermedium versamel was. Alhoewel sensitiwiteit nie optimaal was nie, is die tegniek baie spesifiek, eenvoudig en vinnig. Dit kan in roetine mikrobiologie laboratoriums gebruik word om vinnige diagnose te bewerkstellig terwyl daar gewag word vir die kultuur se resultaat.

Hierdie studie bied omvattende bewys ter ondersteuning van die gebruik van FNAB in veral kinders met vermoedelike mikobakteriële limfadenopatie in lande met beperkte hulpbronne. Die toepassing van die diagnostiese metodes wat in hierdie studie identifiseer is sal spesiebepaling bespoedig en vroegtydige en toepaslike behandeling verseker. Dit stem ooreen met Millennium Ontwikkelingsdoelwit 6: om TB te beveg deur vroeë opsporing van nuwe gevalle en effektiewe behandeling.

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This mortal world is fickle and unstable like unto a shifting shadow and the human life is like unto a mirage and a reflection on the water. (Abdu'l-Baha)

Our lives, and what we achieve, are but a reflection of the lives around us, therefore

I would like to thank

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This work is dedicated to my family, without whose support and understanding
this would not have been completed.

Ariel, my son, who has survived a lifetime of a mother as a student and of whom
I am so proud.

Thandiwe, my daughter, beloved child of the light, who brings such joy into my
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Alethe, Richard and Jonathan, may your lives be blessed and protected
and

Steve, my husband, partner and friend.

You will always be the centre of my world.

And to the children of Africa who continue to bear the burden of this disease
May you say of us, that we tried.....

*There can be no keener revelation of a society's soul than the way in which it
treats its children.*

Nelson Mandela

ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BCG	Bacillus Calmette-Guerin
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
DOTS	Directly observed therapy, short-course
ELISA	Enzyme-linked immunosorbent assay
FNAB	Fine Needle Aspiration Biopsy
HAART	highly active anti-retroviral therapy
HIV	Human Immunodeficiency virus
HRM	High Resolution Melting
IRIS	Immune reconstitution syndrome
LED	Light emitting diode
LIP	Lymphocytic interstitial pneumonitis
MDR	Multi drug resistant
MGIT	Mycobacterial growth indicator tube
MODS	Microscopic observation drug susceptibility assay
<i>M.tuberculosis</i>	Mycobacterium tuberculosis
<i>M.bovis-BCG</i>	Mycobacterium bovis BCG
MVP	Mercury vapor lamp
NAAT	Nucleic acid amplification tests
NHLS	National Health Laboratory Services
NTM	Non - tuberculous mycobacteria
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPL	Persistent peripheral lymphadenopathy
SA	South Africa
TB (PTB)	Tuberculosis (Pulmonary tuberculosis)
TBH	Tygerberg Hospital
TST	Tuberculin skin test
WHO	World Health Organization
ZN	Ziehl-Neelson
XDR	Extensively drug resistant

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CHAPTER 1

INTRODUCTION

Tuberculosis (TB) was declared a global emergency in 1993 by the World Health Organisation (WHO) when it was estimated that a third of the world's population is infected with TB.¹ The developing world bears the brunt of this disease burden; 95% of TB cases and 98% of TB deaths occur in the developing world, with sub-Saharan Africa reporting the highest TB incidence in the world.² In 2005 there were an estimated 8.8 million new TB cases globally and 7.2 million of these were in Asia and sub-Saharan Africa. Despite efforts to contain the epidemic new TB cases increased by 6% between 2000 and 2005, predominantly in sub-Saharan Africa and Asia.

The problem is compounded by the parallel human immunodeficiency virus (HIV) pandemic with 33.2 million adults and children living with HIV in 2007, and 68% of these in sub-Saharan Africa.³ Infection with the HIV virus is estimated to increase the lifetime risk of a patient infected with *M tuberculosis* of developing TB by 10 fold to 50%.⁴ South Africa with 5.5 million people leads the world in the number of patients living with HIV, as well as TB/HIV co-infection.⁵

TB in childhood

TB is a major cause of childhood morbidity and mortality in developing countries.^{6,7} It is estimated that 11% of the 8.3 million new TB cases diagnosed globally in 2000 occurred in children,⁸ and in endemic areas children contribute at least 15-20% of the total disease burden.⁹ In the Western Cape Province, a high burden region in South Africa, the TB notification rate in children less than 14 years of age was reported at 620 /100,000 in 2007.¹⁰

Official child TB figures almost certainly represent a gross underestimate due to problems in obtaining an accurate diagnosis in resource-limited settings, poor record-keeping and under reporting.⁸ Children rarely have sputum smear-positive TB, although this reduces

the risk of actively spreading the disease, it makes it more challenging to establish a definitive diagnosis. Expecterated sputum specimens are difficult if not impossible to obtain in children under the age of 7-8 years. Collection of induced sputum and gastric aspirate specimens are possible, but difficult to collect and reported bacteriological yields are low (30-40%).^{8, 11}

A clinical diagnosis of TB in children is problematical and although chest radiography is regarded as the most practical test to provide a reasonably accurate diagnosis of intrathoracic TB in children with suspicious symptoms, it has multiple limitations.¹² In HIV-infected children the diagnosis of both intrathoracic and extra thoracic TB is difficult due to underlying chronic pathology such as lymphocytic interstitial pneumonitis (LIP) or bronchiectasis and opportunistic infections. Chest radiography can also not provide diagnostic confirmation in children with extra thoracic TB. In a study in the Western Cape, South Africa 10.7% of children treated for active TB had extra thoracic disease manifestations only; cervical TB lymphadenitis was the most common condition in these children being present in 48.6%. TB lymphadenitis is a common cause of persistent cervical adenopathy in TB endemic areas. Persistent peripheral lymphadenopathy in HIV-infected children is a criterion for the clinical classification of HIV itself. It may also be associated with multiple other pathological entities, from infections to neoplasia.

Mycobacterium tuberculosis is not the only mycobacterial organism which involves peripheral lymph nodes. In developed countries non-tuberculous mycobacteria (NT M) is the commonest mycobacterial organism isolated and BCG vaccination may be associated with local complications such as abscess formation at the vaccination site and regional lymphadenitis. It is also the most common manifestation of immune reconstitution syndrome (IRIS) if vaccinated HIV-infected infants are commenced on highly active anti-retroviral therapy (HAART). It is therefore important to develop methods that will provide a rapid and definitive mycobacterial diagnosis in children with persistent peripheral lymphadenopathy.

Fine needle aspiration biopsy (FNAB) is a specimen collection technique which is widely used in the diagnosis of palpable masses including peripheral lymphadenopathy. Its value in the diagnosis of mycobacterial lymphadenitis in adults is well documented. FNAB is a simple effective and safe modality for obtaining a representative sample of material from a lymph node and the diagnosis of mycobacterial adenitis can be confirmed utilising a number of different investigations, including cytomorphology, specific stains to identify the organism, culture and polymerase chain reaction (PCR).

FNAB is not widely used for the diagnosis of mycobacterial disease in children. As this is a rapid and minimally invasive technique which can be performed on an outpatient basis, in a primary health care setting it is ideal for use in resource-limited countries with the highest incidence of mycobacterial disease as well as HIV infection.

Study objectives

The main study objective was to evaluate and develop laboratory-based diagnostic techniques to establish a rapid, accurate and cost effective diagnosis of mycobacterial lymphadenitis in children, using FNAB as the sampling method. We applied a stepwise approach using multiple studies with specific aims to achieve this objective.

To establish that TB lymphadenitis is a common clinical problem in children from TB endemic areas

This aim is addressed in chapter 3:

A prospective community-based study documented the contribution of TB lymphadenitis to persistent lymphadenopathy among children who present to primary health care clinics with persistent cervical adenopathy in a TB endemic area.

To evaluate the diagnostic utility and feasibility of FNAB in a resource limited setting

These aims are addressed in chapter 4:

A laboratory-based retrospective study documented FNAB adequacy rates among various health care professionals who performed the procedure and described the spectrum of pathology seen.

A laboratory-based retrospective study compared FNAB to more established specimen collection.

A laboratory-based retrospective study evaluated the utility of FNAB in HIV-infected children (supplementary manuscript).

To determine optimal methods for cytopathological diagnosis of mycobacterial disease

These aims are addressed in chapter 5 and 7:

A prospective laboratory-based study conducted in adults and children to determine the value of incorporating autofluorescence into cytopathological evaluation of lymph node aspirates in patients with suspected mycobacterial disease.

A prospective laboratory-based study in children assessing the ability of FNAB to diagnose mycobacterial lymphadenitis in children, using cytomorphology, autofluorescence on Papanicolaou stained smears, Ziehl-Nielsen (ZN) staining and/or culture.

A retrospective laboratory-based study to assess cytomorphological patterns of *M. bovis BCG* and *M. tuberculosis* on FNAB (supplementary manuscript).

A retrospective laboratory-based study to evaluate fluorescence microscopy in the diagnosis of mycobacterial infection using FNAB samples and a rewind able (power independent) LED light source (supplementary abstract).

To explore novel methods for optimal culture-based and molecular diagnosis

These aims are addressed in chapters 6 and 7:

A prospective pilot study, collecting residual material from fine needle aspiration biopsy in 200 µL of sterile water, to investigate the feasibility of utilising PCR amplification techniques with fractionation in agarose gel and visualisation using ethidium bromide directly from FNAB samples (supplementary study).

A prospective hospital-based study to assess the value of using a simple mycobacterial transport medium for bedside inoculation of FNAB specimens.

A prospective hospital-based study utilizing FNAB combined with High Resolution Melt Analysis to reduce diagnostic delay in patients mycobacterial adenitis.

A simplified technique using FNAB and collection of residual material on FTA paper and real-time PCR/HRM for the diagnosis of mycobacterial infection (supplementary manuscript in progress).

References

- [1] Corbett EL, Watt CJ, Walker N, et al. The Growing Burden of Tuberculosis: Global Trends and Interactions With the HIV Epidemic. *Arch Intern Med* 2003;163:1009-1021.
- [2] World Health Organization. Global Tuberculosis Control: Surveillance, Planning, Financing: WHO Report 2007. Geneva: World Health Organization; 2007. Report No.: WHO/HTM/TB/2007.376.
- [3] Harries AD. HIV/AIDS: the long haul ahead [Editorial]. *Int J Tuberc Lung Dis* 2008;12:1347-1348.
- [4] Harries A, Maher D, Graham S. *TB/HIV: A Clinical Manual*. 2nd ed. Geneva: WHO 2004.
- [5] UNAIDS. Regional fact sheets. AIDS epidemic update - Regional Summary:Sub-Saharan Africa. 2007 [cited 10 March 2009]; Available from: http://data.unaids.org/pub/FactSheet/2008/epi07_fs_regionalsummary_subsafrica_en.pdf

- [6] Beyers N, Donald PR. A prospective evaluation of children under the age of five years living in the same household as adults with recently diagnosed pulmonary tuberculosis. *Int J Tuberc Lung Dis* 1997;1:38-43.
- [7] Marais BJ, Hesselning AC, Gie RP, Schaaf HS, Beyers N. The burden of childhood tuberculosis and the accuracy of community-based surveillance data. *Int J Tuberc Lung Dis* 2006;10:259-263.
- [8] Marais BJ, Graham SM, Cotton MF, Beyers N. Diagnostic and management challenges for childhood tuberculosis in the era of HIV. *J Infect Dis* 2007;196:76-85.
- [9] Marais BJ, Gie RP, Schaaf HS, et al. Childhood pulmonary tuberculosis: old wisdom and new challenges. *Am J Respir Crit Care Med* 2006;173:1078.
- [10] Hesselning AC, Cotton MF, Jennings T, et al. High Incidence of Tuberculosis among HIV-Infected Infants: Evidence from a South African Population-Based Study Highlights the Need for Improved Tuberculosis Control Strategies. *Clin Infect Dis* 2009;48:108-114.
- [11] Zar HJ, Hanslo D, Appoles P. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. *Lancet* 2005;365:130-134.
- [12] Marais BJ. Tuberculosis in children. *Pediatric Pulmonology* 2008;43:322-329

CHAPTER 2

COMPREHENSIVE LITERATURE REVIEW

Fine needle aspiration Biopsy (FNAB) - an undervalued diagnostic modality in pediatric mycobacterial disease

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Fine needle aspiration biopsy (FNAB) - an undervalued diagnostic modality in pediatric mycobacterial disease

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Unstructured summary

Mycobacterial disease, particularly tuberculosis, is an escalating problem in developing countries, fuelled by the parallel HIV pandemic. In tuberculosis (TB) endemic countries children carry a very high burden of disease, which may be unrecognised due to the difficulty in making a diagnosis based on clinical, radiological or laboratory methods. One of the main hurdles is the difficulty of obtaining adequate specimens for bacteriological confirmation of disease in children.

TB lymphadenitis is the most common extra-pulmonary manifestation of TB and up to up to 22% of children with persistent cervical lymphadenopathy and no local cause may have TB adenitis Therefore fine needle aspiration biopsy (FNAB), which is a simple and safe outpatient procedure that can be performed by nurses in resource-limited settings and provides material for direct microscopy as well as culture and susceptibility testing, provides an excellent opportunity to obtain bacteriologic confirmation. However, it remains a greatly underutilized specimen collection modality.

This review provides a comprehensive overview of the diagnostic difficulties faced in the diagnosis of paediatric TB in resource-limited settings and suggests ways to utilise FNAB

as a practical modality for the rapid and effective diagnosis of mycobacterial disease in the significant subset of patients who present with peripheral lymphadenopathy. It also provides detail on how best to perform the technique and suggests ways of making it more widely available in resource-limited settings that carry the brunt of the paediatric TB disease burden.

The scale of the problem

Mycobacterium tuberculosis

In 2005, there were an estimated 8.8 million new tuberculosis (TB) cases globally, 7.4 million in Asia and sub-Saharan Africa. More than 4000 people died every day from TB related illnesses in that year, with the developing world bearing the brunt of this massive disease burden; 95% of TB cases and 98% of TB deaths occurred in the developing world.¹ What is even more alarming is the projected scale of the epidemic to come; the World Health Organization (WHO) has estimated that between 2000 and 2020 1 billion people will be newly infected with *M. tuberculosis*, resulting in 200 million TB cases and 35 million deaths.²

TB is a major cause of childhood morbidity and mortality in developing countries.³⁻⁴ Accurate figures on the extent of paediatric TB are not available due to inadequate health information systems in endemic countries and the limited attention paid to children who contribute little to TB transmission within affected communities. WHO estimates of disease incidence are based on sputum smear-positive cases, but more than 80% of children with TB are sputum smear-negative. Indications are that children contribute at least 15-20% of the total disease burden in endemic areas and that they suffer severe TB related morbidity and mortality.⁵ Recognition of this high disease burden and the fact that highly effective treatment is available has led to increased emphasis on the diagnosis and management of paediatric TB. WHO research priority guidelines for paediatric TB identified the evaluation of new techniques to improve the diagnosis and management of paediatric TB as an urgent research priority.⁶

According to 2005 WHO figures, South Africa, despite its relatively good infrastructure and health care services, reported a TB incidence of 600/100,000 population/year with a mortality rate of 71/100,000 population/year.¹ The Western Cape Province, a very high burden region in 2007 reported the adult TB incidence as 1005.7 per 100,000 compared to the National incidence rate of 739.6/100,000 population.⁷ In 2007 the Western Cape Department of Health reported a TB notification rate of 620 per 100 000 in children (0-14 years of age).⁸ A prospective observational study conducted in Cape Town demonstrated that children less than 13 years of age contributed 13.7% of the total TB caseload with a calculated TB incidence of 407/100,000/year; 52.2% of that in adults (\geq 13 years of age).

Mycobacterium bovis BCG

BCG vaccination is recommended by the WHO for infants in TB-endemic areas as it affords protection against disseminated disease in children.⁹ However, BCG vaccination may be associated with local complications, such as abscess formation at the vaccination site and/or regional lymphadenitis. Prior to the emergence of HIV as a cause of immunosuppression, disseminated disease was rare (0.19–2 cases/1 million vaccinated infants) and was associated with congenital immunodeficiency syndromes.¹⁰

In South Africa, a change in vaccine policy in July 2000 from percutaneous Tokyo strain BCG to intradermal Danish strain BCG vaccine has seen a number of adverse events reported in both HIV-infected and immune competent children.^{9, 11-12} Distant or disseminated disease has become a serious concern in human immunodeficiency virus (HIV)-infected children. In 2007 it was estimated that 417 per 100 000 HIV-infected infants, are affected by disseminated BCG disease,¹² a rate that is about 1000 times the rate in HIV-uninfected infants, and with a mortality rate of 75–86%.¹³

WHO recommended in 2007 that HIV-infected infants should not receive BCG vaccination,¹⁴ but this policy is very difficult to implement and may not be beneficial in countries where both HIV and TB are endemic, as the HIV status of the baby is not known at the time of routine vaccination (at birth). Where possible however every effort should be made to introduce selective BCG vaccination based on known HIV status.¹³

Non-tuberculous mycobacteria (NTM)

Non-tuberculous mycobacterial infection (NTM) is due to mycobacterial organisms other than *Mycobacterium leprae* or members of the *Mycobacterium tuberculosis* complex¹⁵ and differ from the former in their habitat, contagiousness and susceptibility to chemotherapy.

In developed countries such as the USA, as the incidence of tuberculosis has fallen, NTM are more frequently isolated than those of the *M. tuberculosis* complex. In immune competent adults NTM infection presents as pulmonary disease in a patient with pre-existing chronic pulmonary disease,¹⁵ whilst in immune competent children the organs involved are the lymph nodes and the skin. These children are generally under the age of six years and are otherwise healthy with no constitutional symptoms.¹⁶⁻¹⁷

In countries with a high prevalence of TB and HIV, localized or disseminated disease from non-tuberculous mycobacteria may occur, but is uncommon.¹⁸ However, a case study from Thailand showed that of the 153 HIV-infected children who had initiated antiretroviral therapy, nine developed the immune reconstitution syndrome (IRIS) due to non-tuberculous mycobacterial organisms.¹⁹ This highlights the need for bacteriological confirmation and speciation in suspected cases of mycobacterial infection.

Contribution of HIV

One of the millennium development goals of the United Nations is to reduce TB-related mortality to 50% of the 1990 levels. Increasingly it appears that this goal will not be achieved,

particularly in sub-Saharan Africa,²⁰ due in part to HIV infection, which is a major contributor to the escalating TB epidemic in this region and the rest of the world.²¹⁻²² It is estimated that in 2007 about 420 000 new HIV infections occurred in children, predominantly in sub-Saharan Africa, through perinatal transmission, in a population vulnerable to early contact with tuberculosis.²³ A recent study in the Western Cape Province, South Africa, recorded a TB incidence of 1596 (disseminated TB 240)/100 000 in HIV-infected and 66 (disseminated 14)/100 000 in HIV-uninfected infants.⁸

Autopsy studies in sub-Saharan Africa have shown that 30 to 40% of deaths in HIV-infected adults and up to 20% in HIV-infected children are due to TB.³ In Zambia,³ Cote d'Ivoire,²⁴ Malawi,²⁵ and South Africa,^{22, 26} TB-related mortality is considerably higher in HIV-infected compared to HIV-uninfected children. Despite this high disease risk experienced by HIV-infected children, the majority of child TB cases are still in HIV-uninfected children, as compared to adults relatively fewer children are HIV-infected and young children are highly susceptible to develop TB irrespective of their HIV status.²⁷ When children are co-infected with HIV and TB however, they show more rapid progression of the disease and increased morbidity and mortality.^{28 29}

The diagnostic dilemma

Children rarely have sputum smear-positive TB. Although this reduces their risk of actively spreading the disease, it makes it more challenging to establish a definitive diagnosis. In contrast to adults where the sensitivity of sputum culture approximates 80-90%, young children are unable to expectorate, alternative specimens such as gastric aspirates or induced sputa are difficult to collect and culture yields are low (widely reported as 30-40%).³⁰⁻³¹ Chest radiography is regarded as the most practical test that provides a reasonably accurate diagnosis of intra-thoracic TB in children with suspicious symptoms.³² However, it has numerous limitations including limited availability, expertise is required to interpret child

radiographs,³³ and it has no value in the diagnosis of extra-thoracic TB. A recent survey demonstrated that 10.7% of children treated for active TB had extra-thoracic disease manifestations only.³⁴ TB lymphadenitis was the most common form of extra-thoracic tuberculosis in these children (50%).

Traditionally, three fasting gastric aspirates samples are collected on three consecutive mornings, requiring hospitalization of the child and frequently the caregiver. In developing countries this may create severe hardship as caregivers usually have more than one child in their care. Caregivers may also compromise their employment by remaining in hospital for a prolonged period of time. Hospitalization incurs cost and occupies beds which may be needed for other children. Sputum induction using mobilization and hypertonic saline have shown improved yield compared to gastric aspirates,³⁰ but more recent studies demonstrated equivalence with gastric aspirates.³⁵ The technique requires specialized training and equipment and may present a nosocomial transmission risk.^{27, 32, 36}

Novel culture methods have been developed in an attempt to circumvent the slow turnaround times, poor sensitivity and cost of conventional automated liquid broth systems. The most feasible alternative to date has been the microscopic observation drug susceptibility assay (MODS) that uses an inverted light microscope to rapidly detect “spindle and cord formation” in selective broth culture that is indicative of mycobacterial growth.³² Phage amplification assays that use bacteriophages to detect the presence of live *M. tuberculosis* have been less successful.²⁷

There is abundant literature utilizing commercial and in-house PCR for the diagnosis of mycobacterial infection in sputum and body fluids, such as cerebral spinal fluid, but results in the literature are highly variable and have not been well validated in children.²⁷ Recent reviews and meta-analyses of PCR in TB meningitis, pleuritis and sputum smear-negative pulmonary TB demonstrated poor sensitivity.³⁷ In patients with sputum smear-positive TB, however, PCR may play an invaluable role in offering rapid species identification and

detection of drug resistance.³² The application in patients with sputum smear-negative or extra-pulmonary TB requires further evaluation and use in low income countries remains limited due to cost constraints..

T-cell assays that measure interferon- γ released by lymphocytes in peripheral blood after exposure to *M. tuberculosis* specific antigens, have been hailed as reliable TB tests; two commercial tests are available T-SPOT.TB and QuantiFERON –TB GOLD. However, study results are highly variable and the experience in children remains limited. Current consensus is that these tests are unable distinguish latent infection (a third of the world population is latently infected) from active disease and adds little to the traditional tuberculin skin test (TST). Tests are also too expensive and complex for routine use in low- income countries.^{27,}

32, 36, 38

Fine Needle Aspiration Biopsy (FNAB)

History

FNAB has been used since the early 20th century to diagnose infectious and neoplastic disease.³⁹ Prior to the 1930s there were isolated case reports documenting its history, and the first large scale report on needle aspiration biopsies was published from Memorial Hospital, New York in 1930.⁴⁰ In the 1960s this technique was championed by the Scandinavians and was widely practiced, with many publications on its utility, mainly in the Scandinavian literature.⁴¹ Gradually the use of FNAB spread through Europe and interest was rekindled in the United States in the 1980's. FNAB is now widely utilized as a first line diagnostic procedure in the diagnosis of palpable masses, including peripheral lymphadenopathy. Its value in the diagnosis of mycobacterial lymphadenitis in adults is well documented.⁴²⁻⁴⁴ FNAB is utilized throughout the developed and developing world, although the indications differ in these two groups. In a review of MEDLINE by Das from 1966 to 2002,³⁹ 849 journals published 5609 articles on FNAB, the number increasing sharply from the 1980s

onwards. Of the developed or high income countries, 52.8% (28/53) published articles compared to 29.7% (46/155) of the low income or developing economies. South Africa was one of the top 10 countries in the developing world with publications on FNAB. Although the developed countries publish significantly more articles than the developing countries, these tend to concentrate on breast and pancreatic disease, while the developing countries publish significantly more articles on small round cell tumors and infectious diseases. This may reflect the health care priorities and diagnostic needs of these developing countries.

Children with pulmonary TB have extra-thoracic disease manifestations in 10 to 30% of cases.^{45, 2} TB lymphadenitis is the most common form of extra-thoracic TB in endemic areas,⁴⁶ where up to 50% of extra-thoracic disease manifests as peripheral lymphadenopathy. In fact, TB lymphadenitis is the most common (22-48%) cause of persistent cervical lymphadenopathy in TB endemic areas.⁴⁷⁻⁵⁰ This is rarely recognized as a potential means for specimen collection. Therefore it is important to develop methods that will provide a rapid and definitive mycobacterial diagnosis in children with persistent cervical lymphadenopathy. In these high burden TB endemic areas, where children may contribute up to 40% of the TB caseload,⁵¹ and assuming a conservative estimate of 5-10% of children with TB have peripheral lymphadenopathy, FNAB could make a significant contribution to the diagnosis of TB and other mycobacterial infections.

A recent retrospective study comparing FNAB to gastric aspirates and induced sputum in children with pulmonary TB and peripheral lymphadenopathy showed FNAB to have a superior diagnostic yield and a significantly reduced time to diagnosis.⁵² An accurate bacteriological diagnosis was made within 3 days in the vast majority of patients, which has important benefits for patient management. FNAB should be regarded as a first line diagnostic modality in paediatric TB suspects with peripheral lymphadenopathy.

TB and HIV have many features in common which contribute to diagnostic difficulties, particularly in children. In this population both are chronic diseases that

commonly present with pulmonary symptoms/signs and/or lymphadenopathy.⁵³ Persistent peripheral lymphadenopathy is itself a criterion for the classification of HIV related disease in children.⁵⁴ Radiological features may be impossible to interpret with certainty and in children with CD4 T-cell depletion, alternative tests such as the TST and or novel T-cell assays offer little diagnostic assistance due to poor sensitivity.⁵⁵

A study from South Africa demonstrated that the majority of HIV-infected children with persistent lung disease have persistent peripheral lymphadenopathy (PPL) and PPL was present in many children with pulmonary TB.⁵⁶ In HIV-infected children, peripheral lymphadenopathy may be associated with many pathological entities, from infections to neoplasia.⁵⁶ Thus, enlarged peripheral lymph nodes were identified as important specimen collection sites to consider, in establishing a definitive diagnosis of TB in HIV-infected children. Another important disease presentation results from the immune reconstitution inflammatory syndrome (IRIS), which may exacerbate the symptoms and signs of mycobacterial disease and needs to be distinguished from TB treatment failure.^{57 55} BCG-related right sided axillary adenitis is the most common IRIS manifestation in BCG-vaccinated infants recently started on highly active anti-retroviral therapy (HAART).

FNAB in children

FNAB has been less widely utilized in pediatrics as a diagnostic modality, although the literature demonstrates a steady increase in the use of FNAB in children, achieving sensitivity and specificity rates of over 90% in these studies.⁵⁸⁻⁶⁰ The majority of the studies were in pediatric oncology patients, where they play a role in triage, as childhood malignancy can be difficult to diagnose, with signs and symptoms which often mimic other common paediatric conditions especially viral infections. With significant mass lesions, FNAB proved to be the diagnostic tool of choice in the triage of these patients.⁶¹ However, in developing countries with a high burden of infectious diseases such as TB and HIV, FNAB can be of inestimable

value in confirming the diagnosis of mycobacterial infection, permitting early appropriate therapy, as well as a means to obtain specimens for culture, bacterial species determination and sensitivity testing.^{60, 62-64}

FNAB is a simple and minimally invasive technique, performed at the bedside, and is well tolerated by children. Table 1 provides a summary of the equipment required and how to perform the technique. Children under the age of six years are given oral or intranasal sedation for amnesia, but children older than six years tolerate aspiration with no sedation. The technique is explained to the child, the caregiver is asked to remain with the child during the procedure and the permission of the child is requested to proceed. If the child understands that this is a very quick minimally painful procedure that will enable them to return home after a short period of observation, they are willing to cooperate.

Most aspirates in children are from axillary or cervical lymph nodes that are easily accessible. If the procedures are performed correctly, with a small gauge needle (22-23 G or smaller) complications such as a small hematoma are rare. If the correct technique is utilized the yield as well as the acceptance of the procedure by the patients will be excellent and nursing sisters can be taught this technique with excellent results. In developing countries where TB is endemic and specimens for bacteriological diagnosis are difficult if not impossible to obtain from children, FNAB provides the means for accurate diagnosis in a significant percentage of children with mycobacterial infection. This is particularly important in countries with a high HIV rate, as well as increasing rates of multi drug-resistant (MDR) and extensively drug-resistant (XDR) TB.

FNAB is a cost-effective diagnostic modality that may be performed at the bedside as an outpatient procedure.⁶² It requires no infrastructure or sterile environment and no sophisticated or expensive equipment. Superficial aspirates may be performed by clinicians, pathologists or trained nursing personnel and do not require local or general anesthesia.⁶¹

FNAB is defined as an aspirate done using a cutting needle no larger than 22G.⁶⁵ This ensures minimal complications, the commonest of which is a small hematoma and thus FNAB is the safest method of obtaining a tissue diagnosis.

A number of different techniques have been used to obtain a tissue and/or bacteriological confirmation of TB lymphadenitis. These include core needle biopsy,⁶⁶ wide needle aspiration biopsy⁶⁶⁻⁶⁷ and surgical lymph node biopsy.^{50, 56} None are as simple, safe and cost effective to perform, as FNAB (Table 2). Compared to core needle biopsy, the risk of sinus or fistula formation, permanent damage to nerves or seeding of tumor along the needle track is minimal.⁶⁵ It permits sampling of the entire mass (node) as multiple excursions of the needle are performed in the lesion without withdrawing the needle, and is no more painful than venepuncture. No more than one ml of suction is applied during the aspiration as the cutting needle will ensure material enters the needle by capillary action, while more suction would increase the risk of bleeding and hematoma formation.

Most aspirates yield a very small amount of material which is expressed onto a glass slide and a monolayer smear prepared. Preparation and staining of the smears can be done in a very basic laboratory using manual methods. Although screening and reporting of the aspirates should be performed by a cytopathologist. The smears are robust and may be transported or even couriered to a central laboratory service for diagnosis and reporting. FNAB is an effective and safe modality for obtaining a representative sample of material from a lymph node, but laboratory confirmation of mycobacterial adenitis can be made utilising a number of different investigations.

