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DISSERTATION

Introducing liquid culture medium for tuberculosis in northeast Thailand: an evaluation of changes in culture yield and speed of drug susceptibility testing

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AFB	acid-fast bacilli
BBL MGIT PANTA	polymyxin B, amphotericin, nalidixic acid, trimethoprim and azlocillin containing antimicrobial agent mixture
BMA City Lab	Bangkok municipal laboratory
CDC	United States Centers for Disease Control and Prevention
DOTS	directly observed therapy, short-course
DST	drug susceptibility testing
HIV	human immunodeficiency virus
INH	isoniazid
IUATLD	International Union Against Tuberculosis and Lung Disease
LJ	Löwenstein-Jensen solid culture medium
M +	positive sputum smear microscopy and/or growth of mycobacteria on culture
M.	mycobacterium
MDGs	United Nations Millennium Development Goals
MDR-TB	multi drug resistant tuberculosis
MGIT	BACTEC™ Mycobacteria Growth Indicator Tube 960, Becton Dickinson, Franklin Lakes, NJ, USA
ml	milliliters
MOPH	Ministry of Public Health
MTB	M. tuberculosis complex
NALC-NaOH	N-acetyl-L-cysteine sodium hydroxide
NaOH	sodium hydroxide
NTM	non-tuberculous mycobacteria
NTRL	National Tuberculosis Reference Laboratory
OADC	oleic acid, albumin, dextrose, catalase
ODPC 7th	Office of Disease Prevention and Control Region 7
Ogawa	Ogawa solid culture medium
PAS	para-amino salicylic acid
PNB	para-nitrobenzoic acid
RCT	randomized controlled trial

smear (+/-)	acid-fast bacilli found/not found on sputum smear microscopy
STD	sexually transmitted diseases
TB	tuberculosis
TCH	thiophene-2-carboxylic acid hydrazide
TSA	tryptic soy agar
TTAT	total turnaround time
TTD	time to detection
TTG	time to growth
TUC	Thai Ministry of Public Health – United States Centers for Disease Control and Prevention Collaboration
U.S.	United States of America
VCT	voluntary counseling and testing
WHO	World Health Organization
YDR	yield of drug susceptibility test results
YIID	yield of isolates for identification and drug susceptibility testing
μg	micrograms

1 Introduction

1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease that is transmitted by airborne particles. With an estimated 1.6 million deaths per year globally it remains one of the top causes of death from infectious diseases (Robert Koch Institut 2005). The infectious pathogen is Mycobacterium tuberculosis, a gram-positive bacterium. The roles of its complex mycobacterial cell wall envelope in growth and host interactions and the cell wall's main constituents (mycolylarabinogalactan, lipoarabinomannan, and mycolic acids) have been well described. Their three-dimensional distribution and the exact molecular mechanisms of the most effective anti-tuberculosis drugs that target the cell wall are not yet fully understood (Alsteens 2007). Acid-fast stains of the mycobacterial cell wall such as Ziehl-Neelsen have been used for more than 120 years to detect M. tuberculosis through direct sputum smear microscopy, but have not yet been standardized (Murray 2003), and researchers are searching for novel, more efficient and/or safer staining methods (Tripathi 2001, Selvakumar 2006).

There are two further mycobacteria that can cause tuberculous disease in humans: Mycobacterium africanum and Mycobacterium bovis. The three different mycobacteria M. tuberculosis, M. africanum and M. bovis (M. microti is non-pathogenic in humans and not discussed further) are referred to as M. tuberculosis complex (MTB). All mycobacteria other than MTB are referred to as non-tuberculous mycobacteria (NTM) and may cause pulmonary disease resembling tuberculosis (U.S. Department of Health and Human Services 2000). This differentiation is important, because NTM are intrinsically resistant to several anti-tuberculosis drugs.

M. tuberculosis primary affects the lungs where it can escape total immunologic elimination and persist in a dormant state, so-called latent or subclinical infection (Colston 1999). In only 10% of all infections and particularly in persons in whom the immune system is weakened by aging, systemic disease (i.e. HIV, cancer), or iatrogenic immunosuppression, TB manifests as either pulmonary TB or extrapulmonary TB (which is frequently accompanied by pulmonary TB) (U.S. Department of Health and Human Services 1995a). The most dangerous disease process is miliary TB which is a systemic spread of bacilli with multi-organ infection. It develops in about 1-2% of all cases of tuberculosis in immunocompetent individuals, but is more often found in immunosuppressed patients (Sharma 2005).

