

**The optimisation of laboratory cultivation in
childhood mycobacterial disease in South
Africa**

by

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DECLARATION

I, Wendy Brittle, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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Date

ABSTRACT

The role of the mycobacteriology laboratory in the diagnosis of childhood tuberculosis has become increasingly important in the human-immunodeficiency virus era. Due to the paucibacillary nature of childhood mycobacterial disease, laboratory optimisation of mycobacterial cultivation is necessary for paediatric clinical management and epidemiological surveillance. Previous studies have shown that growth supplements markedly improve the recovery rate and time-to-detection in mycobacterial cultures.

In this study, we hypothesised that specialised culture media and meat-based growth supplements would improve the recovery rate and time-to-detection in clinical samples from paediatric patients.

Pulmonary sputa and gastric aspirates and extra pulmonary fine needle aspiration biopsies were processed from children less than 15 years of age routinely investigated for mycobacterial disease. The processed clinical samples were split into a control aliquot that was cultured in liquid and solid media without growth supplement, and an intervention aliquot cultured on supplemented media. The effect of enrichment of the culture media was then calculated by comparison to the control.

These results indicated a significant reduction in the time-to-detection, 18.5 to 12.4 days, and an improved primary recovery rate of 14% in paediatric samples when cultured in liquid media enriched with a nutrient meat broth growth supplement. The findings of this study confirm the value of optimising mycobacterial cultivation with the use of growth supplements to enhance the detection of childhood mycobacterial disease.

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DEDICATION

**To my father and Dr Dave Hanslo
with love and gratitude**

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LIST OF ABBREVIATIONS

AFB	acid-fast bacilli
ATCC	American type culture collection
BCG	<i>Mycobacterium bovis</i> bacille Calmette-Guèrin
BSA	bovine serum albumin
BSL2	biosafety level 2 laboratory
CDC	Center for Disease Control and Prevention
CFU	colony-forming unit
DNA	deoxyribonucleic acid
DOTS	Directly Observed Therapy Short course
DST	drug susceptibility testing
HEPA	high efficiency particulate air
HIV	human immunodeficiency virus
INH	Isoniazid
IUATLD	International Union Against Tuberculosis and Lung Disease
LJ	Löwenstein-Jensen
MDR-TB	multidrug-resistant tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
ml	milliliter
NaOH-NALC	sodium hydroxide-N-acetyl-L-Cysteine
NB	nutrient broth
NHLS	National Health Laboratory Service
NTM	Nontuberculous mycobacteria
NTP	National Tuberculosis Programme
OADC	oleic acid albumin dextrose catalase complex

PLJ	Pyruvate LJ
PANTA	Polymyxin B, Amphotericin B, Naladixic acid, Trimethoprim, Azlocillin
PCR	polymerase chain reaction
QC	quality control
RIF	Rifampicin
rpm	revolutions per minute
SSI	Staten Serum Institute
TST	tuberculin skin test
TTD	time-to-detection
WHO	World Health Organisation
ZN	Ziehl-Neelsen

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Children account for a significant proportion of the global tuberculosis disease burden, yet the accurate diagnosis of childhood mycobacterial disease remains a major challenge due to the paucibacillary nature of the disease (Marais & Pai, 2006a; Marais *et al.*, 2006b). Previous studies have shown that growth supplements optimise laboratory cultivation of mycobacteria significantly (Frankel *et al.*, 1970; Power & McCuen, 1988; Siddiqi, 2005). Recommendations for laboratory media to cultivate mycobacteria include egg-based Löwenstein-Jensen (LJ) media and a liquid broth-based system, Mycobacteria Growth Indicator Tubes (MGIT™), as not all strains of mycobacteria can be recovered from a single medium (Power & McCuen, 1988; Saito, 1998; Hanna *et al.*, 1999; Maconda *et al.*, 2000; Lee *et al.*, 2003). The MGIT culture system has shown substantial improvement in the time-to-detection and recovery rate of primary cultures compared to solid culture media (Saito, 1998; Lu *et al.*, 2002; Chien *et al.*, 2000; Abe, 2003; Siddiqi, 2005).

1.2 Literature review

1.2.1 History of tuberculosis

Tuberculosis remains a major cause of mortality among the poor, even though Robert Koch discovered the tubercle bacillus over a hundred years ago (Coovadia & Benatar, 1991). It has become one of the most important diseases in

the history of human society, as no other disease has been so prevalent over such an extensive period of time (Coovadia & Benatar, 1991). Tuberculosis is the most common notifiable disease in South Africa having reached epidemic proportions in some areas and has now become the number one cause of death in South Africa (Coovadia & Benatar, 1991).

The origins of tuberculosis however remain unknown, but available evidence suggests that the disease predates written records and has occurred for several millennia (Coovadia & Benatar, 1991). According to Porter and McAdam (1994), tuberculosis is an ancient disease found in Egypt in early dynastic times (3700 BC). Human tuberculosis may have evolved during the seventh and sixth millennia BC due to population increases (Coovadia & Benatar, 1991; Porter & McAdam, 1994).

By the time of Hippocrates, 460 to 377 BC, tuberculosis was well recognised and called “galloping consumption”. The study of tuberculosis only really began during the period of Renaissance, 1483 to 1553, by Fracastoro who was regarded as the first epidemiologist having recognised the contagious nature of the disease (Porter & McAdam, 1994).

In 1861 Oliver Wendell Holmes called it “the white plague”. The following year Pasteur’s experiments suggested tubercle bacilli were airborne and in 1881 Robert Koch isolated *Mycobacterium tuberculosis*. This proved the communicable nature of tuberculosis to the world. Early and accurate diagnosis of tuberculosis became a reality when Ehrlich developed an acid-fast stain in 1885 and Roëntgen discovered X-rays in 1895 (Porter & McAdam, 1994; Coovadia & Benatar, 1991).

Significant advances then occurred during the 20th century that documented the natural history of tuberculosis in children (Marais *et al.*, 2004). The French bacteriologists, Calmette and Guèrin, used specific culture media to lower the virulence of the bovine tuberculosis bacterium to create the *Mycobacterium bovis* bacille Calmette-Guèrin (BCG) vaccine used worldwide today (Bannon, 1999).

After the First World War, the tuberculin skin test (TST) and chest radiography became available to detect tuberculosis infection in children. The introduction of the first anti-tuberculous drugs after World War II changed the history of the disease, with more effective drugs following in the early 1950's (Marais *et al.*, 2004). Between 1920 and 1950, observational studies on childhood pulmonary tuberculosis were first documented to guide effective disease management (Marais *et al.*, 2004).

1.2.2 Global epidemiology of paediatric mycobacterial disease

According to Nelson and Wells (2004), the estimate for total tuberculosis cases in 1994 was 7,500,000 of which 650,000 (9%) occurred in children. In low-income countries, paediatric cases escalated to 15% of the total tuberculosis cases. In 2000, an analysis by Corbett and colleagues (2003) estimated 8,300,000 new tuberculosis cases worldwide of which 884,019 (10.7%) were children. As indicated in **Figure 1.1**, the highest incidence of childhood tuberculosis was found in South Africa as compared to other sub-Saharan African states (Corbett *et al.*, 2003; Corbett *et al.*, 2006). More recently, it was reported that approximately 11% of tuberculosis cases occur annually in children less than 15 years of age, the standard World Health Organisation (WHO) categorisation of tuberculosis in children (WHO, 2006). Tuberculosis is thus an important cause of morbidity and

mortality in children due to diagnostic difficulties and the onset of human immunodeficiency virus (HIV) infection particularly in sub-Saharan Africa (Datta & Swaminatham, 2001; Cotton *et al.*, 2005; Chintu, 2007; Rekha & Swaminatham, 2007).

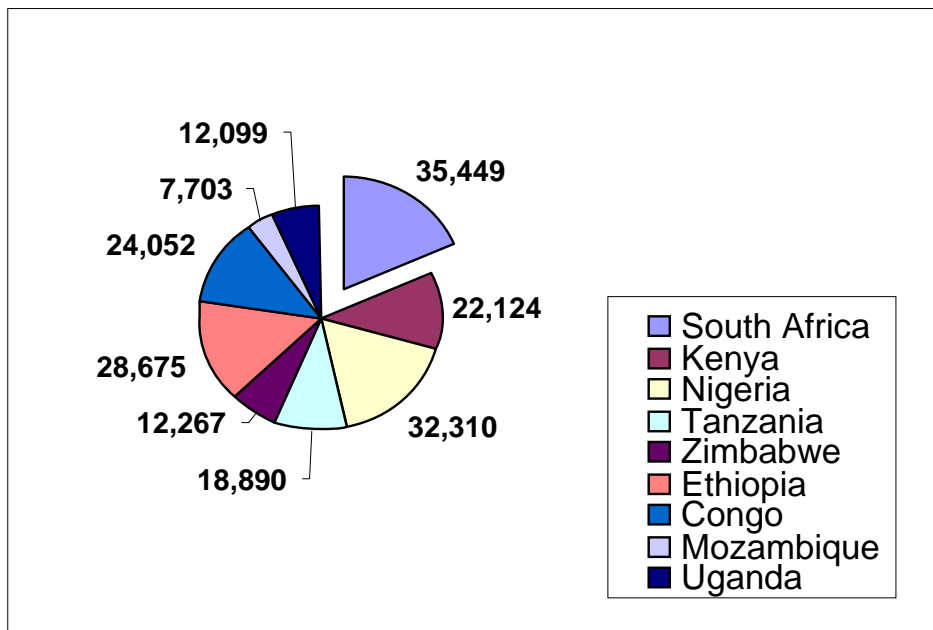


Figure 1.1 Estimated number of tuberculosis infected children, 0-14 years, in the highest burden sub-Saharan African states. Paediatric tuberculosis incidence in South Africa was 16.1% with the highest number of cases reported (n=35,449). According to the United Nations population estimates, South Africa's case rate is 237 / 100,000 children and 501 (all ages) per 100,000 population (modified from Corbett *et al.*, 2003).

The Stop TB Strategy, which supports the Directly Observed Therapy Short course (DOTS) strategy developed by the WHO and the International Union Against TB and Lung Disease (IUATLD), plays an important role in reducing the burden of disease and protecting children from infection and disease (Raviglione & Uplekar, 2006; WHO, 2006).

South African children with drug-susceptible tuberculosis are treated with Isoniazid, Rifampicin and Pyrazinamide for 6 months according to the DOTS policy of the South African National Tuberculosis Programme (NTP).

Tuberculosis strains resistant to either rifampicin or isoniazid are termed drug-resistant, while those resistant to both rifampicin and isoniazid are defined as multidrug-resistant tuberculosis (MDR-TB). Drug-resistant tuberculosis is treated individually according to the specific drug susceptibility pattern of the child (Donald, 2007; Schaaf *et al.*, 2007a; Schaaf *et al.*, 2007b). In South Africa, an astounding 20-39% of the total tuberculosis caseload occurs in children due to poverty, crowding and malnutrition (Van Rie *et al.*, 1999; Theart *et al.*, 2005; WHO, 2007).

1.2.3 *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria

The most common causative agent of mycobacterial disease in children is the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex. According to Frothingham (1995), a bacterial species includes strains with 70% or greater DNA-DNA relatedness. By this criterion, the members of the *M. tuberculosis* complex, that comprises *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti* and *M. africanum*, belong to one species as they share a high level of DNA-DNA-relatedness (Frothingham, 1995; Lennette *et al.*, 1980; Frankel *et al.*, 1970). The genomes of *M. bovis* BCG and *M. tuberculosis*, in particular, share 99.9% of their DNA. It is important, however, to differentiate BCG from other members of the *M. tuberculosis* complex to establish the rate of BCG complications which are relevant in the HIV era (Mahairas *et al.*, 1995; Talbot *et al.*, 1996; Brosch *et al.*, 2002).

The phenotypic characteristics to identify *M. tuberculosis* in the laboratory include, the formation of nonpigmented, rough, buff colonies on LJ slants within 14-28 days of incubation at 37°C, the appearance of small, white colonies on Middlebrook 7H10 agar within 5-10 days of incubation at 37°C and selective growth in broth-based cultures within 7-14 days at 37°C in the fully automated BACTEC MGIT™ 960 System (Difco Laboratories, 1984; Siddiqi, 2005; Koneman *et al.*, 2006).

The typical cell morphology of *M. tuberculosis* in acid-fast stains is a thin, slightly curved bacillus that stains a deep red with a distinctive beaded appearance. In broth cultures, typical serpentine cords from aggregates of acid-fast bacilli can be seen microscopically (Frankel *et al.*, 1970; Kleeberg *et al.*, 1980; Wayne & Kubica, 1986; Koneman *et al.*, 2006).

The BCG strain of *M. bovis* has been used as a vaccine for infants in highly endemic areas of the world for 50 years (Bannon, 1999; Hesselning *et al.*, 2006). This phenotypic strain is known to resemble the *M. tuberculosis* rough, buff colonies growing on LJ media without the pyruvate supplement necessary for the wild-type *M. bovis* (Keating *et al.*, 2005). The microscopic morphology of bovine tubercle bacilli is straight and stubby, unlike the serpentine cording found in *M. tuberculosis*. Poor, slow growth of flat, smooth, white colonies is observed on LJ slants when *M. bovis* BCG is cultured (Frankel *et al.*, 1970; Kleeberg *et al.*, 1980).

On the other hand, atypical mycobacteria or nontuberculous mycobacteria (NTM) are organisms isolated from environmental sources such as soil, water, animals and birds (Frankel *et al.*, 1970; Kleeberg *et al.*, 1980; Koneman *et al.*, 2006). Moreover, the incidence of these organisms has increased substantially since the

HIV era as several NTM species are considered to be potential sources of disease in immunosuppressed children (Frankel *et al.*, 1970; Lennette *et al.*, 1980).

The phenotypic characteristics of NTM have been characterised into different groups according to the Runyon system of classification depending on colony morphology, growth rate and pigmentation (Kleeberg *et al.*, 1980; Koneman *et al.*, 2006). Colonies are typically small, round and orange or golden when grown on LJ slants (Difco Laboratories, 1984).

1.2.4 *Mycobacterium bovis* BCG disease

Prevention and control of childhood tuberculosis relies partly on the protective effect of the BCG vaccination. As the BCG organism is a live strain of *M. bovis*, attenuated in 1921, there is concern for newborn HIV-infected infants (Bannon, 1999; Castanon-Arreola & Lopez-Vidal, 2004; Hesselning *et al.*, 2006; Hesselning *et al.*, 2007). BCG vaccination can cause injection-site complications, adenitis and disseminated disease which is mostly seen in HIV-infected children less than one year of age and in infants with primary immunodeficiency. Surveillance of BCG-related disease and determination of drug sensitivities of *M. bovis* BCG are therefore a research priority as the mortality rate amongst infants is 75% in the Western Cape. (Hesselning *et al.*, 2004; Hesselning *et al.*, 2006; Donald, 2007; Hesselning *et al.*, 2007).

WHO policy currently recommends vaccination of asymptomatic children with the Danish strain BCG as soon after birth as possible in high-burden settings (WHO, 2006). While BCG contributes to protecting infants and young children,

vaccination alone will not adequately reduce the burden of childhood tuberculosis (Nelson & Wells, 2004).

M. bovis BCG vaccine reduces the risk of tuberculosis by 50%, which is insufficient to prevent transmission in endemic settings (Bannon, 1999; Castanon-Arreola & Lopez-Vidal, 2004). The efficacy of BCG varies due to the use of different BCG preparations, levels of nutrition and environmental factors such as climate, socio-economic issues and environmental mycobacteria. Certain species of environmental mycobacteria can block the multiplication of BCG and therefore the subsequent protective immunity to tuberculosis (Chen *et al.*, 2002; Bannon, 1999; Castanon-Arreola & Lopez-Vidal, 2004).

1.2.5 Disease pathology

Tuberculosis is an infectious disease caused by the *M. tuberculosis* complex (that comprises *M. tuberculosis*, *M. bovis* BCG, *M. bovis*, *M. microti* and *M. africanum*) and NTM (Lennette *et al.*, 1980; Wayne & Kubica, 1986; Koneman *et al.*, 2006).

Mycobacteria are usually transmitted from person-to-person. When an infected individual coughs, sneezes or laughs, the mucous droplets containing micro-organisms are expelled. The airborne droplets are then inhaled via the lungs by an individual in close contact and can lead to pulmonary tuberculosis (Enarson *et al.*, 2000). Severity of the disease depends on the pathogen's ability to persist, the virulence of the organisms and the number of organisms inhaled (Marais *et al.*, 2005b). Extrapulmonary tuberculosis occurs when the micro-organisms spread to other parts of the body via the airways, blood stream, lymphatic system or directly to

other organs (Enarson *et al.*, 2000; Mandalakas & Starke, 2005). Children are most often infected by an adult in their household or close environment (Enarson *et al.*, 2000).