Laboratory analysis

Cytomorphology

Cytomorphology is simple, but not specific, as other opportunistic infections may present with similar cytological changes and the quality of the smear is operator dependent.⁴²⁻⁴³ A

number of reactions have been described in the literature: epithelioid granulomas without necrosis, epithelioid granulomas with necrosis and necrosis without granuloma formation.⁶⁸

Ziehl-Neelson (ZN) staining

ZN staining, even in good laboratories, is insensitive, identifying organisms in only about 20% of culture positive samples.⁴⁴ Identification of the mycobacterial organism may be dependent on the immune status of the patient and is reflected in the cytomorphological pattern. Smears with poor granuloma formation are more likely to be positive on Ziehl-Neelsen staining than those showing good epithelioid granulomas without necrosis.⁶⁸

Autofluorescence

Autofluorescence of mycobacteria is simple, sensitive and inexpensive, but is not widely used. It is not highly specific, as the specificity is dependent on the experience of the operator and it cannot differentiate between the various mycobacteria.^{62,69} The recent development of light-emitting diode (LED) technology provides a cheap and reliable light source with a usable life of more than 50,000 hrs using a fraction of the energy required by conventional light bulbs and can run on batteries or inexpensive low-voltage power supplies. Preliminary studies have indicated that LED-fluorescence microscopy offers a valid alternative to conventional fluorescence microscopy⁷⁰ and studies are underway to investigate the diagnostic utility of LED-auto fluorescence microscopy to detect Mycobacteria in fine needle aspiration samples.

Culture

Direct inoculation into MGIT or Bactec tubes (Becton Dickinson, USA) at the bedside gives a high yield,⁶² but it takes 1-6 weeks for a result, and fails to differentiate the various mycobacteria. Additional PCR-based testing is required for accurate speciation.. Direct

inoculation however is not feasible as the MGIT and Bactec collection media are expensive, and are not readily available in clinics and wards for use by clinicians. We have developed an inexpensive transport bottle which is easy to prepare “in house” and does not require refrigeration.⁷¹ The positive culture yield and the time to positive culture from the transport medium, even after 7 days at room temperature, is statistically no different to direct bedside inoculation. The production and distribution of these bottles to clinics and hospitals, will increase the feasibility of diagnosing tuberculosis at primary health care level

Nucleic acid amplification tests (NAATs)

A small number of recent studies have shown considerable promise in the use of NAATs in the diagnosis of *M. tuberculosis* on FNAB specimens.⁷²⁻⁷⁵ These studies have been predominantly in adults. There is limited data on the use of FNAB for the diagnosis of mycobacterial disease in children. Rapid and minimally invasive techniques such as FNAB, which can be performed on an outpatient basis, in a primary health care setting, when combined with a rapid and sensitive diagnostic technique such as PCR, may substantially contribute to the effective management of mycobacterial infection in HIV-infected and uninfected children. The implications of a rapid and accurate diagnosis include expediting access to appropriate and adequate therapy as well as potentially limiting further costly investigation.

Conclusion

It is difficult to understand why such an effective, simple and safe diagnostic modality is so underutilized in the diagnostic workup of pediatric tuberculosis. It is ideally suited for widespread use in resource limited countries which carry the dual burden of TB and HIV. FNAB may be perceived as a procedure to be carried out by specialized medical personnel, but it can safely be carried out by junior medical staff and nursing staff, capable and trusted with inserting intravenous lines. It has been demonstrated that samples collected by nurse aspirators trained during a Burkitt's lymphoma study conducted in Malawi is of similar quality as those obtained by trained pathologists.

Adequate training is essential to develop confidence in the technique and to ensure a good yield.⁵⁶ This can be done through initial outreach teaching and training programs, followed by cascade training to facilitate widespread dissemination of FNAB skills. Pathology departments from South Africa and organizations such as the British Division of the International Academy of Pathology and the Friends of Africa through the United States and Canadian Academy of Pathology (USCAP) offer ongoing education in FNAB and pathology in African and other TB-endemic countries. Most training programs incorporate tuition in the cytopathological diagnosis of mycobacterial infection by pathologists, as the advent of HIV has changed the cytomorphological appearance of these infections.

In addition to making a rapid and definitive tissue diagnosis, widespread use of FNAB would provide ready access to material ideally suited to develop alternative methods for rapid detection of mycobacteria, species determination and drug resistance testing. In order to reduce the massive TB disease burden suffered by children in endemic areas we have to utilize creative and effective means to ensure rapid and accurate diagnosis, such as FNAB, that would facilitate early and appropriate treatment.

REFERENCES

- [1] World Health Organization. Global Tuberculosis Control: Surveillance, Planning, Financing: WHO Report 2007. Geneva: World Health Organization; 2007. Report No.: WHO/HTM/TB/2007.376.
- [2] Shingadia D, Novelli V. Diagnosis and treatment of tuberculosis in children. *Lancet Infect Dis* 2003;3:624-632.
- [3] Chintu C, Mudenda V, Lucas SB. Lung diseases at necropsy in African children dying from respiratory illnesses; a descriptive necropsy study. *Lancet* 2002;360:985-990.
- [4] McNally LM, Jeena PM, Gajee K, et al. Effect of age, polymicrobial disease, and maternal HIV status on treatment response and cause of severe pneumonia in South African children: a prospective descriptive study. *The Lancet* 2007;369:1440-1451.
- [5] Marais BJ, Gie RP, Schaaf HS, et al. Childhood pulmonary tuberculosis: old wisdom and new challenges. *Am J Respir Crit Care Med* 2006;173:1078.
- [6] World Health Organization. A research agenda for childhood tuberculosis. Geneva: World Health Organization; 2007. Report No.: WHO/HTM/TB/2007.381.
- [7] Health Systems Trust. 2007 [cited 6 March 2009]; Available from: <http://www.hst.org.za/healthstats/16/data>
- [8] Hesselning AC, Cotton MF, Jennings T, et al. High Incidence of Tuberculosis among HIV-Infected Infants: Evidence from a South African Population-Based Study Highlights the Need for Improved Tuberculosis Control Strategies. *Clin Infect Dis* 2009;48:108-114.
- [9] Hesselning AC, Schaaf HS, Hanekom W, et al. Danish Bacille Calmette-Guerin Vaccine-Induced Disease in Human Immunodeficiency Virus-Infected Children. *Clin Infect Dis* 2003;37:1226-1233.
- [10] Casanova JL, Blanche S, Emile JF, et al. Idiopathic disseminated bacillus Calmette-Guerin infection: a French national retrospective study. *Pediatrics* 1996;98:774-778.

- [11] Hesselning AC, Rabie H, Marais BJ, et al. Bacille Calmette-Guérin Vaccine-Induced Disease in HIV-Infected and HIV-Uninfected Children. *Clin Infect Dis* 2006;42:548–558.
- [12] Hesselning AC, Marais BJ, Gie RP, et al. The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. *Vaccine* 2007;25:14-18.
- [13] Mak TK, Hesselning AC, Hussey GD, Cotton MF. Making BCG vaccination programmes safer in the HIV era. *Lancet* 2008;372:786-787.
- [14] Safety. GACoV. Revised BCG vaccination guidelines for infants at risk for HIV infection; 2007 accessed 08 03 2009.
- [15] Piersimoni C, Scarparo C. Pulmonary infections associated with non-tuberculous mycobacteria in immunocompetent patients. *Lancet Inf Dis* 2008;8:323-334.
- [16] Hazra R, Robson CD, Perez-Atayde AR, Husson RN. Lymphadenitis Due to Nontuberculous Mycobacteria in Children: Presentation and Response to Therapy. *Clin Infect Dis* 1999;28:123-129.
- [17] Flint D, Mahadevan M, Barber C, Grayson D, Small R. Cervical lymphadenitis due to non-tuberculous mycobacteria: surgical treatment and review. *Int J Pediatr Otorhinolaryngol* 2000;53:187-194.
- [18] Zar HJ. Chronic lung disease in human immunodeficiency virus (HIV) infected children. *Pediatr Pulmonol* 2008;43:1-10.
- [19] Puthanakit T, Oberdorfer P, Ukarapol N, et al. Immune reconstitution syndrome from nontuberculous mycobacterial infection after initiation of antiretroviral therapy in children with HIV infection. *Pediatr Infect Dis J* 2006;25:645.
- [20] Chaisson RE, Martinson NA. Tuberculosis in Africa - combating an HIV-driven crisis. *N Engl J Med* 2008;358:1089-1092.
- [21] Braun MM, Cauthen G. Relationship of the human immunodeficiency virus epidemic to paediatric tuberculosis and Bacillus Calmette-Guerin vaccination. *Pediatr Infect Dis J* 1992;11:220-227.

- [22] Madhi SA, Huebner RE, Doedens L, et al. HIV-1 coinfection in children hospitalised with tuberculosis in South Africa. *Int J Tuberc Lung Dis* 2000;4:448-454.
- [23] UNAIDS WHO. AIDS epidemic update: . December 2007 [cited 10 March 2009]; 1-60]. Available from: http://data.unaids.org/pub/EPISlides/2007/2007_epiupdate_en.pdf
- [24] Mukadi YD, Wiktor SZ, Coulibaly IM, al e. Impact of HIV infection on the development, clinical presentation and outcome of tuberculosis among children in Cote d'Ivoire. *Aids* 1997;11:1151-1158.
- [25] Kiwanuka J, Graham SM, Coulter JBS, et al. Diagnosis of pulmonary tuberculosis in children in an HIV-endemic area, Malawi. *Ann Trop Paeds* 2001;21:5-14.
- [26] Jeena PM, Pillay P, Pillay T, Coovadia HM. Impact of HIV-one coinfection on presentation and hospital-related mortality in children with culture proven pulmonary tuberculosis in Durban, South Africa. *Int J Tuberc Lung Dis* 2002;6:672-678.
- [27] Marais BJ, Pai M. New approaches and emerging technologies in the diagnosis of childhood tuberculosis. *Paediatr Resp Rev* 2007;8:124-133.
- [28] Marais BJ, Graham SM, Cotton MF, Beyers N. Diagnostic and management challenges for childhood tuberculosis in the era of HIV. *J Infect Dis* 2007;196:76-85.
- [29] Walters E, Cotton MF, Rabie H, et al. Clinical presentation and outcome of Tuberculosis in Human Immunodeficiency Virus infected children on anti-retroviral therapy. *BMC Pediatrics* 2008;8:1.
- [30] Zar HJ, Hanslo D, Appoles P. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. *Lancet* 2005;365:130-134.
- [31] Gie RP, Beyers N, Schaaf HS, Goussard P. The challenge of diagnosing tuberculosis in children: a perspective from a high incidence area. *Paediatr Respir Rev* 2004;5:S147-S149.
- [32] Marais BJ, Pai M. Recent advances in the diagnosis of childhood tuberculosis. *Arch Dis Child* 2007;92:446-452.

- [33] Theart AC, Marais BJ, Gie RP, Hesselning AC, Beyers N. Criteria used for the diagnosis of childhood tuberculosis at primary health care level in a high-burden, urban setting. *Int J Tuberc Lung Dis* 2005;9:1210-1214.
- [34] Marais BJ, Gie RP, Schaaf HS, et al. The spectrum of childhood tuberculosis in a highly endemic area. *Int J Tuberc Lung Dis* 2006;10:732-738.
- [35] Hatherill M, Hawkrigde T, Zar H, et al. Induced sputum or gastric lavage for community-based diagnosis of childhood pulmonary tuberculosis? *Arch Dis Child* 2009;94:195-201.
- [36] Coulter JBS. Diagnosis of pulmonary tuberculosis in young children. *Ann Trop Paediatr* 2008;28:3-12.
- [37] Pai M, Flores LL, Pai N. Diagnostic accuracy of nucleic acid amplification tests for tuberculous meningitis: a systematic review and meta-analysis. *Lancet Infect Dis* 2003;3:633-643.
- [38] Pai M, Riley LW, Colford JMJ. Interferon-gamma assays in the immune diagnosis of tuberculosis: a systematic review. *Lancet Infect Dis* 2004;4:761-776.
- [39] Das DK. Fine Needle Aspiration Cytology: its Origin, Development, and Present Status with Special Reference to a Developing Country, India. *Diagn Cytopathol* 2003;28:345-351.
- [40] Martin HE, Ellis, E.B. Biopsy by fine needle puncture and aspirations. *Ann Surg* 1930;92:169-181.
- [41] Zajicek J. Aspiration biopsy cytology. Cytology of supradiaphragmatic organs. Basel: S Karger 1974.
- [42] Nayak S, Mani R, Kavatkar AN, Puranik SC, Holla VV. Fine-needle aspiration cytology in lymphadenopathy of HIV-positive patients. *Diagn Cytopathol* 2003;29:146-148.

- [43] Kocjan G, Miller R. The cytology of HIV-induced immunosuppression. Changing pattern of disease in the era of highly active antiretroviral therapy. *Cytopathology* 2001;12:281-296.
- [44] Gupta AK, Nayar M, Chandra M. Critical appraisal of fine needle aspiration cytology in tuberculous lymphadenopathy. *Acta Cytol* 1992;36:391-394.
- [45] Cruz AT, Starke JR. Clinical manifestations of tuberculosis in children. *Paediatric Respiratory Reviews* 2007;8:107-117.
- [46] Rameshkumar K. Tuberculous lymphadenitis in children--role of fine needle aspiration cytology. *J Assoc Physicians India*; 1999:976-979.
- [47] Marais BJ, Gie RP, Schaaf HS, et al. The clinical epidemiology of childhood pulmonary tuberculosis: a critical reading of literature from the pre-chemotherapy era. *Int J Tuberc Lung Dis* 2004;8:278-285.
- [48] Marais BJ, Wright CA, Schaaf HS, et al. Tuberculous Lymphadenitis as a Cause of Persistent Cervical Lymphadenopathy in Children From a Tuberculosis-Endemic Area. *Pediatr Infect Dis J* 2006;25:142-146.
- [49] Marais BJ, Gie RP, Schaaf HS, et al. The clinical epidemiology of childhood pulmonary tuberculosis: a critical reading of literature from the pre-chemotherapy era. *Int J Tuberc Lung Dis* 2004;8:278-285.
- [50] Moore SW, Schneider JW, Schaaf HS. Diagnostic aspects of cervical lymphadenopathy in children in the developing world: a study of 1,877 surgical specimens. *Pediatr Surg Int* 2003;19:240-244.
- [51] Marais BJ, Obihara CC, Warren RM, et al. The burden of childhood tuberculosis: a public health perspective [Review Article]. *Int J Tuberc Lung Dis* 2005;9:1305-1313.
- [52] Wright CA, Hesselning A, Bamford C, et al. Fine-needle aspiration biopsy: a first-line diagnostic procedure in paediatric tuberculosis suspects with peripheral lymphadenopathy. *Int J Tuberc Lung Dis* 2009;in press.

- [53] Graham SM, Coulter JB, Gilks CF. Pulmonary disease in HIV-infected African children. *Int J Tuberc Lung Dis* 2001;5:12-23.
- [54] Centres for Disease Control and Prevention. Revised classification system for human immunodeficiency virus infection in children less than 13 years of age; 1994. Report No.: MMWR 1994; 43: RR-12.
- [55] Cotton MF, Schaaf HS, Hesselning AC, Madhi SA. HIV and childhood tuberculosis: the way forward. *Int J Tuberc Lung Dis* 2004;8:675-682.
- [56] Jeena PM, Coovadia HM, Hadley LG, et al. Lymph node biopsies in HIV infected and non infected children with persistent lung disease. *Int J Tuberc Lung Dis* 2000;4:139 -146.
- [57] Zampoli M, Kilborn T, Eley B. Tuberculosis during early antiretroviral-induced immune reconstitution in HIV-infected children. *Int J Tuberc Lung Dis* 2007;11:417-423.
- [58] Wakely PE, Kardos TF, Frable WJ. Application of fine needle aspiration biopsy to pediatrics. *Hum Pathol* 1988;19:1383-1386.
- [59] Drut R, Drut RM, Pollono D, et al. Fine-Needle Aspiration Biopsy in Paediatric Oncology Patients. A Review of Experience with 829 Patients. *Paediatr Haematol Oncol* 2005;27:370-376.
- [60] Thomas JO, Adeyi D, Amanguno H. Fine Needle Aspiration in the Management of Peripheral Lymphadenopathy in a Developing Country. *Diagn Cytopathol* 1999;21:159-162.
- [61] Wright CA, Pienaar JP, Marais BJ. Fine needle aspiration biopsy: diagnostic utility in resource-limited settings. *Ann Trop Paediatr* 2008; 28:65-70.
- [62] Wright CA, van der Burg M, Geiger D, et al. Diagnosing Mycobacterial lymphadenitis in children using Fine Needle Aspiration Biopsy: Cytomorphology, ZN staining and Autofluorescence – making more of less. *Diagn Cytopathol* 2008;36:245-251.
- [63] Bezabih M, Marium DW, Selassie SG. Fine needle aspiration cytology of suspected tuberculous lymphadenitis. *Cytopathology* 2002;13:284-290.

- [64] Lau SK, Wei WI, Hsu C, Engzell UCG. Efficacy of fine needle aspiration cytology in the diagnosis of tuberculous cervical lymphadenopathy. *J Laryngol Otol* 1990;104:24-27.
- [65] DeMay RM. *The Art and Science of Cytopathology*. Chicago: ASCP Press 1996.
- [66] Wilson D, Nachega JB, Chaisson RE, Maartens G. Diagnostic yield of peripheral lymph node needle-core biopsies in HIV-infected adults with suspected smear-negative tuberculosis. *Int J Tuberc Lung Dis* 2005;9:220-222.
- [67] Bem C, Patil PS, Elliott AM, et al. The value of wide-needle aspiration in the diagnosis of tuberculous lymphadenitis in Africa. *AIDS* 1993;7:1221-1226.
- [68] Bezabih M, Mariam DW, Selassie SG. Fine needle aspiration cytology of suspected tuberculous lymphadenitis. *Cytopathology* 2002;13:284-290.
- [69] Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Auto fluorescence of Mycobacteria on Lymph Node Aspirates – A glimmer in the dark? *Diagn Cytopathol* 2004;30:257-260.
- [70] Marais BJ, Brittle W, Painczyk K, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. *CID* 2008;47:203-207.
- [71] Wright CA, Bamford C, Prince Y, et al. Mycobacterial Transport medium for routine culture of Fine Needle Aspiration Biopsies. *Arch Dis Child* 2009;In press.
- [72] Parsons LM, Brosch R, Cole ST, et al. Rapid and simple approach for identification of Mycobacterium tuberculosis complex isolates by PCR-based genomic deletion analysis. *J Clin Microbiol* 2002;40:2339-2345.
- [73] Singh KK, Muralidhar M, Kumar A, et al. Comparison of in house polymerase chain reaction with conventional techniques for the detection of Mycobacterium tuberculosis DNA in granulomatous lymphadenopathy. *J Clin Pathol* 2000;53:355-361.
- [74] van Coppenraet ESB, Lindeboom JA, Prins JM, et al. Real-Time PCR Assay Using Fine-Needle Aspirates and Tissue Biopsy Specimens for Rapid Diagnosis of Mycobacterial Lymphadenitis in Children. *J Clin Microbiol* 2004;42:2644-2650.

[75] Portillo-Gomez L, Murillo-Neri MV, Gaitan-Mesa J, Sosa-Iglesias EG. Nested polymerase chain reaction in the diagnosis of cervical tuberculous lymphadenitis in Mexican children. *Int J Tuberc Lung Dis* 2008;12:1313-1319.

Table 1**Summary of the Fine Needle Aspiration Biopsy Technique****Equipment**

22 or 23G cutting needles
10ml disposable plastic syringes
Glass cytology slides (ground edges)
Commercial spray fixative or 95% alcohol
Non Sterile gloves
Alcohol swabs

Procedure

Clean skin, immobilize the mass, position needle to access entire mass and avoid passing through muscles such as sternocleidomastoid
Insert needle firmly and apply no more than 1ml suction, applying constant suction throughout aspirate
Aspirate moving the needle in a fan like fashion through the mass.
When material is in the hub of the needle, release suction and withdraw needle.
Ask parent or assistant to apply pressure to puncture wound

Preparation of smears

Remove needle from syringe, pull 10 ml of air into syringe and reattach needle.
This air is used to express the material in needle onto a glass slide. Place second glass slide face down on first, allow material to spread gently, and keeping both slides together, pull gently apart.
Fix one slide with alcohol and allow the second to air dry

Culture

If liquid culture or transport medium is available, withdraw an aliquot of liquid media into the syringe and then expel it back into the tube or bottle, thereby using the culture media to rinse the syringe and needle in a sterile fashion.

Table 2

Comparison of different superficial mass lesion sample collection methods to achieve a definitive tissue diagnosis

	Open surgical biopsy	Core needle biopsy	Standard Needle Aspiration (greater than 22G)	Fine Needle Aspiration Biopsy (22G or smaller)
Suitable for small lesions (1x1cm)	Yes	No	Yes	Yes
Entire mass sampled	Yes, if excision	No	No	Yes
Complications	Risk of anaesthesia, hospitalization, sinus formation, infection	Sinus formation, infection	Rare	Extremely rare
Cost	Hospitalization, theatre time	High cost of needle	Minimal	Minimal
Hospitalisation	Yes	No	No	No
Anaesthetic required	General	Local	Local	None
Time for entire procedure	1-2 days	30 minutes	30 minutes	10 minutes
Tissue diagnosis	Yes	Yes	Yes	Yes
Microscopy for organism	Yes	Yes	Yes	Yes
Culture	Yes	Yes	Yes	Yes
Time for initial result	1-2 days	1-2 days	12-24 hours (Possible in <1 hr)	12-24 hours (Possible in <1hr)

Table 3**Comparison of methods to confirm a mycobacterial diagnosis in children**

Method	Hospitalization required	Suitable ages	Local/general anaesthetic	Equipment / infrastructure
FNAB	No	All	No	None
Gastric aspirates	Yes	All	No	None
Induced sputum	Yes	All	No	Nebulizer, suction, saturation monitor
Sputum	No	>7-8yrs only	No	None
Surgical biopsy	Yes	All	Yes	Full theatre

CHAPTER 3

TB LYMPHADENITIS: A COMMON CLINICAL PROBLEM

Tuberculous Lymphadenitis as a Cause of Persistent Cervical Lymphadenopathy in Children From a Tuberculosis-Endemic Area

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Tuberculous Lymphadenitis as a Cause of Persistent Cervical Lymphadenopathy in Children From a Tuberculosis-Endemic Area

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Background: Cervical lymphadenitis is the most common form of extrapulmonary tuberculosis in children, although its relative contribution as a cause of persistent cervical adenopathy is not well-documented. The aim of this study was to determine the relative contribution of tuberculous lymphadenitis as a cause of persistent cervical adenopathy in a tuberculosis-endemic setting and to document its clinical presentation at the primary health care level.

Methods: A prospective descriptive study was conducted from February 2003 through October 2004 at 5 primary health care clinics in Cape Town, South Africa. The study included all children younger than 13 years presenting with persistent cervical adenopathy to the local primary health care clinic.

Results: A total of 158 children were evaluated of whom 35 (22.2%) were diagnosed with tuberculous lymphadenitis. Bacteriologic confirmation was achieved in 27 of 35 (77.1%) children; all 35 responded to standard antituberculosis treatment. The majority of those without tuberculous lymphadenitis (105 of 123, 85.4%) had a visible superficial lesion in the area drained by the affected nodes. In children with persistent lymphadenopathy $\geq 2 \times 2$ cm, tuberculosis lymphadenitis was diagnosed in 31 of 33 (93.9%); specificity was 98.4%, sensitivity was 88.6% and the positive predictive value was 93.4%.

Conclusion: Children commonly present with persistent cervical adenopathy to the primary health care clinic. The use of a simple clinical algorithm provided an accurate diagnosis of tuberculous lymphadenitis in the study setting. Fine needle aspirations provided a rapid and definitive diagnosis in the majority of children and will have added diagnostic value in settings where alternative diagnoses are more likely.

Key Words: tuberculosis, lymphadenitis, children, persistent cervical adenopathy

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Peripheral tuberculous lymphadenitis predominantly involves the cervical lymph nodes^{1–5} and is the most common form of extrapulmonary tuberculosis in children from tuberculosis-endemic areas.^{1,2} However, its relative contribution as a cause of persistent cervical adenopathy in these communities is not well-documented.

Cervical lymphadenitis, caused by *Mycobacterium tuberculosis*, is generally considered to have its origin in the lymphatic spread of organisms from a primary pulmonary focus, but in a minority of cases it can originate from a primary focus in the mouth, tonsils, oropharynx or tissues of the head and neck.⁶ Other mycobacteria can also cause cervical lymphadenitis; the relative contribution of different mycobacteria is influenced by the control of bovine tuberculosis,⁷ the use of BCG vaccination,⁸ the presence of environmental mycobacteria and the prevalence of tuberculosis within a particular setting.⁷

The diagnosis of tuberculosis in children is often difficult, given that symptoms and signs might be nonspecific, the collection of bacteriologic specimens problematic and bacteriologic yields low.^{9,10} In children with peripheral tuberculous lymphadenitis, however, clinical signs are usually apparent, and fine needle aspiration (FNA) provides excellent bacteriologic yields.^{11–13} Although the diagnostic value of FNA has been demonstrated in resource-limited settings,^{11–13} to date it remains underutilized as a routine diagnostic modality in most endemic areas.

The aim of this study was to determine the relative contribution of tuberculous lymphadenitis as a cause of persistent cervical adenopathy in children from a tuberculosis-endemic area and to document its clinical presentation at the primary health care level.

METHODS

A prospective descriptive study was performed from February 2003 through October 2004 in Cape Town, the Western Cape Province, South Africa.

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Setting. The study was conducted at 5 primary health care clinics served by one referral hospital. The incidence of all tuberculosis in Cape Town was 678/100,000,¹⁴ and the prevalence of human immunodeficiency virus (HIV) infection among women attending public antenatal clinics in the Western Cape Province was 13.1% (95% confidence interval, 8.5–17.7%), in 2003.¹⁵ Bovine tuberculosis is well-controlled within the study communities, and children receive routine neonatal Calmette-Guérin bacillus (BCG) vaccination. The study communities rarely use private medical services, and children diagnosed with tuberculosis are routinely referred to the local primary health care clinic, where supervised antituberculosis treatment is provided free of charge. Pediatric services are extended only to children younger than 13 years of age.

Study Population. All children (younger than 13 years of age) who presented with persistent cervical adenopathy; defined as lymph nodes $\geq 1 \times 1$ cm, persisting for >4 weeks despite a course of oral antibiotics (usually amoxicillin), were referred to the investigator for evaluation.

Data Collection and Surveillance. The principal investigator visited each clinic on a weekly basis to screen referred children, whereas a study nurse recorded children referred directly to hospital. The areas surrounding the affected cervical lymph nodes were inspected to exclude a visible superficial lesion within their drainage area (visible local cause), such as impetigo of the scalp, tinea capitis or traction folliculitis. Those with a visible local cause were given appropriate therapy and instructed to return if the lymph nodes persisted or increased in size. Surveillance was continued at all 5 clinics and the referral hospital throughout the study period, and for an additional 3 months after enrollment was stopped, to document any child who subsequently returned with symptom deterioration or a possible diagnosis of tuberculosis.

Children with no visible local cause of cervical lymphadenopathy received a Mantoux (2 tuberculin units of purified protein derivative RT23 intradermal) tuberculin skin test (TST) and a chest radiograph (anteroposterior and lateral). The Mantoux TST was interpreted as positive if induration was ≥ 10 mm. Two independent experts evaluated the chest radiographs for intrathoracic signs of tuberculosis. Symptoms and signs as well as possible tuberculosis contact were documented. Children diagnosed with tuberculous lymphadenitis were offered a rapid HIV test (Determine HIV 1/2; Abbott) after appropriate counseling. Standard 3-drug antituberculosis treatment was initiated,¹⁶ and response to antituberculosis therapy was monitored after a period of 3 months. In the group not diagnosed with tuberculous lymphadenitis, symptom resolution in the absence of antituberculosis therapy was monitored.

Case Definition. Tuberculous lymphadenitis was defined as cervical lymphadenopathy $\geq 1 \times 1$ cm persisting for >4 weeks despite a course of oral antibiotics, in the presence of at least 1 of the following criteria: (1) bacteriologic confirmation: isolation of *M. tuberculosis* from a lymph node, or microscopically visible acid-fast or autofluorescent bacilli associated with amorphous necrosis on cytology; or (2) clinical diagnosis: significant therapeutic response (lymph node size decreased from $\geq 2 \times 2$ cm to $<1 \times 1$ cm after 3 months of standard antituberculosis treatment). All cultures positive

for *M. tuberculosis complex* were routinely speciated by polymerase chain reaction to differentiate *M. tuberculosis* from *Mycobacterium bovis* and *M. bovis* BCG.¹⁷

FNA and/or Biopsy. Of the children enrolled, only those with a positive TST (≥ 10 mm) and/or a suggestive chest radiograph and/or a cervical mass $\geq 2 \times 2$ cm were referred to the hospital for FNA. FNA was performed in the pediatric outpatient department with sedation and pain relief (chloral hydrate 20–50 mg/kg and paracetamol 10–20 mg/kg, administered orally 30–60 minutes before the procedure). The largest discrete node was identified by careful palpation of the cervical mass. Two needle passes were performed with a 23-gauge needle attached to a 10-mL syringe, applying 2 mL of suction. Aspirated material was placed on standard microscope slides, thinly smeared and air-dried or fixed with commercial spray fixative for Giemsa and Papanicolaou stains, before flushing the syringe and needle directly into a MGIT (Becton Dickinson, Cockeysville, MD) mycobacterial culture bottle. Microscopic evaluation included cytology, acid-fast staining and autofluorescence.¹⁸ Excision biopsies were occasionally performed if children presented with persistent cervical adenopathy to the referral hospital without a letter from the investigator or if the FNA was inconclusive.

Statistical Analysis. Descriptive analysis was conducted with SPSS (version 11.5). The sensitivity and specificity of a simple clinical algorithm were calculated with the use of either bacteriologic confirmation and/or the clinical case definition (see case definition) as the “reference” test.