In 1882, on 24 March (today's World Tuberculosis Day), M. tuberculosis was first described by Robert Koch, who later received the "Nobel prize for Physiology or Medicine 1905 for his investigations and discoveries in relation to tuberculosis" (Kaufmann 2005). With the knowledge about the etiologic agent, its bacterial characteristics and virulence factors, drugs effective against MTB were sought, but MTB's cell wall characteristics, its slow replication rate of fifteen to twenty hours, and its intracellular dormant phase are responsible for its resistance to many antibiotics, a phenomenon also known as "antibiotic indifference" (Dickinson 1977).

For a long time, there was no treatment and control of this disease possible other than the isolation of patients in sanatoriums. Finally, in 1944, streptomycin was first shown to be effective for treatment of TB in guinea pigs and later was confirmed to improve the treatment of the disease in humans through a randomized controlled trial (RCT), conducted by the British Medical Research Council beginning in 1946. However, in a five-year follow up of this trial, monotherapy with streptomycin was found to have little benefit for the intervention group compared to the control group (Medical Research Council 1948). Most patients had developed streptomycin-resistant strains (Mitchison 2005). In a subsequent RCT conducted by the British Medical Research Council and published in 1952, combinations of streptomycin with para-amino salicylic acid (PAS) were shown to reduce the emergence of drug resistance from 79% to 11% after six months of treatment (Medical Research Council 1952). With this step, the principle of combination therapies was first established and the focus was placed on the development of new drug regimens that would be more effective, less toxic, cheaper and shorter. Isoniazid, introduced in 1952 and rifampicin, introduced in the late 1960s, are still in use as the main pillars of the current short-course regimen of six months' duration recommended by the World Health Organization (WHO) (Toman 2004e). One example of the most recent search for new effective drugs and the reduction of time needed to treat through new regimens is the promising eradication of TB in a mouse model after only two months through a new diarylquinoline drug (Andries 2005) and the evaluation of such novel ofloxacin derivatives concerning their synthesis and their antimycobacterial and toxicological properties (Dinakaran 2008).

1.2 Epidemiology

1.2.1 Global

The infectious form of TB is pulmonary TB with positive sputum for acid-fast bacilli (AFB). In most tuberculosis control programs, AFB are detected on sputum smear microscopy, and infectious cases are therefore referred to as smear-positive cases.

In the Global Tuberculosis Report 2007, WHO announced that in the year 2005 5.1 million new and relapse cases were notified by the 199 countries reporting to WHO, of which 2.4 million (47%) were new smear-positive cases (WHO 2007b). Based on surveillance and survey data from 2004 a prevalence of 14.1 million cases of all forms of TB (217 per 100,000) and an incidence of 8.8 million new cases of TB (136 per 100,000), including 3.9 million (60 per 100,000) new smear-positive cases constituted the estimated global TB burden (WHO 2007b).

"The African Region (23%), South East Asia Region (35%), and Western Pacific Region (25%) together accounted for 83% of all notified new and relapse cases and similar proportions of new smear-positive cases in 2005" (WHO 2007b). Global mortality from TB was 24 per 100,000 people (a total of 1,577,000 deaths) (WHO 2007b).

1.2.2 Thailand

When analyzing the global situation in an annual global report, WHO discusses the data reported in the specific year with a focus on the so-called 22 high burden countries. These are countries that "account for approximately 80% of the total number of new TB cases (all forms) arising worldwide each year. These countries are subject to intensified efforts in DOTS expansion [as explained in chapter 1.4] [...] [and] are not necessarily those with the highest incidence rates per capita" (WHO 2007a). Thailand was 17th in a ranking of these 22 high-burden countries in 2005.

The estimated number of new TB cases in Thailand in 2005 was 91,000 (total population: 64,233,000; incidence: 142 per 100,000 people), of which 41,000 were smear-positive cases (incidence: 63 per 100,000 people). The prevalence of all forms of TB was 204 per 100,000 people (130,000 cases).

The TB mortality rate was 19 per 100,000 people (a total of 12,000 deaths from TB in Thailand in 2005) (WHO 2007a).

1.3 Interactions between the HIV and TB epidemic

Co-infection of tuberculosis with HIV increases the infected person's risk to proceed from latent infection to active TB. While an HIV-negative person's usual lifetime risk for developing active TB is reported to be about 10%, an HIV-positive person is likely to have an annual risk of more than 10% (Corbett 2003). It was shown that "spatial and temporal variation in TB incidence is strongly associated with the prevalence of HIV infection" (Corbett 2003).

Corbett further "calculated that 31% of adult TB cases were attributable to HIV in the entire African Region in 2000 [...]. In the same region, HIV-infected cases were responsible for an estimated 7% of all TB transmission" (Corbett 2003).