The immune response, which develops 4-6 weeks after infection, usually stops the multiplication of tubercle bacilli, but a few dormant bacilli may persist. The presence of these dormant micro-organisms in the body is indicated by a reaction to a TST and may be evidence of infection or past exposure to tuberculosis (Enarson *et al.*, 2000; WHO, 2006). Infants younger than 2 to 3 years of age with immature immune systems, and adolescents aged 10 or above, are at high risk of developing progressive disease (Marais *et al.*, 2004; Marais *et al.*, 2005a). From the ages of 5 to 10 years, children enter the “safe school age”. Thereafter, an increasing incidence of adult-type disease is found in 10 to 14 year old children (Marais *et al.*, 2005a; WHO, 2007).

1.2.6 Diagnosis of childhood tuberculosis

Reliable diagnosis of childhood tuberculosis depends on laboratory confirmation, yet traditional diagnostic methods are less than optimal due to poor sensitivity and delayed results (Enarson *et al.*, 2000; Gray, 2004; WHO, 2006). Diagnosis is further complicated by the difficulty of collecting clinical specimens from children and the low bacteriological yield (Marais *et al.*, 2006a). As the accuracy of smear microscopy is reduced by HIV co-infection, the optimisation of existing diagnostic tests is therefore necessary for children (Cotton *et al.*, 2004; Corbett *et al.*, 2007). Fine needle aspiration biopsy (FNAB), however, provides exceptional mycobacterial yields and remains a valuable diagnostic tool for children with tuberculous lymphadenitis (Marais *et al.*, 2006c). Continuing research into new

diagnostic methods by developing affordable tests in low-resource settings should be a priority for researchers. The WHO has recently updated its reporting practice to include childhood tuberculosis (WHO, 2006). Positive paediatric cultures are therefore sent to reference laboratories for identification and drug susceptibility testing as recommended for Biosafety Level 2 (BSL2) laboratories (Frankel *et al.*, 1970; Kleeberg *et al.*, 1980; Wayne & Kubica, 1986; Koneman *et al.*, 2006).

The WHO reports only smear-positive tuberculosis cases by age and as about 95% of cases in children are smear-negative, the burden of childhood tuberculosis is vastly underestimated (Nelson & Wells, 2004). The value of the mycobacteriology laboratory lies in diagnosing smear-positive or infectious cases of childhood tuberculosis by examining clinical specimens microscopically for the presence of micro-organisms (Enarson *et al.*, 2000). Gastric aspirate microscopy for acid-fast bacilli is not recommended as it only has a 5 to 10% yield in children and does not distinguish between *M. tuberculosis* complex and NTM. Only about 30-40% of paediatric cases are confirmed bacteriologically using *M. tuberculosis* culture as the gold standard of diagnosis (Schaaf *et al.*, 2000; Marais *et al.*, 2006b). Bacterial yields however may differ depending on HIV status and extent of childhood mycobacterial disease (Schaaf *et al.*, 2005).

Mycobacterial cultures in children are mainly done at referral hospitals, as most children are clinically diagnosed before culture results become available. Culture confirmation however remains valuable for drug susceptibility testing to establish treatment regimens (Engelbrecht *et al.*, 2006). According to Schaaf and colleagues (2006), drug-resistant and multidrug-resistant tuberculosis poses a major threat to patients and the tuberculosis control programmes.

The two mechanisms for developing drug resistance are defined firstly as primary drug resistance where no previous tuberculosis treatment occurred and therefore implies transmitted resistance. The second mechanism is acquired drug resistance which develops in a previously treated patient due to inappropriate treatment (Schaaf *et al.*, 2006). Drug resistance in children is an important indicator of transmitted disease within communities, as children mostly have primary MDR-TB transmitted to them by an adult with MDR-TB (Schaaf *et al.*, 2000; Schaaf *et al.*, 2006). Bacteriological follow-up is therefore necessary after antituberculous treatment is completed as HIV-infected children are at a high risk of developing recurrent tuberculosis (Schaaf *et al.*, 2005). For this reason, drug resistance has become an important reason for optimisation of tuberculosis culture in paediatric specimens.

Rapid molecular systems, such as polymerase chain reaction (PCR), spoligotyping and DNA fingerprinting, are used for further diagnostic testing of mycobacteria (Buck & Eid, 1995; Negi *et al.*, 2005; Koneman *et al.*, 2006).

1.2.7 History of bacterial culture media

Due to Pasteur and Koch's research, the study of bacteria emerged as a defined branch of modern science (Difco Laboratories, 1984). Since then, a variety of methods and media have been investigated to reduce the time required to the diagnosis and ultimate treatment of patients (Power & McCuen, 1988). In 1876, Robert Koch introduced a way of investigating disease by propagating pure cultures of pathogenic bacteria outside the host's body (Difco Laboratories, 1984). *In vitro* culture techniques were then introduced due to their safety, accuracy and simplicity (Power & McCuen, 1988).

Koch then pioneered further research of pure culture techniques to isolate bacteria on solid media by using clear liquid media solidified with agar. The agar was superior to the gelatine previously used as it was also resistant to microbial digestion and liquefaction.

The general constituents found in microbiological media today have been used for over one hundred years. In 1881 Fanny Eilshemius Hesse, a technician in Robert Koch's laboratory in Germany, introduced the concept of agar to bacteriology, having used it for many years in the preparation of homemade jellies (Lindquist, 2006). Agar is a polysaccharide gum extracted from red marine algae and is used as a solidifying agent, not as a nutrient, in media for the culture of microorganisms. Prior to agar, potato slices and gelatine were used to form solid substrates for microbial colonies to be grown and studied. These materials were unacceptable for general use, as gelatine liquefies in a warm room, and potato slices are opaque (Difco Laboratories, 1984; Lindquist, 2006).

Koch's research was then advanced by manipulating culture media using meat extracts and infusions to mimic the infected host's tissue. These serendipitous discoveries led to the evolution of correct methods for the *in vitro* cultivation of bacteria (Frankel *et al*, 1970; Difco Laboratories, 1984).

Essential requisites to study and isolate specific organisms involve understanding the nutritional requirements of bacteria and developing culture media capable of producing rapid and confluent growth. From 1868 to 1880, Naegeli published his reports on determining the forms of carbon, hydrogen and nitrogen used by bacteria for their development. In 1879 he was the first to reference the use of peptone, which contains amino acids and nitrogen necessary to cultivate various microorganisms. Many workers have since reported that *M. tuberculosis* depends on amino acids to grow (Difco Laboratories, 1984).

In 1931, Löwenstein originally formulated an egg-based medium to cultivate mycobacteria which incorporated congo red and malachite green to partially inhibit other bacteria. Jensen then modified Löwenstein's recipe in 1932 to produce the present formula which contains more malachite green and supplements, but no congo red (Difco Laboratories, 1984; Power & McCuen, 1988).

From 1945 to 1946, Dubos developed an enrichment medium called Dubos broth for culturing tubercle bacilli. This nutrient or meat broth was recommended for the rapid culture of fastidious bacteria which did not grow well on solid media (Kleeberg *et al.*, 1980; Difco Laboratories, 1984). In 1947, Dubos and Middlebrook were instrumental in developing media formulas which contained oleic acid and albumin as key ingredients to enhance the growth of tubercle bacilli (Difco Laboratories, 1984; Power & McCuen, 1988). According to Merck (1952), the use of meat broth to prepare a nutrient medium was originally suggested by Loeffler who developed the media for *Corynebacterium diphtheriae* (Oxoid Laboratories, 1976; Difco Laboratories, 1984).

From 1958 to 1960, Cohn and Middlebrook improved the formula of oleic acid-albumin agar to obtain a faster, more confluent growth of *Mycobacterium* species. This Middlebrook 7H10 agar was not affected by proteolytic organisms and allowed for less contamination, unlike an egg-based medium. A modification of this agar, Middlebrook 7H11 which included casein, was described by Cohn and colleagues in 1968 (Kleeberg *et al.*, 1980; Difco Laboratories, 1984; Power & McCuen, 1988).

In 1967, the Tuberculosis Advisory Committee of the Department of Health, South Africa, recognised the need to update and revise laboratory methodology to monitor tuberculosis in South Africa. This led to standardisation of routine

diagnostic methods and precise laboratory techniques to diagnose tuberculosis (Kleeberg *et al.*, 1980).

1.2.8 Optimisation of laboratory cultivation

The survival of micro-organisms in the laboratory depends on their ability to grow under certain chemical and physical conditions. An understanding of these conditions enables one to characterise isolates and differentiate between types of mycobacteria (Frankel *et al.*, 1970; Lennette *et al.*, 1980; Lindquist, 2006).

Media used in the laboratory to cultivate mycobacteria must supply the necessary carbon and energy sources. Nutrients or growth factors are also required for cellular growth and maintenance of the organisms (Difco Laboratories, 1984; Power & McCuen, 1988; Lindquist, 2006).

Blood, plasma, serum or other body fluids also contribute growth factors to culture media for isolation and cultivation of pathogens. Buffers are compounds incorporated to maintain the optimum pH range for the growth of organisms. Furthermore, substances like sodium and potassium phosphates, trisodium citrate and calcium carbonate prevent changes in pH which otherwise would result from microbial production of organic acids or bases (Difco Laboratories, 1984; Lindquist, 2006).

Organisms such as yeast, and tissue such as beef muscle, liver, brain or heart are extracted by boiling and then concentrated into a paste or dried to a powder. These extracts are used as a source of amino acids, vitamins and coenzymes needed as growth factors by organisms which are difficult to grow. Trace elements, minerals

and sugar are also commonly present in growth media (Kleeberg *et al.*, 1980; Difco Laboratories, 1984).

Peptones are compounds obtained by digestion of protein-containing tissues of animals and plant such as meat scraps, beef muscle, gelatine and milk protein (casein). Peptones such as tryptone or trypticase contain peptides and amino acids, but they may be deficient in certain minerals and vitamins. (Oxoid Laboratories, 1976; Difco Laboratories, 1984; Lindquist, 2006).

Additionally, selective or antimicrobial agents such as malachite green, bile salts and antibiotics can be used in selective media to inhibit the growth of certain micro-organisms while allowing growth of desired organisms. These agents are usually bacteriostatic (Kleeberg *et al.*, 1980; Siddiqi, 2005; Lindquist, 2006).

Incubation conditions that are appropriate for mycobacteria include a suitable atmosphere of 5% oxygen or carbon dioxide and a temperature range of 35-37°C.

As indicated in **Table 1.1**, various culture media are utilised for the isolation, growth and maintenance of mycobacteria from clinical specimens, as a single medium will not support the growth of all micro-organisms (Kleeberg *et al.*, 1980; Lindquist, 2006).

TABLE 1.1 Mycobacterial culture media (ingredients in grams per litre). Liquid media, nutrient broth and MGIT, Mycobacteria Growth Indicator Tube, 7H9 broths were assessed as well as solid media, Middlebrook 7H11 agar slants, LJ, Löwenstein-Jensen slants and Pyruvate LJ slants for enhanced growth of mycobacteria from paediatric diagnostic specimens (Difco Laboratories).

Ingredients (g/l)	Nutrient Broth (Difco)	MGIT (7H9)	Middlebrook 7H11 agar	LJ slant	Pyruvate LJ slant
pH		6.6	6.6		
Malachite green			0.001	0.4	0.4
Bacto asparagine				3.6	3.6
Monosodium phosphate	1	1	1.5	2.4	2.4
Magnesium sulphate	0.6	0.05	0.05	0.24	0.024
Magnesium citrate				0.6	0.6
Potato flour				30	30
87% Glycerol				7.5	
Pyruvic acid					4.1
Egg yolk				present	present
Egg white				present	present
Ammonium sulphate		0.5	0.5		
Disodium phosphate	2.5	2.5	1.5		
Sodium citrate	1.5	0.1	0.4		
Calcium chloride		0.0005			
Zinc sulphate		0.001			
Copper sulphate		0.001			
L-glutamic acid		0.5	0.5		
FAC		0.04	0.04		
Pyridoxine hydrochloride		0.001	0.001		
Biotin		0.0005	0.0005		
Bacto agar			15		
Casein			1		
Tween 80	0.5				
Casitone	2				
Proteose peptone	2				
Bacto meat extract*	2				

*This substance represents a unique growth supplement not contained in other media preparations

1.2.9 Mycobacterial nutrient requirements

A growth factor is defined as a specific organic compound that is required by a particular organism as it cannot be synthesised by that organism (Lindquist, 2006). Micro-organisms termed fastidious or difficult to grow, such as *M. bovis* BCG, tend to require different or additional growth factors to *M. tuberculosis* (Kleeberg *et al.*, 1980; Fritz *et al.*, 2001; Lindquist, 2006). According to Talbot and colleagues (1996), “BCG has a unique history. From 1908 to 1921, a virulent strain of *M. bovis* isolated from bovine milk was maintained on potato bile and potato with glycerin beef broth”.

A standard reference saline solution, 0.9% (w/v) sodium chloride, was routinely used for sub-culturing *M. bovis* BCG isolates. Three recommended mycobacterial growth supplements were investigated to enhance the growth of *M. bovis* BCG (Difco Laboratories, 1984; Bosman, 2006; Brittle, 2006).

Tween 80 is a 10% (v/v) polyoxyethylene sorbitan mono-oleate solution (**Appendix D**) that allows necessary compounds to enter the bacterial cell more rapidly. This may result in enhanced growth of bacteria (Difco Laboratories, 1984; Wayne & Kubica, 1986). Polyoxyethylene stearate is a growth promoting substance that minimises the inhibitory effect of antimicrobials on mycobacteria and enhances growth of slow growing *M. tuberculosis* (Siddiqi, 2005). Nutrient Broth, a modified Dubos liquid medium containing beef extract and peptone, is recommended for the rapid cultivation of *M. tuberculosis* specifically from inocula containing fewer organisms (Difco Laboratories 1984; Bosman, 2006; Brittle, 2006). This meat stock, obtained by boiling, was supplemented with salt and peptone to make a nutrient broth. Subsequently, meat extract has been commonly used to prepare meat broths for bacterial culture. The ingredients of the Bacto TB nutrient broth growth

supplement used are reflected in **Table 1.1** (Difco Laboratories, 1984). This nutrient broth growth supplement contains a unique substance, beef extract, not found in standard growth media or in other growth supplement preparations. The growth supplement was prepared by dissolving 1.6g Bacto Nutrient broth in 200ml distilled water and adding 0.2ml Tween 80 before dispensing in 5ml aliquots in screw cap tubes, which were autoclaved at 121°C for 15 minutes and stored at 4-6°C (Difco Laboratories, 1984; Bosman, 2006).

The MGIT™ OADC, containing 10% (w/v) oleic acid, albumin, dextrose and catalase, is an enrichment medium developed in 1986 that provides substances essential for the rapid growth of mycobacteria (**Appendix D**). While oleic acid plays an important role in the metabolism of mycobacteria, albumin acts as a protective agent that binds free fatty acids often found to be toxic to mycobacteria, thereby enhancing their recovery. Dextrose is included as an energy source, while catalase destroys any toxic peroxides that may be present in the medium (Siddiqi, 2005).

1.3 Problem statement

Difficulties are often experienced in culturing clinical paediatric specimens due to limited documentation regarding their culturability and the paucibacillary nature of childhood mycobacterial disease.

1.4 Objectives of the study

We hypothesised that the use of growth supplements and enrichment media would enhance the recovery of mycobacteria from paediatric clinical samples.

The main objectives of this study were to evaluate different growth supplements that would increase mycobacterial yield using *M. bovis* BCG cultures as a model to optimise growth supplements for *M. tuberculosis* culturing. The selected nutrient broth growth supplement was then used for testing paediatric clinical samples for improved mycobacterial yield and reduced time-to-detection.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study setting and laboratory safety

This prospective, hospital-based, laboratory study was carried out in the Biosafety Level 2 (BSL2) Microbiology laboratory, National Health Laboratory Service, (NHLS), Tygerberg Hospital, South Africa, and the BSL3 facility in the Department of Biomedical Sciences, Stellenbosch University, South Africa.