Parents gave written informed consent for study participation and ethics approval was obtained from the Institutional Review Board of Stellenbosch University, the City of Cape Town Health Department and local health committees.

RESULTS

Persistent Cervical Adenopathy. Of the 167 children who were identified with persistent cervical adenopathy, 9 (5.4%) did not return to the clinic for evaluation by the investigator (Fig. 1). Of the 158 children evaluated, 53 had no visible cause of whom 40, with either a positive TST or a cervical mass $\geq 2 \times 2$ cm, were referred to hospital to establish a histologic diagnosis. None of the 13 TST-negative children had radiographic or other clinical signs indicative of possible tuberculosis, and all children judged not to have tuberculosis on clinical grounds showed symptom resolution in the absence of antituberculosis treatment.

Table 1 summarizes the demographics and etiology of persistent cervical adenopathy in the 158 children who were evaluated. The majority of children (105, 66.5%) had lymph nodes $<2 \times 2$ cm with a visible local cause; 28 returned for reevaluation within 1 month; the lymphadenopathy resolved in 18 and decreased to $<1 \times 1$ cm in 9. Multiple discrete lymph nodes, 1–2 cm in diameter, remained in 1 child who was TST-negative and asymptomatic. The lymphadenopathy showed slow resolution, decreasing to $<1 \times 1$ cm within 3 months. None of the children with a visible local cause returned to the clinic with symptom deterioration or with a possible tuberculosis diagnosis from any hospital, during the study period.

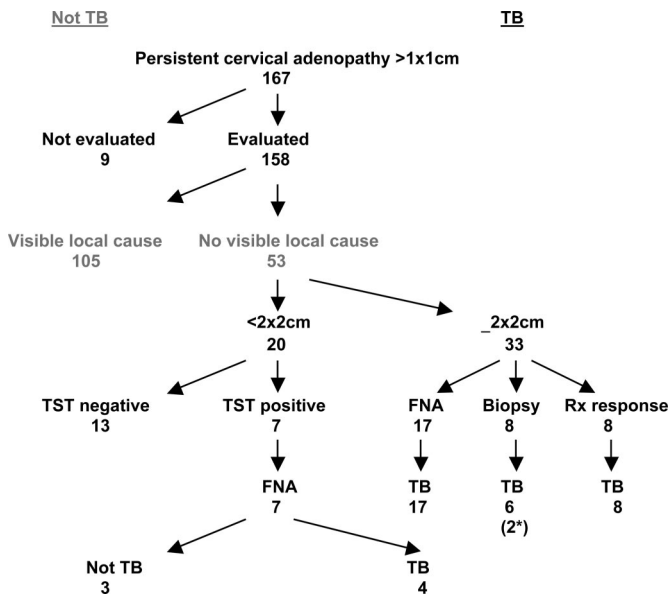


FIGURE 1. Flow diagram of children referred with persistent cervical adenopathy. Not TB indicates symptom resolution in the absence of antituberculosis chemotherapy. TB, bacteriologic confirmation: isolation of *M. tuberculosis* from a lymph node, or microscopically visible acid-fast or autofluorescent bacilli associated with caseating necrosis on cytology or clinical diagnosis; significant therapeutic response (lymph node size decreased from $\geq 2 \times 2$ cm to $< 1 \times 1$ cm after 3 months of standard antituberculosis treatment); Not evaluated, did not return to the clinic for evaluation by the investigator; Rx response, clinical diagnosis together with significant therapeutic response in the absence of FNA or biopsy-based diagnosis; *classified as not TB: 1 chronic inflammatory process diagnosed after excision, 1 nonacute bacterial abscess diagnosed after incision and drainage.

TABLE 1. Demographics and Etiology of Persistent Cervical Adenopathy in Children (n = 158)

	No. of Instances
Demographics	
Gender	
Male	69 (43.7)*
Female	89 (56.3)
Age groups	
<5 yr	93 (58.9)
5–9 yr	51 (32.2)
≥10 yr	14 (8.9)
Etiology	
Visible local cause 105 (66.5)	
Bacterial infection (crusted impetigo)	26 (16.5)
Tinea capitis (with secondary infection)	34 (21.5)
Traction folliculitis	44 (27.8)
Otitis externa	1 (0.6)
No visible local cause 53 (33.5)	
Tuberculous lymphadenitis	35 (22.2)
Reactive nodes [†]	13 (8.2)
Nonspecific inflammation	4 (2.5)
Nonacute bacterial abscess	1 (0.6)
Malignancy	0

*Numbers in parentheses, percent.

[†]Cervical mass $< 2 \times 2$ cm, tuberculin skin test-negative and natural symptom resolution.

Of the 53 (33.5%) children without a visible local cause, tuberculous lymphadenitis was diagnosed in 35 (66.0%). Of those with a cervical mass $\geq 2 \times 2$ cm, tuberculous lymphadenitis was diagnosed in 31 of 33 (93.9%). Of the 2 remaining children, 1 had an unidentified chronic inflammatory process diagnosed through excision biopsy, and the other developed a chronic bacterial abscess after repeat courses of oral antibiotics, requiring eventual incision and drainage. Two children presented with acute bacterial adenitis; both had a history of persistent lymph node enlargement that preceded the acute event. Both these children were TST-positive and were diagnosed with secondary bacterial infection, as *M. tuberculosis* was cultured after incision and drainage. One had signs suggestive of tuberculosis on the chest radiograph and reported a prolonged cough and night sweats, whereas the other had no additional suspect symptoms or signs apart from the cervical mass and a positive TST.

Tuberculous Lymphadenitis. Of the 35 children diagnosed with tuberculous lymphadenitis (Fig. 1), bacteriologic confirmation was achieved in 27 (77.1%) children. Of the 8 children without bacteriologic confirmation, 7 failed to attend the referral hospital, and 1 refused permission for FNA. All 8 had a TST response ≥ 15 mm and showed excellent response to standard antituberculosis treatment. In the 27 children with bacteriologic confirmation; FNA was performed in 21 children and formal biopsies in 6. No cases caused by *M. bovis*, *M. bovis* BCG, infection with environmental mycobacteria such as *Mycobacterium avium-intracellulare* complex or *Mycobacterium scrofulaceum* or malignancies were identified.

Of the 21 children with tuberculous lymphadenitis in whom FNA was performed, 16 of 21 (76.2%) were acid-fast or autofluorescent smear-positive, which allowed rapid and definitive diagnosis. Culture confirmation was achieved in 19 of 21 (90.5%), of whom 2 had no microscopic features indicative of tuberculosis. One of the 2 children who were culture-negative had received antituberculosis treatment before FNA was performed. Either a positive culture or typical microscopic features were present in all 21 cases. One of the 2 children, who had no microscopic features indicative of tuberculosis on FNA, had an excision biopsy performed that established the diagnosis, before the FNA culture result became known. No immediate complications relating to the FNA procedure, apart from minimal bleeding, were noted. No long term complications, such as sinus formation, were recorded during the 3-month follow-up period.

Table 2 reflects the lymph node characteristics and associated findings in the 35 children diagnosed with tuberculous lymphadenitis. Using the clinical algorithm of a persistent cervical mass $\geq 2 \times 2$ cm, without a visible local cause or response to antibiotics, accurately identified children with tuberculous lymphadenitis; sensitivity was 88.6%, specificity was 98.4% and the positive predictive value of this clinical algorithm was 93.4%.

Tuberculous lymphadenitis occurred in children of all ages, except in infants. In 18 (51.4%), lymph nodes occurred in the anterior triangle, with involvement of multiple regions in 5 (14.3%). Other regions involved were: posterior triangle, 8 (22.9%); submandibular, 2 (5.7%); and supraclavicular, 2 (5.7%). Lymph nodes occurred more regularly on the right

TABLE 2. Clinical Characteristics of Children With Tuberculous Lymphadenitis (n = 35)

	No. of Instances
Lymph node characteristics	
Persistence (present for >4 wk, no response to antibiotics)	35 (100)*
Size [†]	
<2 × 2 cm	4 (11.4)
(2–4) × (2–4) cm	25 (71.5)
>4 × 4 cm	6 (17.1)
Character	
Single	5 (14.3)
Multiple	
Discreet	14 (40.0)
Matted	16 (45.7)
Solid	28 (80.0)
Fluctuant	
Without secondary bacterial infection	5 (14.3)
With secondary bacterial infection (red and warm)	2 (5.7)
Associated findings	
Tuberculin skin test	
0 mm	2 (5.7)
1–9 mm	0
≥10 mm	33 (94.3)
≥15 mm	32 (91.4)
Mean response 19.1 mm (standard deviation 2.9 mm)	
Constitutional symptoms	
Any symptom	21 (60.0)
Fever	7 (20.0)
Cough	9 (25.7)
Night sweats	8 (22.8)
Fatigue [‡]	19 (54.3)
Failure to thrive [§]	10 (28.6)
Chest radiograph	
Suggestive of tuberculosis	13 (37.1)
Lymph node disease	
Uncomplicated	8 (22.8)
With airway compression	1 (2.9)
With parenchymal consolidation	4 (11.4)

*Numbers in parentheses, percent.

[†]Transverse diameter of the largest cervical mass.

[‡]Less playful and active since the mass was first noted.

[§]Crossing at least 1 centile line in the preceding 3 months or having lost >10% of body weight (minimum, 1 kg) over any time interval.

side: right, 19 (54.3%); left, 13 (37.1%); bilateral, 3 (8.6%). Most lymph nodes were multiple, either discreet or matted (30, 85.7%), either discreet or matted, and solid (28, 80.0%). All lymph nodes were nontender, either discreet or matted, except 2 with secondary bacterial infection. A sinus developed in 2 children, one before presentation and the other after surgical incision and drainage.

Associated findings in children with tuberculous lymphadenitis included a positive TST in 33 of 35 (94.3%). Two (5.7%) children had a nonreactive TST; 1 was HIV-infected, the other was severely ill with coexistent pulmonary tuberculosis. The TST in the latter patient measured 16 mm when repeated after 3 months of antituberculosis treatment. Twenty-nine (82.9%) children were tested for HIV, and 1 (3.5%) was HIV-infected. Contact with an adult index case was reported in 19 (54.3%), whereas in a further 2 cases, adults with suspicious symptoms were subsequently diagnosed with tuberculosis. Constitutional symptoms were present in 21 (60.0%) children, and the chest radiograph was suggestive of primary pulmonary tuberculosis in 13 (37.1%).

The response to standard antituberculosis therapy was good, and most children showed considerable improvement, with reduction in lymph node size to <1 × 1 cm after 3 months of standard antituberculosis treatment. Three (8.6%) children deteriorated initially; 1 developed upper airway obstruction that required surgical lymph node enucleation and the addition of corticosteroids. All 3 children showed good response after completing 6 months of standard antituberculosis therapy, although a cervical mass of >1 × 1 cm remained in 1 child.

DISCUSSION

Children commonly presented with persistent cervical adenopathy to primary health care clinics in the study setting. The majority of these children had visible local lesions such as crusted impetigo, infected tinea capitis or traction folliculitis. The hair of most small girls in this community is tightly braided, which often leads to irritation and bacterial infection of the hair follicles. This also explains the overrepresentation of girls in the study population. It is understandable that none of these conditions responded to a short course of oral antibiotics, in the absence of etiology-specific systemic and/or local treatment.

A simple clinical algorithm that identified children with persistent (>4 weeks) cervical lymphadenopathy, no visible local cause or response to antibiotics and a cervical mass ≥2 × 2 cm showed excellent diagnostic accuracy within the study setting. The addition of a positive TST may be of value in settings where infection with *M. tuberculosis* is less common or where conditions other than tuberculosis, such as malignant lymph node involvement or other chronic infections, are more common and may present with a similar clinical picture. The fact that accurate clinical diagnosis is possible at the primary health care level might allow the initiation of antituberculosis treatment without hospital referral, which should improve access to care for children in extremely resource-limited settings. However, regular follow-up (at least monthly) is essential so that children who do not respond to standard antituberculosis treatment are referred as soon as possible to establish a definitive diagnosis. The value of this clinical approach requires further evaluation.

FNA proved to be a robust and simple technique, which provided an excellent bacteriologic yield. No significant side effects were noted. FNA provided a definitive bacteriologic diagnosis with the ability to speciate *M. tuberculosis* complex and to perform drug susceptibility testing. A definitive diagnosis is always desirable, but the diagnostic value of FNA will be even greater in settings where a clinical diagnosis is expected to be less accurate, such as areas where malignant lymph node involvement and/or other chronic infections are more common.^{11,12}

The lymph node characteristics and constitutional symptoms recorded in this study correlate well with findings from Papua New Guinea and India.^{3,4,19} The most distinctive features were the chronic persistent course, the lymph node size (≥2 × 2 cm) and the involvement of multiple, discrete or matted, lymph nodes. The study also emphasizes the value of

clinical follow-up and serial measurement of enlarged lymph nodes if the diagnosis is not apparent.²⁰

Only 1 child with tuberculous lymphadenitis was HIV-infected. This reflects the low HIV prevalence among children in the study setting but might also illustrate a decreased tendency in HIV-infected children to develop peripheral tuberculous lymphadenitis, as suggested in a comparative study from Zambia.²¹ FNA may have increased diagnostic value in HIV-infected children, because the relative contribution of tuberculous lymphadenitis to persistent cervical adenopathy in this group is expected to be smaller.

It is interesting that no environmental mycobacteria were isolated, although it is reported to be the most common cause of persistent cervical adenopathy in the developed world.²² Paucity of disease caused by environmental mycobacteria has also been described in other tuberculosis-endemic countries, such as India.^{23,24} This may result from the protective effect afforded by routine neonatal BCG vaccination,⁸ whereas natural infection with *M. tuberculosis* might provide additional protection against disease caused by environmental mycobacteria.

An important study limitation is that bacteriologic confirmation was achieved in only 77.1% of the study patients, as it was not attempted in 8 children. However, we are confident that the diagnosis was accurate, given the fact that these 8 children all had a TST response ≥ 15 mm and showed excellent clinical response to standard antituberculosis treatment.

In conclusion, the use of a simple clinical algorithm identified tuberculous lymphadenitis with a high degree of accuracy in the study setting, whereas FNA provided a rapid and definitive diagnosis in the majority of children.

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REFERENCES

- Coovadia HM. Tuberculosis in children. In: Coovadia HM, Benatar SR, eds. *A Century of Tuberculosis*. Cape Town, South Africa: Oxford University Press; 1991:91–100.
- Seth V. Antituberculous therapy in children. *Indian J Pediatr*. 1986;53:174–198.
- McMaster P, Ezeilo N, Freisen H, Pomat N, Vince JD. Ten-year experience with paediatric lymph node tuberculosis in Port Moresby. *J Trop Pediatr*. 2001;47:160–164.
- Seth V, Kabra SK, Jain Y, et al. Tubercular lymphadenitis: clinical manifestations. *Indian J Pediatr*. 1995;62:565–570.
- Reddy MP, Moorchung N, Chaudrey A. Clinico-pathological profile of paediatric lymphadenopathy. *Indian J Pediatr*. 2002;69:1047–1051.
- Miller FLW, Cashman JM. The natural history of peripheral tuberculous lymphadenitis associated with a visible primary focus. *Lancet*. 1955;1:1286–1289.
- Grzybowski S, Allen EA. History and importance of scrofula. *Lancet*. 1995;346:1472–1474.
- Romanus V. Tuberculosis in BCG immunized and unimmunized children in Sweden: a ten-year evaluation following the cessation of BCG immunization of the newborn in 1975. *Pediatr Infect Dis J*. 1987;6:272–280.
- Eamranond P, Jamarillo E. Tuberculosis in children: reassessing the need for improved diagnosis in global control strategies. *Int J Tuberc Lung Dis*. 2001;5:594–603.
- Starke JR. Pediatric tuberculosis: time for a new approach. *Tuberculosis*. 2003;83:208–212.
- Handa U, Palta A, Mohan H, Punia RP. Fine needle aspiration diagnosis of tuberculous lymphadenitis. *Trop Doct*. 2002;32:147–149.
- Lau S, Ignace W, Kwan S, Yew W. Combined use of fine needle aspiration cytologic examination and tuberculin skin test in the diagnosis of cervical tuberculous lymphadenitis. *Arch Otolaryngol Head Neck Surg*. 1991;117:87–90.
- Thomas J, Adeyi AO, Olu-eddo AO, Nwachokor N. Fine needle aspiration cytology in the management of childhood palpable masses: Ibadan experience. *J Trop Pediatr*. 1999;45:378.
- Cape Town TB Control. Progress Report 1997–2003. Available at www.hst.org.za/publications/618.
- Department of Health. National HIV and Syphilis antenatal sero-prevalence survey 2003. Available at www.doh.gov.za/docs/reports/2003/hiv/p1-23.
- Department of Health. *The South African Tuberculosis Control Programme: Practical Guidelines*. 2000:32–37.
- de Wit D, Steyn L, Shoemaker S, Sogin M. Direct determination of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. *Clin Microbiol*. 1990;28:2437–2441.
- Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Mycobacterial autofluorescence in Papanicolaou-stained lymph node aspirates: a glimmer in the dark? *Diagn Cytopathol*. 2004;30:257–260.
- Narang P, Narang R, Narang R, et al. Prevalence of tuberculous lymphadenitis in children in Wardha district, Maharashtra State, India. *Int J Tuberc Lung Dis*. 2005;9:188–194.
- Knight PJ, Mulne AF, Vassy LE. When is lymph node biopsy indicated in children with enlarged peripheral nodes? *Pediatrics*. 1982;69:391–396.
- Chintu C, Bhat G, Luo C, et al. Seroprevalence of human immunodeficiency virus type 1 infection in Zambian children with tuberculosis. *Pediatr Infect Dis J*. 1993;12:499–504.
- Lai KK, Stottmeier KD, Sherman IH, McCabe WR. Mycobacterial cervical lymphadenopathy. *JAMA*. 1984;251:1286–1288.
- Krishnaswami H, Koshi G, Kulkarni KG, Job CK. Tuberculous lymphadenitis in South India: a histopathological and bacteriological study. *Tubercle*. 1972;53:215–220.
- Aggarwal P, Wali JP, Singh S, et al. A clinico-bacteriological study of tuberculous lymphadenitis. *J Assoc Physicians India*. 2001;49:808–812.

CHAPTER 4

DIAGNOSTIC UTILITY AND FEASIBILITY OF FINE NEEDLE ASPIRATION BIOPSY

Fine needle aspiration biopsy: diagnostic utility in resource-limited settings

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**Fine Needle Aspiration Biopsy - A First Line Diagnostic Procedure in
Pediatric Tuberculosis suspects with Peripheral Lymphadenopathy?**

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Fine needle aspiration biopsy: diagnostic utility in resource-limited settings

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(Accepted November 2007)

Abstract

Background: Little information is available on the value of fine needle aspiration biopsy (FNAB) in routine paediatric practice in resource-limited settings.

Aim: To provide an overview of all paediatric FNAB samples received at Tygerberg Hospital, Cape Town, South Africa over a 3-year period, including the determinants of sample adequacy and the diagnoses.

Methods: Samples were analysed from three locations: Tygerberg Hospital (TBH) where pathologists performed all the procedures, surrounding clinics where aspirates were mostly performed by doctors with no formal training in FNAB technique, and Queen Elizabeth Hospital, Blantyre, Malawi where FNABs were performed by trained nurse aspirators.

Results: A total of 830 aspirates were reviewed: 464 (56%) from TBH, 264 (32%) from local clinics and 102 (12%) from Blantyre. The main diagnoses at TBH were mycobacterial infection (31%), normal/reactive tissue (27%) and malignancy (14%); malignancy dominated (74%) in the select group from Blantyre. Sample adequacy rates were similar between pathologists and nurse aspirators [399/464 vs 82/102, odds ratio (OR) 1.4, 95% confidence interval (CI) 0.8–2.6]. Results were significantly better in the group who received formal training (TBH and Malawi) than in the clinics where clinicians had no formal training (481/566 vs 171/264, OR 3.1, 95% CI 2.2–4.4).

Conclusions: FNAB provides a definitive tissue diagnosis in the majority of patients. Well-trained nurse aspirators perform as well as pathologists, indicating the feasibility of FNAB in resource-limited settings.

Introduction

Fine needle aspiration biopsy (FNAB) has been used as a diagnostic modality for more than a century. It was initially applied to infective and inflammatory lesions only, but the advent of cytological investigation (around 1925) expanded its diagnostic application to confirm or exclude malignancy also.¹ FNAB is well established as the diagnostic procedure of choice for

superficial mass lesions.^{2–4} However, few data are available on its routine use in paediatric practice, especially in resource-limited settings.

FNAB provides a simple, fast, cost-effective and safe method of obtaining a definitive tissue diagnosis.^{3–7} In experienced hands, a high level of diagnostic accuracy is possible, averting the need for a formal biopsy and all its associated risks.⁸ Apart from providing a cytological diagnosis, FNAB also provides tissue for ancillary tests, such as microbiological cultures, flow cytometry, cytogenetics, electron microscopy and polymerase chain reaction (PCR).^{2,5}

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If the procedure is performed according to established protocols, the disadvantages are minimal.⁹ Potential complications include pain, bleeding, infection, vasovagal reactions, haemothorax, pneumothorax, air embolism, seeding along the needle tract and perforation of organs.⁹ As a general rule, the rate of complications rises with use of larger calibre needles and deep organ aspiration. Contra-indications for FNAB are few but include an abnormal clotting profile (especially for deep organ aspirations) and suspected vascular lesions, and aspiration in the region of the thorax is best avoided in patients with cardiovascular and/or respiratory dysfunction.⁹ The risk associated with the aspiration of superficial masses using small gauge needles (22G or less) is minimal.^{2,9}

Previously at Tygerberg Hospital, clinicians without formal training in the correct technique performed routine FNAB, but since July 2001 trained pathologists perform all paediatric fine needle aspirates. The study aims were to provide an overview of all diagnoses made during the 1st 3 years of this new policy and to investigate possible determinants of sample adequacy.

Methods

Study population

This retrospective descriptive study reviewed all paediatric FNAB samples received by the cytology laboratory at Tygerberg Hospital (TBH) from 1 July 2001 to 30 June 2004. These included samples received from TBH and surrounding clinics and specimens couriered from Queen Elizabeth Hospital, Blantyre, Malawi. The cases referred from Malawi were all enrolled in a paediatric oncology study and represent a very select group. Children were recruited into this study and underwent FNAB if they presented with a mass lesion suspected of being Burkitt's lymphoma.¹⁰

Fine needle aspiration biopsy

FNAB were usually performed in the clinic, in the paediatric outpatient department or in the ward. Children received sedation and pain relief (usually chloral hydrate 20–50 mg/kg and paracetamol 10–20 mg/kg) orally 30–60 minutes before the procedure. Before performing the aspiration, the largest discrete mass was identified by careful palpation, (usually using a 23G-needle attached to a 10-ml syringe and applying 2 ml of suction). Aspirated material was placed on standard microscope slides, thinly smeared and air-dried or fixed with commercial spray fixative for Giemsa and Papanicolaou stains. If tuberculosis was clinically suspected, the needle and syringe were rinsed in mycobacterial growth tube indicator (MGIT, Beckton Dickinson, USA) medium for mycobacterial culture. All mycobacterial isolates were identified as *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG or non-tuberculous mycobacteria (NTM) by polymerase chain reaction (PCR) testing.

Data collection and analysis

Cytology results were captured in a computerised database. We reviewed the results of all FNAB samples received during the 3-year study period from patients ≤ 16 years of age. Relevant information including patient demographics, specimen characteristics and final diagnosis was transferred to a Microsoft Excel spreadsheet. Descriptive statistical analysis was performed using Statistica 7.

Written, informed consent to the FNAB procedure was obtained from the parent or legal guardian. Ethics clearance was obtained from the institutional review board of the University of Stellenbosch.

Results

A total of 830 aspirates were reviewed, 464 (56%) from TBH, 264 (32%) from local

clinics and 102 (12%) from Malawi. In general, the age and gender distribution was uniform (406, 51% female), but Malawi had more males (63% male *vs* 37% female) and the clinics had more females (57% female *vs* 43% male).

Peripheral lymph nodes were most frequently aspirated (376, 45%); of the lymph nodes specified, 70% were cervical and 30% axillary. Aspiration sites for the remaining 454 samples were distributed as follows: head and neck (124, 15%), chest and abdomen (98, 12%), breast (66, 8%), site not specified (166, 20%). All 66 breast tissue samples were collected from pubertal girls and came from the clinics, constituting 25% of clinic samples. In Malawi where all the children were clinically suspected of having Burkitt's lymphoma, the bulk of aspirates (44, 43%) came from the head and neck area, 28 (27%) from the chest, abdomen or pelvis and in 30% of cases the site was not specified.

The cytological diagnosis was assessed in conjunction with the clinical and radiological findings and all clinically or cytologically suspected lymphomas at TBH were aspirated for flow cytometry. Where appropriate, immunocytochemistry was performed on neoplasms. All solid neoplasms were managed according to SIOP (International Society for Paediatric Oncology) protocols, and histology was done after chemotherapy. Where there was any uncertainty as to the precise diagnosis after FNAB, the mass was excised or biopsied prior to chemotherapy.

All cases of suspected mycobacterial infection received bedside inoculation for mycobacterial culture and subsequent speciation.

All aspirates from Malawi were referred after clinical assessment as Burkitt's lymphoma and, when confirmed by cytology, patients were commenced on limited chemotherapy according to the study protocols and response to treatment was carefully monitored.

The most common diagnostic category was normal/reactive tissue, followed by

mycobacterial infection and lymphoma, in particular Burkitt's lymphoma. However, the distribution of diagnostic categories showed marked variation between the different centres. Mycobacterial disease was the most common diagnosis in children from TBH and Burkitt's lymphoma in the select group from Malawi. Among the children with mycobacterial infection, *M. bovis* BCG was identified in 20 (22.5%) cases. All these children had right-sided axillary lymph node enlargement and were <2 years of age (average age <1 y). *M. tuberculosis* was identified in 67 (75.3%) cases, mostly from cervical lymph nodes, and was equally prevalent in all age groups (average age 5.7 y). In Malawi, Burkitt's lymphoma was seen predominantly between the ages of 2 and 12 years (average age 6.3), corresponding to the age of maximum prevalence of endemic Burkitt's lymphoma.¹¹ No significant seasonal or other time-related variation was recorded on reviewing the temporal profile of cases with TB or Burkitt's lymphoma.

Of all aspirates received, 464 (56%) were performed by pathologists (TBH), 264 (32%) by clinicians (clinics) and 102 (12%) by nurse aspirators (Malawi). Pathologists and nurse aspirators received formal training in FNAB technique but few of the clinicians. Fig. 1 indicates the adequacy of samples received; in total, 174 (21%) samples were regarded as inadequate. The proportion of inadequate samples varied widely between centres, TBH 14%, Malawi 20% and clinics 35%. This reflects differences between the aspirator groups, adequate samples being 86% in the pathologist group, 80% in the nurse aspirator group and 65% in the untrained clinician group. Sample adequacy rates were similar between pathologists and nurse aspirators (399/464 *vs* 82/102; OR 1.4, 95% CI 0.8–2.6) but were significantly better in the group who received formal training (TBH and Malawi) than in the clinics where clinicians did not receive formal training (481/566 *vs* 171/264, OR 3.1, 95% CI

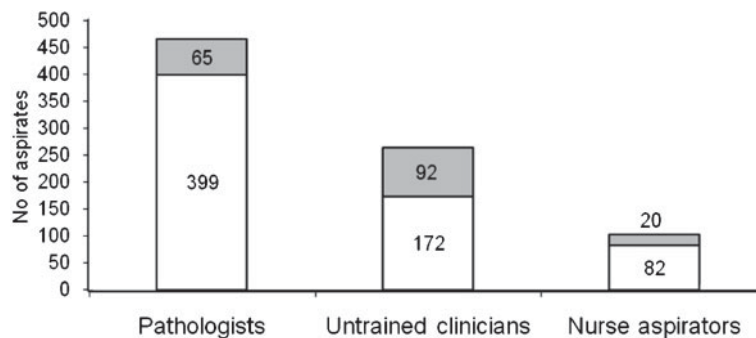


FIG. 1. Adequacy rates of FNAB by aspirator: pathologists 86%, untrained clinicians 65%, nurse aspirators 80% (■ inadequate, □ adequate).

2.2–4.4). The sample adequacy rate also differed according to the number of passes performed, single pass 75% and more than one pass 85% (85% *vs* 75%, OR 1.9, 95% CI 1.3–2.8).

Discussion

FNAB is widely used as a first-line diagnostic procedure for the diagnosis of mass lesions in adults, but it is used less frequently in children, especially so in resource-limited settings where it has the greatest diagnostic potential. This study demonstrates that FNAB is a feasible option in resource-limited settings; well trained nurse aspirators performed as well as trained pathologists and the procedure can be done on an outpatient basis.

Nurse aspirators from Malawi who attended a short training course at TBH and received clear operating procedure guidelines achieved an adequacy rate of 80%, despite the fact that multiple needle passes were restricted. The technique shown was subsequently implemented within the units. This was a highly select patient group with clinically diagnosed Burkitt's lymphoma who were enrolled in a trial that investigated cost-effective management of Burkitt's lymphoma in resource-limited countries.¹⁰ In the absence of a local cytopathologist, the slides were fixed and sent to TBH for review. This trial demonstrated that diagnostic slides

can be transported easily and safely using standard courier services, enabling cytopathologists to provide a long-distance diagnostic service for countries without cytopathology services.

The diagnostic value of FNAB is completely dependent on the quality of the sample presented to the cytopathologist. Optimal FNAB technique together with adequate slide preparation and preservation is taught poorly, if at all, to medical students in South Africa. The technique is usually acquired by observing and assisting senior clinicians, most of whom receive no formal training, resulting in the propagation of incorrect techniques and a high percentage of inadequate samples. Samples are frequently bloody, inadequately spread and poorly fixed, making it impossible for the pathologist to interpret the smears. The resulting non-diagnostic reports promote the misconception that FNAB is a technique with a poor diagnostic yield.

Before January 2001, FNAB was not widely used by clinicians at TBH. This was partly due to inadequate training in FNAB technique and under-recognition of the valuable contribution that FNAB could make to the management of infective and neoplastic lesions in children. Since 2001, cytopathologists have provided the FNAB service at TBH, and the diagnostic use and yield of FNAB in children has increased; an outreach programme has been established offering short practical tutorial sessions to

interested clinicians, combined with follow-up of subsequent aspirates received, providing positive feedback and constructive advice.

The advanced training and experience of the cytopathologists might partly explain the excellent results achieved, although nurse aspirators in Malawi who received training in the correct technique achieved comparable results. The performance of more than one needle pass also improved sample adequacy and diagnostic yield. In total, more than one needle pass was performed in 40% of patients, 59% at TBH, 18% in the clinics and 11% in Malawi. In Malawi, multiple needle passes were restricted in an attempt to minimise the risk of needle tract spread, a precaution recommended in children suspected of having a malignant neoplasm.

This lack of training is not unique to developing countries. In a study of breast aspirates, Ljung showed that physicians trained in FNAB technique missed significantly fewer malignant lesions than physicians who had received no training (2% vs 25%, $p < 0.0001$).¹² This was not affected by the number of aspirates performed by individual aspirators.

Few studies have reported on the value and limitations of FNAB in routine paediatric practice. In the USA, Wakely reported a sensitivity and specificity rate of 97% and a positive predictive value of 95% throughout the entire age range of infancy and childhood.³ Handa showed FNAB to be an excellent procedure for the triage of patients with significant lesions requiring treatment or referral to specialised services in India.⁸ A paediatric oncology study in the USA demonstrated that FNAB is an excellent tool for the diagnosis of primary and recurrent malignant neoplasms.⁶ These results were supported by a South African study of paediatric oncology patients where FNAB showed a sensitivity of 96.1% and a specificity of 100%.¹³

FNAB is used increasingly for the diagnosis of infectious disease, particularly tuberculosis.^{14,15} Poor countries bear the

brunt of paediatric tuberculosis,¹⁶ necessitating optimal use of limited resources. It has been reported that cervical lymphadenitis is the most common extra-thoracic manifestation of paediatric tuberculosis in India and South Africa,¹⁷⁻¹⁹ but differentiation from other infections such as *M. bovis* BCG, non-tuberculous mycobacteria, fungal infections or malignancies such as Burkitt's lymphoma or Kaposi's sarcoma is important.^{20,21} FNAB is a minimally invasive procedure that offers a definitive tissue diagnosis and can be performed by well trained nurses on an outpatient basis. It has particular diagnostic value in HIV-infected children in whom the diagnosis of intra-thoracic tuberculosis is often complicated by atypical manifestations and other HIV-associated conditions.²² In addition to providing rapid diagnostic confirmation by cytomorphology and/or direct visualisation of the infecting organism, FNAB samples may be inoculated into relevant culture that allows more accurate organism speciation and drug sensitivity testing.^{20,23}

This study confirms the diagnostic value of FNAB in routine paediatric practice. It also demonstrates that FNAB, using a small-gauge needle, is rapid and safe even in resource-limited settings. Well trained nurse aspirators can perform superficial aspirates as an outpatient procedure, reducing the need for hospitalisation. Cytology slides, once prepared and fixed, can be transported easily for expert evaluation elsewhere. FNAB is of particular value to countries with limited resources and a high prevalence of diseases such as tuberculosis which can be treated successfully at primary health care level, while identifying patients who require referral to a regional or tertiary care centre.

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References

- 1 Silverman JF, Gay M. Fine-needle aspiration and surgical pathology of infectious lesions. Morphologic features and the role of the clinical microbiology laboratory for rapid diagnosis. *Clin Lab Med* 1995; **15**:251–78.
- 2 Buchino JJ, Lee HK. Specimen collection and preparation in fine-needle aspirations in children. *Am J Clin Pathol* 1998; **109** (4 suppl 1):S4–8.
- 3 Wakely PE, Kardos TF, Frable WJ. Application of fine needle aspiration biopsy to paediatrics. *Hum Pathol* 1988; **19**:1383–6.
- 4 Howell LP. Changing role of fine needle aspiration in the evaluation of paediatric masses. *Diagn Cytopathol* 2001; **24**:65–70.
- 5 van Coppenraet ESB, Lindeboom JA, Prins JM, Peeters MF, Claas ECJ, Kuijper EJ. Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children. *J Clin Microbiol* 2004; **42**:2644–50.
- 6 Smith MB, Katz R, Black CT, Cangir A, Andrassy RJ. A rational approach to the use of fine-needle aspiration biopsy in the evaluation of primary and recurrent neoplasms in children. *J Pediatr Surg* 1993; **28**:1245–7.
- 7 Martins MR, da Cunha Santos G. Fine needle aspiration in the diagnosis of superficial lymphadenopathy: a 5 year Brazilian experience. *Diagn Cytopathol* 2006; **34**:130–4.
- 8 Handa U, Mohan H, Bal A. Role of fine needle aspiration cytology in evaluation of paediatric lymphadenopathy. *Cytopathology* 2003; **14**:66–9.
- 9 DeMay RM. *The Art and Science of Cytopathology*. Chicago, IL: ASCP Press, 1996.
- 10 Hesseling PB, Broadhead R, Molyneux E, et al. Malawi pilot study of Burkitt's lymphoma treatment. *Med Pediatr Oncol* 2003; **41**:532–40.
- 11 Jaffe ES, Harris NL, Stein H, Vardiman JW. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. In: *World Health Organization Classification of Tumours*. Lyon, France: International Agency for Research on Cancer, 2001; 120–53.
- 12 Ljung BM, Drejet A, Chiampi N, et al. Diagnostic accuracy of fine-needle aspiration biopsy is determined by physician training in sampling technique. *Cancer* 2001; **93**:63–8.
- 13 Wright CA, Michelow P, Harnekar A, Poole J, Leiman G. *FNA in Paediatric Oncology*. XV International Academy of Cytology Congress, Santiago, Chile, 2004.
- 14 Wright CA, Burgess SM, Geiger D, Wasserman E, van den Burg M. *The Diagnosis of Mycobacterial Lymphadenitis in Children: Is Fine Needle Aspiration the Way to Go?* 46th Conference of the Federation of South African Societies of Pathology, Durban, South Africa, 2006.
- 15 Ersöz C, Polat A, Serin MS, Soylu L, Demircan O. Fine needle aspiration (FNA) cytology in tuberculous lymphadenitis. *Cytopathology* 1998; **9**:201–7.
- 16 Marais BJ, Gie RP, Schaaf HS, Donald PR, Beyers N, Starke J. Childhood pulmonary tuberculosis—old wisdom and new challenges. *Am J Resp Crit Care Med* 2006; **173**:1078–90.
- 17 Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Enarson DA, Beyers N. The spectrum of diseases in children treated for tuberculosis in a highly endemic area. *Int J Tuberc Lung Dis* 2006; **10**:732–8.
- 18 Seth V, Kabra SK, Semwal OP, Mukhopadhyaya S, Jensen RL. Tubercular lymphadenitis: clinical manifestations. *Indian J Pediatr* 1995; **62**:565–70.
- 19 Marais BJ, Wright CA, Schaaf HS, et al. Tuberculous lymphadenitis as a cause of persistent cervical lymphadenopathy in children from a tuberculosis-endemic area. *Pediatr Infect Dis J* 2006; **25**:142–6.
- 20 Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Autofluorescence of mycobacteria on lymph node aspirates—a glimmer in the dark? *Diagn Cytopathol* 2004; **30**:257–60.
- 21 Marais BJ, Pienaar J, Gie RP. Kaposi sarcoma with upper airway obstruction and bilateral chylothoraces. *Pediatr Infect Dis J* 2003; **22**:926–8.
- 22 Jeena PM, Coovadia HM, Hadley LG, Wiersma R, Grant H, Chrystal V. Lymph node biopsies in HIV-infected and non-infected children with persistent lung disease. *Int J Tuberc Lung Dis* 2000; **4**:139–46.
- 23 Silverman JF, Gay RM. Fine-needle aspiration and surgical pathology of infectious lesions. Morphologic features and the role of the clinical microbiology laboratory for rapid diagnosis. *Clin Lab Med* 1995; **15**:251–78.

Fine Needle Aspiration Biopsy - A First Line Diagnostic Procedure in Pediatric

Tuberculosis suspects with Peripheral Lymphadenopathy?

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SUMMARY

OBJECTIVE To evaluate the diagnostic yield and time to diagnosis of fine needle aspiration biopsy (FNAB) versus routine respiratory specimens collected from children with a palpable peripheral lymph node mass and symptoms suspicious of tuberculosis.

DESIGN We performed a retrospective review of laboratory records at Tygerberg Hospital over a 4-year period, from January 2003 to December 2006. All children (<13 years) in whom an FNAB and other mycobacterial specimens were collected as part of their diagnostic workup were included.

RESULTS In 95 children the following specimens were collected; FNAB 95, gastric aspirates 142, other respiratory specimens 36, non-respiratory specimens 26. Mycobacterial disease was diagnosed in 70/95 (73.3%) patients. Children without respiratory specimens (n = 6) and/or with *M bovis BCG* disease (n = 15) were excluded from comparative analysis. In the remainder, FNAB was positive in 45/74 (60.8%) versus any respiratory specimen in 29/74 (39.2%; p <0.001). The mean time to bacteriologic diagnosis with FNAB was 7.1 days (95% CI 4.2-10.1) compared to 22.5 days (95% CI 15.8-29.1) for any respiratory specimen.

CONCLUSION FNAB is a simple, rapid and effective modality to achieve confirmation of mycobacterial disease in pediatric tuberculosis suspects with a palpable peripheral lymph node mass.

INTRODUCTION

Pediatric tuberculosis (TB) contributes significantly to the global TB disease burden.¹⁻² Children suffer severe TB-related morbidity and mortality in endemic areas, but contribute little to disease transmission and the maintenance of the TB epidemic.¹ Consequently, treatment of children has been a low priority in global TB control efforts, but the World Health Organization (WHO) produced guidance for the management of childhood TB in 2006 calling for the evaluation of new techniques to improve the diagnosis.³ The Global Drug Fund made child-friendly TB treatment formulations available to poor countries since 2008.⁴

The accurate diagnosis of pediatric TB remains a significant challenge, due to the low specificity of signs and symptoms, especially in human immunodeficiency virus (HIV)-infected children, the difficulty of obtaining bacteriologic specimens and the paucibacillary nature of pediatric disease.⁵ New diagnostic tests for TB have been developed, such as T cell assays and nucleic amplification tests, but these are all poorly validated in children.⁶ The

ability to distinguish latent infection from active disease remains problematic, particularly in endemic areas with heavy disease burdens.

An accurate bacteriologic diagnosis in children with TB is needed to initiate effective therapy and has become even more important with the transmission of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB to children.⁷⁻⁸ Obtaining bacteriologic specimens from children is difficult and diagnostic yield is usually low. Sputum smear microscopy is positive in less than 10-15% of children with probable pulmonary TB, with reported culture yields between 30-40%.⁹⁻¹¹ Various methods have been described to obtain respiratory specimens from children who are unable to expectorate,¹²⁻¹⁴ with no international consensus on what is best.

TB lymphadenitis is the most common extrapulmonary manifestation of TB in children from endemic areas,¹⁵ accounting for almost 50% of extrapulmonary disease.¹⁶ Fine needle aspiration biopsy (FNAB) is the diagnostic modality of choice in children with persistently enlarged cervical nodes in TB endemic areas.¹⁷ FNAB is increasingly utilized in paediatric oncology patients,¹⁸⁻¹⁹ and multiple studies have reported the value of FNAB for TB diagnosis in adults,²⁰⁻²² but it remains underutilized as a diagnostic tool in TB suspects. The aims of this study were to compare the diagnostic yield and time to diagnosis of FNAB compared to routine respiratory specimens collected in child TB suspects with a palpable peripheral lymph node mass.

METHODS

We performed a retrospective review of laboratory records at Tygerberg Hospital, Cape Town South Africa, from January 2003 to January 2007. Only children less than 13 years of age (eligible for access to pediatric services) were included. Since this was a laboratory-based study we were unable to review clinical information and all diagnostic tests were requested at

the discretion of the attending clinician. Standard practice at our institution is to refer children with a persistent peripheral lymph node mass (>1-2x1-2cm) for FNAB, while respiratory specimens are collected in most pediatric TB suspects.

Children who underwent FNAB were identified using laboratory records of the National Health Laboratory Services. Those in whom another specimen for mycobacterial culture was obtained within a 30 day time window either side of the FNAB collection date were included in the analysis. Cases with *Mycobacterium bovis* bacille Calmette-Guerin (BCG) disease were excluded from the comparative analysis. These children typically present with right axillary adenitis ipsilateral to the vaccination site, which is nearly pathognomonic.²³ Inclusion of these children would have biased results in favour of FNAB.

FNAB was done in the ward or as an outpatient procedure by a trained pathologist following standard protocol.²⁴ The lymph node mass was stabilized by the pathologist performing the aspiration biopsy using a 23g or 25g needle attached to a 10ml syringe, applying constant suction of no more than 2ml. Two smears were prepared from each aspirate: one fixed with commercial cytology fixative for Papanicolaou staining and the other air-dried for Giemsa and ZN staining. Thereafter the needle and syringe was rinsed by withdrawing an aliquot of liquid growth media into the syringe and discharging the contents back into a mycobacterial growth indicator tube (MGIT, Beckton Dickinson, USA). The inoculated MGIT tubes were transported to the microbiology laboratory within 2 hours, where standard PANTA supplement was added and the tubes incubated for 42 days. Positive cultures were identified as *M. tuberculosis* complex by polymerase chain reaction (PCR).²⁵

In the cytology laboratory, smears were evaluated for adequacy, defined as sufficient cells and/or necrotic material to allow a definitive diagnosis. A modified ZN stain was used on the Giemsa stained smears to detect acid fast bacilli.²⁴ Fluorescence microscopy was carried out on Papanicolaou stained smears using a Zeiss Axiophot microscope with a

fluorescent attachment and a wide-band blue excitation filter (450–480 nm). Papanicolaou stained mycobacteria fluoresce as brilliant yellow bacilli, thin and slightly curved with polar enhancement and a length of 2.0–2.7 microns.²⁶ *M. tuberculosis*, *M. bovis*, *M. bovis* BCG and non-tuberculous mycobacteria (NTM) are morphologically indistinguishable, but in this TB-endemic setting *M. bovis* is hardly ever detected on culture, *M. bovis* BCG usually has a clinical pathognomonic presentation,²⁷ and disease caused by NTM is relatively rare.

Early morning gastric aspirates were obtained following overnight admission to the pediatric ward. A nasogastric tube was passed into the stomach and the contents aspirated; 5–10 ml of normal saline inserted if required until a total volume of at least 5ml was aspirated. Gastric aspirates were placed in a sterile tube and neutralized with an equal volume of sodium carbonate. Induced sputa and/or nasopharyngeal aspirates were collected by a trained physiotherapist, but this was rarely done. Sputum induction was undertaken after 3 hours of fasting. Children were pre-treated with 200ug of salbutamol via metered dose inhaler and spacer. A jet nebulizer attached to oxygen at a flow rate of 5 l/min delivered 5 ml of 5% sterile saline for 15 minutes. Thereafter chest physiotherapy was applied before obtaining sputum by expectoration or by nasopharyngeal suctioning, using a mucus extractor. Oropharyngeal suction was used if the child had excessive nasal secretions.

All microbiology specimens were submitted to the laboratory within 6 hours. Smear microscopy, using either Ziehl-Neelsen or auramine stains, was only performed on expectorated sputum samples. Following N-acetyl-L-cysteine - sodium hydroxide decontamination, sputum specimens were inoculated into MGIT and incubated for 42 days. Further procedures for identification of mycobacteria were as described for the FNAB samples. All procedures were carried out according to standard operating procedures in a Biosafety Level 2 laboratory.

The reference standard for disease on FNAB was cytomorphology consistent with mycobacterial infection plus mycobacteria visualized with ZN stain and/or autofluorescence, and/or if *M. tuberculosis* was identified on culture. Given the low rates of NTM in the study population culture negative cases with cytomorphological and mycobacterial proof of disease were included in the TB group. For respiratory specimens the reference standard was acid fast bacilli (AFB) detected on sputum smear microscopy or identification of *M. tuberculosis* by culture in at least one specimen. For comparative purposes multiple respiratory specimens were regarded as a single test procedure, since we aimed to compare the value of routine respiratory specimen collection as a diagnostic procedure to FNAB.

We assessed whether the comparative bacteriological yield of FNAB (cytology and/or culture) was higher than other methods using Pearson's chi-square (X^2) and Fisher's exact tests²⁸. All analyses were conducted using Statistica Version 8.²⁹ Ethics approval was obtained from the Institutional Review Board of Stellenbosch University (N08/09/240).

RESULTS

FNAB and additional mycobacterial specimens were collected from 95 children, ranging in age from 2 months to 11 years. Figure 1 provides a flow diagram of patients and specimens included in the study. There was no significant difference in gender distribution (49 male vs. 46 females) and 48% of children were less than one year of age. The HIV-infection status was unknown in 28 (29.5%) children while 33 (34.7%) were HIV-infected and 34 (35.8%) were HIV-uninfected. Mycobacteria were identified in 70 (73.7%) children. Table 1 reflects the biopsy site, bacteriologic yield and mycobacterial species identified on FNAB; no NTM were identified.

Table 2 reflects the bacteriologic yield and mycobacterial species identified for all specimen types; of the 57 specimens speciated 41 (71.9%) were *M. tuberculosis*, 15 (26.3%)

M. bovis BCG and 1 (1.8%) NTM. A total of 143 gastric aspirates were collected from 80 (84.2%) children, with only 11 children (13.8%) submitting the desired 3 specimens. Additional respiratory specimens were collected in 15 patients; of whom 7 (mean age 9.3 years) provided expectorated sputum. Non-respiratory specimens, including cerebrospinal fluid, pus swabs and blood, were collected in 21 patients (26 specimens). All non-respiratory specimens, including 6 patients in whom no respiratory specimens were collected, and *M bovis BCG* cases were excluded from the comparative analysis. *M bovis BCG* was isolated in 15 children; in 2 cases the site of aspiration was soft tissue rather than an axillary lymph node. One third of these children (5 cases) had distant or disseminated BCG disease as evidenced by the isolation of *M. bovis* BCG from gastric and/or nasopharyngeal aspirates. All were immunocompromised: 4 were HIV-infected and the other had Severe Combined Immune Deficiency.

Of the 45 patients diagnosed with TB using the defined FNAB reference standard, 36 (80.0%) were confirmed to be *M. tuberculosis* on culture. Of the 30 diagnosed from respiratory specimens, 29 (96.7%) were confirmed to be *M. tuberculosis* on culture and 1 adolescent child was sputum smear (AFB) positive. For comparative purposes the collection of multiple respiratory specimens was regarded as a single test procedure. The bacteriological yield of FNAB compared to any combination of respiratory specimens is reported in Table 3; being higher compared to gastric aspirates ($p < 0.001$) and all respiratory specimens combined ($p < 0.001$).

We also assessed differences in the mean time to diagnosis for FNAB versus the other specimen types (where time to diagnosis is time to bacteriologic confirmation from onset of the test procedure). Whether the timing of additional mycobacterial cultures occurred before, after or simultaneously with FNAB collection did not influence the significance of the yield or time to diagnosis comparisons. Collecting multiple respiratory specimens was regarded as

a single test procedure. Accepting an average time to microscopy diagnosis of 2 days, FNAB (7.1 days, 95% confidence interval = 4.2 - 10.1 days) requires less than one-third the time of respiratory cultures (22.5 days, 95% CI = 15.8 – 29.1 days). This difference is illustrated in Figure 2. The one outlier is the child whose TB was confirmed by sputum smear-microscopy. The mean time to diagnosis for gastric aspirate culture (the most commonly performed test) was 21.4 days (95% CI = 15.2 – 27.6 days).

DISCUSSION

In the current study FNAB provided a better yield in child TB suspects with palpable peripheral lymph node masses than the collection of multiple respiratory specimens.

Children referred with suspected mycobacterial lymphadenitis routinely have a tuberculin skin test (TST) performed and return to the clinic or hospital for this to be read within 48–72 hours. If FNAB is performed with the initial visit, microscopy results should be available at the time of the return visit 48-72 hours later, enabling the commencement of TB therapy pending the results of culture and speciation.

The true extent of the pediatric TB disease burden is poorly documented, since more than 80% of children with TB are sputum smear-negative and direct smear microscopy is often the only diagnostic modality available in countries with limited resources. The diagnostic dilemma is further compounded by the HIV pandemic,²⁷ HIV-associated disease and TB may have common clinical and radiological presentations, in addition tests for TB infection such as the TST and newer T-cell assays have poor sensitivity in immunocompromised children.^{2, 6, 10} Two to three fasting gastric aspirates or a single induced sputum specimen have traditionally been advised for bacteriological confirmation of TB.¹² However, the yields observed in everyday practice deviate considerably from those achieved under trial conditions, which emphasizes the value of “real-life” retrospective analysis.

The low number of patients in whom gastric aspirates were collected on 3 consecutive days reflects practical difficulties. Severe limitations of in-patient beds and staff and the reluctance of parents to remain in hospital with their children for more than 1-2 days, since they often have other children at home to care for. In 5 children induced sputa and/or nasopharyngeal aspirates were collected as part of a separate specimen collection study, comparing the yield of gastric aspirates, nasopharyngeal aspirates and induced sputum. We included these specimens to provide a comprehensive overview of all the respiratory specimens collected. Inclusion of these specimens may have introduced bias, but numbers were small and did not influence the outcome. If anything these additional specimens would have favored the bacteriological yield of routine respiratory specimens. Development of improved respiratory specimen collection techniques that can be performed on an outpatient basis require further exploration. However our study demonstrates the value of FNAB as an outpatient diagnostic procedure in TB suspects with a palpable peripheral lymph node mass.

Enlarged peripheral lymph nodes provide a valuable opportunity to establish a definitive diagnosis of TB in HIV-infected children.³⁰ The majority of HIV-infected children with persistent lung disease have concurrent persistent peripheral lymphadenopathy,³⁰ which provides a neglected sample collection opportunity in this problematic group. In a recent South African study, 22 of 95 FNAB performed in mass lesions in HIV-infected children were positive for mycobacterial infection.³¹ FNAB has been established as an easy and reliable outpatient procedure for the diagnosis of palpable superficial masses in children, and is ideally suited for use in resource limited settings. It is simple, inexpensive, requires limited infrastructure and can be taught to medical and nursing staff.³² More widespread utilization is limited by a lack of experienced pathologists, but slides are easily transportable to regional or even international centers for diagnosis.³³

If the correct technique is followed, it is associated with minimal complications.³³

There were no adverse events or complications recorded with any of the FNAB's performed during this study. In a previous study we demonstrated that using FNAB with cytomorphology, autofluorescence and culture, provided a rapid and definitive diagnosis in the vast majority of children.²⁴ Culture alone produced a significantly lower yield, since it is influenced by factors such as inefficient sampling, contamination, variable bacterial load, and prior commencement of TB therapy. Rapid microscopy confirmation was achieved in 34/45 (75.5%) patients ultimately diagnosed with *M tuberculosis* using a combination of microscopy and/or culture positivity. Previous FNAB studies from this TB endemic setting, as well as the current study, demonstrated no NTM disease,^{17, 24, 26, 31} while *M bovis BCG* was only cultured in children less than 2 years age with a clinically pathognomonic presentation. Although rapid microscopy diagnosis allows a fairly accurate diagnosis and permits immediate commencement of therapy, culture is advisable as this enables speciation and drug susceptibility testing.

Compared to conventional respiratory specimens FNAB provided superior diagnostic yields and a significant reduction in time to TB diagnosis. Rapid bacteriological diagnosis has important benefits for patient management, also in settings not endemic for TB where NTM adenitis may be more common.³⁴⁻³⁵ This study reviewed laboratory records only, therefore, we are unable to provide detailed clinical descriptions or explore potential differences in patient characteristics that may have influenced the sequencing and/or yield of various diagnostic tests. However, it provides an assessment of every day ("real life") practice without any intentional bias. Despite these limitations, we believe FNAB should be regarded as a first line diagnostic modality in child TB suspects with a palpable peripheral lymph node mass.

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REFERENCES

- [1] Marais BJ, Gie RP, Schaaf HS, et al. Childhood pulmonary tuberculosis: old wisdom and new challenges. *Am J Respir Crit Care Med* 2006;173:1078.
- [2] Cotton MF, Schaaf HS, Hesselning AC, Madhi SA. HIV and childhood tuberculosis: the way forward. *Int J Tuberc Lung Dis* 2004;8:675-682.
- [3] World Health Organization. A research agenda for childhood tuberculosis. Geneva: World Health Organization; 2007. Report No.: WHO/HTM/TB/2007.381.
- [4] Gie CP, Matiru RH. Supplying quality-assured child-friendly anti-tuberculous drugs to children. *Int J Tuberc Lung Dis* 2009;13:277-278.
- [5] Eamranond P, Jaramillo E. Tuberculosis in children: reassessing the need for improved diagnosis in global control strategies. *Int J Tuberc Lung Dis* 2001;5:594-603.
- [6] Marais BJ, Pai M. New approaches and emerging technologies in the diagnosis of childhood tuberculosis. *Paediatr Resp Rev* 2007;8:124-133.
- [7] Schaaf HS, Marais BJ, Whitelaw A, et al. Culture-confirmed childhood tuberculosis in Cape Town, South Africa: a review of 596 cases. *BMC Infectious Diseases* 2007:140.
- [8] Chaisson RE, Martinson NA. Tuberculosis in Africa - combating an HIV-driven crisis. *N Engl J Med* 2008;358:1089-1092.
- [9] Newton SM, Brent AJ, Anderson S, Whittaker E, Kampmann B. Paediatric tuberculosis. *Lancet Infect Dis* 2008;8:499-510.
- [10] Shingadia D, Novelli V. Diagnosis and treatment of tuberculosis in children. *Lancet Infect Dis* 2003;3:624-632.
- [11] Marais BJ, Graham SM, Cotton MF, Beyers N. Diagnostic and management challenges for childhood tuberculosis in the era of HIV. *J Infect Dis* 2007;196:76-85.
- [12] Coulter JBS. Diagnosis of pulmonary tuberculosis in young children. *Ann Trop Paediatr* 2008;28:3-12.

- [13] Owens S, Abdel-Rahmen IE, Balyejusa S, et al. Nasopharyngeal aspiration for diagnosis of pulmonary tuberculosis. *Arch Dis Child* 2007;92:693-696.
- [14] Zar HJ, Hanslo D, Appoles P. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. . *Lancet* 2005;365:130-134.
- [15] Marais BJ, Gie RP, Schaaf HS, et al. The spectrum of childhood tuberculosis in a highly endemic area. *Int J Tuberc Lung Dis* 2006;10:732-738.
- [16] Cruz AT, Starke JR. Clinical manifestations of tuberculosis in children. *Paediatr Respir Rev* 2007;8:107-117.
- [17] Marais BJ, Wright CA, Schaaf HS, et al. Tuberculous Lymphadenitis as a Cause of Persistent Cervical Lymphadenopathy in Children From a Tuberculosis-Endemic Area. *Pediatr Infect Dis J* 2006;25:142-146.
- [18] Drut R, Drut RM, Pollono D, et al. Fine-Needle Aspiration Biopsy in Paediatric Oncology Patients. A Review of Experience with 829 Patients. *Paediatr Haematol Oncol* 2005;27:370-376.
- [19] Wakely PE, Kardos TF, Frable WJ. Application of fine needle aspiration biopsy to pediatrics. *Hum Pathol* 1988;19:1383-1386.
- [20] Nayak S, Mani R, Kavatkar AN, Puranik SC, Holla VV. Fine-needle aspiration cytology in lymphadenopathy of HIV-positive patients. *Diagn Cytopathol* 2003;29:146-148.
- [21] Kocjan G, Miller R. The cytology of HIV-induced immunosuppression. Changing pattern of disease in the era of highly active antiretroviral therapy. *Cytopathology* 2001;12:281-296.
- [22] Gupta AK, Nayar M, Chandra M. Critical appraisal of fine needle aspiration cytology in tuberculous lymphadenopathy. *Acta Cytol* 1992;36:391-394.

- [23] Hesselning AC, Rabie H, Marais BJ, et al. Bacille Calmette-Guérin Vaccine-Induced Disease in HIV-Infected and HIV-Uninfected Children. *Clin Infect Dis* 2006;548-558.
- [24] Wright CA, van der Burg M, Geiger D, et al. Diagnosing Mycobacterial lymphadenitis in children using Fine Needle Aspiration Biopsy: Cytomorphology, ZN staining and Autofluorescence – making more of less. *Diagn Cytopathol* 2008;36:245-251.
- [25] De Wit D, Steyn L, Shoemaker S, Sogin M. Direct detection of Mycobacterium tuberculosis in clinical specimens by DNA amplification. *J Clin Microbiol* 1990;28:2437-2441.
- [26] Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Auto fluorescence of Mycobacteria on Lymph Node Aspirates – A glimmer in the dark? *Diagn Cytopathol* 2004;30:257-260.
- [27] Hesselning AC, Cotton MF, Jennings T, et al. High Incidence of Tuberculosis among HIV-Infected Infants: Evidence from a South African Population-Based Study Highlights the Need for Improved Tuberculosis Control Strategies. *Clin Infect Dis* 2009;48:108-114.
- [28] Agriesti A. *Categorical Data Analysis*. 2nd ed. Hoboken, New Jersey: Wiley 2002.
- [29] Statistica. Version 8 ed. Tulsa, Oklahoma: Statsoft, Inc.
- [30] Jeena PM, Coovadia HM, Hadley LG, et al. Lymph node biopsies in HIV infected and non infected children with persistent lung disease. *Int J Tuberc Lung Dis* 2000:139-146.
- [31] Michelow P, Meyers T, Dubb M, Wright C. The utility of fine needle aspiration in HIV positive children. *Cytopathology* 2008;19:86-93.
- [32] Leiman G. The Registered Nurse as Aspirator: Experience in a Busy Cytopathology Unit. *Acta Cytol* 1995;39:1010.
- [33] Wright CA, Pienaar JP, Marais BJ. Fine needle aspiration biopsy: diagnostic utility in resource-limited settings. *Ann Trop Paediatr* 2008; 28:65-70.