According to Kleeberg *et al.* (1980), BSL2 laboratories can perform direct microscopic examinations and culture of specimens for *M. tuberculosis*. Positive cultures were submitted to the NHLS Microbiology laboratory, Groote Schuur Hospital, South Africa, for identification and drug susceptibility testing. Safety precautions were strictly observed by processing all infectious specimens, preparing smears and inoculating media in a Class II biological safety cabinet under sterile conditions in a room dedicated for mycobacterial work as protection against airborne infectious particles (Shinnick & Good, 1995; Nyirenda *et al.*, 1998; Carroll *et al.*, 2002; Siddiqi, 2005). The biosafety cabinet working area was cleaned and sterilised with an appropriate mycobacterial disinfectant such as a 2% (v/v) solution of Hycolin (William Pearson Chemicals, Coventry, UK, summarised in **Appendix A**) before and after processing each batch of clinical specimens.

As indicated in **Figure 2.1**, protective clothing such as blue theatre gowns and sterile latex gloves were worn while handling specimens and cultures of potential pathogens (Kleeberg *et al.*, 1980; Siddiqi, 2005). Rigorous procedures were

followed to avoid cross-contamination between specimens processed daily by ensuring only one tube was open at a time and reagent bottles remained closed between steps (Bird *et al.*, 1996; Carroll *et al.*, 2002; Siddiqi., 2005; Martinez *et al.*, 2006).



Figure 2.1 Processing of paediatric clinical specimens was performed under sterile conditions in a Class II Biosafety cabinet. Protective clothing such as blue theatre gowns and latex gloves were worn at all times to prevent contamination when handling potentially pathogenic specimens.

2.2 Ethical consideration

Ethical approval for this study was given by the Institutional Review Board, Faculty of Health Sciences, Stellenbosch University, Western Cape. Study number 2003/005.

2.3 Optimisation of growth supplements for *M. bovis* BCG

M. bovis BCG is a fastidious organism that does not grow on media recommended for the wild-type *M. bovis* and was therefore suspected to have different nutritional requirements (Beste *et al.*, 2004; Keating *et al.*, 2005). Different growth supplements were therefore investigated to find a suitable growth supplement and identify *in vitro* growth conditions for *M. bovis* BCG, to be used ultimately for paediatric diagnostic specimens. The fastidious *M. bovis* BCG organism was used as a model to optimise growth supplements for *M. tuberculosis* culturing.

2.3.1 Establishment of a *M. bovis* BCG liquid culture

A comparative analysis was carried out to assess the culture yield and time-to-detection using a variety of growth media on pure cultures of *M. bovis* BCG test strains and vaccine reference strains. The *M. bovis* BCG test strains were isolated from 12 HIV-infected infants who were vaccinated at birth with the Danish strain BCG vaccine (intradermal vaccination 1331, Staten Serum Institute; SSI). These infants subsequently developed local and/or disseminated *M. bovis* BCG disease.

Two rapid growers that grew confluent on solid media in 9 to 12 days, and 10 slow growers with light growth observed in 24 to 30 days, were selected from the organism bank (Desmond Tutu Tuberculosis Centre, Stellenbosch University) based on previously observed growth patterns on LJ slants. Two BCG vaccine reference strains, the Danish (1331, SSI) and Tokyo strain (Tokyo-172 BCG, South African Vaccine Institute), were included as positive controls. After reconstitution

according to the manufacturer's instructions, the reference strains contained 1-4 x 10⁵ colony-forming units (CFU) (Staten Serum Institute).

A MGIT tube (Becton Dickinson, Maryland, USA) inoculated with processed sterile phosphate buffer at pH 6.8 (**Appendix D**) (NHLS, Green Point, South Africa) was included as a negative control in each batch of processed samples (varying in size from 5 to 25 samples), to monitor possible cross-contamination (Bird *et al.*, 1996; Carroll *et al.*, 2002; Siddiqi, 2005; Martinez *et al.*, 2006).

A standard reference saline solution, 0.9% sodium chloride (w/v) routinely used for sub-culturing *M. bovis* BCG isolates, and three recommended mycobacterial growth supplements were assessed: 1) Tween 80 (NHLS, Green Point, South Africa) that consists of a 10% (w/v) polyoxyethylene sorbitan mono-oleate solution; 2) polyoxyethylene stearate (Becton Dickinson, Maryland, USA), and 3) TB Nutrient Broth (NHLS, Green Point, South Africa), a modified Dubos liquid medium containing beef extract (Difco Laboratories, 1984; Bosman, 2006).

Using sterile plastic loops (Lasec, South Africa) to measure a standardised inoculum size for all stock cultures, a loopful of *M. bovis* BCG colonies were harvested from LJ slants (NHLS, Green Point, South Africa) that were previously sub-cultured from positive MGIT cultures. Each *M. bovis* BCG test strain was then transferred to a sterile screw-capped tube (Lasec, South Africa) containing 1ml of each sterile growth supplement. In addition, 0.25ml of each reference strain (Tokyo and Danish) was aspirated directly from the reconstituted BCG vaccination inoculation vials, into 1ml of each sterile growth supplement as indicated in **Figure 2.2**. MGIT tubes containing 7ml of Middlebrook 7H9 liquid broth and 0.8ml Middlebrook OADC enrichment medium (Becton Dickinson) were used as liquid culture media for each of the growth supplement tubes. Standard antimicrobials,

Polymyxin B (10, 000 units), Amphotericin B (1000µg), Naladixic acid (4000µg), Trimethoprim (1000µg) and Azlocillin (1000µg) (PANTA, Becton Dickinson) were added to each MGIT tube prior to inoculation according to manufacturers' instructions (Siddiqi, 1986).

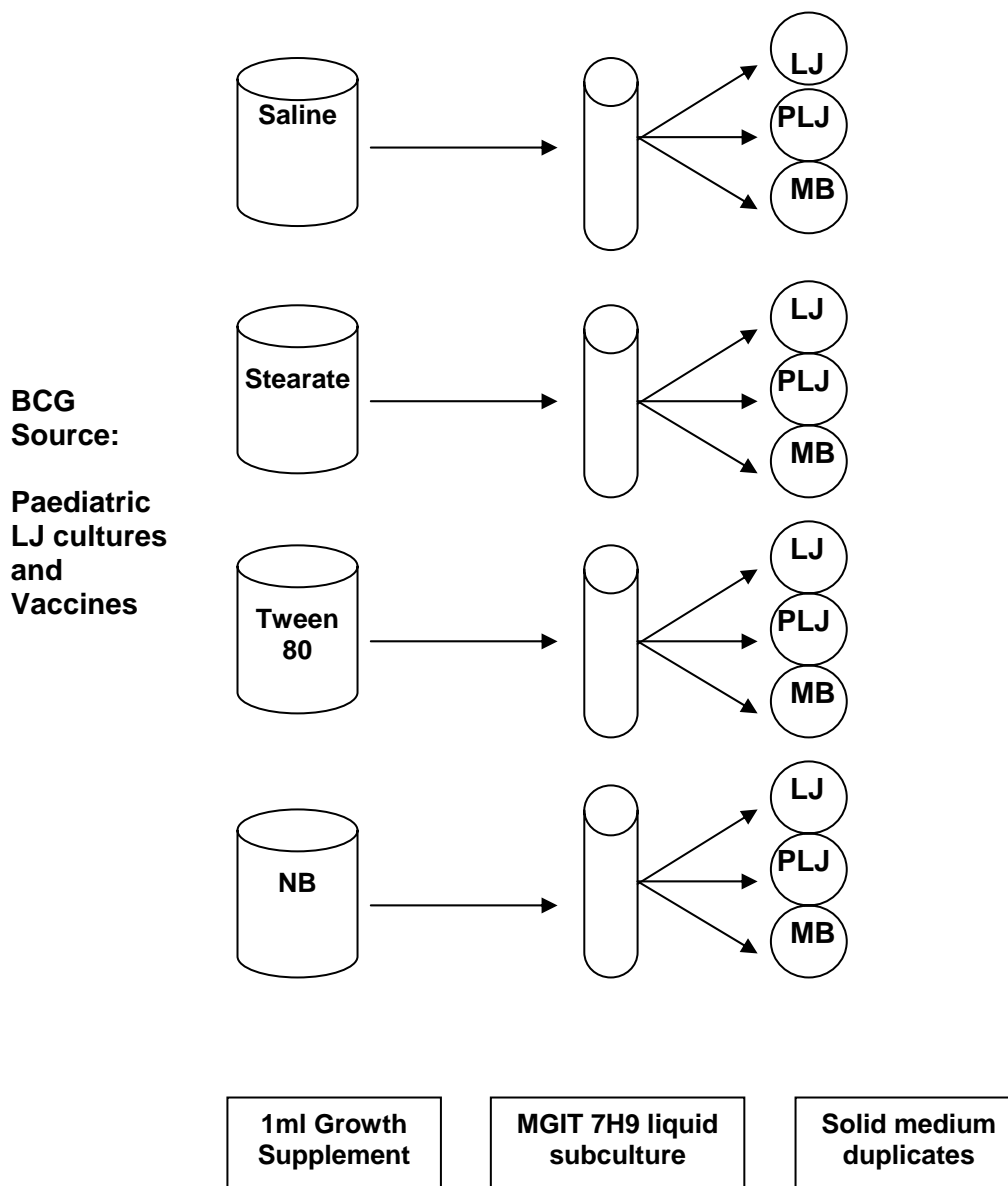


Figure 2.2 Methodology flow chart of establishing an *M. bovis* BCG culture to assess different growth supplements. *M. bovis* BCG colonies were diluted in saline, polyoxyethylene stearate, Tween 80 and nutrient broth (NB) growth supplements before culturing in MGIT 7H9 liquid medium. Time-to-detection was noted for all positive MGIT cultures before duplicate sub-culturing onto solid Löwenstein-Jensen (LJ), Pyruvate LJ, and Middlebrook 7H11 slants.

Each MGIT tube containing a modified Middlebrook 7H9 broth, supplemented with PANTA, was incubated at 37°C in the fully automated BACTEC MGIT™ 960 System (Becton Dickinson, Maryland, USA). Unlike prior culture systems, this fluorimetric system is non-invasive and nonradiometric. An orange fluorescent compound, embedded in silicone on the bottom of the MGIT tubes, is sensitive to the presence of oxygen dissolved in the broth. Mycobacteria present in the broth, consume the oxygen and the lack of oxygen allows the fluorescence in the sensor to be detected. MGIT tubes were automatically monitored hourly for fluorescence until a positive signal developed. The MGIT tubes displaying no growth after 42 days were reported as negative. The time-to-detection was recorded for each positive culture to compare the different growth supplements. All positive cultures underwent a purity check by staining with ZN to confirm the presence of acid-fast bacilli, and culturing on blood agar plates to exclude bacterial contamination, as recommended by the manufacturer (Becton Dickinson, Maryland, USA).

2.3.2 Establishment of a *M. bovis* BCG solid culture

Once a positive signal was recorded for the MGIT tubes, 0.5ml of these positive liquid medium cultures were sub-cultured onto duplicate LJ, pyruvate LJ and Middlebrook 7H11 agar slants (NHLS, Green Point, South Africa). The slants were incubated at 37°C in 5% oxygen (Scientific Series 9000 incubator, Lasec, South Africa) and the time-to-detection of successful growth or confluence was observed visually over a period of 8 weeks as indicated in **Figure 2.3**.

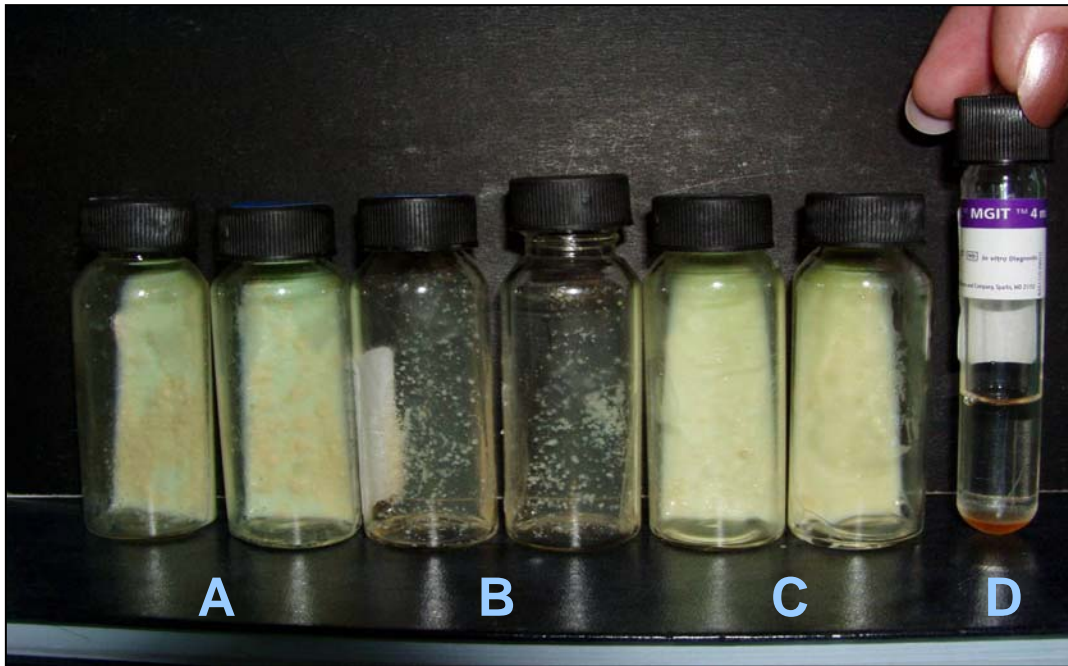


Figure 2.3 Solid and liquid culture media used to optimise paediatric *M. bovis* BCG cultures. Duplicate slants of (A) Standard LJ, Löwenstein-Jensen slants, (B) Middlebrook 7H11 agar slants and (C) Pyruvate LJ slants, were inoculated from (D) positive Mycobacterial Growth Indicator Tubes. TTD, time-to-detection, was recorded as the earliest growth of colonies visualised on the slants.

2.4 Processing of paediatric sputa, gastric aspirates and fine needle aspiration biopsies

The objective of this part of the study was to test the selected nutrient broth growth supplement on paediatric diagnostic samples for improved mycobacterial yield and reduced time-to-detection, using standard laboratory methods.

2.4.1 Specimen collection

Sputa and gastric aspirates were collected from children in sterile screw-capped containers (Lasec, South Africa) and transported from the wards to the routine

mycobacteriology laboratory by medical staff. An equal volume of 10% (Na_2CO_3) Sodium carbonate (NHLS, Tygerberg Hospital, South Africa) was routinely added to the gastric aspirates in the clinical area to neutralise stomach acids which could kill any mycobacteria present. If delays were anticipated, specimens were stored at 4°C as transport time is proportional to the contamination rate (Siddiqi, 2005). The time taken between collection and processing of specimens in the laboratory was therefore noted. FNAB were aseptically collected into a MGIT tube by a clinician, and brought directly to the laboratory by medical staff.

2.4.2 Paediatric specimen processing

Once consecutive paediatric specimens were received in the laboratory, the patient data was captured from the hospital form received with the clinical specimen and entered onto the hospital computer system for direct access to wards and doctors for results. Relevant patient details included the specimen type, date of birth, folder number, laboratory number, date of sample and test requested.

2.4.2.1 Principle of the digestion and decontamination procedure

Contaminated specimens such as sputa and gastric aspirates were digested or liquefied using a mucolytic agent, N-acetyl-L-cysteine, NALC (Merck, Darmstadt, Germany), to free the trapped bacilli from the mucus, cells or tissue. A decontamination step followed for the selective elimination of bacteria other than mycobacteria as contaminating bacteria grow more rapidly than mycobacteria. Alkaline digestant procedures must be critically timed to minimise exposure of acid-

fast bacilli because the acids, alkalis and detergents usually used for digestion and decontamination of specimens could be toxic for the contaminating bacteria as well as the mycobacteria present in the clinical specimens (Siddiqi, 2005). Sodium hydroxide and Trisodium citrate (NHLS, Green Point, South Africa) act as decontaminating agents and emulsifiers (**Appendix D**). The correct concentration and time these agents are exposed to a contaminant must therefore be controlled to ensure the optimal recovery of mycobacteria from clinical samples (Kleeberg *et al.*, 1980; Siddiqi, 2005).