[34] Hazra R, Robson CD, Perez-Atayde AR, Husson RN. Lymphadenitis Due to Nontuberculous Mycobacteria in Children: Presentation and Response to Therapy. *Clin Infect Dis* 1999;28:123-129.

[35] Benson-Mitchell R, Buchanan G. Cervical lymphadenopathy secondary to atypical mycobacteria in children. *J Laryngol Otol* 2007;110:48-51.

Table 1**Mycobacteria isolated on culture and site of fine needle aspiration biopsy (FNAB)**

Mycobacteria isolated ^a	Number (%)	Site of FNAB							
		Axillary node			Sub-mandibular node	Cervical node	Salivary gland	Soft tissue	Site not stated
		R	L	Not stated					
All	51/95 (54)	11	5	6	8	31	4	5	25
<i>M. Tuberculosis</i>	36/51 (71)	5	5	2	8	31	4	3	22
<i>M. bovis</i> BCG ^b	15/51 (29)	6	0	4	0	0	0	2	3

^a No *M. bovis* or Non Tuberculous Mycobacteria (NTM) were isolated

^b 13 (87%) of children <1 year of age

Table 2**Specimen type, bacteriological yield and organisms cultured in 95 patients**

Specimen type	Total number	Consecutive specimens			Culture positive No (%)	<i>M. tb</i> ^a No (%)	BCG ^b No (%)	NTM ^c No (%)
		1st	2nd	3rd				
FNAB	95	95	0	0	51 (54)	36 (71)	15 (30)	0
Gastric aspirates	143	80	52(65)	11(14)	39 (27)	30 (77)	8 (21)	1 (3)
Induced sputum	15	10	5(50)	0	6 (40)	6 (100)	0	0
Expectorated sputum	11	7	3(43)	1(14)	5 (45)	5 (100)	0	0
Nasopharyngeal aspirates	10	7	3(43)	0	5 (50)	4 (80)	1 (20)	0
Non-respiratory	26 ^d	26	0	0	3 (12)	2(67) ^e	1 (33) ^f	0

^a*M.tb* – *Mycobacterium tuberculosis*;

^bBCG – *Mycobacterium bovis* bacilli Calmette-Guerin;

^cNTM – Non-tuberculous mycobacteria

^d21 patients, pus swabs, urine, CSF, blood cultures;

^eCerebrospinal fluid;

^fPus swab.

Table 3

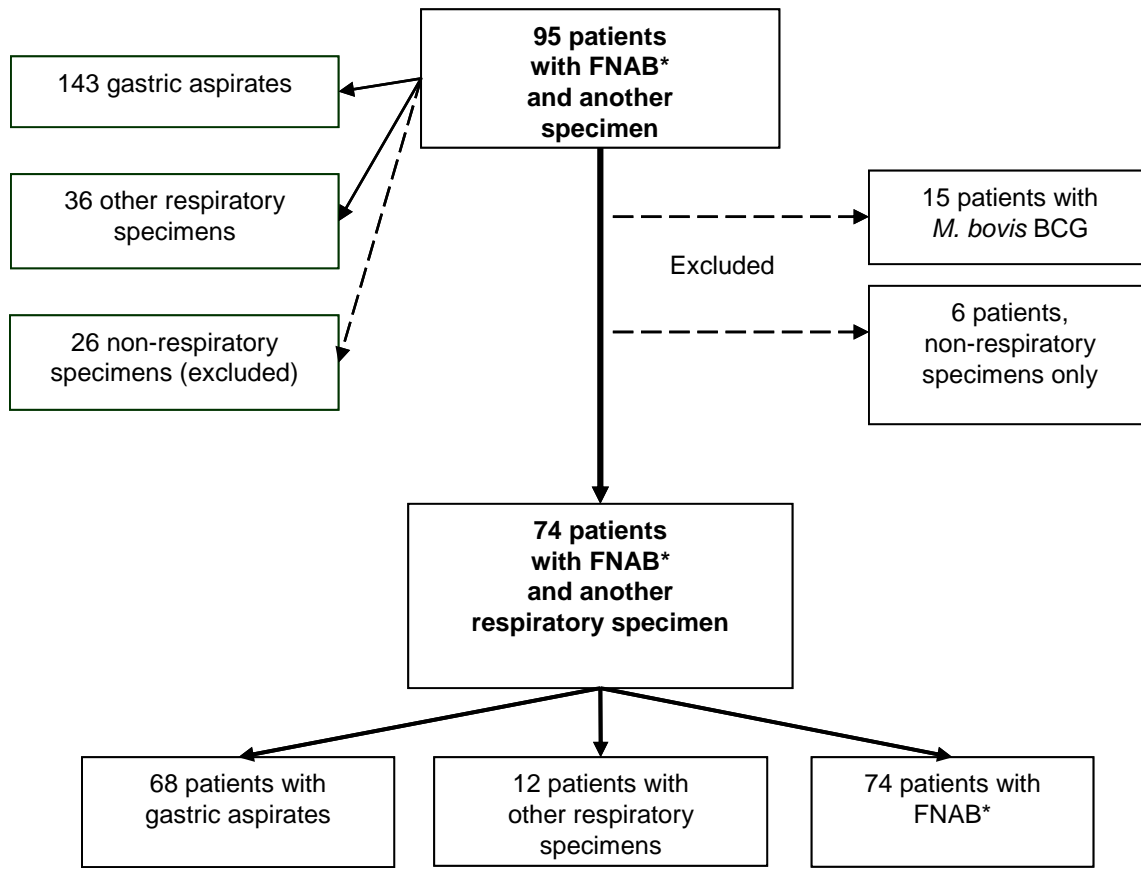
Bacteriologic yield of fine needle aspiration biopsy (FNAB) compared to routine respiratory specimens (*M bovis* BCG excluded)

Routine respiratory specimens	FNAB ^a negative	FNAB ^a positive	Total
All gastric aspirates negative	25	19	44
Any gastric aspirate positive	3	21	24
Total	28	40	68
All respiratory specimens ^b negative	26	19	45
Any respiratory specimen positive	3	26	29
Total	29	45	74

^aCytology and/or culture

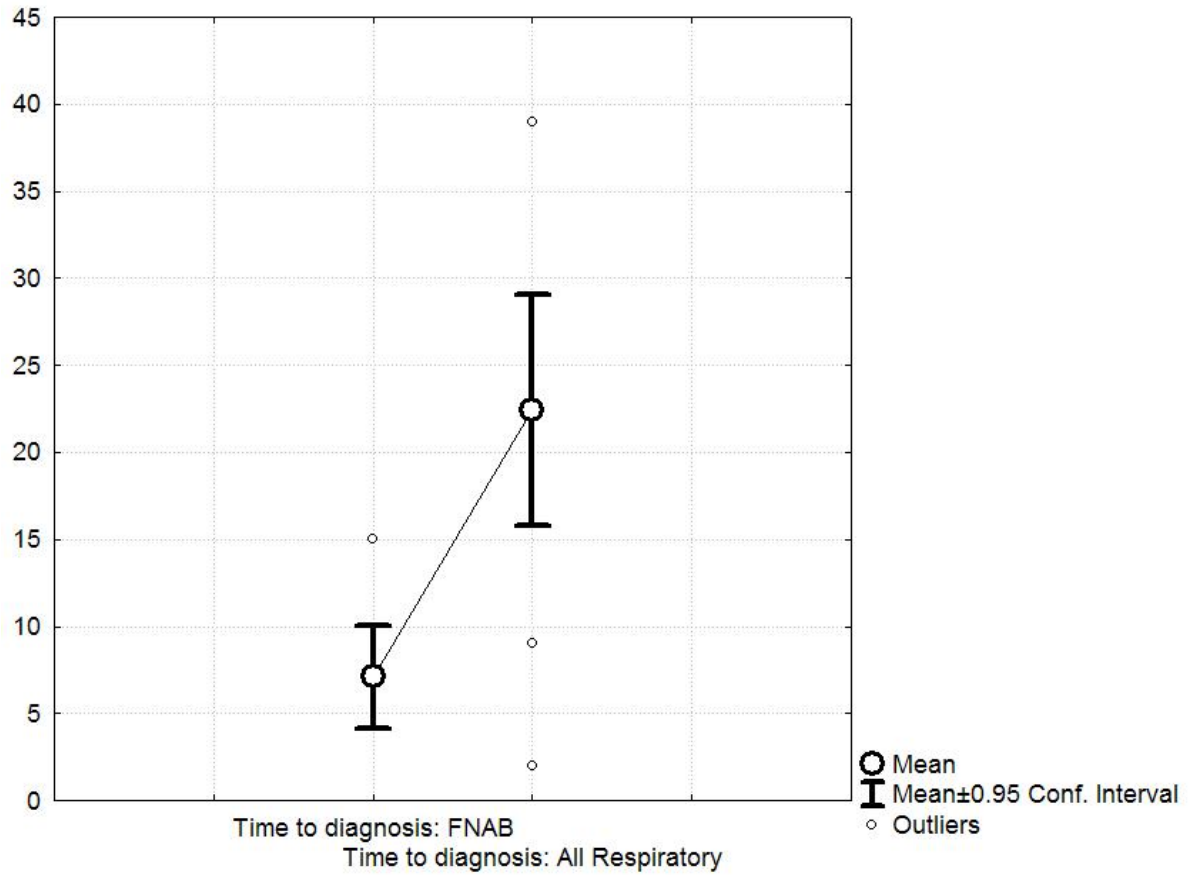
^bIncludes gastric and nasopharyngeal aspirates, as well as sputum and induced sputum specimens

Figure 1
Breakdown of patients and specimens included in the analysis



*FNAB – Fine needle aspiration biopsy

Figure 2
Time to diagnosis Fine Needle Aspiration Biopsy (FNAB) versus All respiratory specimens



Y-axis – specify units “Days”

X-axis – only “FNAB” & “All respiratory”

CHAPTER 5

OPTIMISING CYTOPATHOLOGICAL DIAGNOSIS

Mycobacterial Autofluorescence in Papanicolaou-Stained Lymph Node Aspirates: a Glimmer in the Dark?

Colleen A Wright, Yvonne van Zyl, Steven M Burgess, Lucille Blumberg, Gladwyn Leiman.

Diagnostic Cytopathology. 2004;30:257-60.

Diagnosing Mycobacterial Lymphadenitis in Children Using Fine Needle Aspiration Biopsy: Cytomorphology, ZN Staining and Autofluorescence-making more of less.

Colleen A Wright, Steven M Burgess, Mirjam van der Burg, D Geiger, Jeroen G Noordzij, Ben J Marais

Diagnostic Cytopathology: 2008; 36: 245-251

Mycobacterial Autofluorescence in Papanicolaou-Stained Lymph Node Aspirates: A Glimmer in the Dark?

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and Gladwyn Leiman, FRCPath¹

This study was undertaken to determine the value of incorporating fluorescence into cytopathological evaluation of lymph node fine-needle aspiration (FNA) specimens suspected of harboring mycobacterial species. The study population consisted of 1,044 HIV-positive and -negative patients referred for FNA to the cytopathology unit of a South African medical school located in a very high HIV prevalence region. Each aspirate was assessed on routine Papanicolaou-stained slides for morphologic characteristics of mycobacterial infection. The same glass slides were then viewed under fluorescent microscopy to determine the presence or absence of mycobacterial autofluorescence. Using multivariate analysis, results of both cytology and fluorescence were compared with mycobacterial culture as the final arbiter of the presence of organisms. In this large clinical study, compared with culture, cytomorphology showed sensitivity of 84.9%, but low specificity of only 50.9%. Fluorescence demonstrated lower sensitivity of 65.9%, but improved specificity of 73.0%. Taken together, positivity of both cytology and fluorescence improved specificity to 81.8%. Fluorescent microscopy is rapid, inexpensive, and cost-

effective; neither radioactive materials nor further staining are required. It is felt that this methodology would be of diagnostic benefit if used on morphologically suspicious samples in areas with a high prevalence of HIV and mycobacterial infections. Appropriate therapy could be commenced within hours of FNA, with reduction in the current number of patients lost to follow-up while awaiting results of culture. The technique is readily extended to other FNA types such as deep organ aspirates. Autofluorescence of organisms specifically requires usage of Papanicolaou staining; the technique cannot be used in histopathologic specimens stained with hematoxylin-eosin. Diagn. Cytopathol. 2004;30:257-260.

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Key Words: mycobacteria; tuberculosis; fluorescence; cytodiagnosis; HIV

The human immunodeficiency virus (HIV) epidemic in South Africa has attracted considerable attention. Prevalence figures in the general population are unknown, but 24.5% of women attending antenatal clinics nationwide are HIV-positive; the corresponding figure for the province in which this study was undertaken is 29.4% (Department of Health National HIV and Syphilis seroprevalence survey of women attending public antenatal clinics in South Africa, 2000; www.doh.gov.za/docs/reports/2000/hivreport.html). It is estimated that more than 60% of medical admissions in academic hospitals are due to opportunistic infections in patients with acquired immunodeficiency disease (AIDS). The most common of these opportunistic infections in South African adults with AIDS is *Mycobacterium tuberculosis* (MTB). In the year 2000, 150,696 new MTB cases were reported to the national registry, including 31,058 extrapulmonary infections (personal communication, Tuberculosis Directorate, SA National TB Control Program). By 2001, MTB incidence in the general population was approximately 300 per 100,000; at least 50% of new MTB cases were in retroviral-positive patients (personal communication, SA National Department of Health). Dual HIV and

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MTB epidemics place overwhelming demands on health care resources in any developing country. Expected life span is reduced by AIDS (down from 68 years to 48 years in South Africa between 1990 and 2000).^{1,2} By dominating hospital admissions, the HIV/MTB population limits investigation and treatment of non-HIV patients with potentially curable disease entities compatible with normal longevity. MTB diagnosis may be challenging, even in high prevalence areas; it is modified by HIV status and compounded by atypical symptoms, overlap in presentation of other opportunistic infections, frequency of extrapulmonary involvement, and rapid progression.³

Traditionally, laboratory diagnosis of mycobacterial infection has been the purview of the microbiologist; it has relied on positive culture or microscopic recognition of the organism by Ziehl-Nielsen staining.⁴ Fluorescence microscopy using auramine-rhodamine was described as early as 1937 and generally has been shown to be superior to Ziehl-Nielsen staining,⁵⁻⁷ but involves the use of toxic and carcinogenic substances. A substantial benefit would derive from any new, inexpensive, exposure-free diagnostic procedure, enabling early commencement of anti-MTB treatment. Mycobacteria (as well as *Pneumocystis carinii*, fungi, and certain bacteria) show brilliant autofluorescence in Papanicolaou-stained smears.⁸⁻¹⁰ The method is quick and inexpensive and can be focussed on those specimens felt to harbor the above infections on morphologic grounds. This suggests that fluorescence microscopy of Papanicolaou-stained smears could provide a rapid, safe, and inexpensive technique for confirmation of mycobacterial infection in cytological specimens. The present study explores this opportunity by investigating the ability of cytomorphology and fluorescence microscopy of Papanicolaou-stained smears from superficial lymph nodes obtained by fine-needle aspiration (FNA), individually and together, to identify mycobacterial infections.

Materials and Methods

A prospective study was performed in the teaching hospitals of the University of the Witwatersrand in Johannesburg, South Africa, during the period January 1998 to December 2001. The sample consisted of 1,044 superficial lymph node aspirates from patients with clinically suspected mycobacterial lymphadenopathy. In this center, retroviral serology is not performed without patient consent; serology was thus not available in many cases. In the 330 cases in which retroviral status was known, 318 (96%) were positive; a high proportion of the remaining patients were clinically suspected to be retrovirus-positive. Patients were referred to FNA clinics operated by the cytopathology unit at four teaching hospitals. Procedures were performed using 22 gauge needles attached to 10 ml syringes. At least two needle passes were performed on each node aspirated. Direct smears were made and were either air-dried for Diff-Quik staining or fixed with commercial spray fixative

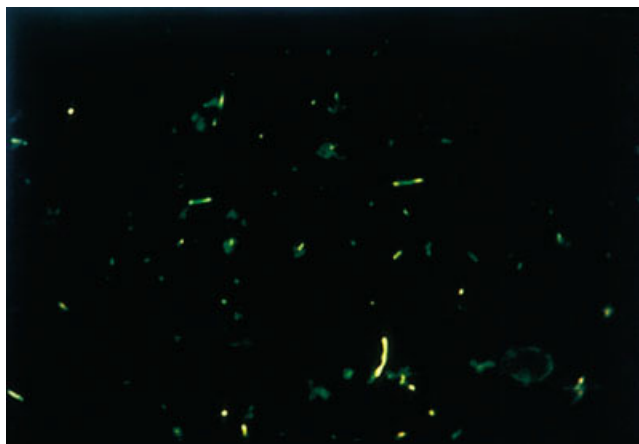


Fig. C-1. Dark-field illumination of characteristic MTB organism demonstrating autofluorescence in a lymph node aspirate (Papanicolaou, $\times 1,000$).

for Papanicolaou staining. Onsite rapid staining was not undertaken. The needle was rinsed in BACTEC 12B medium (Becton-Dickinson, Mountain View, CA), a commercially manufactured mycobacterial culture system. Slides were returned to the central cytopathology laboratory for same-day processing.

The air-dried Diff-Quik-stained and alcohol-fixed Papanicolaou-stained smears were screened and evaluated for adequacy and content. All neoplastic samples were excluded from the study, as were inadequate slides and those showing benign nonreactive, noninflammatory morphology. The remainder were reported as cytologically suggestive (positive) or nonsuggestive (negative) of mycobacterial lymphadenitis. This cytomorphologic evaluation was made on previously established criteria: amorphous granular necrotic debris, epithelioid histiocytes, and multinucleated Langhans-type giant histiocytes, in a background population of reactive lymphocytes and plasma cells. In the presence of AIDS, the cytological picture is more frequently that of necrosis only, or necrosis associated with an acute inflammatory infiltration, without attendant lymphocytic or histiocytic components.

Without any additional processing, the Papanicolaou-stained smears were then screened by one author (Y.v.Z.) using an Olympus BX40 microscope with a fluorescent attachment and the following filters: wide-band blue excitation filter (450–480 nm), dichromatic splitter (500 nm), and barrier filter (515 nm). Slides were viewed at $\times 400$ magnification. This displayed the MTB organisms as brilliant yellow fluorescent rods, which were thin and slightly curved with light ends and a uniform length of 2.0–2.7 microns (Fig. C-1). *Mycobacterium tuberculosis* is differentiated from *Mycobacterium avium*, which is shorter (1.2–1.8 microns) and variably fluorescent.

Table I. Comparative Results of MTB Detection by Cytodiagnosis and by Culture*

Cytodiagnosis	Culture		
	Negative	Positive	Total
Negative	228 (22%)	90 (8%)	318 (31%)
Positive	220 (21%)	506 (49%)	726 (70%)
Total	448 (43%)	596 (57%)	1,044 (100%)

*Sensitivity, 84.9%; specificity, 50.9%; negative predictive value, 71.7%; positive predictive value, 69.7%; accuracy, 69.3%.

Table II. Comparative Results of Detection of MTB by Autofluorescence and by Culture*

Fluorescence	Culture		
	Negative	Positive	Total
Negative	327 (31%)	203 (19%)	530 (50%)
Positive	121 (12%)	393 (38%)	514 (50%)
Total	448 (43%)	596 (57%)	1,044 (100%)

*Sensitivity, 65.9%; specificity, 73.0%; negative predictive value, 67.7%; positive predictive value, 76.5%; accuracy, 69.3%.

Table III. Summary of Statistical Analyses for Detection of MTB by Cytodiagnosis and by Autofluorescence, Separately and Together (%)

	Cytodiagnosis	Autofluorescence	Both
Sensitivity	84.9	65.9	56.7
Specificity	50.9	73.0	81.8
Negative predictive value	71.7	61.7	58.7
Positive predictive value	69.7	76.5	80.5
Accuracy	69.3	69.3	69.4

Results

Retroviral status (where known) and the diagnostic outcome of each of the three tests (i.e., cytomorphology, fluorescence, and culture) were code-captured in a standard portable computer spreadsheet using a standard indicator coding scheme (1 = positive; 0 = negative). The laboratory's unique case reference number was also captured for quality control purposes. Detailed results appear in Tables I–III. The results indicate a strong and highly significant association between cytology and culture diagnoses (Cramer's $V = 0.59$; $P < 0.001$) and a virtually equivalent association between fluorescence and culture (Cramer's $V = 0.57$; $P < 0.001$). Thus, the overall accuracy of the techniques, separately and together, is virtually identical at almost 70%. Cytology and fluorescence offer differing benefits as regards sensitivity and specificity. Cytology is suggestive of 84.9% of culture-positive cases, yields few false negatives, but has low specificity of 50.9%. Fluorescence has lower sensitivity of 65.9%, yields few false positive readings, and thereby raises specificity to 73.0%. Taken together, cytology and autofluorescence refine the diagnostic group, incorporating lower sensitivity at 56.7%, but significantly improving specificity to 81.8%. Clinical implications are that three of five TB patients undergoing FNA could be placed

on appropriate and immediate antituberculous therapy. At a later stage of patients so medicated, one of four would not be confirmed on traditional culture as harboring active mycobacterial infection.

Discussion

The diagnosis of tuberculosis by cytomorphology is not new. It is a necrotizing granulomatous infection, which cytologically demonstrates the microscopic equivalent of caseous necrosis, i.e., a granular-appearing necrotic background, together with mature lymphocytes, tangles of epithelioid histiocytes, and giant multinucleated Langhans-type histiocytes. This cytological appearance, however, is neither sensitive nor specific for MTB. In recent years, the advent of HIV-associated tuberculosis has altered the characteristic cytologic picture, mainly by paucity or even absence of cellular response, i.e., fewer epithelioid and multinucleated histiocytes. Recent literature has shown that FNAs, particularly from large nodes with necrotic centers, may contain no cellular response at all, i.e., no epithelioid or multinucleated histiocytes, and may even lack lymphocytes if the entire node has undergone necrosis.^{11,12} Aspirates may then be entirely necrotic or may contain debris together with a secondary influx of neutrophils. The cytology then resembles an abscess more than it does classic tuberculosis. However, in high-incidence regions, sufficient experience has now been gained with new and varied presentations of tuberculosis in lymph node aspirates to raise the cytological suspicion of MTB in appropriate clinical settings. Ziehl-Nielsen staining may be performed on FNAs; while very specific, this test lacks sensitivity, as organisms may be extremely sparse and difficult to visualize. Therefore, it has become routine to submit needle rinses or separate needle passes in appropriate media to microbiology, where direct microscopy is performed, together with traditional culture. If all tests are negative prior to culture becoming positive, definitive diagnosis may be delayed for up to 6 weeks. In a country or region in which MTB is so prevalent, treatment delays of this nature are unacceptable, as they result in continued presence of infected and infectious patients in their communities, where further spread is likely.

To alleviate this, fluorescence, utilizing fixed Papanicolaou-stained cytologic material, becomes appealing for rapid and cost-effective diagnosis of MTB. When judged against traditional culture, the sensitivity in this study of cytodiagnosis (84.9%), and that of autofluorescence of Papanicolaou-stained material (65.9%), may not reach levels of sensitivity as attained in the diagnosis of malignancy by FNA. These sensitivity values are, however, higher than those quoted by Kupper et al.⁹ in their work on *Mycobacterium kansasii*. In terms of providing clinicians in overcrowded hospital settings with same-day working diagnoses on which immediate antituberculous treatment can be commenced, it is our feeling that the routine addition of fluo-

rescence to microscopy in FNAs has much to offer. It can be regarded as a one-stop test, performed if necessary on one glass slide, seen in one laboratory unit, with the final report available within hours of the FNA procedure. Diagnosis is accomplished without reprocessing from transport medium for direct evaluation and for culture, as occurs in microbiology. It also avoids the use of radioactive substances that, as indicated previously, carries some degree of morbidity for laboratory workers.⁹ The percentage of cytology⁺/fluorescence⁺ patients subsequently found to be culture-negative (18.2% of those who might be placed on anti-MTB therapy using this dual positivity as an indication; 8% of the entire group referred for FNA) would appear to justify the immediate commencement of such therapy, if clinically consistent. Antituberculous therapy can be stopped if culture is found to be negative, or if clinoradiological assessment deems the disease process not to be tuberculous in nature.

Culture was used in this study as the final arbiter of MTB infection. However, while it is the most accurate means of diagnosis available, it too is beset by an irreducible false negative rate. It is thus possible that some patients who are positive by cytology and/or fluorescence in this study, but negative by subsequent culture, did in fact have tuberculosis. Only a long-term prospective clinoradiologic and cytologic study incorporating review of patient records and ultimate response to therapy over time could answer this dilemma. An investigation of that nature is outside the capabilities of this laboratory-based study.

There are ways in which the sensitivity of diagnostic fluorescence could be improved. A recent study¹³ utilized automated Papnet screening technology for the identification of small rare mycobacterial organisms stained by the auramine-rhodamine method. However, for most laboratories, an innovation of that type would be financially unattainable. On the other hand, the mere addition of careful dark-field illumination, searching for fluorescence as reported here, is feasible in most laboratories.

Compared with culture techniques, fluorescence yielded a number of false positive cases in this study. Bacterial forms other than MTB are known to exhibit autofluorescence; it is thus possible that incorrect identification of these very small organisms will be made. Kupper et al.⁸ has suggested that a minimum number of eight organisms be required for positive diagnosis. In our experience in retroviral positive patients, MTB organisms are profuse and theoretically would be easier to identify. The fluorescent staining properties of *Mycobacterium avium intracellulare* are more variable than of MTB. In 596 culture-positive cases in this study, only 6 were mycobacteria other than *Mycobacterium tuberculosis*. There is, therefore, no replacement for experience and familiarity with the morphology of the organisms and scrupulous attention to detail when evaluating fluorescent bacteria.

It cannot be sufficiently emphasized that fluorescence of organisms is appropriate only to material stained with the

Papanicolaou stain. The results of the above study cannot, for example, be transferred to cell block or histopathologic sections stained with hematoxylin-eosin. The particular ingredient of the Papanicolaou stain that is thought to permit autofluorescence is most likely EA50 or EA65. Because of the capacity of the Papanicolaou stain to permit autofluorescence, cytopathologists find themselves in the unique position of extending the usual capability of cytodagnosis to include identification of fluorescent organisms, such as *Mycobacterium tuberculosis*, *Pneumocystis*, and *Cryptococcus*. In high-incidence areas of tuberculosis and of AIDS, this method may prove to be diagnostically useful on a scale larger than previously anticipated.

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References

1. Wilkinson D, Moore DA. HIV-related tuberculosis in South Africa: clinical features and outcome. *S Afr Med J* 1996;86:64-67.
2. Connolly C, Davies GR, Wilkinson D. Impact of the human immunodeficiency virus epidemic on mortality among adults with tuberculosis in rural South Africa, 1991-1995. *Int J Tuberc Lung Dis* 1998;2:919-925.
3. Del Amo J, Malin AS, Pozniak A, De Cock KM. Does tuberculosis accelerate the progression of HIV disease? evidence from basic science and epidemiology. *AIDS* 1999;13:1151-1158.
4. Shinnink TM, Good RC. Diagnostic mycobacteriology laboratory practices. *Clin Inf Dis* 1995;21:291-299.
5. Kommareddi S, Abramowski CR, Swinehart GL, Hrabak L. Nontuberculous mycobacterial infections: comparison of the fluorescent auramine-0 and Ziehl-Neelsen techniques in tissue diagnosis. *Hum Pathol* 1984;15:1085-1089.
6. Kumar N, Tiwari MC, Verma K. AFB staining in cytodagnosis of tuberculosis without classical features: a comparison of Ziehl-Neelsen and fluorescent methods. *Cytopathology* 1998;9:208-214.
7. Wright PW, Wallace RJ, Wright NW, Brown BA, Griffith DE. Sensitivity of fluorochrome microscopy for detection of *Mycobacterium tuberculosis* versus nontuberculous mycobacteria. *J Clin Microbiol* 1998;36:1046-1049.
8. Kupper TH, Steffen U, Wehle K, Richartz G, Pfitzer P. Morphological study of bacteria of the respiratory system using fluorescence microscopy of Papanicolaou-stained smears with special regard to the identification of mycobacteria. *Cytopathology* 1995;6:338-402.
9. Kupper TH, Wehle K, Marzahn S, Pfitzer P. The cytologic diagnosis of *Mycobacterium kansasii* tuberculosis by fluorescence microscopy of Papanicolaou-stained specimens. *Cytopathology* 1995;6:331-338.
10. Zaharopoulos P. Demonstration of parasites in toxoplasma lymphadenitis by fine-needle aspiration cytology: report of two cases. *Diagn Cytopathol* 2000;22:11-15.
11. Kocjan G, Miller R. The cytology of HIV-induced immunosuppression: changing pattern of disease in the era of highly active antiretroviral therapy. *Cytopathology* 2001;12:281-296.
12. Jannotta FS, Sidawy MK. The recognition of mycobacterial infections by intraoperative cytology in patients with acquired immunodeficiency syndrome. *Arch Pathol Lab Med* 1989;113:1120-1123.
13. Veropoulos K, Learmonth G, Campbell C, Knight BK, Simpson J. Automated identification of tubercle bacilli in sputum: a preliminary investigation. *Analyt Quant Cytol Histol* 1999;21:277-282.

Diagnosing Mycobacterial Lymphadenitis in Children Using Fine Needle Aspiration Biopsy: Cytomorphology, ZN Staining and Autofluorescence—Making More of Less

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Although the incidence of TB has stabilized or declined in most world regions, it is increasing in Africa, Southeast Asia, and the Eastern Mediterranean, fuelled by the HIV pandemic. More than 4,000 people died daily from TB-related illnesses in 2005. TB is a major cause of childhood morbidity and mortality in these developing countries, and there is an urgent need for rapid and definitive modalities for mycobacterial diagnosis in children. This prospective study in Tygerberg Hospital, Cape Town, South Africa, evaluates the ability of fine needle aspiration biopsy (FNAB) to diagnose mycobacterial lymphadenitis in children, using cytomorphology, autofluorescence on Papanicolaou stained smears, Ziehl-Nielsen (ZN) staining and/or culture. FNABs were performed on 200 children, and 25 (12.5%) aspirates were inadequate. Cultures were positive in 79/175 (45%); *Mycobacterium tuberculosis* was identified in 61 and *Mycobacterium bovis* BCG in 18 cases. Using culture as the gold standard, the concordance of the different techniques was as follows: cytomorphology 70%, ZN staining 73%, and autofluorescence 68%. Using an alternative gold standard (culture positive and/or suggestive cytomorphology plus positive autofluorescence or ZN smear), the "true" diagnostic performance of the various techniques was as follows: cytomorphology—sensitivity 78%, specificity 91%, positive predictive value (PPV) 93%, ZN staining - sensitivity 62%, specificity 97%, PPV 97%; autofluorescence—

sensitivity 67%, specificity 97%, PPV 97%; and culture—sensitivity 75%, specificity 100%, and PPV 100%. FNAB was shown to provide a rapid and definitive diagnosis in the majority of cases of suspected tuberculous lymphadenitis in children, based on cytomorphology and identification of the organism. *Diagn. Cytopathol.* 2008;36:245–251. © 2008 Wiley-Liss, Inc.

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Tuberculosis (TB) was declared a global emergency by the World Health Organization (WHO) in 1993.¹ Since then, the incidence of TB has stabilized or declined in most world regions, except Africa, Southeast Asia, and the Eastern Mediterranean, where the impact of the pandemic is staggering.² Between 2000 and 2005, new TB cases increased worldwide by 6%, to more than 24,000 new TB cases daily. More than 84% of these cases were located in sub-Saharan Africa and Asia. Even though TB-related deaths declined by 11% between 2000 and 2005, more than 4,000 people died daily from TB-related illnesses in 2005. It is fair to say that TB remains an emergency in those countries that can least afford the costs of diagnosing and treating its victims.

TB is a major cause of childhood morbidity and mortality in developing countries.^{3,4} Children below 3 years of age are particularly vulnerable because their immature immune systems make them susceptible to more severe disease manifestations, such as miliary TB and TB meningitis.^{5,6}

In 1989, the WHO estimated the worldwide incidence of TB in children to be 1.3 million per year with 450,000 children dying from TB-related illnesses in that year.⁷ In 2000 there were 8.3 million new cases of TB estimated worldwide, with 11% occurring in children younger than 15 years of age.⁸ As alarming as these estimates are, they

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may grossly underestimate the incidence of TB in children because the WHO estimates are based on smear positive cases and more than 80% of children with TB are not smear positive.⁹ In South Africa, as many as 40% of all cases of TB occur in children.⁸

The diagnosis of TB in children is particularly challenging for three reasons.^{10–12} First, sputum and alternative specimens (e.g., gastric aspirates) are difficult to obtain and have a poor diagnostic yield (i.e., typically as low as 30–40% even with more sensitive mycobacterial culture).⁹ Second, culture has limitations. Although direct culture inoculation at the bedside gives a high yield,¹³ it takes 1–6 weeks to deliver a final result. Culture also fails to differentiate between the various mycobacteria and, thus, requires additional polymerase chain reaction (PCR)-based testing for accurate speciation. Third, although chest radiography is considered reasonably accurate and practical for diagnosis of intrathoracic TB in children with suspicious symptoms, it has many limitations. Most importantly, it is not helpful in the diagnosis of extrathoracic TB without concurrent intrathoracic manifestations, a combination common in children.^{2,10} For example, a recent South African national study reported that 39,739/270,178 (14.7%) of new TB cases in 2005 were extrapulmonary TB.² A Cape Town study reported that 72/439 (16.4%) of children treated for active TB had extrathoracic disease and 65.4% of the positive cases had no concurrent intrathoracic disease manifestations.¹¹ TB lymphadenitis was the most common form of extrathoracic TB in the Cape Town study and nearly all cases with peripheral TB lymphadenitis presented with enlarged cervical lymph nodes,¹¹ consistent with previous research.^{12,14} TB lymphadenitis is the most common cause of persistent cervical lymphadenopathy in this TB endemic area.¹³ The challenge of diagnosing TB in children highlights the urgent need for rapid and definitive mycobacterial diagnosis in children with persistent cervical lymphadenopathy, which is the focus of our research.

Fine-needle aspiration biopsy (FNAB) has many qualities that make it an excellent diagnostic procedure in children with persistent superficial lymphadenopathy, especially in TB-endemic countries with limited resources. FNAB is a simple technique to perform. It is minimally invasive, safe and has very limited side effects when performed with a small gauge needle (e.g., no sinus formation). It can be performed as an outpatient procedure by trained medical or paramedical staff.^{13,15} Collecting a representative sample of material from a lymph node allows cytomorphological analysis and identification of the organism by direct microscopy and/or culture.

The diagnostic value of various microscopy techniques used to evaluate an FNAB sample has not been fully evaluated in children. A pilot study of 65 aspirates in

children using cytomorphology, ZN staining, and autofluorescence as diagnostic modalities had showed 92.3% concordance with culture when all three modalities were combined.¹⁶ The current study aimed to compare the diagnostic performance of cytomorphology, ZN staining, autofluorescence, and culture in a large cohort of children.

Materials and Methods

A prospective study was performed in Tygerberg Hospital during the period of January 2003 to June 2005. All children (a) less than 13 years of age (eligible for access to pediatric services), (b) with persistent superficial lymphadenopathy not responsive to first line antibiotic therapy, and (c) with a clinical suspicion of mycobacterial infection routinely referred for diagnostic FNAB were included in the study.

The referring clinician was requested to give oral sedation and analgesia 30 minutes prior to the procedure, which was performed in the ward or outpatient clinic with the assistance of the referring doctor and/or nursing staff. Consent was obtained from the legal guardian accompanying the child. The FNAB was performed using a 23-g or 25-g needle attached to a 10-cc syringe. The skin was cleaned using an alcohol swab, the child firmly immobilized and the node stabilized by the aspirator while performing the aspirate using no more than 2-cc suction. Standard precautions were taken to minimize any complications. Two smears were prepared from each aspirate and the needle and syringe rinsed in mycobacterial growth tube indicator (MGIT, Beckton Dickinson, USA) medium for mycobacterial culture. One smear was spray fixed with commercial cytology fixative for Papanicolaou staining and the other air-dried for Giemsa staining. Smears were sent to cytology for cytomorphology, autofluorescence, and ZN staining.

The stained smears were screened and evaluated for adequacy and diagnosis using each of the modalities described. Cytological results were reported as nonspecific reactive lymphadenopathy or cytologically consistent with mycobacterial infection, based on specific morphological criteria which have been previously described.^{17–19} Immune competent patients with TB present with the more classical morphological picture of epithelioid granulomata and epithelioid histiocytes in a background of reactive lymphocytes and plasma cells (Fig. C-1). A small amount of amorphous necrosis and occasional giant cells may be present. Patients with tuberculosis who are immune compromised have smears showing abundant “dirty” necrosis in which neutrophils and cellular debris are prominent (Fig. C-2). There is a spectrum of morphology between these extremes, and aspirates from patients with mycobacterial lymphadenitis may occasionally show morphological features of suppurative acute lymphadenitis.

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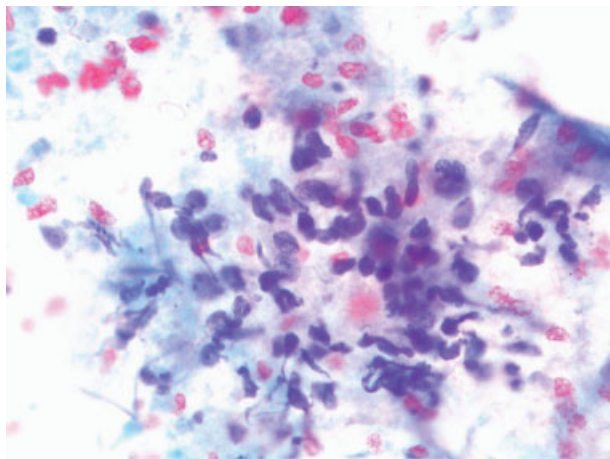


Fig. C-1

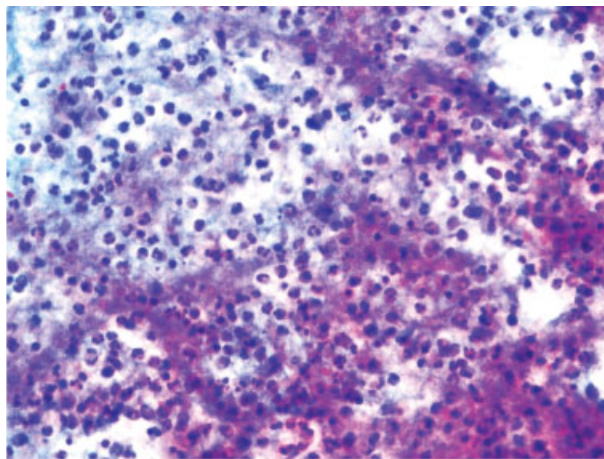


Fig. C-2

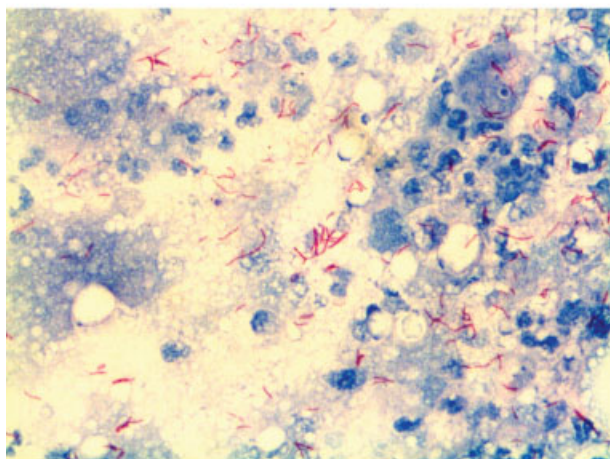


Fig. C-3

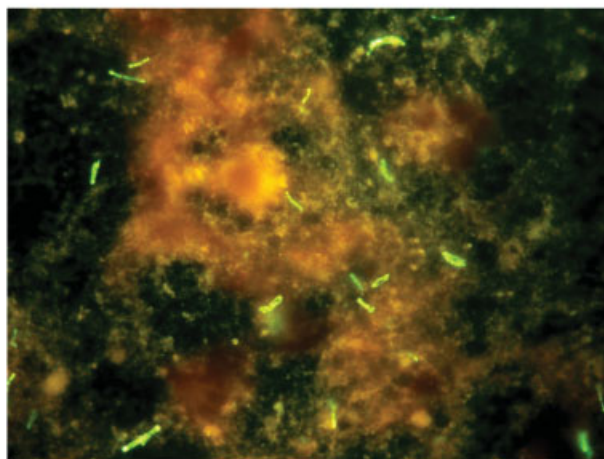


Fig. C-4

Fig. C-1–C-4. **Fig C-1.** Poorly formed granuloma consisting of epithelioid histiocytes and lymphocytes in a clean background, consistent with tuberculous lymphadenitis in an immune competent child (Papanicolaou, $\times 400$). **Fig. C-2.** Numerous neutrophils and karyorrhectic debris in a necrotic background, consistent with tuberculous lymphadenitis in an immune compromised child. (Papanicolaou, $\times 400$). **Fig. C-3.** Modified ZN stain in a lymph node aspirate from an immune compromised child, demonstrating the overwhelming load of mycobacterial organisms present (Papanicolaou, $\times 400$). **Fig. C-4.** Autofluorescence of mycobacteria in a lymph node aspirate (Papanicolaou, $\times 1,000$ with a wide-band blue excitation filter).

In patients with lymphadenitis due to *M bovis* BCG, histiocytes with abundant foamy cytoplasm are present. Because of the considerable morphological variation, the fact that patients with mycobacterial infection may present with aspirates showing nonspecific lymphadenitis and that other organisms such as fungal infections may present a similar clinical and morphological picture, staining for mycobacteria was performed on all aspirates, irrespective of the cytological diagnosis. ZN stains were performed on one of the Giemsa stained slides according to a slightly modified technique. The smears were differentiated in 3% acid alcohol for 1 minute and counterstained with 1% methylene blue for 10 seconds only (Fig. C-3).

One of the Papanicolaou stained slides was screened using a Zeiss Axiophot microscope with a fluorescent attachment and a wide-band blue excitation filter (450–

480 nm). Mycobacteria auto fluoresce as brilliant yellow bacilli, thin and slightly curved with polar enhancement and a uniform length of 2.0–2.7 μm (Fig. C-4).²⁰ Despite identification of the mycobacteria on ZN staining and/or autofluorescence, *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, and *Mycobacterium avium-intracellulare* are morphologically indistinguishable.

Culture samples were inoculated into 7H9 liquid growth medium at the bedside and sent directly to microbiology, according to standard protocol. Cultures were incubated at 37°C for 8 weeks or until indicated as positive, according to standard protocol using the MGIT system (Becton Dickinson, Cockeysville, MD). All mycobacterial isolates were identified as *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG or nontuberculous mycobacteria (NTM) by polymerase chain reaction (PCR) testing.

Parents gave written informed consent for study participation and ethics approval was obtained from the Institutional Review Board of Stellenbosch University.

Results

FNABs were collected from 200 children, and 25 (12.5%) aspirates were inadequate. Samples from 175 children were included in the analysis. Patient demographics and sample data are reported in Table I.

The results for the three diagnostic techniques were as follows. Cultures were positive in 79 cases (45%): 61 *Mycobacterium tuberculosis*, 18 *Mycobacterium bovis* BCG and no NTM positive samples. The median age of children with positive cultures was similar to those with negative cultures (2 years). However, all children with *Mycobacterium bovis* BCG were less than 2 years of age, the majority 16/18 (89%) being infants.

Table II compares the diagnostic performance of the three microscopy techniques cytomorphology, autofluorescence, and ZN staining alone and in combination to culture as the gold standard. However, culture is probably not the optimal gold standard, as a false negative culture

is not uncommon. The previous pilot study investigated the culture negative cases in which any of the other modalities were positive and identified eight false culture negative cases that were clinically assessed as having TB and responded to TB therapy.¹⁶ Patients may commence TB treatment prior to referral for FNAB. Therefore, using culture alone as the gold standard may be flawed, and we identified an alternative gold standard as culture positive or positive cytomorphology plus positive autofluorescence or ZN staining.

Using this alternative gold standard improved the diagnostic performance of all three modalities. The concordance rate with the new standard is 83% for cytomorphology, 79% for autofluorescence, 76% for ZN staining, and 84% for culture (Table III). The sensitivity, specificity, and overall efficacy of these diagnostic modalities are shown in Table IV.

Discussion

In many low- and middle-income countries with limited resources, the diagnosis of TB is still based on poorly validated symptom-based algorithms, often not resulting in a definitive diagnosis.²¹ FNAB provides a rapid and definitive tissue diagnosis in the majority of children with superficial lymphadenopathy. This study demonstrates that it also permits confirmation of the presence of mycobacteria, with fluorescence microscopy being more sensitive than ZN staining. Recent studies have demonstrated that light emitting diode (LED) technology provides a cheap and reliable light source with minimal energy requirements that performs as well as the traditional mercury vapour lamp used in this study.^{22,23} To date, the high cost and short half-life of the mercury vapour lamp has limited the availability of fluorescence microscopy in healthcare systems with constrained financial resources.²³

The 2007 WHO report noted that over half of the populations in the African, South-East Asia, and Western Pacific regions had limited coverage of culture services. Most countries had neither national policies to expand culture and sensitivity testing services nor the technical capacity to implement and support such services. In South Africa., there were 143 laboratories performing smear microscopy and 18 laboratories performing culture.² This is mainly due to centralization of the more expensive and

Table I. Demographics and Sample Characteristics

	Numbers	Percentage
Number of aspirates	200	
Inadequate samples	25	12.5
Specimens included in analysis	175	87.5
Age		
≤1 year	75	42.9
2–4 years	48	27.4
5–9 years	32	18.3
≥10 years	20	11.4
Gender		
Male	82	46.9
Female	93	53.1
Diagnosis		
Malignancy	0	–
Nonspecific node 1	86	49.1
Consistent with mycobacterial infection	89	50.1
Fluorescence positive	73	41.7
ZN positive	68	38.9
Culture positive mycobacterial infection	79	45.0
Organism isolated		
<i>M. Tuberculosis</i>	61	77.0
<i>M. bovis</i> BCG	18	23.0
Nontuberculous mycobacteria	0	–

Table II. Concordance of Diagnostic Modalities with Culture as Gold Standard

	Cytology	Fluorescence	ZN	Any diagnostic modality	Any two diagnostic modalities
Positive agreement	33%	27%	29%	39%	30%
Negative agreement	37%	41%	45%	34%	39%
Concordant	70%	68%	73%	73%	69%
False positive	18%	14%	10%	21%	15%
False negative	12%	18%	17%	6%	15%
Pearson χ^2	29.33	21.48	36.19	43.21	24.86
Phi	0.41	0.35	0.46	0.50	0.38

$n = 175$; all Pearson χ^2 statistics are significant at $P \leq 0.0001$.

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Table III. Concordance of Diagnostic Modalities with New Gold Standard Culture Positive or Cytomorphology Positive Plus Positive Autofluorescence or ZN

	Cytology	Fluorescence	ZN	Culture
Positive agreement	47%	41%	38%	45%
Negative agreement	36%	38%	38%	39%
Concordant	83%	79%	76%	84%
False positive	3%	1%	1%	0%
False negative	13%	20%	23%	15%
Pearson χ^2	81.02	70.59	61.99	93.74
Phi	0.68	0.64	0.60	0.73

$n = 175$; all Pearson χ^2 statistics are significant at $P \leq 0.0001$.

sophisticated culture techniques. Centralization is also a feasible option in resource constrained healthcare systems because FNAB can be done as an outpatient procedure by trained medical or paramedical personnel and correctly fixed slides can be sent to specialized diagnostic facilities for cytological evaluation and fluorescent microscopy.

The diagnosis of TB lymphadenitis is increasingly complicated by the HIV pandemic. HIV infection, in children and adults, is a major contributor to the escalating TB epidemic worldwide.²⁴⁻²⁶ At the end of 2000, it was estimated that there were 11.5 million HIV-infected people coinfecting with TB in sub-Saharan Africa.¹ In 2005, South Africa with 0.7% of the world's population had 19% of all cases of TB in adult HIV-positive people.² Infection with the HIV virus is estimated to increase the lifetime risk of a patient infected with *M tuberculosis* of developing TB by 10-fold to 50%.¹ Children are not exempt from this burden.

A hospital-based study from Zambia demonstrated a HIV seroprevalence rate in children with TB to be >70%, while the minority of childhood TB cases were HIV negative.²⁷ In HIV-infected children the diagnosis of intra- and/or extrathoracic TB is even more difficult due to concomitant chronic diseases such as lymphocytic interstitial pneumonitis (LIP) and other opportunistic infections.^{27,28} Persistent peripheral lymphadenopathy in children is common and is itself a criterion for the classification of HIV-related disease in children.^{29,30} In HIV-infected children, peripheral lymphadenopathy may be associated with many pathological entities, from infections to neoplasia.

A study from South Africa demonstrated that the majority of HIV-infected children with persistent lung disease (PLD), due to a variety of causes, have persistent peripheral lymphadenopathy (PPL) and PPL was present in many children with pulmonary TB (PTB).³¹ Thus, enlarged peripheral lymph nodes were identified as important specimen collection sites to consider in establishing a definitive diagnosis of TB in HIV-infected children.³¹ In HIV-infected children, it is even more important to establish a mycobacteriologic diagnosis, as tuberculin skin tests, symptom-based approaches, and radiology are less

Table IV. Summary of All Diagnostic Modalities Using an Alternative Gold Standard: Culture Positive or Cytomorphology Positive Plus Positive Autofluorescence or ZN

	Cytology (%)	Fluorescence (%)	ZN (%)	Culture (%)
Sensitivity	78	67	62	75
Specificity	91	97	97	100
PPV	93	97	97	100
NPV	73	66	63	72
Efficacy	83	79	76	84

helpful,²⁸ and these children may be at an increased risk of exposure to drug resistant strains of TB.

Disseminated BCG disease is a rare complication of BCG vaccination, classically only described in children with severe congenital immune deficiencies. However, HIV-infected children also appear to be at high risk.^{32,33} BCG vaccination may also associated with local complications such as abscess formation at the vaccination site and/or regional lymphadenitis, with reported rates of lymphadenitis in BCG recipients ranging from 0.5% to 17.6%.²⁴ In South Africa, the recent change in vaccine policy in July 2000 from percutaneous Tokyo strain BCG to intradermal Danish strain BCG vaccine has seen a number of adverse events reported in both HIV-infected and immune competent children.³² In children with axillary or other regional lymph node enlargement, FNAB offers a simple first line diagnostic procedure to confirm a diagnosis of BCG disease.

FNAB is widely utilized in the diagnosis of palpable masses including peripheral lymphadenopathy, and its value in the diagnosis of mycobacterial lymphadenitis in adults is well documented.^{17-20,34,35} It offers a simple, effective, and safe modality for obtaining a representative sample of material from a lymph node, permitting cytological evaluation, identification of the organism by morphology, culture and molecular techniques such as the polymerase chain reaction (PCR). Cytomorphology is simple, but not specific, as other opportunistic infections may present with similar cytological changes and the quality of the smear is operator dependent.^{17,19} With good clinical triage of patients, its sensitivity and specificity can be increased. In our study, cytomorphology achieved a sensitivity of 78% and a specificity of 91%.

ZN staining, even in good laboratories, is generally regarded as insensitive, identifying organisms in only about 20% of culture positive samples.³⁵ This may be dependent on experience, the staining, and the bacterial load. In our study, ZN staining achieved sensitivity of 62% but with a specificity of 97%. Although autofluorescence is simple, sensitive, and inexpensive, it is not widely used. It requires a fluorescent microscope, which may not be readily available, but has the advantage of not requiring additional stains and is therefore inexpensive and rapid. In our study, it performed similar to ZN stain-

ing in identifying mycobacterial infection with a sensitivity of 67% and a specificity of 97%. Its specificity is dependant on the experience of the operator and, as ZN staining, it cannot differentiate between the various mycobacteria.²⁰ These results may be partly due to the high incidence of mycobacterial disease in South Africa and good clinical selection of patients.

Culture, where feasible and affordable, is an important part of the investigation of suspected mycobacterial lymphadenitis. Direct inoculation into MGIT or Bactec™ medium at the bedside gives a high yield,¹⁴ but it takes 1–6 weeks for a result, and fails to differentiate the various mycobacteria unless additional PCR-based testing is available for accurate speciation of *M. tuberculosis* complex. Culture has inherent problems and may be influenced by many factors such as inefficient sampling, contamination, variable bacterial load, and commencement of therapy prior to the diagnostic procedure. If the cytomorphology is consistent with mycobacterial infection, and the organism is identified by ZN staining or autofluorescence, the probability of a false positive diagnosis is small, and patients may safely commence therapy. FNAB of superficial lymph nodes is an outpatient procedure and requires little infrastructure and equipment and is therefore ideal for resource-limited countries, which are the very same countries that bear the burden of HIV and TB.¹⁵ The basic diagnostic modalities of cytomorphology and subsequent morphological identification of the organism are readily available and relatively inexpensive. Although culture adds to the cost of the diagnosis, its value lies in providing an opportunity for speciation and sensitivity testing, as well as surveillance of prevalent strains within a community. Although radiometric methods such as MGIT have reduced the time to culture mycobacterial organisms, this may still be unacceptably long for patients who have difficulty in returning for results. Other innovative methods for diagnosing TB are being developed but remain mainly experimental and are still relatively expensive.²¹

Most children referred with suspected mycobacterial lymphadenitis have a tuberculin skin test performed and must return to the clinic for this to be read with in 48–72 hours. If FNAB is performed at the time of the first visit, the results of the aspirate can be available at the return visit 48 hours later. This enables children to be put on appropriate therapy with a high degree of certainty, pending the results of culture and speciation. In countries where culture is not available, FNAB provides the clinician with the ability to make a definitive diagnosis based on cytomorphology and identification of the organism, particularly in immune compromised children, who require urgent and appropriate treatment. If cytology services are not available, children with suspected mycobacterial lymphadenopathy may have an FNAB performed and

smears made for direct TB microscopy. Although this is not ideal, it provides reasonable diagnostic accuracy, with the additional option to courier-fixed slides to a center with adequate cytology services. This will help health care workers in TB-endemic countries with limited resources to navigate the diagnostic minefield of persistent lymphadenopathy in children.

In conclusion, FNAB proved highly feasible and provided a rapid and definitive diagnosis in the majority of cases, irrespective of the diagnostic modality used.

References

1. Harries A, Maher D, Graham S. TB/HIV: A clinical manual. Geneva: World Health Organization; 2004. WHO/HTM/TB/2004.329.
2. World Health Organization. Global tuberculosis control: Surveillance, planning, financing. Geneva: World Health Organization; 2007. WHO Report 2007, WHO/HTM/TB/2007.376.
3. Beyers N, Gie RP, Schaaf HS, Van Zyl S. A prospective evaluation of children under the age of five years living in the same household as adults with recently diagnosed pulmonary tuberculosis. *Int J Tuberc Lung Dis* 1997;1:38–43.
4. Starke JR. Tuberculosis in children. In: Reichman LB, Herschfield ES, editors. Tuberculosis. A comprehensive international approach. New York: Marcel Dekker; 1993. p 329–367.
5. Marais BJ, Gie RP, Schaaf HS, et al. The natural history of childhood intra-thoracic tuberculosis: A critical review of literature from the pre-chemotherapy era. *Int J Tuberc Lung Dis* 2004;8:392–402.
6. Marais BJ, Donald PR, Gie RP, Schaaf HS, Beyers N. Diversity of disease manifestations in childhood pulmonary tuberculosis. *Ann Trop Paediatr* 2005;25:79–86.
7. Kochi A. The global tuberculosis situation and new control strategy of the WHO. *Tubercle* 1991;72:1–6.
8. Nelson LJ, Wells CD. Tuberculosis in children: Considerations for children from developing countries. *Semin Pediatr Infect Dis* 2004;15:150–154.
9. Walls T, Shingadia D. Global epidemiology of paediatric tuberculosis. *J Infect* 2004;48:13–22.
10. Theart AC, Marais BJ, Gie RP, Hesselting AC, Beyers N. Criteria used for the diagnosis of childhood tuberculosis at primary health care level in a high-burden, urban setting. *Int J Tuberc Lung Dis* 2005;9:1210–1214.
11. Marais BJ, Gie RP, Schaaf HS, Hesselting AC, Enarson DA, Beyers N. The spectrum of disease in children treated for tuberculosis in a highly endemic area. *Int J Tuberc Lung Dis* 2006;10:732–738.
12. Miller FGW, Seale RME, Taylor MD. Tuberculosis in children. Boston: Little Brown; 1963.
13. Marais BJ, Wright CA, Schaaf HS, et al. Tuberculous lymphadenitis as a cause of persistent cervical lymphadenopathy in children from a tuberculosis-endemic area. *Pediatr Infect Dis J* 2006;25:142–146.
14. Kumar RK. Tuberculous lymphadenitis in children—Role of fine needle aspiration cytology. *J Assoc Physicians India* 1999;47:976–999.
15. Wright CA, Pienaar JPP, Marais BJ. Fine needle aspiration biopsy in children—Diagnostic utility in resource-limited settings. *Ann Trop Paedr* 2008;28 (in press).
16. Wright CA, Burgess SM, Geiger D, Wasserman E, van den Burg M. The diagnosis of mycobacterial lymphadenitis in children: Is fine needle aspiration the way to go? In: The 46th Conference of the Federation of South African Societies of Pathology, Durban, South Africa, 2006.

FINE NEEDLE ASPIRATION BIOPSY IN CHILDREN WITH MYCOBACTERIAL INFECTION

17. Kocjan G, Miller R. The cytology of HIV-induced immunosuppression. Changing pattern of disease in the era of highly active antiretroviral therapy. *Cytopathology* 2001;12:281–296.
18. Purohit SD, Purohit V, Mathur ML. A clinical scoring system as useful as FNAC in the diagnosis of tuberculous lymphadenitis in HIV positive patients. *Curr HIV Res* 2006;4:459–462.
19. Nayak S, Mani R, Kavatkar AN, Puranik SC, Holla VV. Fine-needle aspiration cytology in lymphadenopathy of HIV-positive patients. *Diagn Cytopathol* 2003;29:146–148.
20. Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Autofluorescence of mycobacteria on lymph node aspirates—A glimmer in the dark? *Diagn Cytopathol* 2004;30:257–260.
21. Marais BJ, Pai M. Recent advances in the diagnosis of childhood tuberculosis. *Arch Dis Child* 2007;92:446–452.
22. Anthony RM, Kolk AH, Kuijper S, Klatser PR. Light emitting diodes for auramine O fluorescence microscopic screening of *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 2006;10:1060–1062.
23. Hung NV, Sy DH, Anthony RM, Cobelens FGJ, van Soolingen D. Fluorescence microscopy for tuberculosis diagnosis. *Lancet Infect Dis* 2007;7:238–239.
24. Braun MM, Cauthen G. Relationship of the human immunodeficiency virus epidemic to pediatric tuberculosis and Bacillus Calmette-Guerin-vaccination. *Pediatr Infect Dis J* 1992;11:220–227.
25. Jeena PM, Mitha T, Bamber S, Wesley A, Coutoudis A, Coovadia HM. Effects of human immunodeficiency virus on tuberculosis in children. *Tuberc Lung Dis* 1996;77:437–443.
26. Madhi SA, Huebner RE, Doedens L, Aduc T, Wesley D, Cooper PA. HIV-1 coinfection in children hospitalized with tuberculosis in South Africa. *Int J Tuberc Lung Dis* 2000;4:448–454.
27. Graham SM, Coulter JB, Gilks CF. Pulmonary disease in HIV—Infected African children. *Int J Tuberc Lung Dis* 2001;5:12–23.
28. Marais BJ, Cotton M, Graham S, Beyers N. Diagnosis and management challenges of childhood TB in the era of HIV. *J Infect Dis* 2007;196 (Suppl 1):S76–S85.
29. World Health Organization. Provisional guidelines for the diagnosis and classification of the EPI target diseases for primary health care, surveillance and special studies. Geneva: WHO; 1983. EPI/GEN/83/4.
30. Centers for Disease Control and Prevention. 1994. Revised classification system for human immunodeficiency virus infection in children less than 13 years of age. *MMWR* 1994;43:RR-12.
31. Jeena PM, Coovadia HM, Hadley LG, Wiersma R, Grant H, Chrystal V. Lymph node biopsies in HIV infected and noninfected children with persistent lung disease. *Int J Tuberc Lung Dis* 2000;4:139–146.
32. Hesselting AC, Rabie H, Marais BJ, et al. Bacille Calmette-Guerin vaccine-induced disease in HIV-infected and HIV-uninfected children. *Clin Infect Dis* 2006;42:548–558.
33. Hesselting AC, Marais BJ, Gie RP, et al. The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. *Vaccine* 2006;25:14–18.
34. Ersoz C, Polat A, Serin MS, Soyulu L, Demircan O. Fine needle aspiration (FNAB) cytology in tuberculous lymphadenitis. *Cytopathology* 1998;9:201–207.
35. Gupta AK, Nayar M, Chandra M. Critical appraisal of fine needle aspiration cytology in tuberculous lymphadenopathy. *Acta Cytol* 1992;36:391–364.