2.4.2.2 Sodium hydroxide-N-Acetyl-L-Cysteine decontamination procedure

All processing of paediatric clinical specimens for culturing mycobacteria was performed in a biological safety cabinet. The Sodium Hydroxide-N-Acetyl-L-Cysteine (NALC) (**Figure 2.4**) is the standard recommended decontamination procedure used with the BACTEC MGIT™ 960 System (Kleeberg *et al.*, 1980; Siddiqi, 2005). The NaOH-NALC reagents were freshly prepared for each day's cultures as shown in **Appendix D**. Equal volumes of the sputa or gastric aspirates were added to the 4% NaOH-NALC in a 30ml polypropylene centrifuge tube (Merck, Darmstadt, Germany) to make a final concentration of 2% NaOH before incubating at 37°C in 5% oxygen for twenty minutes. The centrifuge tubes were then filled with refrigerated sterile phosphate buffer, pH 6.8, (NHLS, Green Point, South Africa) to neutralise the NaOH-NALC and minimise heat formed during centrifuging as this may kill any mycobacteria present. This was followed by centrifugation at 3000 rpm for twenty minutes at room temperature (22-24°C) in order to maximise the mycobacterial yield. After centrifugation, the supernatant fluid was gently poured off into a sterile hecolin container, leaving a pellet or sediment for smear preparation and culture (Kleeberg *et al.*, 1980; Siddiqi, 2005).

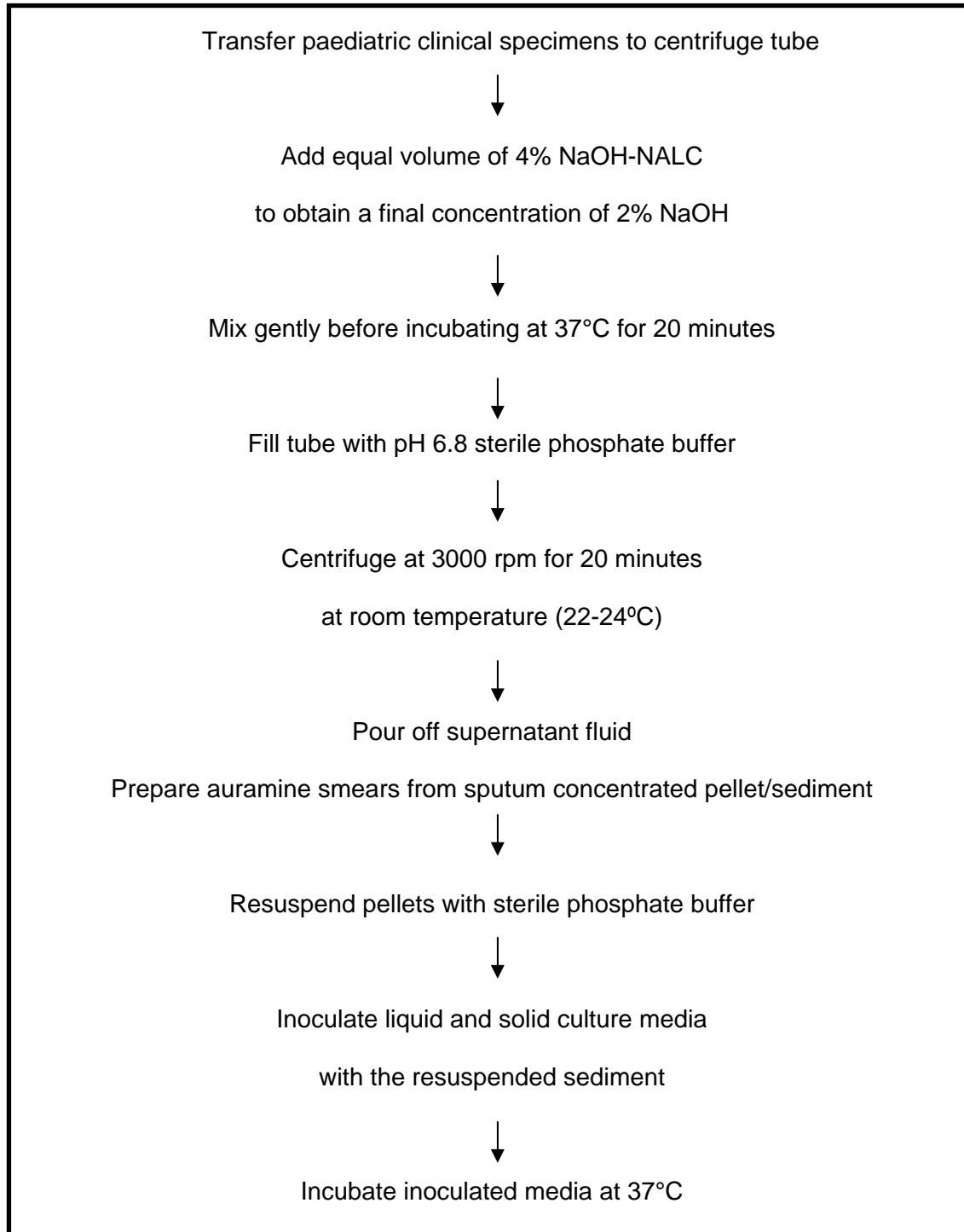


Figure 2.4 Flow chart of the Sodium hydroxide-N-acetyl-L-Cysteine, NaOH-NALC, method for digestion and decontamination of clinical paediatric specimens. The processing of contaminated specimens such as sputum and gastric aspirates from children is necessary to selectively eliminate bacteria other than mycobacteria. After digestion and decontamination with NaOH-NALC, specimens are centrifuged to concentrate any mycobacteria that may be present. The remaining sediments are reconstituted for culture in liquid and solid media.

2.4.3 Smear microscopy for paediatric sputum specimens

Smears were prepared for Auramine-O (NHLS, Gauteng, South Africa) fluorescent staining on sputum specimens only, according to the standard operating procedure (NHLS, Tygerberg Hospital, South Africa), to determine the presence of acid-fast bacilli (AFB) (Enarson *et al.*, 2000). One drop of the concentrated sputum pellet was transferred to a clean frosted glass slide (Lasec, South Africa) using a sterile 3ml graduated plastic pipette (Lasec, South Africa).

A drop of fixative, 0.2% bovine-serum-albumin, BSA, (NHLS, Green Point, South Africa), was mixed with the sputum to allow the sputum to adhere to the slide. The prepared smear was 1.5 x 1.5cm in size, thin, evenly spread out and then placed on a hot tray (Lasec, South Africa) at 80°C for two hours for further fixing to the slide. The smear was left to air-dry in the biosafety cabinet before being heat-fixed by passing the slide through the flame of a Bunsen burner (Kleeberg *et al.*, 1980; Siddiqi, 2005).

The fluorochrome staining procedure was used to examine the paediatric sputum smears. Acid-fast organisms emitted a bright yellow fluorescence, while the non-specific fluorescence from the potassium permanganate counter stain was a pale yellow background colour (Kleeberg *et al.*, 1980; Enarson *et al.*, 2000) as seen in **Figure 2.5**. The advantage of fluorescence microscopy is that low-power objectives may be used to scan large areas in a short time. This method also provides better contrast and minimal eye-strain compared to the ZN stain evaluation (Marais *et al.*, 2008).

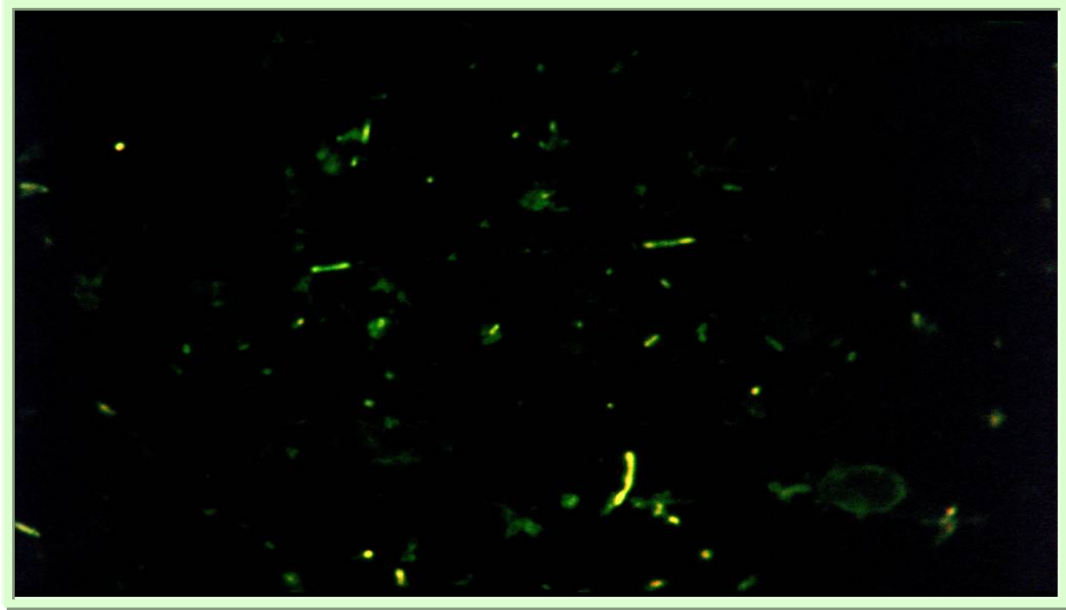


Figure 2.5 Auramine-O fluorescent stain prepared from a paediatric sputum sample displaying bright yellow fluorescent acid-fast bacilli against a dark background. (magnification, 40X).

The Auramine-O staining procedure consisted of flooding the smear with the Auramine-O fluorescent stain (**Appendix B**) for twenty minutes without heating, followed by a rinsing step with running tap water before decolourising with 0.5% acid alcohol (NHLS, Gauteng, South Africa) for two minutes. After rinsing the slide with tap water, the potassium permanganate counter stain (NHLS, Gauteng, South Africa) was applied for one minute, followed by a final rinsing step with running tap water (Kleeberg *et al.*, 1980; Siddiqi, 2005).

The smear was then drained and air-dried before being examined using a fluorescence microscope equipped with a nonhazardous blue light source. The light-emitting diode lamp (Royal Tropical Institute, Netherlands) is more cost effective and lasts longer than the standard mercury vapour lamp routinely used in the mycobacteriology laboratory (Marais *et al.*, 2008).

The numbers of AFB observed were quantified according to the Centres for Disease Control and Prevention (CDC) guidelines (**Table 2.1**) as it relates to the infectivity of the patient and the severity of disease (Kleeberg *et al.*, 1980; Enarson *et al.*, 2000).

Table 2.1 Sputum smear grading for acid-fast bacilli (AFB) according to CDC guidelines

Number of AFB* seen	CDC# guideline	Study definition
0	Negative	Negative
1-2 AFB / whole smear	Doubtful positive	Scanty
1-9 AFB / 100 fields	1+	Positive
1-9 AFB / 10 fields	2+	
1-9 AFB / single field	3+	
>9 / single field	4+	

*AFB – acid-fast bacilli

#CDC – Centres for Disease Control and Prevention

2.4.4 Processing of paediatric samples in liquid media

The pellet or sediment of each sputum and gastric aspirate was resuspended in 1.5ml sterile phosphate buffer. The resuspension was mixed very well by aspirating gently several times with a sterile 3ml graduated plastic pipette, as even distribution of AFB from paucibacillary specimens may have been problematic. As indicated in **Figure 2.6**, the resuspended sample was then split into 4 aliquots. The first 0.5ml aliquot was inoculated into a MGIT tube without the nutrient broth (NHLS, Green Point, South Africa) growth supplement (unsupplemented). The second or intervention 0.5ml aliquot was inoculated into a MGIT tube enriched with the nutrient broth growth supplement (supplemented).

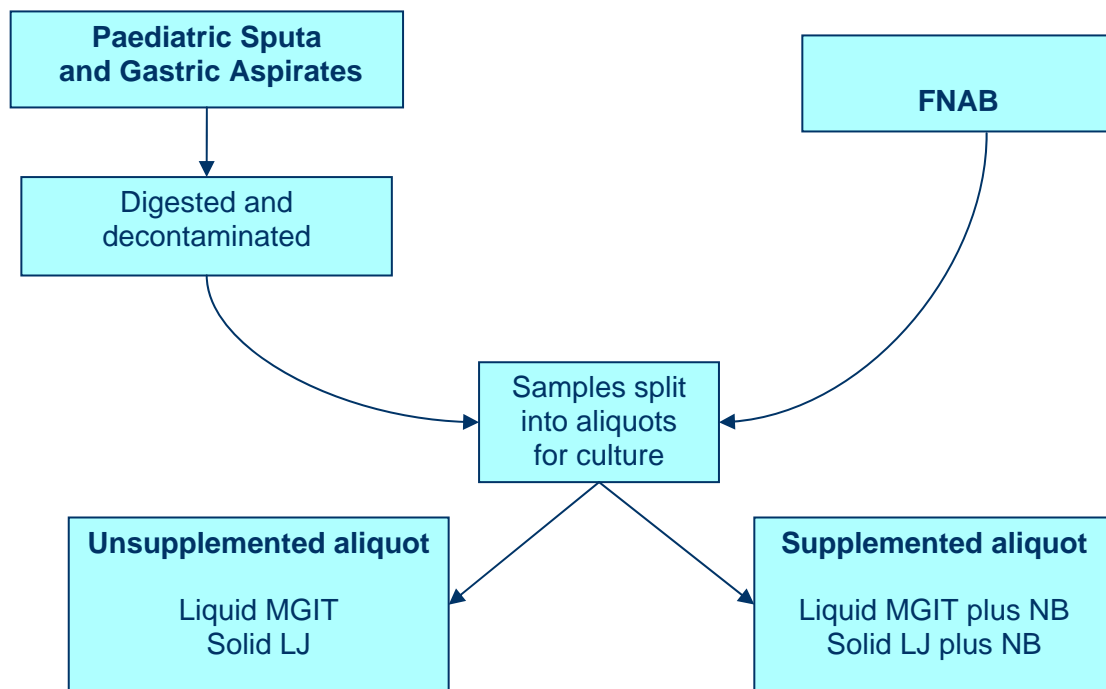


Figure 2.6 Methodology flow chart of paediatric clinical specimens processed for liquid and solid culture. Gastric aspirates and sputa were decontaminated to eliminate bacterial contamination while fine needle aspiration biopsies (FNAB), were collected aseptically and inoculated directly into MGIT tubes. After processing, samples were split into four aliquots for culture in liquid MGIT 7H9 medium and solid LJ, Löwenstein-Jensen, slants as well as in liquid and solid media supplemented with the nutrient broth growth supplement.

The sterile FNAB received in MGIT tubes were supplemented with 0.8ml of the antibiotic mixture, PANTA, and then split by aspirating 0.5ml for inoculation into a second MGIT tube enriched with 0.5ml nutrient broth. Both tubes were incubated at 37°C in the fully automated BACTEC MGIT™ 960 System (Becton Dickinson, Maryland, USA) until a positive signal was recorded. MGIT tubes were reported as negative if there was no growth signal after 42 days. The BACTEC MGIT™ 960 system monitored the tubes hourly for increasing fluorescence that determined whether the tube was instrument positive. Instrument positive tubes contained approximately 10^5 to 10^6 colony-forming units (CFU) per ml (Siddiqi, 2005).

The ZN staining procedure was used as a purity check to confirm the presence of AFB in the positive MGIT tubes, according to manufacturer's recommendations (Becton Dickinson, Maryland, USA), to rule out bacterial and fungal contamination. A smear was prepared on a clean frosted glass slide with one drop of the positive MGIT culture and one drop of fixative, 0.2% BSA, to allow the culture to adhere to the slide. The ZN stain (**Appendix C**) was performed after the smear was air-dried in the biosafety cabinet on a hot tray for 2 hours.

The positive culture smear was flooded with ZN Carbol Fuchsin (NHLS, Gauteng, South Africa) for 5 to 10 minutes and heated very gently with a low Bunsen flame till steam occurred, to melt the wax layer surrounding the AFB. The smear was rinsed with running tap water and then decolourised with 5% acid alcohol (NHLS, Gauteng, South Africa) for 1 to 2 minutes. After each smear was rinsed with tap water again, a 0.3% methylene blue counter stain (NHLS, Gauteng, South Africa) was applied for 1 minute and finally rinsed with water. The smear was then air-dried before being examined microscopically using a light microscope (Olympus X41) fitted with an oil immersion lens. A drop of immersion oil (Merck, Darmstadt,

Germany) was placed on the stained smear before lowering the immersion objective into the drop of oil to focus the image. A magnification of 500 (10x eyepiece and 50x objective lens) was used to scan the morphological detail of acid-fast bacilli present. Acid-fastness, the mycobacterial property of retaining dye after exposure to acid alcohol, was indicated by red-stained bacteria against a blue background (**Figure 2.7**) (Kleeberg *et al.*, 1980; Marais *et al.*, 2008).