CHAPTER 6

OPTIMISING CULTURE-BASED AND MOLECULAR DIAGNOSIS

Mycobacterial Transport medium for routine culture of Fine Needle Aspiration Biopsies

CA Wright, C Bamford, Y Prince, A Vermaak, KGP Hoek, BJ Marais, RM Warren,

Archives of Disease in Childhood: in press

**Combining Fine Needle Aspiration Biopsy (FNAB) and High Resolution Melt Analysis
to reduce diagnostic delay in Mycobacterial Lymphadenitis**

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Mycobacterial Transport medium for routine culture of Fine Needle Aspiration Biopsies

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SUMMARY

Fine needle aspiration biopsy (FNAB) offers a simple outpatient technique for specimen collection in paediatric tuberculosis (TB) suspects with peripheral lymphadenopathy. Culture facilities are usually centralized; to perform FNAB with mycobacterial culture on an outpatient basis requires use of a sterile transport medium to facilitate bedside inoculation, maintain organism viability and reduce contamination risk en route to the laboratory.

We compared the mycobacterial yield and time to positive culture following bedside inoculation into standard MGIT tubes versus initial inoculation into an inexpensive “in house” liquid growth medium used for transport to the laboratory followed by immediate and delayed MGIT inoculation (laboratory inoculation).

Over the period of one year 150 FNAB's were performed; 57 (38%) cultured *M. tuberculosis* complex. There was one case each of NTM and *M bovis BCG*, with the remaining 55 being *M tuberculosis*. Results were concordant in 142 (94.7%) bedside and laboratory inoculation pairs. There was no significant difference in time to positive culture between the bedside and laboratory inoculation tubes (16.2 days S.D. 0.87 versus 17.1 days S.D. 0.85). There were 31

pairs with matched immediate and delayed laboratory inoculation culture results, of which 29 were concordant (93.6 %). The 2 discordant pairs showed no growth in the delayed culture; both had required repeated decontamination due to bacterial contamination introduced in the laboratory.

The use of inexpensive “in house” liquid growth medium transport bottles, combined with practical tuition in FNAB, will improve cost effective diagnosis of TB at primary health care level.

INTRODUCTION

Tuberculosis (TB) may be extrapulmonary in 10-30% children,¹ and TB lymphadenitis is the most common extra-pulmonary manifestation of TB in endemic areas;² 5-10% of children may have TB adenitis in association with pulmonary involvement.³ This provides an excellent opportunity to obtain bacteriologic confirmation using fine needle aspiration biopsy (FNAB).⁴ FNAB is a simple and safe outpatient procedure that can be performed by nurses in resource-limited settings⁵ and provides material for direct microscopy as well as culture and susceptibility testing. Cultures from FNAB specimens in children give a greater yield in a shorter time, compared to conventional respiratory specimens such as gastric washings.⁶

Direct bedside inoculation of FNAB specimens is ideal and excellent results have been obtained with commercial liquid media systems such as Mycobacterial Growth Indicator Tubes (MGIT, Beckton Dickinson, USA).⁴ This practice however, is limited by cost and availability.⁴ We aimed to develop a simple and cheap mycobacterial transport medium and then to compare the bacteriologic yield and time to positive culture achieved with direct bedside inoculation into MGIT versus initial use of the transport medium.

MATERIALS AND METHODS

All adults and children referred to the FNAB Clinic at Tygerberg Hospital, Cape Town, South Africa (June 2007-May 2008) with clinical mycobacterial lymphadenitis and in whom written informed consent to participate in the study was obtained (following ethics approval) were included.

TB transport bottles, containing 1 ml aliquots of Middlebrook 7H9 broth (with 0.2% glycerol and 0.05% Tween 80 added) in 10ml headspace glass vials, were prepared “in-house” (Biomedical TB Research Unit by a study scientist, KGPH) in a laminar flow cabinet, sealed with 20mm TFE/Sil Septa and 20mm Aluminium open top seals and autoclaved at 120° C / 20min (Figure 1). Unlike the MGIT tube, the transport bottle does not require removal of the lid, minimizing the risk for contamination.

FNAB was done following standard protocol.⁴ Two needle passes were performed: from the first, cytology slides were prepared before the syringe and needle were rinsed by withdrawing an aliquot of liquid growth media into the syringe and then expelling the contents back into the MGIT tube (bedside inoculation or control). After transport to the laboratory within the same hospital, within 2-24 hours, the MGIT tube was incubated in a BACTEC MGIT 960 machine for 42 days.

From the second FNAB pass, slides were prepared for cytology and the syringe and needle were rinsed into the TB transport bottle. In the laboratory, 0.5ml was aspirated from the transport bottle, inoculated into a separate MGIT tube (immediate laboratory inoculation) and incubated as above. In a subset of consecutive samples, the transport bottle was held at room temperature (12-30 deg Celsius) for 7 days after which subculture was performed as described

above (delayed laboratory inoculation). The delayed inoculation group was designed to mimic the situation of rural clinics where specimens may take some days to reach the laboratory.

PANTA antibiotic mixture (containing polymixin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) (Becton Dickinson, New York, NY, USA) was added to all MGIT tubes before incubation. All positive MGIT tubes were confirmed to contain acid fast bacilli in the absence of bacterial contamination, followed by accurate polymerase chain reaction (PCR) speciation.⁷ The mycobacterial yield and time to positive culture in the respective MGIT tubes were compared using Statistica Version 8 software and the Chi² test.

RESULTS

Over the period of one year 150 FNAB's were performed, and 24.5% of these were in patients less than 16 years, the youngest being 5 months old. *M. tuberculosis* complex was cultured in 57 patients (38%). There was one case each of NTM and *M bovis BCG*, with the remaining 55 being *M tuberculosis*; consistent with the profile of disease in our population.^{3,4,8} In the TB culture negative patients there were an additional 12 cases (11.4%) positive on cytology (morphological evidence of mycobacterial infection and identification of the organism). In 17 patients there was a specific diagnosis other than mycobacterial infection, made on cytology, 13 of these malignant neoplasms.

Results of culture were concordant in 142 (94.7%) bedside and laboratory inoculation pairs, with 8 discordant pairs: 3 were positive on bedside inoculation only and 5 were positive on laboratory inoculation only. In one of the discordant pairs, positive on bedside inoculation and negative on laboratory inoculation there was inadequate material on the cytology slides.

There was no significant difference in time to positive culture between the bedside and laboratory inoculation tubes (16.2 days S.D. 0.87 versus 17.1 days S.D. 0.85).

There were 31 pairs with matched immediate and delayed laboratory inoculation culture results, of which 29 were concordant (93.6 %). The 2 discordant pairs showed no growth in the delayed culture, compared to growth in bedside and immediate laboratory cultures; both had required repeated decontamination due to bacterial contamination introduced in the laboratory. There was no significant difference in time to positive culture between the immediate and delayed laboratory inoculation groups (Table 1).

DISCUSSION

Although FNAB has been shown to be an excellent method for obtaining specimens for culture as well as cytology, the method for transporting specimens to the laboratory is rarely specified.⁹ Mycobacteria need to remain viable during transportation if culture is to be successful and traditional methods for the preservation of specimens, e.g. refrigeration¹⁰ are not applicable to FNAB specimens in resource- poor settings. In most patients FNAB yields insufficient material to express into a sterile tube, and commercial liquid culture media, e.g. BACTEC™ MYCO/F LYTIC are recommended only for blood and body fluids.¹¹

Submission of the syringe (with or without the needle attached) directly to the laboratory compromises the quality of the specimen and puts health care workers at risk. This practice is unacceptable, especially in settings with a high prevalence of MDR TB and/or HIV.

Rinsing the needle and syringe in commercial liquid culture medium, such as MGIT, is very successful in obtaining a positive culture.^{4,5} However, the widespread distribution of MGIT tubes is limited by issues of cost and storage requirements. These tubes need to be stored in the dark and removal of the cap for inoculation outside the laboratory increases the risk for contamination. This study suggests that subculture from a TB transport bottle, even after a 7 day delay, gives similar yields in a similar time period, to direct bedside inoculation. The

few discrepancies (8/150) noted between the bedside and laboratory cultures may be due to random sampling differences as the tubes were inoculated from separate needle passes.

The transport medium described in this study is inexpensive (MGIT tube \$4 vs. \$1 TB transport bottle, 2008 prices in South Africa), easy to prepare “in house”, does not require refrigeration as it is stable through a range of temperatures, and does not require removal of the lid for inoculation. Transport of these specimens from rural clinics may follow the same route as other laboratory specimens such as sputum and peripheral blood. The positive culture yield and the time to positive culture from the transport medium, even after 7 days at room temperature, are statistically comparable to direct bedside inoculation into MGIT tubes. The production and distribution of these bottles to clinics and hospitals, combined with practical tuition in the optimal performance of FNAB, will have a positive impact on the cost effective diagnosis of TB at primary health care level.

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What is already known about this topic.

FNAB is used as to diagnose suspected mycobacterial lymphadenopathy.

Mycobacteria may be seen on staining FNAB material, but this does not allow speciation (TB or non-tuberculous mycobacteria) or antimicrobial sensitivity testing.

What this study adds

A simple and inexpensive means to obtain mycobacterial culture from FNAB specimens was developed using an “in house” liquid growth medium for transport to the laboratory.

Transporting FNAB material to the laboratory in the “in house” medium was similar to a commercial medium with respect to culturing mycobacteria and in time to positive culture.

Similar results were obtained when the ‘in house’ transport bottle was kept at room temperature for 7 days before culturing for mycobacteria.

REFERENCES

- [1] Cruz AT, Starke JR. Clinical manifestations of tuberculosis in children. *Paediatr Respir Rev.* 2007;8(2):107-17.
- [2] Marais BJ, Gie RP, Schaaf HS, et al. The spectrum of childhood tuberculosis in a highly endemic area. *Int J Tuberc Lung Dis.* 2006;10:732-8.
- [3] Marais BJ, Wright CA, Schaaf HS, et al. Tuberculous Lymphadenitis as a Cause of Persistent Cervical Lymphadenopathy in Children From a Tuberculosis-Endemic Area. *Pediatr Infect Dis J.* 2006;25:142-6.
- [4] Wright CA, van der Burg M, Geiger D, et al. Diagnosing Mycobacterial lymphadenitis in children using Fine Needle Aspiration Biopsy: Cytomorphology, ZN staining and Autofluorescence – making more of less. *Diagn Cytopathol.* 2008;36:245-51.

- [5] Wright CA, Pienaar JP, Marais BJ. Fine needle aspiration biopsy: diagnostic utility in resource-limited settings. *Ann Trop Paediatr*. 2008; 28:65-70.
- [6] Wright C, Hesselning A, Marais B. A comparison of Fine Needle Aspiration Biopsy and other specimen collection methods in the diagnosis of Paediatric Tuberculosis. *48th Conference of the Federation of South African Societies of Pathology*. Cape Town, South Africa 2008.
- [7] De Wit D, Steyn L, Shoemaker S, Sogin M. Direct detection of Mycobacterium tuberculosis in clinical specimens by DNA amplification. *J Clin Microbiol*. 1990;28:2437-41.
- [8] Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Auto fluorescence of Mycobacteria on Lymph Node Aspirates – A glimmer in the dark? *Diagn Cytopathol*. 2004;30:257-60.
- [9] Hall GS. Primary processing of specimens and isolation and cultivation of mycobacteria. *Clin Lab Med*. 1996;16:551-66.
- [10] Kent P, Kubica G. Public Health Mycobacteriology A guide for the Level III laboratory. Atlanta: USDHHS, Centers for Disease Control 1985.
- [11] Becton Dickinson Microbiology Systems. BACTEC™ Myco/F Lytic Medium 2009 [cited 02/08/09]; Available from: <http://www.bd.com/ds/productCenter/442288.asp>

Table 1

The mycobacterial yield and time to positive culture achieved with fine needle aspiration biopsy and variable timing of MGIT* inoculation

Timing of MGIT* inoculation	Total no specimens	Mycobacterial culture		Time to positive culture		
		Positive	Negative	Mean days	Standard deviation	95% CI [#]
Bedside vs. immediate laboratory inoculation from transport bottle						
Bedside	150	53 (35.3%)	97 (64.7%)	16.2	0.87	16.8 - 20.3
Immediate laboratory	150	55 (36.7%)	95 (63.3%)	17.1	0.85	15.7-19.1
Subset - matched pairs: delayed vs. immediate laboratory inoculation from transport bottle						
Delayed laboratory (day 7)	31	11 (35.5%)	20 (64.5%)	17.1	1.36	14.1-20.1
Immediate laboratory (day 0)	31	13 (41.9%)	18 (58.1%)	16.2	1.31	13.3-19.2

*MGIT- Mycobacterial Growth Indicator Tube

[#]CI- Confidence Interval

Figure 1. TB Transport Bottle



**Combining Fine Needle Aspiration Biopsy (FNAB) and High Resolution Melt Analysis
to reduce diagnostic delay in Mycobacterial Lymphadenitis**

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ABSTRACT

Tuberculous lymphadenitis is the most common cause of extra-pulmonary tuberculosis (TB) in developing countries. Lymphadenitis caused by non-tuberculous mycobacteria (NTM) requires consideration, particularly in immunocompromised patients and children in developed countries. Fine Needle Aspiration Biopsy (FNAB) offers a valuable specimen collection technique, but culture confirmation, mycobacterial speciation and drug resistance testing (if indicated) is often unavailable in TB endemic areas and result in unacceptable diagnostic delay.

We evaluated the diagnostic value of high-resolution DNA melting (HRM) analysis in the diagnosis of mycobacterial lymphadenopathy using FNAB and an inexpensive transport medium.

Specimens were collected from patients referred to the FNAB Clinic at Tygerberg Hospital (June 2007-May 2008) with clinical mycobacterial lymphadenitis. Cytology, culture and HRM were performed on all specimens. The reference standard for disease was defined as positive cytology (morphological evidence plus mycobacterial visualization) and/or a positive culture

Specimens were collected from 104 patients and mycobacterial disease was confirmed in 54 (51.9 %); 46 *M. tuberculosis*, 1 *M. Bovis BCG*, 1 NTM, and 8 not speciated. Cytology was positive in 83.3 % (45 /54) and culture in 72.2 % (39/54) of patients. HRM identified 57.4 % (31/54) of cases. Using the defined reference standard we recorded 51.9 % sensitivity and 94.0 % specificity (positive predictive value 90.3%) with HRM analysis.

HRM analysis allowed rapid and species specific diagnosis of mycobacterial lymphadenitis in the majority of patients, permitting early institution of appropriate therapy. Optimization of this technique requires further study.

INTRODUCTION

Resource limited countries carry the brunt of the global tuberculosis (TB) epidemic, particularly those affected by the parallel human immunodeficiency virus (HIV) pandemic.¹ According to the most recent World Health Organisation (WHO) estimates, in 2007 there were 9.3 million incident (newly diagnosed) TB cases of whom 1.4 million (14.8%) were HIV-infected. The African region accounted for 79% of HIV-infected TB cases.¹ Although TB incidence rates seem to plateau and/or decline in most regions, absolute numbers continue to rise due to increases in population size. The projected scale of the epidemic and ongoing transmission of drug resistant TB remains alarming.¹ WHO estimated that between 2000 and 2010 1 billion people will be newly infected with *M. tuberculosis*, resulting in 200 million TB cases and 35 million deaths.²

Peripheral lymphadenitis is the most common extra-pulmonary manifestation of TB.³⁻⁴ TB lymphadenitis is also the most common cause of persistent cervical lymphadenopathy in children from TB endemic areas.⁵⁻⁷ In developed countries where the incidence of TB is low, non-tuberculous mycobacteria (NTM) are frequently responsible for mycobacterial lymphadenitis, particularly in children and HIV-infected immune compromised adults.^{3, 8-9} In patients with a peripheral lymph node mass fine needle aspiration biopsy (FNAB) is a

valuable and underutilized specimen collection technique. This simple and safe procedure allows rapid confirmation of mycobacterial disease using cytomorphology and direct mycobacterial visualization with either Ziehl-Neelsen (ZN) staining or fluorescence microscopy.

Mycobacterial culture is required for accurate species determination (speciation) and drug susceptibility testing,¹⁰⁻¹³ since organisms in the *M. tuberculosis* complex are morphologically indistinguishable. The amount of material harvested during FNAB is minimal, and the needle needs to be rinsed at the bedside in liquid medium to facilitate culture. Although FNAB can be safely performed as an outpatient procedure by well trained nurses¹⁴, the need for direct inoculation and unavailability of liquid culture tubes limited decentralization. Use of an inexpensive transport medium for direct inoculation has been described,¹⁵ which should facilitate mycobacterial culture from FNAB's performed in rural clinics and hospitals. Direct bedside inoculation at the time of FNAB collection provides an excellent diagnostic yield but culture results may take up to 6 weeks and requires additional speciation.^{10, 16}

Performing nucleic acid amplification tests (NAATs) on FNAB specimens may provide a rapid species specific diagnosis and expedite access to appropriate therapy. A recent systematic review demonstrated highly variable results with NAATs to diagnose TB lymphadenitis, reported sensitivities ranged between 2-100% (specificities 28-100%).¹⁷ Most NAATs analyze the polymerase chain reaction (PCR) products by gel electrophoresis or other open tube formats, which afford the opportunity for laboratory cross contamination. These technically challenging techniques will pose problems in countries with limited laboratory resources. High resolution DNA melt analysis (HRM) is a simple "closed-tube" technique that reduces the risk of cross contamination. Specific PCR amplicons are identified according to their characteristic DNA melting profile. The amplicons are combined with a saturating dye that fluoresces in the presence of double stranded DNA. It is heated through a range of

temperatures while fluorescence is monitored.¹⁸ As the double stranded DNA dissociates (melts) into single strands the fluorescence decreases. The melting peak of the specific infectious agent is identified. The amplicon specific for *M.tuberculosis* melt at 90.5°C and other members of the *M.tuberculosis* complex at 86°C. This simple technique can be used in routine diagnostic laboratories.¹⁸⁻¹⁹

The aim of our study was to evaluate the value of PCR-based HRM analysis,¹⁸ to provide a rapid and accurate diagnosis of mycobacterial disease using routinely collected FNAB specimens directly inoculated into liquid transport medium.

MATERIALS AND METHODS

All adults and children referred to the FNAB Clinic at Tygerberg Hospital (June 2007-May 2008) with a superficial lymph node mass suggestive of possible mycobacterial lymphadenitis and in whom written informed consent to participate in the study was obtained, were included.

Specimen Collection

FNAB was done following standard protocol as an outpatient procedure by a pathologist.¹⁰ The lymph node was stabilized by the aspirator and two needle passes were performed using a 23g or 25g needle attached to a 10 ml syringe while applying a constant suction of no more than 2 ml. From each aspirate two smears were prepared: one fixed with commercial cytology fixative for Papanicolaou staining and the other air dried for the Giemsa and subsequent ZN staining. The residual material in the syringe and needle was collected by withdrawing an aliquot of liquid growth media from the TB transport bottle into the syringe and then expelling the contents back into the bottle. No additional needle passes were performed to collect material for microbiology or PCR. The TB transport bottles were prepared “in-house” in a laminar flow cabinet: 10ml headspace glass vials containing 1 ml of Middlebrook 7H9

broth (with 0.2% glycerol and 0.05% Tween 80 added), sealed with 20mm TFE/Sil Septa and 20mm Aluminium open top seals and autoclaved at 120° C for 20min.²⁰

Cytology

Stained smears were evaluated for adequacy and to make a morphological diagnosis. If any specific lesion such as a lymphoepithelial cyst or neoplasia was present, this was reported. Alternatively we reported “non-specific reactive lymphadenopathy” or “cytologically consistent with mycobacterial infection”, based on specific morphological criteria.²¹⁻²³ In immunocompetent patients with TB, epithelioid granulomata and epithelioid histiocytes may be identified in a background of reactive lymphocytes and plasma cells (Fig 1). A small amount of amorphous necrosis may also be identified. In immunocompromised patients, the cytological picture is that of abundant necrosis in which neutrophils and cellular debris are prominent (Fig 2). In patients with lymphadenitis due to *M bovis* BCG or NTM infection, histiocytes with abundant foamy cytoplasm may be present.^{8, 24-25}

Mycobacteria were visualized using a ZN stain performed on a Giemsa stained slide according to a slightly modified technique (Fig 3). Smears were differentiated in 3% acid alcohol for one minute and counterstained with 1% methylene blue for 10 seconds only. In addition, one of the Papanicolaou stained slides was screened using a Zeiss Axiophot microscope with a fluorescent attachment and a wide-band blue excitation filter (450–480 nm); mycobacteria fluoresce as brilliant yellow bacilli, thin and slightly curved with polar enhancement and a uniform length of 2.0–2.7 microns (Fig 4).¹¹

Microbiology

In the laboratory, 0.5ml of the media was aspirated from the TB transport bottle, inoculated into a separate MGIT tube containing the PANTA antibiotic mixture and incubated in a BACTEC MGIT 960 machine for 42 days. All positive MGIT tubes were confirmed to contain acid fast bacilli in the absence of bacterial contamination by ZN staining.

Mycobacteria were identified as *M. tuberculosis* or other by standard PCR.²⁶

Extraction of mycobacterial DNA

The TB transport bottles containing the remaining 0.5ml media were stored at -20°C until further analysis. Mycobacteria within a 250µl aliquot were pelleted by centrifugation at full speed (14 000rpm) for 10 minutes. The supernatant was discarded and the bacterial pellet was resuspended in 1ml phosphate buffered saline and re-centrifuged at full speed (14 000rpm) to remove residual blood. Thereafter the bacterial pellet was resuspended in 30µl ddH₂O and heat inactivated at 100°C for 20 minutes. The lysed bacterial extract was stored on filter paper (FTA[®] Classic Card Collection, Storage and Purification system, Whatman, UK). Genomic DNA was eluted from the filter paper cards following manufacturers' instructions (high pH, low pH protocol) and served as the template for subsequent PCR amplification.

Speciation and HRM Analysis

Primers were designed to amplify the Region of Deletion 9 (RD9), present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the *M. tuberculosis* complex. PCR was performed as previously described¹⁹ using an annealing temperature of 62°C and the following primers RD9Fs1 5'-CAA GTT GCC GTT TCG AGC C-3', RD9FR 5'-GCT ACC CTC GAC CAA GTG TT-3' and RD9INT 5'-CAA TGT TTG TTG CGC TGC-3'.²⁷

Resulting amplification products underwent HRM analysis in a Rotorgene[™]6000 real-time rotary analyzer (Corbett Life Science, Australia). The thermal denaturation profiles were measured as previously described.¹⁹ The infectious agent was identified by the Rotorgene[™]6000 software according to the presence of derivative melt peaks located within defined temperature bins. *M. tuberculosis* PCR products melt at 90°C (*M. tuberculosis* could not be differentiated from *M. canettii*, but *M. canettii* is very rarely observed and has not been recorded in the study setting) while PCR products of other members of the *M. tuberculosis* complex melted at 86°C. (Fig.4)

Reference standard for mycobacterial disease

The reference standard for the presence of mycobacterial disease¹⁷ was defined as positive cytology (morphology consistent with mycobacterial infection plus direct visualization of the organism) and/or positive culture with speciation.

Statistical Analysis

We assessed the diagnostic accuracy of PCR-based HRM analysis compared to cytology, mycobacterial culture and the reference standard as defined using Pearson's chi-square (X^2) and Fisher's exact tests.²⁸ All analyses were conducted using Statistica Version 8.²⁹ Ethics approval was obtained from the Institutional Review Board of Stellenbosch University (N05/03/043).

RESULTS

FNAB specimens were collected from 104 patients with possible mycobacterial lymphadenitis in whom complete cytopathology and mycobacterial culture results were available together with PCR-based HRM analysis. The median age was 30 years with a range from 4 months to 62 years. Children less than 13 years of age comprised 21.2 % of the study population. There was no significant difference in gender distribution. HIV status was known in 46.1% of patients (48 /104) and 75% of these were HIV positive (36/48). There were 23 HIV-positive patients and 6 HIV negative patients with mycobacterial disease as defined by the reference standard above. Table 1 summarizes the demographics and diagnostic outcome of the study population.

Applying the defined reference standard 54 of the 104 patients (51.8 %) were diagnosed with mycobacterial lymphadenitis. Mycobacterial culture was positive in 39/54 (72.2 %) patients; 37 *M. tuberculosis*, one *M. bovis* BCG and one NTM. The child with *M. bovis* BCG was less than two years old; four others in this age group were positive for *M. tuberculosis*. Cytology was positive in 83.3.9 % (45/54) of the cases. Cytology and culture

were both positive in 30/54 (55.6%) cases, while HRM analysis was positive in 57.4 % (31/54) of cases identified using the reference standard. The calculated sensitivity of HRM analysis was 51.9 % and the specificity 94.0 % (Fig 2) with a negative predictive value of 64.4 % and a positive predictive value of 90.3 % (Fig 5). Concordance between the different diagnostic modalities and HRM analysis was poor, with Kappa values of 0.39 vs. cytology, 0.27 vs. mycobacterial culture, 0.19 vs. both cytology and culture, and 0.45 vs. cytology and/or culture (the defined reference standard).

To investigate a possible association between mycobacterial load and the result of PCR-based HRM analysis we tested for any correlation between the time to positivity (TTP, often used as a surrogate of mycobacterial load) and PCR outcome. Among those with positive mycobacterial cultures no correlation between the time to positivity and PCR-based HRM analysis could be demonstrated; mean TTP in PCR positive cases was 18 days compared to 22 days in PCR negative cases ($p=0.22$). (Fig 5)

DISCUSSION

With global coordination of control efforts TB incidence rates seem to be stabilizing and showing signs of decline in all six world regions. However, the TB disease burden in areas plagued by the concurrent HIV pandemic remains at unprecedented levels.¹ Although there is some assistance for the development of strong laboratory networks in developing countries, the existing infrastructure remains poor in most TB endemic areas. South Africa, despite its relatively good infrastructure and health care services, in 2007 reported a national TB incidence of 739.6/100,000 population and an adult TB incidence in the Western Cape Province, a high burden region, of 1005.7 /100.000.³⁰

In TB endemic areas with ongoing transmission, children constitute a significant percentage of the total caseload, estimated at 15-20%.³¹ Immune immaturity and/or compromise, most often due to HIV infection,³²⁻³³ influence the risk to develop extra

pulmonary and/or disseminated TB as well as the likelihood of rapid disease progression and TB-related mortality.³⁴⁻³⁵ Autopsy studies in Africa^{33, 36-39} have shown that up to 54% of deaths in HIV-infected adults and 20% in HIV-infected children are due to TB. Reducing this mortality and morbidity necessitates early detection, efficient diagnosis and timely institution of appropriate therapy.

FNAB is a simple, non-invasive specimen collection technique in patients with peripheral lymph node masses¹⁰. It assists with rapid diagnosis of mycobacterial disease, but also helps to rule out alternative diagnoses that may require urgent treatment such as neoplasia. Diagnostic sensitivity is dependent on the experience of the pathologist and for mycobacterial disease varies from 32-78%.^{10, 40-41} Identification of the organism is essential particularly in immune compromised patients, as other opportunistic infections such as fungal organisms may elicit a similar morphological reaction pattern. Mycobacteria may be visualized using ZN staining, which is a very simple stain but has suboptimal sensitivity (20-62%).^{10, 17, 42-43} Autofluorescence, using the ability of mycobacteria on Papanicolaou stains smears to fluoresce using a blue excitation filter requires no additional staining and improves sensitivity (65-67%).¹⁰⁻¹¹ Culture is often regarded as the “gold standard”, but is limited by poor sensitivity (reported to be as low as 2-34%)^{41, 44} and is highly dependent on the culture medium and inoculation technique used. A positive result is delayed by 1-6 weeks and requires additional PCR-based testing for speciation.

Direct NAAT application provides results in 3 to 6 hours and has been evaluated with respiratory and non-respiratory specimens such as FNAB's.^{17, 43-47} Test sensitivity with respiratory specimens is highly dependent on mycobacterial load and use current PCR-based tests are only advised in patients with sputum smear-positive TB. Results with extra-pulmonary disease have been variable but recent studies have shown excellent results. Use of a nested PCR in cervical TB lymphadenitis in Mexican children showed a sensitivity of 96% and a specificity of 93%,⁴⁷ whilst a study utilizing DNA from dried and fixed cytology smears

showed a sensitivity of 85% and specificity of 95% using nested PCR, although reference standards are not necessarily based on bacteriological confirmation of disease.⁴⁸

The majority of these studies use agarose gel electrophoresis to visualize the products, which is not practical in a busy microbiology laboratory. The open tube nature of the procedure also allows the release of amplicons, which pose a real risk of cross-contamination. A recent systematic review evaluating the diagnostic utility of NAATs in TB lymphadenitis identified 36 peer reviewed publications.¹⁷ The authors found marked variation in populations, test techniques, reference standards, volume of material utilized and quality indicators. Few studies controlled for cross contamination and inhibitors in clinical samples, thereby increasing the possibility of both false positive and false negative results. The reference standard used most frequently was culture, although the authors regard the sensitivity of culture from FNAB to be about 62%. Using an imperfect reference standard may lead to an underestimation of test performance and this is a major limitation that is rarely acknowledged.⁴⁹

The current study attempted to address some of the shortcomings noted above. The reference standard used is a combination of positive cytology, defined as cytomorphological features consistent with mycobacterial infection combined with identification of the organism using ZN staining or autofluorescence and/or positive culture. The residual material from the fine needle aspirates was collected in TB transport medium in a sealed bottle, minimizing the possibility of contamination. After washing and concentration of the specimen the pellets were placed on filter paper for storage and to remove inhibitors present in the specimen. After extraction of DNA, the products were amplified using primers designed to amplify the Region of Deletion 9 (RD9), present in *M. tuberculosis* and *M. canetti*, but absent from all other members of the complex.¹⁹ The amplified products were then identified using high resolution melt analysis, which is a closed tube format that minimizes the possibility of cross-contamination.

This technique is rapid and simple, and the equipment required for the HRM such as the Rotorgene 6000™ real-time rotary analyzer (Corbett Life Science, Australia) is relatively inexpensive. Up to 72 samples may be processed in a cycle which takes approximately 3 hours.

The results of PCR using this technique did not differ significantly from that of cytology or culture when these were assessed independently against the reference standard. The sensitivity is relatively low, but the specificity is high at 94% as is the PPV of 90%. This enables appropriate therapy to be implemented early with a high degree of confidence in the majority of patients with disease, while continuing with culture in the PCR negative cases.

Limited sample volume may have accounted for the low sensitivity observed. No additional needle passes or aspirates were performed and the material collected was limited to the residual amount left in the needle. Sensitivity may be improved if an additional needle pass is performed to obtain material for PCR. No single diagnostic modality was adequately sensitive to enable it to be used alone. FNAB provides material for cytology, culture and PCR using the transport medium described. This is of particular value in resource limited countries where laboratories tend to be centralized.¹ Cytology slides once prepared are stable at room temperature, as is the transport medium where the mycobacterial organism has been shown to remain viable for up to 7 days at room temperature, enabling both culture and PCR.²⁰

Ideally, the PCR technique could be refined, increasing the sensitivity and thereby eliminating the need for culture, which is costly and lengthy. Use of the transport bottle facilitates both culture and PCR analysis, but material may be deposited directly onto filter paper for subsequent PCR analysis. Refined techniques need to remain simple inexpensive and appropriate for use in routine laboratories in countries with limited resources and skills that bear the burden of this devastating and persistent disease.

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REFERENCES

- [1] World Health Organization. Global Tuberculosis Control: Surveillance, Planning, Financing: WHO Report 2007. Geneva: World Health Organization; 2007. Report No.: WHO/HTM/TB/2007.376.
- [2] Shingadia D, Novelli V. Diagnosis and treatment of tuberculosis in children. *Lancet Infect Dis* 2003;3:624-632.
- [3] Golden MP, Vikram HR. Extrapulmonary tuberculosis: an overview. *Am Fam Physician* 2005;72:1761-1768.
- [4] Marais BJ, Wright CA, Schaaf HS, et al. Tuberculous Lymphadenitis as a Cause of Persistent Cervical Lymphadenopathy in Children From a Tuberculosis-Endemic Area. *Pediatr Infect Dis J* 2006;25:142-146.
- [5] Marais BJ, Gie RP, Schaaf HS, et al. The natural history of childhood intra-thoracic tuberculosis: a critical review of literature from the pre-chemotherapy era State of the Art. *Int J Tuberc Lung Dis* 2004;8:392-402.
- [6] Marais BJ, Gie RP, Schaaf HS, et al. The clinical epidemiology of childhood pulmonary tuberculosis: a critical reading of literature from the pre-chemotherapy era. *Int J Tuberc Lung Dis* 2004;8:278-285.

- [7] Moore SW, Schneider JW, Schaaf HS. Diagnostic aspects of cervical lymphadenopathy in children in the developing world: a study of 1,877 surgical specimens. *Pediatr Surg Int* 2003;19:240-244.
- [8] Hazra R, Robson CD, Perez-Atayde AR, Husson RN. Lymphadenitis Due to Nontuberculous Mycobacteria in Children: Presentation and Response to Therapy. *Clin Infect Dis* 1999;28:123-129.
- [9] Flint D, Mahadevan M, Barber C, Grayson D, Small R. Cervical lymphadenitis due to non-tuberculous mycobacteria: surgical treatment and review. *Int J Pediatr Otorhinolaryngol* 2000;53:187-194.
- [10] Wright CA, van der Burg M, Geiger D, et al. Diagnosing Mycobacterial lymphadenitis in children using Fine Needle Aspiration Biopsy: Cytomorphology, ZN staining and Autofluorescence – making more of less. *Diagn Cytopathol* 2008;36:245-251.
- [11] Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Auto fluorescence of Mycobacteria on Lymph Node Aspirates – A glimmer in the dark? *Diagn Cytopathol* 2004;30:257-260.
- [12] Thomas JO, Adeyi D, Amanguno H. Fine Needle Aspiration in the Management of Peripheral Lymphadenopathy in a Developing Country. *Diagn Cytopathol* 1999;21:159-162.
- [13] Bezabih M, Marium DW, Selassie SG. Fine needle aspiration cytology of suspected tuberculous lymphadenitis. *Cytopathology* 2002;13:284-290.
- [14] Leiman G. The Registered Nurse as Aspirator: Experience in a Busy Cytopathology Unit. *Acta Cytol* 1995;39:1010.
- [15] Wright CA, Bamford C, Prince Y, et al. Mycobacterial Transport medium for routine culture of Fine Needle Aspiration Biopsies. *Arch Dis Child* 2009;In press.
- [16] Wright CA, Hesseling A, Bamford C, et al. Fine-needle aspiration biopsy: a first-line diagnostic procedure in paediatric tuberculosis suspects with peripheral lymphadenopathy. *Int J Tuberc Lung Dis* 2009;in press.

- [17] Daley P, Thomas S, Pai M. Nucleic acid amplification tests for the diagnosis of tuberculous lymphadenitis: a systematic review. *Int J Tuberc Lung Dis* 2007;11:1166-1176.
- [18] Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics* 2007;8:597-608.
- [19] Hoek KGP, Gey van Pittius NC, Moolman-Smook H, et al. Fluorometric Assay for Testing Rifampin Susceptibility of Mycobacterium tuberculosis Complex. *J Clin Microbiol* 2008;46:1369-1373.
- [20] Vermaak A, Wright CA, Bamford C, et al. TB Transport Medium for Fine Needle Aspiration Biopsies *TB Conference 2008*. Durban, South Africa 2008.
- [21] Purohit SD, Purohit V, Mathur ML. A Clinical Scoring System as Useful as FNAC in the Diagnosis of Tuberculous Lymphadenitis in HIV Positive Patients. *Current HIV Research* 2006;4:459-462.
- [22] Kocjan G, Miller R. The cytology of HIV-induced immunosuppression. Changing pattern of disease in the era of highly active antiretroviral therapy. *Cytopathology* 2001;12:281-296.
- [23] Nayak S, Mani R, Kavatkar AN, Puranik SC, Holla VV. Fine-needle aspiration cytology in lymphadenopathy of HIV-positive patients. *Diagn Cytopathol* 2003;29:146-148.
- [24] Schaaf HS, Zumla A. *TUBERCULOSIS: A Comprehensive Clinical Reference*. Philadelphia: SAUNDERS, Elsevier 2009.
- [25] Al-Bhlal LA. Fine-needle aspiration cytology of postvaccinal disseminated bacillus Calmette-Guerin infection. *Diagn Cytopathol* 2001;24:333-335.
- [26] De Wit D, Steyn L, Shoemaker S, Sogin M. Direct detection of Mycobacterium tuberculosis in clinical specimens by DNA amplification. *J Clin Microbiol* 1990;28:2437-2441.

- [27] Warren RM, Gey PNC, Barnard M, et al. Differentiation of Mycobacterium tuberculosis complex by PCR amplification of genomic regions of difference. *Int J Tuberc Lung Dis* 2006;10:818-822.
- [28] Agriesti A. *Categorical Data Analysis*. 2nd ed. Hoboken, New Jersey: Wiley 2002.
- [29] StatSoft Inc. *Statistica, Version 8*. Tulsa, Oklahoma: Statsoft, Inc. 2007.
- [30] Health Systems Trust. 2007 6 March 2009 [cited 6 March 2009]; Available from: <http://www.hst.org.za/healthstats/16/data>
- [31] Marais BJ, Gie RP, Schaaf HS, et al. Childhood pulmonary tuberculosis: old wisdom and new challenges. *Am J Respir Crit Care Med* 2006;173:1078.
- [32] Braun MM, Cauthen G. Relationship of the human immunodeficiency virus epidemic to paediatric tuberculosis and Bacillus Calmette-Guerin vaccination. *Pediatr Infect Dis J* 1992;11:220-227.
- [33] Madhi SA, Huebner RE, Doedens L, et al. HIV-1 coinfection in children hospitalised with tuberculosis in South Africa. *Int J Tuberc Lung Dis* 2000;4:448-454.
- [34] Marais BJ, Graham SM, Cotton MF, Beyers N. Diagnostic and management challenges for childhood tuberculosis in the era of HIV. *J Infect Dis* 2007;196:76-85.
- [35] Walters E, Cotton MF, Rabie H, et al. Clinical presentation and outcome of Tuberculosis in Human Immunodeficiency Virus infected children on anti-retroviral therapy. *BMC Pediatrics* 2008;8:1.
- [36] Lucas SB, Hounnou A, Peacock C, et al. The mortality and pathology of HIV infection in a West African city. *AIDS* 1993;7:1569-1579.
- [37] Chintu C, Mudenda V, Lucas SB. Lung diseases at necropsy in African children dying from respiratory illnesses; a descriptive necropsy study. *Lancet* 2002;360:985-990.
- [38] Kiwanuka J, Graham SM, Coulter JBS, et al. Diagnosis of pulmonary tuberculosis in children in an HIV-endemic area, Malawi. *Ann Trop Paeds* 2001;21:5-14.

- [39] Jeena PM, Pillay P, Pillay T, Coovadia HM. Impact of HIV-one coinfection on presentation and hospital-related mortality in children with culture proven pulmonary tuberculosis in Durban, South Africa. *Int J Tuberc Lung Dis* 2002;6:672-678.
- [40] Lau SK, Wei WI, Hsu C, Engzell UCG. Efficacy of fine needle aspiration cytology in the diagnosis of tuberculous cervical lymphadenopathy. *J Laryngol Otol* 1990;104:24-27.
- [41] Singh KK, Muralidhar M, Kumar A, et al. Comparison of in house polymerase chain reaction with conventional techniques for the detection of *Mycobacterium tuberculosis* DNA in granulomatous lymphadenopathy. *J Clin Pathol* 2000;53:355-361.
- [42] Gupta AK, Nayar M, Chandra M. Critical appraisal of fine needle aspiration cytology in tuberculous lymphadenopathy. *Acta Cytol* 1992;36:391-394.
- [43] Gong G, Lee H, Kang GH, et al. Nested PCR for diagnosis of tuberculous lymphadenitis and PCR-SSCP for identification of rifampicin resistance in fine-needle aspirates. *Diagn Cytopathol* 2002;26:228-231.
- [44] van Copenraet ESB, Lindeboom JA, Prins JM, et al. Real-Time PCR Assay Using Fine-Needle Aspirates and Tissue Biopsy Specimens for Rapid Diagnosis of Mycobacterial Lymphadenitis in Children. *J Clin Microbiol* 2004;42:2644-2650.
- [45] Ling DI, Flores LL, Riley L, Pai M. Commercial Nucleic-Acid Amplification Tests for diagnosis of Pulmonary Tuberculosis in Respiratory Specimens: Meta-Analysis and Meta-Regression. *PLoS ONE* 2008:e1536.
- [46] Aljafari AS, Khalil EAG, Elsiddig KE, et al. Diagnosis of tuberculous lymphadenitis by FNAC, microbiological methods and PCR: a comparative study. *Cytopathology* 2004;15:44-48.
- [47] Portillo-Gomez L, Murillo-Neri MV, Gaitan-Mesa J, Sosa-Iglesias EG. Nested polymerase chain reaction in the diagnosis of cervical tuberculous lymphadenitis in Mexican children. *Int J Tuberc Lung Dis* 2008;12:1313-1319.

[48] Purohit MRMD, Mustafa TMP, Sviland LMDMP. Detection of Mycobacterium tuberculosis by Polymerase Chain Reaction With DNA Eluted From Aspirate Smears of Tuberculous Lymphadenitis. *Diagnostic Molecular Pathology* 2008;17:174-178.

[49] Pai M, O'Brien R. Tuberculosis diagnostics trials:do they lack methodological rigor? *Expert Rev Mol Diagn* 2006;6:509-514.

Table 1**Demographics and diagnostic outcome**

	Numbers	Percentage
Number of cases	104	
Age		
≤ 2 years	6	5.8
≤13 years	22	21.2
> 13 years	77	74
Unknown	5	5
Gender		
Male	48	46.2.
Female	51	49.0
Unknown	5	4.8
HIV status		
Positive	36	34.6
Negative	12	11.5
Unknown	56	53.9
Cytodiagnoses		
Malignancy	12	11.5
Reactive node	43	41.4
Other (e.g. cyst)	4	3.9
Cytology positive mycobacterial infection	45	43.3
Cases with mycobacterial disease		
Culture and/or cytology positive (Reference Std)	54	100
Cytology positive	45	83.3
Culture positive	39	72.2
Culture positive and cytology positive	30	55.6
PCR positive mycobacterial infection	31	57.4

Figure 1. Epithelioid granuloma in an immune competent patient. (Papanicolaou, x400)

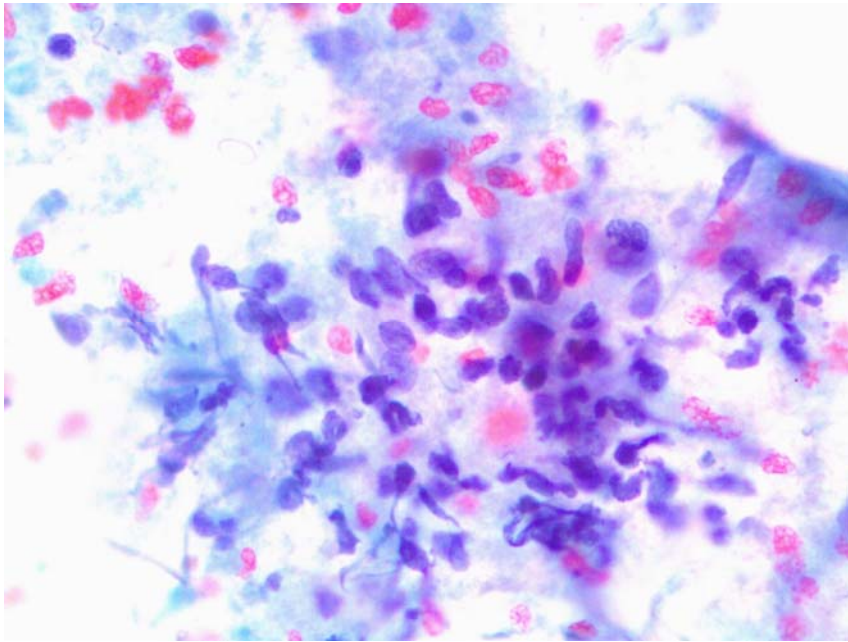


Figure 2. Abundant necrosis in which neutrophils and cellular debris are prominent consistent with tuberculous lymphadenitis in an immune compromised patient. (Papanicolaou, x400)

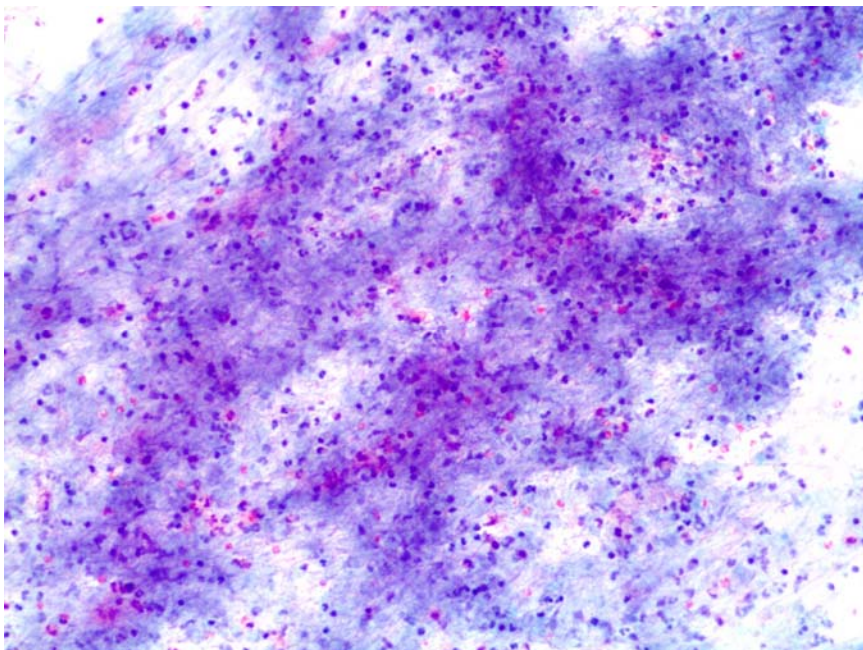


Figure 3. Modified ZN stain in a lymph node aspirate. (Ziehl-Neelson, x400)

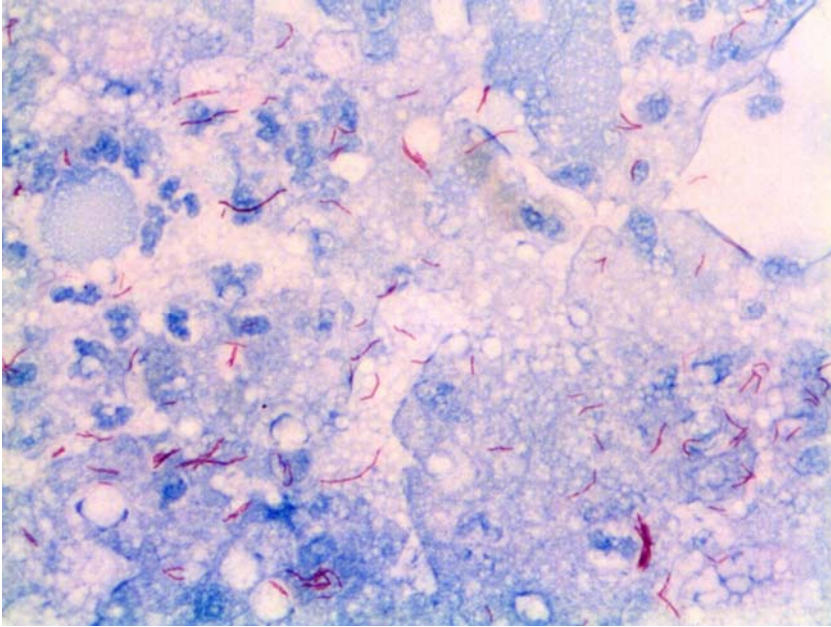


Figure 4. Autofluorescence of mycobacteria in a lymph node aspirate.

(Papanicolaou, x1000 with a wide-band blue excitation filter)

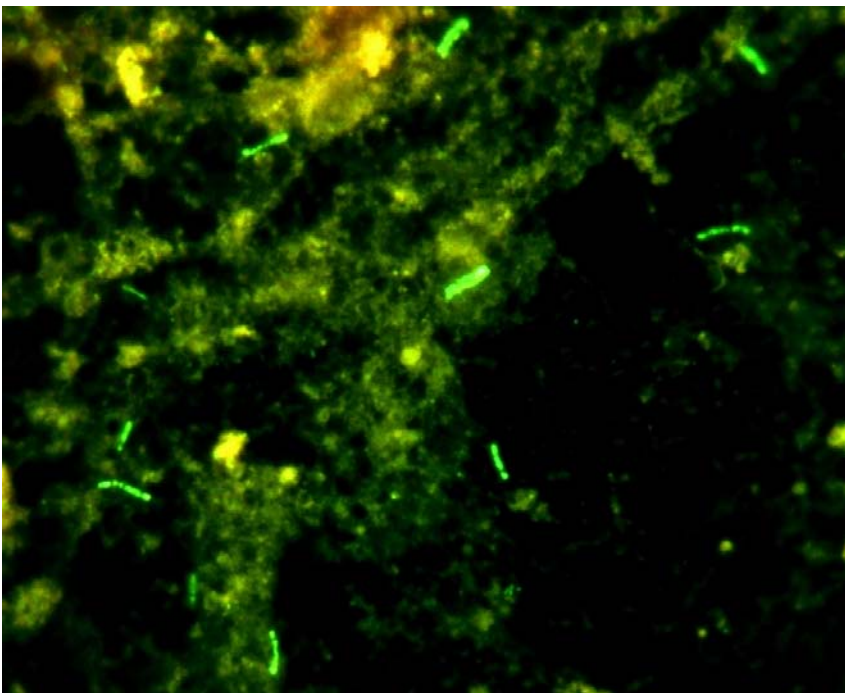
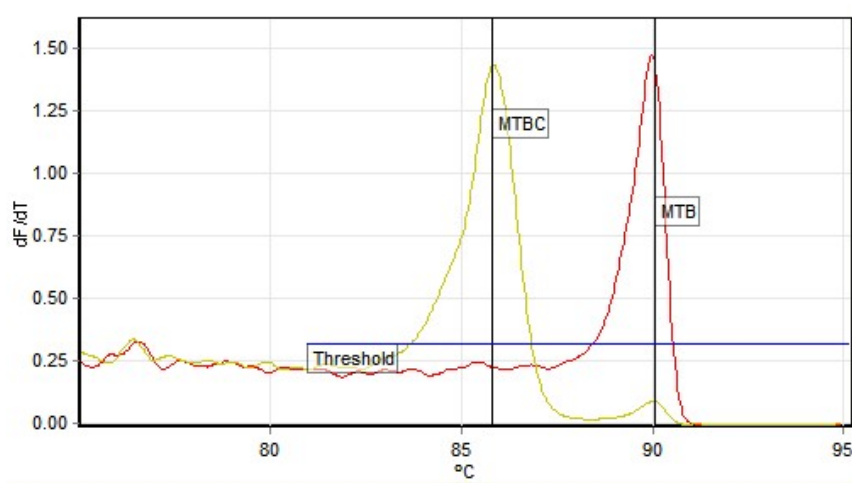


Fig 5 Rotorgene software depicting the derivative melt peaks located within defined temperature bins. *M.tuberculosis* products melted at 90°C and *M.tuberculosis* complex at 86°C.



No.	Colour	Name	Genotype	Peak 1
1	■	<i>M.tuberculosis</i>	MTB	89.98 (MTB)
2	■	<i>M.bovis</i>	MTB complex	85.85 (MTBC)

CHAPTER 7

CONCLUSION

The aim of this dissertation was to demonstrate the value of Fine Needle Aspiration Biopsy in the diagnosis of mycobacterial lymphadenitis with particular reference to children.

Mycobacterial disease in children poses a particular diagnostic and management problem. It is a major cause of childhood morbidity and mortality in developing countries, where it is under reported due to problems in obtaining an accurate diagnosis. The traditional methods used to diagnose tuberculosis (TB) in adults are not feasible in children under the age of 7-8 years, young children experience the greater disease burden and a significant percentage (~10%) of children have extra pulmonary disease manifestations only.

Clinical criteria and chest radiography have diagnostic limitations which are compounded in children who are HIV infected. In young children and in those with HIV/AIDS, early diagnosis is essential, as dissemination may occur rapidly due to their immature or compromised immune system.

We first established that TB lymphadenitis is a common clinical problem in children in TB endemic areas and that FNAB is an efficient, simple and effective diagnostic modality in determining the cause of lymphadenopathy in children.

We showed that FNAB provides superior diagnostic yields and a significant reduction in time to diagnosis when compared with conventional laboratory specimens in children.

Autofluorescence was demonstrated to be an accurate, rapid and inexpensive method for demonstrating mycobacteria on routinely stained cytology smears and this was confirmed in a prospective study in children where FNAB was shown to provide a rapid and definitive diagnosis in the majority of cases with TB lymphadenitis.

We developed a new reference standard for mycobacterial disease using FNAB - positive cytology (cytomorphological evidence of disease and identification of the organism) and/or positive culture.

Lymphadenopathy induced by BCG vaccination needs to be distinguished from TB lymphadenitis. Although not central to this thesis, a small study during this time period demonstrated cytomorphological features on FNAB which may assist in distinguishing between these two organisms, together with clinical features. (See Appendix A)

Although cytomorphology may confirm mycobacterial disease, organisms in the *M tuberculosis* complex are morphologically indistinguishable. This requires submission of material for culture and subsequent speciation or submission of material for direct PCR. As FNAB yields a miniscule amount of material, immediate bedside inoculation is required for culture. We developed an inexpensive liquid transport medium which facilitated culture from FNAB specimens, in which organisms remain viable for up to 7 days at room temperature. This is particularly useful in resource limited countries where laboratories are centralised and transport of specimens may be problematical.

Culture for speciation of mycobacterial organisms takes up to 6 weeks and this may result in inappropriate treatment or delayed commencement of therapy. Using the transport medium developed we established a rapid simple and inexpensive technique using PCR and High Resolution Melt Analysis, which is feasible for implementation in routine diagnostic laboratories.

In summary, the outcome of these studies has demonstrated the need for, and value of, FNAB in the diagnosis of mycobacterial lymphadenitis particularly in children in TB endemic areas.

We have improved the ability of cytology to confirm mycobacterial disease using autofluorescence. We have developed a means to obtain a culture from FNAB using a novel

transport medium and have developed a simple nucleic acid amplification technique which will enable rapid confirmation and speciation of mycobacterial disease.

There are many challenges that remain, but we are working to develop an even simpler technique for collecting material from FNAB for PCR/HRM analysis which would provide higher sensitivity by removing inhibitors in the sample.

Conventional fluorescent microscopy using mercury vapour lamps is expensive and therefore not feasible in resource-limited countries, and we intend to demonstrate the utility of inexpensive rewindable LED light sources in autofluorescence and auramine stained FNAB smears. A retrospective comparative study has already been completed. (see addendum A)

The quest to find and establish the ideal modality for diagnosing mycobacterial disease has been a long one for many researchers. The many clinical manifestations of this elusive disease and the reality that it is a disease that affects the poor and less influential segments of the global population compounds the problem.

Currently a lot of attention is focused on identifying improved methods for TB diagnosis. The field is ever-changing and no sooner has a technique been optimised when it is evident that a better methodology has emerged. We hope that this research has contributed a small step towards success in the fight against this devastating disease.

REFLECTIVE ASSESSMENT OF CONTRIBUTION.

In summary this thesis makes the following theoretical, empirical and methodological contributions to the literature.

Theoretical contribution:

FNAB is utilised in adult patients in neoplastic and infectious diseases, and to a lesser extent in paediatric oncology, but its potential contribution in paediatrics is not sufficiently appreciated. These results demonstrate the utility of FNAB in the diagnosis of TB, especially in children. The studies show that FNAB is equal to or superior to conventional diagnostic specimen types in children and that it is accurate, easy to perform, and yields rapid diagnostic information.

Empirical contribution:

Countries with low economic resources and high healthcare resource constraints bear the brunt of TB and HIV infections. This body of research demonstrates that FNAB can be implemented in these settings in the diagnosis of mycobacterial disease in children. It shows how FNAB and a novel transport medium solve problems in the collection of material for culture and NAAT, and autofluorescence assists in the cytological diagnosis of mycobacterial disease.

Methodological contribution:

The methodologies and concepts used in this thesis have been developed specifically for utilisation in resource limited countries.

A new method of NAAT on FNAB material is developed and its benefits assessed.

This new method is appropriate for use in resource limited countries. Although NAAT's have

been used in the diagnosis of mycobacterial disease on FNAB, the results are extremely variable and impractical for implementation in routine diagnostic laboratories. The new method utilises high resolution DNA melt analysis was developed which is rapid inexpensive, minimises contamination and is applicable for implementation in routine diagnostic laboratories. This was performed on specimens collected in the mycobacterial transport medium.

A novel transport medium was developed to facilitate mycobacterial culture from FNAB specimens, particularly in rural hospitals and clinics. Autofluorescence is used in the research as an additional inexpensive and rapid means of bacteriological confirmation of mycobacterial disease on cytology smears, which was not previously published. This was then applied to fine needle aspirates in children with mycobacterial disease.

“I am not discouraged, because every wrong attempt discarded is another step forward”

Thomas A. Edison

I hope that this body of research will help others remember the inestimable honour we all have in serving humankind by undertaking research into the diseases plaguing our nation. All of us have an invaluable and unique role to play from the clinicians in their day-to-day contact with patients experiencing the frustration of practical problems associated with diagnosis and management of disease, to the biomedical scientists with out whose expertise solutions would not be possible. It is through collaboration and commitment to a common goal that we will achieve the Millennium Developmental Goal 6 of reducing the prevalence and death rate from tuberculosis by 2015 through early diagnoses of new cases.

The primary, the most urgent requirement is the promotion of education. It is inconceivable that any nation should achieve prosperity and success unless this paramount, this fundamental concern is carried forward. The principal reason for the decline and fall of peoples is ignorance. Today the mass of the people are uninformed even as to ordinary affairs, how much less do they grasp the core of the important problems and complex needs of the time.

It is therefore urgent that beneficial articles and books be written, clearly and definitely establishing what the present-day requirements of the people are, and what will conduce to the happiness and advancement of society. These should be published and spread throughout the nation, so that at least the leaders among the people should become, to some degree, awakened, and arise to exert themselves along those lines which will lead to their abiding honor. The publication of high thoughts is the dynamic power in the arteries of life; it is the very soul of the world. Thoughts are a boundless sea, and the effects and varying conditions

of existence are as the separate forms and individual limits of the waves; not until the sea boils up will the waves rise and scatter their pearls of knowledge on the shore of life.

(Abdu'l-Baha, The Secret of Divine Civilization, p. 108)