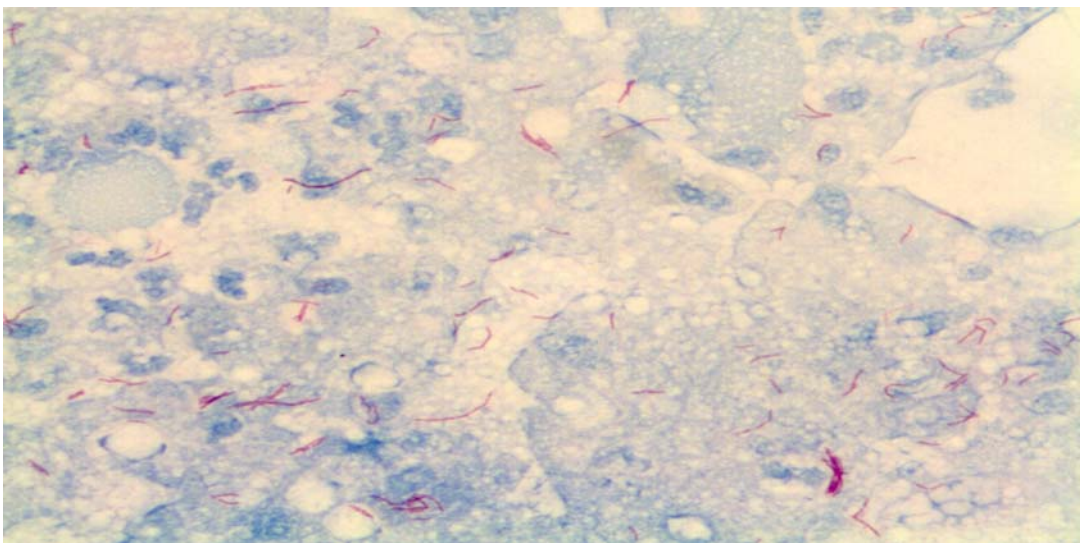


Figure 2.7 Ziehl-Neelsen stain displaying the red acid-fast bacilli that have absorbed the ZN Carbol Fuchsin stain by resisting decolourisation with 3% acid alcohol, against a blue background using a methylene blue counter stain (magnification, 100X).

Culture on blood agar plates for 48 hours at 37°C was also required to rule out bacterial contamination in positive MGIT tubes. Pure MGIT cultures that displayed acid-fastness with ZN staining and no growth on the blood agar plate were sent to the NHLS, Groote Schuur Hospital Mycobacteriology laboratory for identification and drug susceptibility testing (DST). Contaminated MGIT cultures were decontaminated with 4% sodium hydroxide to recover pure cultures of mycobacteria for identification and DST (Kleeberg *et al.*, 1980; Siddiqi, 2005).

2.4.5 Processing of paediatric samples on solid media

After the inoculation of liquid culture media, the remaining 0.5ml resuspended pellet of sputa, gastric aspirates and sterile FNAB were split into two aliquots. The first aliquot of 0.25ml was inoculated onto an LJ slant without enrichment media. The second aliquot of 0.25ml was inoculated onto an LJ slant enriched with 0.5ml nutrient broth growth supplement. Both LJ slants were incubated at 37°C in 5% oxygen for 48 hours with their agar face down to allow for the mycobacteria to adhere to the agar surface. Thereafter, the slants were incubated vertically for 6 weeks, aerated and checked weekly until growth was observed. Time-to-detection and confluence of growth was noted for all positive slants, while LJ slants showing no growth were discarded after 6 weeks.

2.4.6 Quality Control and contamination

Quality control (QC) testing was essential for checking reagents, equipment and the decontamination process. Reagents had to be fresh, pure and reconstituted according to the manufacturer's instructions for the correct concentration. If the recovery of mycobacteria was reduced, the decontamination process was too stringent. For this reason, a 4% sodium hydroxide solution was recommended when decontaminating paediatric sputa and gastric washings. Mycobacteria that were present in clinical samples were preserved by adhering to factors such as time and temperature of the decontamination process. The pH of reagents was checked, as most mycobacteria require an alkaline environment. A high pH could cause a delay in detection time of liquid and solid culture. The correct centrifuge speed of 3000 rpm for 20 minutes at room temperature (22-24°C) was adhered to for optimal pellet

formation. Samples were decanted immediately after centrifuging to avoid the concentrated pellet reconstituting or diluting. MDR strains, treated patients or paucibacillary paediatric samples were handled with care as the inoculum size was important for culture. A negative MGIT control was added to each batch of work by processing phosphate buffer as if it were a specimen, to control cross-contamination within each batch. Laboratory cross-contamination caused by *M. tuberculosis* is responsible for misdiagnosis of tuberculosis and impacts on the clinical and therapeutic management of children. Quality control of microbiological procedures is therefore essential as unnoticed cross-contamination in diagnostic laboratories leads to over-estimation of tuberculosis cases (Carroll *et al.*, 2002; Siddiqi, 2005; Martinez *et al.*, 2006).

2.5 Statistical analysis

Data analysis was done using Statistica (Version 8, Statsoft, USA). The median and mean detection time for each culture method was compared using the paired student's t-test. The rates of recovery were compared and analysed by McNemar's chi-square test and a P value of smaller than 0.05 was considered statistically significant.

CHAPTER THREE

RESULTS

3.1 Optimisation of *M. bovis* BCG culture conditions

Difficulties have been experienced in culturing *M. bovis* BCG as it is a fastidious organism that requires different nutritional requirements to wild-type *M. bovis*. The first part of this study investigated different growth supplements and *in vitro* growth conditions using *M. bovis* BCG as a model to optimise growth supplements for *M. tuberculosis*. The aim of this preliminary experiment was to ultimately enhance the detection of mycobacteria in paediatric clinical specimens.

3.1.1 Time-to-detection of *M. bovis* BCG cultures in liquid media

M. bovis BCG test strains were isolated from 12 HIV-infected infants who developed BCG disease. The pure cultures of these strains were aliquoted and diluted in four different growth supplements, saline, polyoxyethylene stearate, Tween 80 and nutrient broth before culturing in MGIT tubes. After incubation at 37°C in the fully automated BACTEC MGIT™ 960 System, time-to-detection of each enriched MGIT tube was measured and compared. **Figure 3.1** summarises the detection times or days to culture positivity of *M. bovis* BCG cultured in each of the four growth supplements. Positive growth for cultures diluted in saline occurred within 7 days, whereas both those inoculated in polyoxyethylene stearate and nutrient broth were culture positive in 9 days. Tween 80 cultures were detectable in 11 days. The mean time-to-detection for these test strains (n=12) was 3 days for

both polyoxyethylene stearate and nutrient broth cultures, and 4 days for each of the saline and Tween 80 cultures.

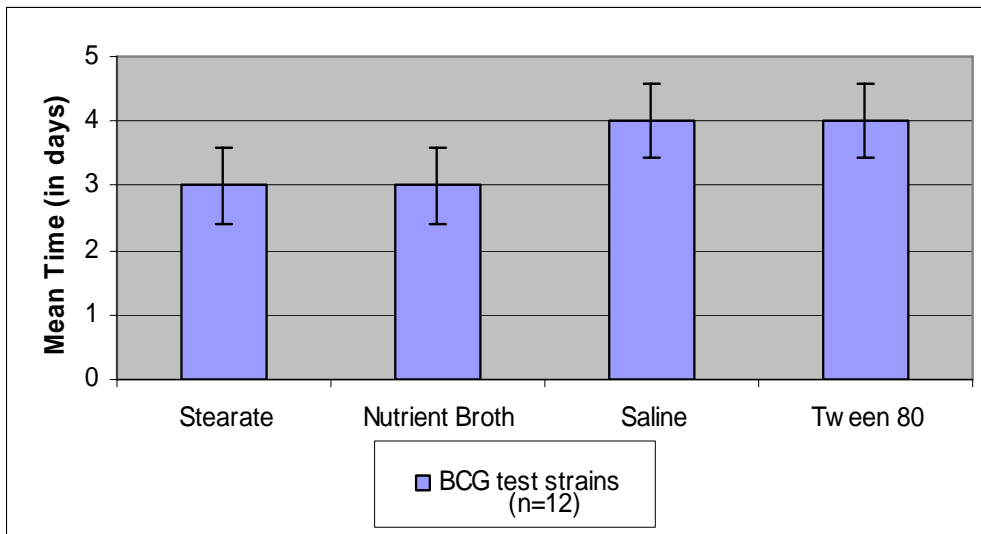


Figure 3.1 Mean time-to-detection, in days, of *M. bovis* BCG test strains enriched with different growth supplements. The bar on the left represents *M. bovis* BCG colonies that were diluted in stearate then cultured in MGIT liquid media. The mean detection times were 3 days for both the stearate and nutrient broth cultures, and 4 days for the saline and Tween 80 cultures.

Two *M. bovis* BCG reference vaccine strains, Danish and Tokyo strains, were included as controls (**Figure 3.2**) since known ATCC strains of *M. bovis* BCG were not available.

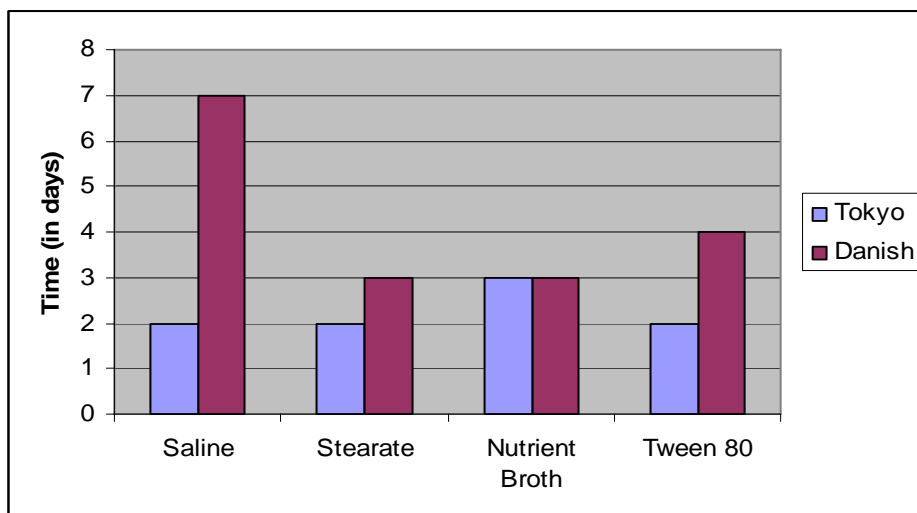


Figure 3.2 Time-to-detection, in days, of the Danish and Tokyo reference vaccine strains enriched with different growth supplements. Both strains were reconstituted and processed similarly to the BCG test strains. The time-to-detection of the four growth supplements were compared. The mean TTD for the Danish strain cultures was 4.3 days and the Tokyo strain TTD was 2.3 days.

3.1.2 Time-to-detection of *M. bovis* BCG cultures on solid media

As soon as the MGIT cultures developed a positive signal, they were sub-cultured onto duplicate solid LJ, Pyruvate LJ and Middlebrook 7H11 slants. The mean visual time-to-detection and confluence of growth were recorded for each of the *M. bovis* BCG solid cultures. As indicated in **Figure 3.3**, Middlebrook 7H11 agar slants and LJ slants performed the best with a confluent growth in the shortest time ($P=0.08$) for both vaccines and test strains, while the recommended pyruvate LJ slants, routinely used for wild-type *M. bovis*, showed poor growth after 24 days of incubation.

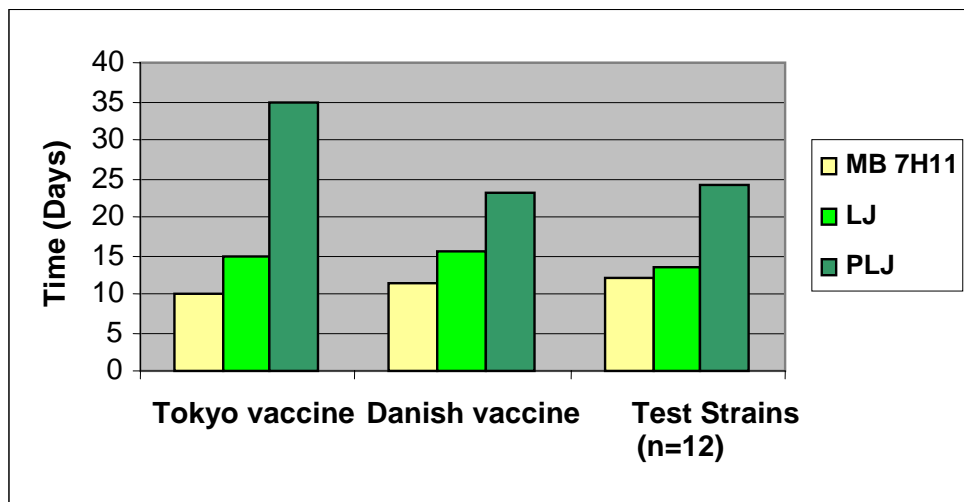


Figure 3.3 Time-to-detection, in days, for Tokyo and Danish *M. bovis* BCG reference vaccine strains and mean time-to-detection for *M. bovis* BCG test strains (n=12) on different solid media. Middlebrook 7H11 (MB 7H11) agar slants showed a confluent growth within 12 days and Löwenstein-Jensen (LJ) slants within 13.5 days. Pyruvate LJ (PLJ) slants showed a light to moderate growth within 24 days ($P=0.08$).

Although the nutrient broth and stearate growth supplements displayed a shorter mean time-to-detection (3 days), that was not significant compared to the saline and Tween 80 of 4 days (**Figure 3.1**), the nutrient broth was selected for availability and cost-effectiveness for the next phase of the study. The selected nutrient broth was subsequently tested on paediatric diagnostic specimens for improved mycobacterial yield and reduced time-to-detection.

3.2 Demographics of paediatric study population

A total of 804 consecutive paediatric clinical specimens were selected as they were received in the routine mycobacteriology laboratory. Gastric aspirates, sputa and FNAB were collected from 493 children, less than 15 years of age, admitted to Tygerberg Children's Hospital with suspected mycobacterial disease from June 2007 to February 2008. **Table 3.1** illustrates the demographics of the children included in this study. Of the 804 samples, 801 samples were cultured as 3 patients were older than 15 years of age and were therefore excluded from the study.

Table 3.1 Demographics of paediatric patients 0-14 years of age.

Sample and patient description	Sample and patient numbers
Total number of paediatric specimens evaluated	801
Total number of children in study	493
Number of paediatric cultures positive for mycobacteria	88
Number of children with positive TB cultures	70
Demographics of children with positive TB cultures	n (%)
Age groups	
<1 year	24 (34.3%)
1-4 years	34 (48.6%)
5-9 years	6 (8.6%)
10-14 years	6 (8.6%)
Gender	
Male	36 (51%)
Female	34 (49%)
HIV status	
HIV positive	14 (25.9%)
HIV negative	40 (74.1%)
Not tested	16 (22.9%)

A total of 88 MGIT liquid cultures from 70 children were positive for mycobacteria, as some children had more than one positive culture. The majority (83%) of the children were less than 5 years of age. The mean and median age of the study population was 18 months, with a range of less than 1 month to 180 months (**Figure 3.4**).

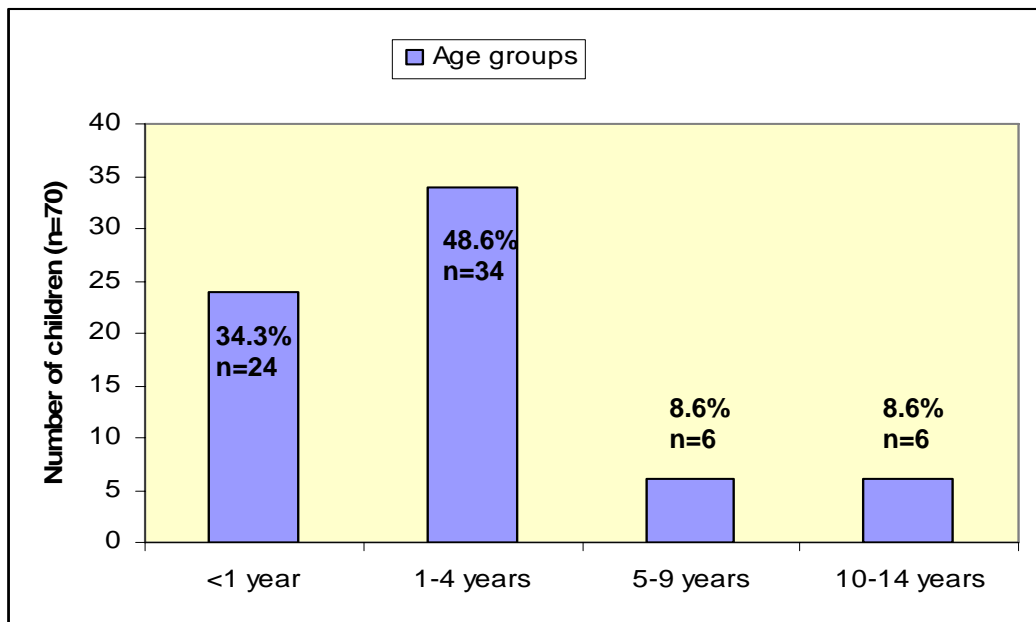


Figure 3.4 Age group distribution of paediatric study population. The majority of children (n=34) with culture-confirmed tuberculosis were in the 1-4 year age group, followed by children less than one year of age (n=24).

As illustrated in **Figure 3.5 (A)** the gender distribution of the total study population (n=493 children) consisted of 55% males (n=271) and 45% females (n=222), whereas **(B)** the 70 culture-confirmed tuberculosis children showed a 51% male (n=36) and 49% female (n=34) distribution. The percentage of positive cultures found in boys was therefore 13.3% (36/271) and for girls was higher at 15.3% (34/222).

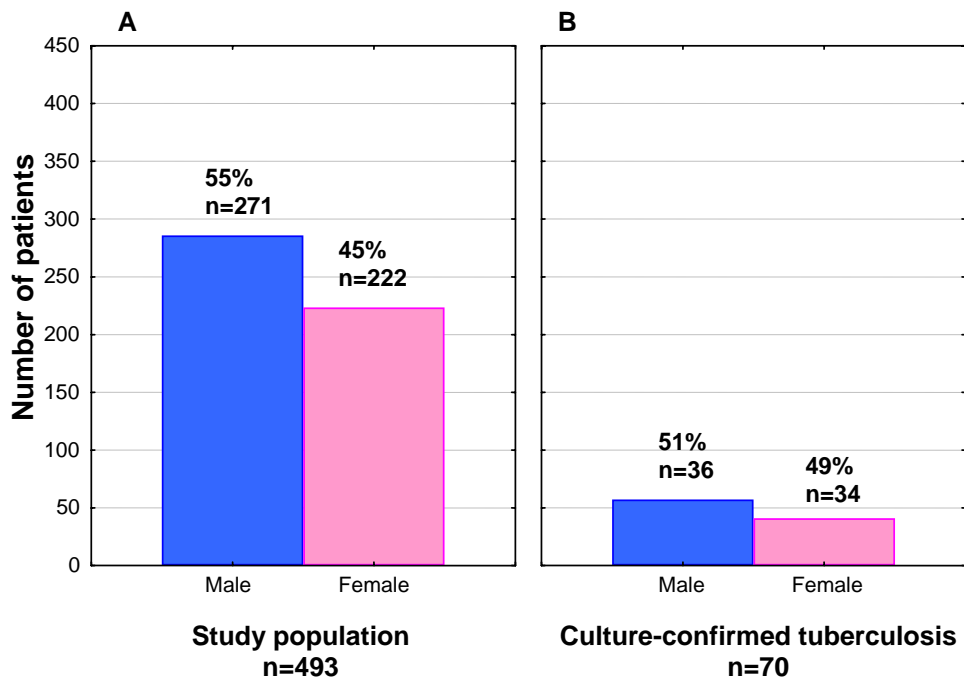


Figure 3.5 Study population gender distribution. (A) The percentage of male (55%) and female (45%) children (n=493) of the total study population are represented here; (B) illustrates the percentage of children, male (51%) and female (49%) with positive mycobacterial cultures (n=70) (P=0.84).

Figure 3.6 illustrates the HIV status of the children with culture-confirmed tuberculosis (n=70). Of these, 22.9% (16/70) were not tested for HIV infection. Of the 54 children tested for HIV infection, 25.9% (n=14) tested positive and were therefore co-infected with HIV and tuberculosis. The HIV positive children had a mean age of 25.6 months and yielded 11 *M. tuberculosis*, 3 *M. bovis* BCG, but no NTM were detected. The remaining 74.1% (n=40) children infected with tuberculosis (35 *M. tuberculosis*, 2 *M. bovis* BCG and 3 NTM) tested negative for HIV infection.

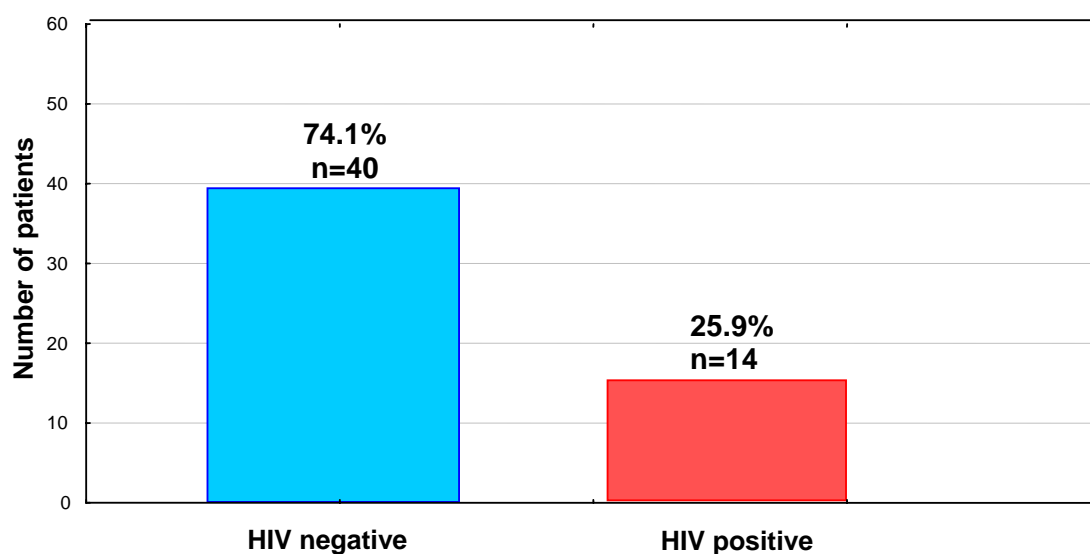


Figure 3.6 HIV status of paediatric study population. Of the 70 children with positive mycobacterial cultures, 77.1% (n=54) were tested for HIV infection. Seventy four percent (n=40) of the children infected with tuberculosis tested negative for HIV infection, whereas 26% (n=14) of the children with tuberculosis tested positive for HIV.

3.3 Source distribution of paediatric diagnostic specimens

Among the 801 clinical samples processed, 98.4% (n=788) were respiratory specimens, consisting of 125 sputa and 663 gastric aspirates, and 1.6% (n=13) were extrapulmonary FNAB, as summarised in **Table 3.3**. Mycobacteria were isolated (from routine and enriched media) from 10% (66/663) gastric aspirates, 13.6% (17/125) sputa and 38.5% (5/13) FNAB.

Table 3.2 Comparison of respiratory and extrapulmonary paediatric diagnostic specimens. Respiratory specimens, sputa and gastric aspirates, and extrapulmonary FNAB were collected from children less than 15 years of age with suspected mycobacterial disease. The majority of specimens, 98.4%, were respiratory, while 1.6% were extrapulmonary. FNAB had the highest absolute yield, 38.5%, whereas 75.0% of the relative yield was from gastric aspirates

Specimen type	Respiratory		Extrapulmonary
	Sputum	Gastric Aspirate	FNAB*
Number of specimens (%)	125/801 (15.6)	663/801 (82.8)	13/801 (1.6)
Absolute mycobacterial culture yield ^α	17/125 (13.6)	66/663 (10.0)	5/13 (38.5)
Relative mycobacterial culture yield [§]	17/88 (19.3)	66/88 (75.0)	5/88 (5.7)
Fluorescent Auramine stain yield	9/125 (7.2%)	No microscopy done [#]	No microscopy done
Total	98.4%		1.6%

*FNAB – Fine Needle Aspiration Biopsy

^αAbsolute Mycobacterial yield: calculated using the number of culture positive specimens as numerator and the total number collected, for each specimen type, as denominator

[§]Relative Mycobacterial yield: calculated using the number of type specific culture positive specimens as numerator and the total number of positive specimens as denominator

[#]No microscopy was performed on gastric aspirates and FNAB according to the NHLS standard method

3.4 Smear preparation of paediatric sputum specimens

Smears were only prepared and stained with Auramine-O fluorescent stain from 125 sputum specimens (see **Table 3.3**) that represented 15.6% of the total study population. Of the 125 stained sputum smears examined for the presence of AFB, 7.2% (n=9) were positive for AFB. This result is not a true reflection, since the

majority of specimens in this study were gastric aspirates (n=663) as children struggle to expectorate sputum. The value of smear microscopy in paucibacillary paediatric samples is questionable considering the reduced sensitivity of the test and the difficulty in collecting good quality samples from children, yet positive AFB smears provide clinicians with results within 24 hours.

3.5 Liquid culturing of paediatric diagnostic specimens

3.5.1 Mycobacterial yield of paediatric specimens in liquid media

Of the 801 samples processed, 88/801 (11.0%) were culture positive in the MGIT 7H9 liquid broth when using the BACTEC MGIT™ 960 System. The recovery rate of mycobacteria from unsupplemented MGIT 7H9 liquid medium was compared to MGIT 7H9 liquid medium enriched with a nutrient broth growth supplement.

Of the 88 positive MGIT cultures, 48 cultures were positive in both the supplemented and unsupplemented MGIT. However, 26 cultures were positive in the supplemented MGIT only and 14 cultures were positive in the unsupplemented MGIT only, as illustrated in **Table 3.3**. Even though the reconstituted sediment was mixed thoroughly during pipetting, equal distribution of acid-fast bacilli may have been problematic when aliquoting paucibacillary specimens.

Table 3.3 Positive mycobacterial liquid cultures. Amongst the 88 positive liquid cultures, 14 cultures were positive in unsupplemented MGIT tubes only, whereas 26 cultures were positive in supplemented MGIT tubes only. However, 48 cultures showed positive growth in both the unsupplemented and supplemented MGIT tubes

	Unsupplemented MGIT (A)	Supplemented MGIT (B)
Positive number of cultures in A or B	n=14	n=26
Positive number of cultures in A and B	n=48	n=48
Total positive liquid cultures	n=62	n=74

Although not statistically significant, the unsupplemented MGIT 7H9 liquid medium yielded 70.5% (62/88) positive cultures, whereas the supplemented MGIT 7H9 broth recovered 84.0% (74/88) positive cultures (P=0.11) as seen in **Figure 3.7**.

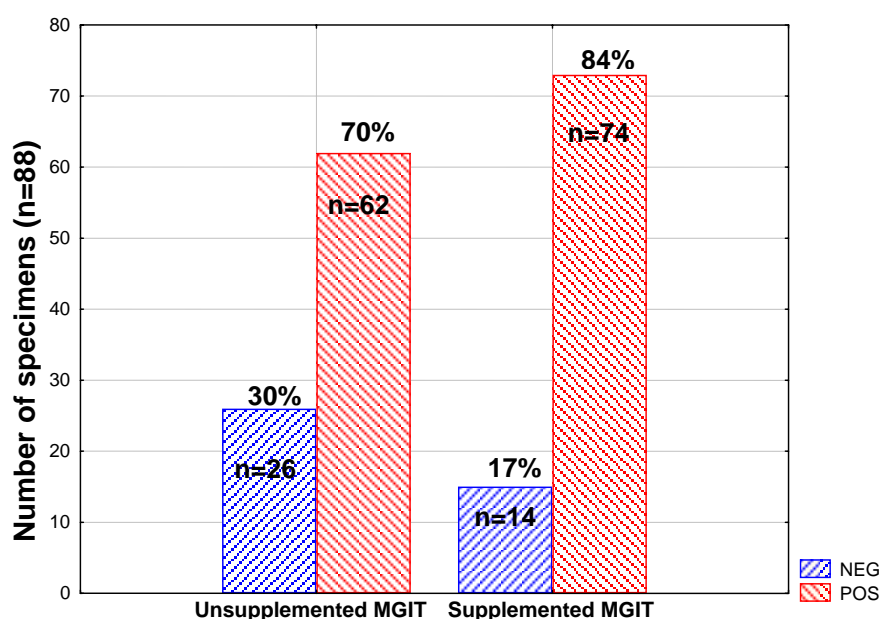


Figure 3.7 Mycobacterial yield for liquid media. Of the 88 positive MGIT liquid cultures, the unsupplemented MGIT tube yielded 70% (n=62) positive cultures. The highest mycobacterial yield of 84% (n=74) was found in the MGIT supplemented with the nutrient broth growth supplement, that recovered 14% (n=12) more positive cultures than the unsupplemented MGIT (P=0.11). The corresponding samples that were negative after 42 days could possibly have been due to the uneven distribution of acid-fast bacilli when aliquoting paucibacillary paediatric specimens.

3.5.2 Time-to-detection of paediatric specimens in liquid media

Figure 3.8 summarises the data for the average time required to detect the growth of mycobacteria in liquid cultures from paediatric diagnostic specimens. The MGIT 7H9 liquid broth showed a significant ($P < 0.001$) reduction in the mean detection time of 18.5 days to 12.4 days when it was enriched with the nutrient broth growth supplement. The addition of the growth supplement to MGIT 7H9 liquid media therefore allowed detection of all supplemented positive mycobacterial cultures 7 days earlier than unsupplemented MGIT cultures.

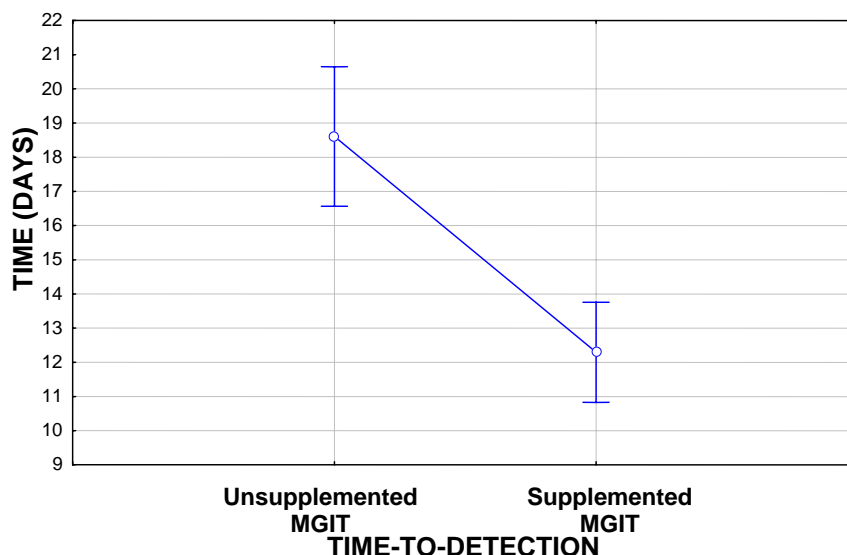


Figure 3.8 Mean time-to-detection, in days, for unsupplemented and supplemented liquid media. The supplemented MGIT that was enriched with nutrient broth ($n=74$), showed a significant difference in a detection time of 12.4 days, compared to the unsupplemented MGIT ($n=62$), with a detection time of 18.5 days ($P < 0.001$). Vertical bars denote 95% confidence intervals.

3.5.3 Contamination rate of paediatric specimens in liquid media

The bacterial contamination rate for the unsupplemented MGIT 7H9 liquid medium was 3.0% (24/801), and 3.4% (27/801) for the supplemented MGIT 7H9 liquid medium. Depending on the specimen and population type, the normal range for bacterial contamination has been reported as 3-7% for liquid media (Siddiqi, 2005).

3.5.4 Cross-contamination rate of paediatric specimens

The cross-contamination rate for the study was 2.9% as two out of the seventy batches of specimens that were processed revealed growth in the MGIT negative control tube due to air flow problems experienced in the biosafety cabinet. Positive cultures from specimens processed in these batches were sent for spoligotyping to confirm which cultures were true positives as contaminants had the same spoligotype pattern, whereas true positives all had different spoligotype patterns. Processing of paediatric specimens was resumed after the cabinet was serviced and the HEPA filters were replaced. The normal rate of laboratory cross-contamination for *M. tuberculosis* ranges from 0.1 to 3.0% (Martinez *et al.*, 2006).

3.6 Solid culturing of paediatric diagnostic specimens

3.6.1 Mycobacterial yield and detection times of paediatric specimens on solid media

Of the 801 clinical specimens, only 1.5% (n=12) cultures were positive on solid LJ slants (P=0.99). Unsupplemented LJ slants yielded 5.7% (5/88) positive cultures, while supplemented LJ slants recovered 11.4% (10/88) positive cultures. The inoculum size for solid media was 50% compared to liquid media according to standard methodologies in published data (Stager *et al.*, 1991; Rivera *et al.*, 1997; Somoskovi & Magyar, 1999; Tortoli *et al.*, 1999; Somoskovi *et al.*, 2000; Chien *et al.*, 2000; Sharp *et al.*, 2001; Lu *et al.*, 2002). The mean detection time was 27 days for both slants, whereas the median detection time was 25 days for supplemented LJ slants and 22 days for unsupplemented slants as shown in **Table 3.4**. No positive cultures were detected on solid media only.

Table 3.4 Mycobacterial yield and time-to-detection for solid Löwenstein-Jensen slants. Of the 12 positive cultures on solid LJ media, 6% (5/88) grew on unsupplemented LJ slants, whereas 11% (10/88) grew on LJ slants supplemented with the nutrient broth growth supplement. The mean and median detection times for unsupplemented LJ cultures was 22 and 27 days respectively, and for supplemented LJ cultures 25 days and 27 days respectively.

Number of patients	Mycobacterial yield		Time-to-detection (days)	
	Unsupplemented LJ (n=5)	Supplemented LJ (n=10)	Unsupplemented LJ	Supplemented LJ
P01	+	-	39	No growth
P02	-	+	No growth	17
P03	-	+	No growth	38
P04	-	+	No growth	27
P05	-	+	No growth	27
P06	+	+	22	22
P07	+	+	21	21
P08	+	+	21	21
P09	-	+	No growth	42
P10	-	+	No growth	22
P11	+	-	30	No growth
P12	-	+	No growth	30
Mean TTD (days)			27	27
Median TTD (days)			22	25
Range TTD (days)			21-39	17-42

3.6.2 Contamination rate of paediatric specimens for solid media

The bacterial contamination rate for the unsupplemented LJ slants was 14.6% (117/801), and 14.9% (119/801) for the supplemented LJ slants. The normal rate of bacterial contamination has been reported as 3 to 5% for solid media (Siddiqi, 2005).

3.7 Comparison of liquid and solid culturing methods

Liquid media yielded 11.0% (88/801) positive cultures in 12.4-18.5 days compared with 1.5% (12/801) positive cultures detected from solid media over a period of 27

days. **Figure 3.9** illustrates the significantly ($P<0.001$) improved mycobacterial yield of liquid media compared to solid media in paediatric mycobacterial cultures. Although a smaller inoculation volume (0.25ml versus 0.5ml) was applied to the LJ slants, which probably biased results in favour of the liquid MGIT media, the difference in mycobacterial yield seems far in excess of what can be attributed to differences in inoculation volume. The superior mycobacterial yield achieved with MGIT broth compared to LJ slants has been well established in previous comparative studies

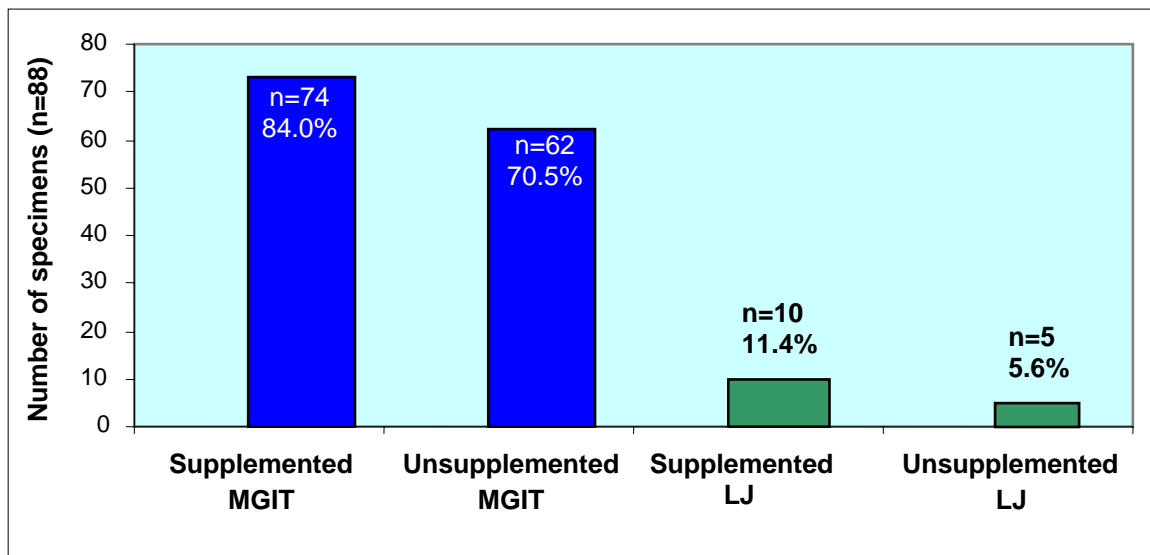


Figure 3.9 Mycobacterial yield in liquid media compared to solid media. The highest yield of mycobacteria (84%) was cultured in the MGIT tubes enriched with the nutrient broth growth supplement, followed by the conventional or unsupplemented MGIT tubes that recovered 70% (n=62) positive mycobacterial cultures. The solid Löwenstein-Jensen slants enriched with nutrient broth, showed a recovery rate of 11% (n=10) positive cultures, whereas the conventional LJ only recovered 6% (n=5) positive cultures.

3.8 Mycobacterial identification of positive paediatric cultures

MGIT 7H9 liquid media that recorded a positive signal in the BACTEC MGIT 960 System were sent to NHLS, Groote Schuur Hospital for mycobacterial identification after a purity check revealed the presence of AFB and any bacterial contamination was excluded. The in-house PCR method of identification yielded 88.6% (78/88) *M. tuberculosis*, 5.7% (5/88) *M. bovis* BCG and 5.7% (5/88) NTM. The majority of mycobacterial isolates (75%) were cultured from gastric aspirates, whereas sputa yielded 19.3% and FNAB recovered 5.7% of the isolates as indicated in **Table 3.5**.

Table 3.5 Mycobacterial yield from various specimen types. The majority, 88.6%, of the isolates identified were *M. tuberculosis* (MTB), followed by 5.7% *M. bovis* BCG (BCG), and 5.7% nontuberculous mycobacteria (NTM). Most of the isolates (75.0%) were recovered from gastric aspirates.

Specimen type	MTB	BCG	NTM	Total
Gastric Aspirates (n)	60	2	4	66 (75.0%)
Sputa (n)	16	0	1	17 (19.3%)
FNA (n)	2	3	0	5 (5.7%)
Total n (%)	78 (88.6%)	5 (5.7%)	5 (5.7%)	88

3.9 Drug susceptibility testing on positive paediatric cultures

NTM cultures (5/88) were not tested for drug susceptibility as patients are only treated with clarithromycin in combination with rifampicin and/or ethambutol for clinically significant NTM infections. Of the 78 *M. tuberculosis* isolates, 73 isolates were drug susceptible and 5 were MDR. Of the 5 *M. bovis* BCG isolates recovered, three were resistant to Isoniazid (INH) but sensitive to Rifampicin (RIF), and two were drug susceptible as illustrated in **Table 3.6**.

Table 3.6 Comparison of mycobacterial yield and drug susceptibility testing. Of the 83 positive mycobacterial cultures tested for drug susceptibility, 73/83 *M. tuberculosis* (MTB) and 2/83 *M. bovis* BCG (BCG) cultures were susceptible to INH and RIF, whereas 3 *M. bovis* BCG cultures were resistant to INH and RIF, whereas 5 *M. tuberculosis* cultures were multidrug resistant.

	MTB	BCG	Total
Susceptible (sensitive to INH and RIF)	73/83 (88.0%)	2/83 (2.4%)	75/83 (90.4%)
Mono-resistant (resistant to INH)	0	3/83 (3.6%)	3/83 (3.6%)
Multidrug resistant (resistant to INH and RIF)	5/83 (6.0%)	0	5/83 (6.0%)

Figure 3.10 summarises the results of the drug susceptibility tests performed on positive paediatric MGIT cultures using an in-house PCR method (De Wit *et al.*, 1990) at the GSH Mycobacteriology laboratory (NHLS). The majority, 90.4% (n=75), were susceptible to the antituberculous drugs INH and RIF, whereas 3.6% (n=3 BCG) were mono-resistant (resistant to either INH or RIF), and 6.0% (n=5 MTB) were multidrug resistant (resistant to INH and RIF).

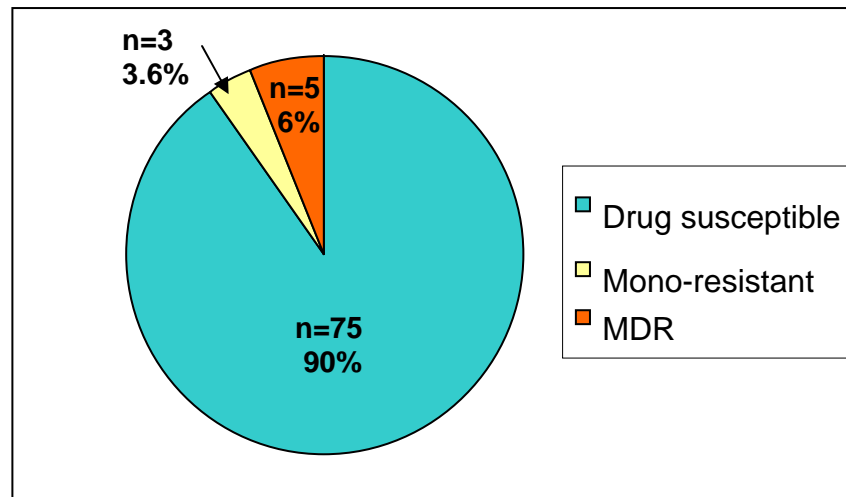


Figure 3.10 Drug susceptibility testing on paediatric positive cultures. Of the 88 positive mycobacterial cultures, 83 were tested for drug susceptibility. The majority of the isolates, 90.4% (75/83), were drug susceptible, 3.6% (3/83) were resistant to either INH or RIF, and 6.0% (5/83) were multidrug resistant.

CHAPTER FOUR

DISCUSSION

4.1 Introduction

This study evaluated the use of a nutrient broth growth supplement to improve the sensitivity and detection time in paediatric specimens. This resulted in improved yield and significantly reduced detection times of cultures from paediatric clinical specimens.

4.2 Growth supplementation of mycobacterial culture media

There is limited documentation regarding paediatric diagnostic tests for tuberculosis, especially the culturing of paucibacillary samples. As the study of micro-organisms is difficult using microscopic single cells, it is general practice therefore to study pure cultures of a single cell type (Difco Laboratories, 1984). Difficulties were experienced with culturing *M. bovis* BCG from paediatric diagnostic specimens, as these mycobacteria did not conform to the conventional media requirements given for wild-type *M. bovis*. An experimental study was therefore conducted to test different growth supplements and mycobacterial culture media using known *M. bovis* BCG cultures as a model for *M. tuberculosis*.

In the first part of this study, *M. bovis* BCG test strains and two reference vaccine strains were diluted in four different growth supplements before inoculation into MGIT 7H9 liquid medium. Once these liquid media showed positive growth, they

were inoculated onto solid LJ, Middlebrook 7H11 and Pyruvate LJ slants, in order to identify suitable media for subsequent paediatric diagnostic specimens.

Of the four growth supplements assessed, the nutrient broth was selected for its availability and cost-effectiveness in preference to polyoxyethylene stearate, saline or Tween 80. *M. bovis* BCG grew well on both LJ and Middlebrook 7H11 culture media, but grew poorly on Pyruvate LJ, although it has been reported that the wild-type *M. bovis* grew well on Pyruvate LJ slants (Keating *et al.*, 2005).

The marked differences in nutritional requirements for wild-type *M. bovis*, where no meat extract is needed, and *M. bovis* BCG, that requires a meat supplement as found in this study, accentuate the value and need for further research in optimising culture conditions for childhood tuberculosis specimens. The selected nutrient broth was subsequently tested on paediatric diagnostic specimens for improved mycobacterial yield and reduced time-to-detection.

4.3 Demographics of paediatric study population

A prospective, hospital-based, laboratory study was conducted for children admitted to Tygerberg Children's Hospital with suspected mycobacterial disease. This study may have introduced a selection bias as it excluded children from community-based clinics and children over 15 years of age and adults. The median age, 18 months, of the 70 children with culture-confirmed tuberculosis was very young, probably due to close contact with a family member with active tuberculosis. The majority of children with tuberculosis, 82.8%, were under 5 years of age because children normally enter the "safe-school age" from 5-10

years as observed in previous studies and in this study (WHO, 2007; Marais *et al.*, 2005b). The gender distribution of the 801 children was fairly even with 55% males and 45% females, yet more females, 49%, proportionate to males were found in the 70 culture-confirmed children ($P=0.84$). Of the 54 children tested for HIV, 14 children were positive with 11 *M. tuberculosis* cultures and 3 *M. bovis* BCG, but no NTM were cultured in this group.

4.4 Processing of paediatric diagnostic specimens

In this study, smear microscopy was performed on paediatric sputum samples only, according to the NHLS standard method. The smear positivity rate of 7.2% found in this study population was comparable to rates of 5-10% that have previously been reported for children of the same age (Nelson & Wells, 2004; Schaaf *et al.*, 2005).

The processed clinical samples from children were initially split into a control aliquot, without growth supplement, as the routine or conventional method, and an intervention aliquot that included growth supplement, and were then cultured in liquid and solid media. This parallel inoculation into supplemented and unsupplemented media was limited by the restricted amount of sediment, as after decontamination, smears were prepared from the concentrated sediments of the paediatric specimens for fluorescent Auramine-O staining. In addition, a 0.3ml increase in volume of phosphate buffered saline, from 1.2 to 1.5 ml, used to resuspend the sediment after decontamination could have decreased the sensitivity due to specimen dilution. However, similar findings regarding decreased

sensitivity due to specimen dilution were reported by Somoskovi and Magyar (1999).

4.4.1 Mycobacterial culture of paediatric diagnostic specimens

Mycobacterial culture allows for a definitive diagnosis of tuberculosis and differentiation between *M. tuberculosis* and NTM (WHO, 2006). As *M. tuberculosis* is a slow-growing bacterium that requires specialised culture media (Frankel *et al.*, 1970; Lennette *et al.*, 1980; Koneman *et al.*, 2006), liquid-based mycobacterial detection systems such as the BACTEC MGIT™ 960 System allow for improved sensitivity and reduced time-to-detection in comparison with the conventional solid LJ media routinely used in the mycobacteriology laboratory (Siddiqi, 2005; Hanna *et al.*, 1999; Tortoli *et al.*, 1999; Maconda *et al.*, 2000). The cost and laboratory infrastructure required, however, remain a major limitation in resource-poor settings (Marais and Pai, 2006a; Pai *et al.*, 2008). The average detection time of growth using conventional solid media is 2 to 4 weeks, while enriched liquid cultures facilitate growth within 1 to 2 weeks. Isolates are then sent for species identification and antibiotic susceptibility testing which delay results by another 2 to 3 weeks (Gray, 2004). According to Bird *et al.* (1996) and Tokars *et al.* (1996), there is a crucial need for diagnostic efficiency by using recommended rapid testing methods for laboratory improvement and effective patient management.

The clinical importance of these results means that not only were more cases detected with the improved mycobacterial yield, but the reduced detection time ensured earlier treatment of paediatric patients with tuberculosis to minimise organ damage and possibly decrease transmission in the community, specifically for the 10 to 14 year old children who present with an adult-type tuberculosis.

4.4.2 Mycobacterial recovery rate of solid paediatric cultures

Recovery rates of mycobacteria in supplemented solid paediatric cultures (10%), were double those of unsupplemented LJ slants (5%). This observation was in contrast to reports by Tortoli *et al* (74.0%), Lee *et al* (87.2%), Rivera *et al* (89.3%), Chien *et al* (75.8%), Somoskovi *et al* (81.8%), Hanna *et al* (69.0%) and Lu *et al* (59.7%) that showed an increased recovery rate using solid media (Rivera *et al*, 1997; Hanna *et al*, 1999; Tortoli *et al*, 1999; Somoskovi *et al*, 2000; Lu *et al*, 2002; Lee *et al*, 2003). The problems experienced with low recovery rates in solid media in this study could possibly be due to the dilution of the concentrated sediment when reconstituted with phosphate buffer for subsequent culture or the smaller volume of processed sample added (0.25ml vs. 0.5ml for MGIT) according to standard methodologies. In addition, the literature not only merits liquid media versus solid media but may also reflect that the high bacillary load found in adult specimens would promote growth on solid media. No previously published data could be found regarding mycobacterial detection times or rates of recovery for children with which to compare these findings.

4.4.3 Mean detection times of solid paediatric cultures

Conventional solid media, such as egg-based LJ slants, have traditionally been used to recover mycobacteria from clinical samples, but the slow growth rate substantially delays the identification process (Rivera *et al*, 1997; Lu *et al*, 2002). The mean detection time found in this study for solid LJ slants was 26.6 days for the supplemented and 26.8 days for the unsupplemented slants (P=0.99). Similar findings were reported by Hanna *et al* (24.1 days), Tortoli *et al* (25.3 days) and

Somoskovi and Magyar (25.8 days), yet extended detection times were published by Chien *et al* (30.6 days), Rivera *et al* (29.9 days) and Somoskovi *et al* (42.2 days). Lee and colleagues (2003), however, reported a shorter mean detection time of 20.1 days for solid LJ slants (Rivera *et al.*, 1997; Hanna *et al.*, 1999; Somoskovi & Magyar, 1999; Tortoli *et al.*, 1999; Chien *et al.*, 2000; Somoskovi *et al.*, 2000).

4.4.4 Mycobacterial recovery rate of liquid paediatric cultures

Similarly to solid media mycobacterial culturing techniques, no previously published data for paediatric diagnostic methods could be found with which to compare these findings.

The MGIT 7H9 liquid medium enriched with nutrient broth growth supplement recovered the most mycobacteria, 84% (74/88) of the total number of cultures recovered by both methods, in a mean detection time of 12.4 days, followed by unsupplemented liquid culture media, 70%, in 18.5 days. The addition of a nutrient broth growth supplement to the MGIT 7H9 liquid media therefore only improved the recovery rate of mycobacteria by 14%, from 70 to 84% ($P=0.11$), which was not statistically significant, yet reduced the detection time significantly ($P<0.001$) from 18.5 to 12.4 days.

The relative mycobacterial recovery rate of 84% (74/88) obtained in this study with the supplemented MGIT was higher than Hanna *et al* (77.0%) and Somoskovi and Magyar (81.3%), yet lower than Somoskovi *et al* (96.4%), Lee *et al* (100%), Chien *et al* (94.4%), Rivera *et al* (99.2%) and Tortoli *et al* (88.0%). The recovery rate of 70.5% (62/88) in unsupplemented MGIT cultures was lower than all the published findings for adults and therefore highlights the need to supplement liquid media for

paediatric diagnostic specimens (Rivera *et al.*, 1997; Hanna *et al.*, 1999; Somoskovi & Magyar, 1999; Tortoli *et al.*, 1999; Somoskovi *et al.*, 2000; Chien *et al.*, 2000).

4.4.5 Mean detection times of liquid paediatric cultures

The mean detection time of 12.4 days (with a range of 6 to 30 days), using the supplemented MGIT 7H9 liquid medium, was comparable to Lee *et al* (11.6 days) and Stager *et al* (12.6 days), but shorter than times reported by Tortoli *et al* (14.3 days), Hanna *et al* (14.4 days), Rivera *et al* (15.7 days) and Somoskovi and Magyar (19.1 days) (Stager *et al.*, 1991; Rivera *et al.*, 1997; Hanna *et al.*, 1999; Somoskovi & Magyar, 1999; Tortoli *et al.*, 1999; Lee *et al.*, 2003). Chien and colleagues (2000), however, reported the shortest mean detection time of 10.7 days, in adult specimens that probably carry a larger bacillary load. Our data, however, indicates that the supplemented MGIT liquid medium method may be much faster than the conventional unsupplemented method routinely used for the recovery of mycobacteria from paediatric samples.

The time-to-detection of 18.5 days for the unsupplemented MGIT was comparable to Somoskovi and Magyar (19.1 days), but longer than all other detection times reported (Somoskovi & Magyar, 1999). However, the detection time study was perhaps biased in favour of the BACTEC MGIT™ 960 System because it was read every 60 minutes compared to the solid LJ media that were read once a week, but it is impossible to eliminate this small bias in a routine clinical laboratory (Chien *et al.*, 2000).

4.4.6 Contamination rate of positive paediatric cultures

In contrast to other reports, the rate of bacterial contamination in this study with supplemented MGIT at 3.4%, and unsupplemented MGIT 7H9 liquid media at 3.0%, was found to be significantly lower ($P < 0.001$) compared to 14.6% when using the solid LJ media (Wilson *et al.*, 1995; Rivera *et al.*, 1997; Somoskovi *et al.*, 2000; Lee *et al.*, 2003). These results could possibly reflect the paucibacillary nature of childhood mycobacterial disease and the quality assurance problems experienced with the production of the solid media used in this study. The majority of LJ slants used in this study either lacked malachite green, which acts as an antibacterial and antifungal agent, or pulverised easily due to problems reported with manufacturing.

Wilson and colleagues (1995) reported that the high contamination rate of 41% with solid LJ slants was responsible for the lower recovery rates found. Sixty percent of their LJ slants were either negative or the slants could not be processed due to bacterial contamination. In fact, more LJ slants were lost to contamination than were positive, as experienced in this study (Wilson *et al.*, 1995). Lee and colleagues (2003), however, reported LJ contamination rates of 10.1%, Rivera *et al.* 4%, and Somoskovi *et al.* 1.2%, whereas Tortoli and colleagues (1999) experienced contamination rates of 17.07% and 22.4% from a multicenter clinical trial. In contrast, Hanna and colleagues (1999) excluded all contaminated specimens from their final data (Rivera *et al.*, 1997; Somoskovi *et al.*, 2000).

The bacterial contamination rates for liquid cultures reported by other authors were generally higher than those found in this study as the suspected study populations were most likely adults only. In addition, the paediatric specimens in this study were

processed within 24 hours of receipt to avoid contamination. Similar contamination rates for MGIT 7H9 liquid media were found by Stager *et al* (3.7%) and Somoskovi *et al* (3.7%). Higher rates of contamination were experienced by Lee *et al* (15.1%), Tortoli *et al* (10%), Hanna *et al* (8.1%) and Chien *et al* (5.5%) (Stager *et al.*, 1991; Hanna *et al.*, 1999; Tortoli *et al.*, 1999; Chien *et al.*, 2000; Somoskovi *et al.*, 2000; Lee *et al.*, 2003).

The laboratory cross-contamination rate of 2.9% (2/70 batches) for this study was within the normal range of 0.1%-3.0% (Carroll *et al.*, 2002; Martinez *et al.*, 2006). The production of aerosolised particles after processing smear positive or culture positive specimens may be responsible for the inoculation of other specimens processed on the same day, resulting in false positives. Problems arise in high-incidence settings where several positive samples are found in one day as experienced in this study when an air-flow problem occurred in the Class II Biosafety cabinet used to process paediatric specimens (Carroll *et al.*, 2002; Martinez *et al.*, 2006).

Cross-contamination was confirmed with spoligotyping when the strains cultured from both truly infected and contaminated specimens shared the same genotypic patterns and no epidemiological links could be found between the cases. Recommendations to minimise laboratory cross-contamination include the use of independent cabinets when working with specimens or positive cultures, including more negative controls for larger batches of samples to monitor cross-contamination, as well as tracking the days on which problems occur with the biosafety cabinet air-flow (Martinez *et al.*, 2006).

4.4.7 Comparison of liquid and solid paediatric cultures

The data for the paediatric samples clearly illustrates the superior performance of enriched liquid culture media that detected 88/801 (11.0%) positive cultures, compared with solid LJ media that only recovered 12/801 (1.6%) positive cultures ($P < 0.001$).

The mycobacterial yields in liquid culture were clearly higher than those with the conventional solid media, probably due to the difference in aliquoted volumes (0.5ml vs. 0.25ml) added to each medium according to standard methodologies in previous studies (Stager *et al.*, 1991; Rivera *et al.*, 1997; Somoskovi & Magyar, 1999; Tortoli *et al.*, 1999; Somoskovi *et al.*, 2000; Chien *et al.*, 2000; Sharp *et al.*, 2001; Lu *et al.*, 2002). The rich mycobacterial ingredients found in MGIT 7H9 liquid media and the unforeseen problems experienced with the LJ slants could also have affected the difference in the mycobacterial yield for each medium.

The comparison between liquid and solid media showed the average time of 15.5 days required for liquid media, was 11.2 days sooner than the solid media, 26.7 days. The mean detection time required for enriched media, 12.4 to 26.6 days, was also shorter than found for conventional media, 18.5 to 26.8 days.

The paucibacillary nature of paediatric samples may explain the loss of positive cultures when using solid media directly from primary specimens. Similarly, Sharp *et al.* (2001) and Somoskovi and Magyar (1999) reported that liquid automated systems alone provided sufficient recovery of mycobacteria without the need for supplemental solid LJ media. This is contrary to recommendations by the CDC to use a liquid broth system in combination with solid media to improve mycobacterial

recovery rate and reduce time-to-detection in clinical samples. This data therefore suggests that for the growth of the *M. tuberculosis* complex from paediatric clinical samples, it is advisable to culture in enriched liquid media before sub-culturing onto solid media.

As these findings were not sufficiently powered to demonstrate statistically significant differences in the mycobacterial yield using supplemented and unsupplemented MGIT liquid medium, further studies should include larger cohorts with paucibacillary disease to confirm the value of growth supplements.

Accurate diagnosis of childhood mycobacterial disease relies on laboratory confirmation, which in itself is less than optimal. New diagnostic techniques need to be developed to improve the speed and sensitivity of traditional diagnostic methods, specifically in HIV-infected children where diagnosis is problematic (Marais & Pai, 2006a; Pai *et al.*, 2008). It would be of great interest to evaluate the performance of supplemented liquid growth media in combination with early detection techniques such as microscopic observed drug susceptibility (MODS), especially for the detection of pauci-bacillary mycobacterial disease.

The rapid genotypic methods now used for species identification and drug susceptibility testing still require positive cultures for DNA extraction. The Hain PCR method, for example, can be used directly on smear positive specimens, yet it has not been evaluated for direct detection in paediatric specimens specifically. In this study, only 9/125 culture positive respiratory specimens were smear positive, which confirms that direct detection is not likely to be useful in children who typically have a low bacillary load. For this reason, culture will still remain a high priority, specifically in children.

CHAPTER FIVE

CONCLUSION

Bacteriological confirmation of childhood tuberculosis is essential, as diagnosis is problematic specifically in HIV-infected children.

The preliminary comparative analysis to identify a suitable growth supplement and enriched medium confirmed that the optimisation of laboratory cultivation enhanced the growth of pure paediatric cultures. The use of a MGIT broth-based method in combination with the standard Löwenstein-Jensen and Middlebrook solid media is therefore strongly recommended for pure BCG cultures used as a model for *M. tuberculosis* culturing from children.

In addition, the selected nutrient broth growth supplement, which was subsequently evaluated on paediatric diagnostic samples, improved the mycobacterial yield and significantly reduced the detection time in liquid culture media.

These findings accentuate the need for rapid, reliable culture methods and diagnostic efficiency in mycobacteriology laboratories to facilitate earlier treatment of childhood tuberculosis.

APPENDICES

APPENDIX A

Materials

- | | |
|---|---------------------------|
| 1. 30 ml polypropylene centrifuge tubes | Merck, Darmstadt, Germany |
| 2. 3 ml sterile plastic pipettes | Lasec SA (Pty) Ltd. |
| 3. Frosted glass slides | Lasec SA (Pty) Ltd. |
| 4. Gilson pipette | Lasec SA (Pty) Ltd. |
| 5. Pipette tips | Lasec SA (Pty) Ltd. |

Equipment

- | | |
|--|---------------------------------------|
| 1. Biological Safety Cabinet | Lab&Air |
| 2. Scientific Series 9000 Incubator (37°C) | Lasec SA (Pty) Ltd. |
| 3. Hot tray (80°C) | Lasec SA (Pty) Ltd. |
| 4. Centrifuge | Heraeus Varifuge F |
| 5. Light/Fluorescent microscope | OlympusX41 |
| 6. LED fluorescent lamp | Royal Tropical Institute, Netherlands |
| 7. BACTEC MGIT 960 System | Becton Dickinson, Maryland, USA |

Reagents

- | | |
|----------------------------------|---|
| 1. Phosphate Buffer | NHLS, GAUTENG |
| 2. NaLC | Merck, Darmstadt, Germany |
| 3. Sodium hydroxide | NHLS, GAUTENG |
| 4. Sodium citrate | NHLS, GAUTENG |
| 5. 2% Hycolin disinfectant | William Pearson Chemicals, Coventry, UK |
| 6. Nutrient broth | NHLS, GAUTENG |
| 7. Tween 80 | NHLS, GAUTENG |
| 8. 0.9% sodium chloride | NHLS, GAUTENG |
| 9. Polyoxyethylene stearate | Becton Dickinson, Maryland, USA |
| 10. Microscope immersion oil | Merck, Darmstadt, Germany |
| 11. 0.2% BSA fixative | NHLS, GAUTENG |
| 12. ZN Carbol Fuchsin | NHLS, GAUTENG |
| 13. Acid alcohol | NHLS, GAUTENG |
| 14. Methylene Blue counter stain | NHLS, GAUTENG |
| 15. Auramine-O stain | NHLS, GAUTENG |
| 16. Potassium permanganate | NHLS, GAUTENG |
| 17. 70% ethanol | NHLS, GAUTENG |

APPENDIX B

Auramine-O fluorochrome stain

Reagents

1. Auramine-O

Auramine-O	0, 1 g
Ethyl alcohol, 95% conc.	10, 0 ml
Phenol crystals	3, 0 g
Distilled water	87, 0 ml

Dissolve the auramine-O and ethyl alcohol. Mix the phenol crystals with distilled water, and then mix all ingredients before storing in a brown bottle away from heat and light.

2. 0.5% acid alcohol decolourising solution

Ethyl alcohol, 70% conc.	100, 0 ml
Concentrated hydrochloric acid	0, 5 ml

3. Counter stain

Potassium permanganate	0, 5 g
Distilled water	100, 0 ml

Dissolve ingredients and store in a brown bottle.

APPENDIX C

Ziehl-Neelsen stain

Reagents

1. Carbol Fuchsin

Basic Fuchsin	3,0g
Phenol crystals	5,0g
Ethanol, 95% conc.	10, 0 ml
Distilled water up to 100 ml	

Basic fuchsin is dissolved in ethanol. The phenol is dissolved in boiling distilled water. The two solutions are mixed before the final volume is made up to 100 ml with distilled water.

2. Decolourising solution: Acid alcohol

Ethanol, 95% conc.	97, 0 ml
Concentrated hydrochloric acid	3, 0 ml

3. Counter stain

Methylene Blue chloride	0, 3 g
Distilled water	100, 0 ml

APPENDIX D

Reagent preparation

1. Tween 80 saline

Sodium chloride	19, 6 g
Tween 80 (10% polyoxyethylene)	0, 4 ml
Distilled water	4, 0 Litres

Mix ingredients and dispense 4, 5 ml aliquots into tubes. Autoclave before use.

2. OADC Middlebrook enrichment media

Oleic acid	0, 05 g
Bovine albumin	5, 0 g
Dextrose	2, 0 g
Beef catalase	0, 004 g
Sodium chloride	0, 85 g
Distilled water	100, 0 ml

Dissolve ingredients in 900 ml distilled water.

3. NALC-NaOH

For daily cultures, add up the total volume of specimens, eg 10, to be treated. Prepare an equal volume of the digestant-decontamination mixture as follows

1 N (4%) NaOH	50 mL
0.1 M (2.94%) trisodium citrate 3H ₂ O	50 mL
NALC powder	0.5 grams

Mix, sterilise and store the NaOH and trisodium citrate in sterile, screw-capped bottles. Once the NALC powder has been added, the solution must be used within 24 hours.

4. 0.67 M phosphate buffer, pH 6.8

Solution A: 0.067 M disodium phosphate

Sodium monohydrogen phosphate	9.47 grams
Distilled water	1000 mL

Solution B: 0.067 M monopotassium phosphate

Potassium dihydrophosphate	9.07 grams
Distilled water	1000 mL

Add 50 mL of the solution B to 50 mL of the solution A, then adjust the pH of the mixture to 6.8

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