Co-infection of TB with HIV leads to a spectrum of manifestations different from TB infection without HIV. Most notably, the number of extrapulmonary TB cases with negative sputum smear results rises and diagnostic testing therefore needs careful revision in these cases (Cohn 2005). Treatment of a patient with HIV infection and TB is very complicated due to drug interactions and overlapping toxicity of anti-TB and antiretroviral therapy (Aaron 2004). One of the policy changes in Thailand in 2005 was the introduction of voluntary counseling and testing (VCT) for HIV in all new TB cases (Varma 2007). This change was a step toward better cooperation between health care for TB and for HIV and was carried out in the light of findings from the "ProTEST" activities coordinated by WHO (Godfrey-Faussett 2002).

The rate of HIV infection among new adult TB cases (aged 15–49 years) in Thailand in 2005 was 7.6% (WHO 2007a). Data available on HIV rates among the general population are difficult to compile and often biased by the acceptance of test availability within the community. In an HIV Sentinel Surveillance in Thailand presented by the Division of Epidemiology, Thai Ministry of Public Health, in 2004, the median provincial HIV seroprevalence among different risk and population subgroups was described for the whole country and then compared by region during the years 1989-2003 (unpublished data, Thai Ministry of Public Health). Within the general population (represented by data available on male army conscripts, pregnant women, and blood donors) the epidemic peaked at 2.3-4% between the years 1993-95 and then was trending down to 0.5-1.2% in 2003. Within high-risk groups (represented by data available on intravenous drug users, female sex workers and male clients at sexually transmitted diseases (STD) clinics) the epidemic peaked at 10-33% around the year 2000. There was a downward trend for female sex workers (26%) and male STD clinic clients (7%) in 2003, whereas the HIV seroprevalence remained as high as 40% in the group of intravenous drug users. Regional differences were

present during the early phase of the observational period in all groups (i.e. all groups including high-risk groups and general population) with the upper northern and central regions dominating and northeast Thailand continuously being among the regions with the lowest seroprevalence.

1.4 Tuberculosis control strategies

Faced with the high numbers of deaths from TB globally despite the knowledge that highly cost effective antibiotic combination therapies were available, two targets for TB control were set at WHO's World Health Assembly in 1991 to be reached by 2000 (WHO 1991): to detect 70% of all new sputum smear-positive cases arising each year and to successfully treat 85% of these cases. This was, when WHO started to promote Directly Observed Therapy, Short-course (DOTS) which "has become the term used to describe a broader public health strategy with five principal elements" (Dye 2005):

1. political commitment,
2. case detection by sputum smear microscopy, mostly among self-referring symptomatic patients,
3. standard short-course chemotherapy with supportive patient management, including DOT,
4. a system to ensure regular drug supplies, and
5. a standard recording and reporting system, including the evaluation of treatment outcomes.

DOTS was first applied under the supervision of Dr. Karel Stýblo in the United Republic of Tanzania, who "developed the technical and managerial principles of effective tuberculosis control based on the management unit of the district" (Smith 2004). The DOTS strategy showed that the successful control of TB is multifactorial (Udwadia 2007), and that improvement of its effectiveness is only possible through a multidisciplinary network (Chaudhury 2003).

In 2000, 149 countries had adopted DOTS, and, even though the World Health Assembly targets had not yet fully been reached and were deferred until 2005, first successes had been seen (e.g. the decline in incidence of 6% per year in Peru and the reduction of the prevalence of culture-positive TB by 30% in 13 provinces of China between 1990 and 2000) (Dye 2005). Despite these successes, it still remained to be proven that DOTS was having the expected effect on the epidemiological markers of improved TB control.

The Year 2000 United Nations Millennium Development Goals (MDGs) provided a suitable framework for this query (United Nations 2001). The MDGs clearly broadened the approach to TB control: While in the pre-MDGs period successful disease control was exclusively attributed to an improvement of DOTS (higher case detection and treatment success rates), the MDGs included incidence, prevalence and mortality as measures to reflect the impact of efforts made in TB control. Between the years 1990 and 2015 TB incidence should be reduced, TB prevalence and deaths should be halved globally. In an analysis of the prospects for these goals based on a worldwide survey on tuberculosis control evolution, Dye et al. estimated that the prevalence could only be halved by 2015 if TB control programs achieved the global DOTS targets (see above) and if incidence decreased by at least 2% annually. The annual decrease in TB incidence would need to be even higher (5-6%) if death rates were to be halved by 2015. In detailed analysis it could be shown that these changes could be achieved in seven out of nine regions worldwide, but that the greatest effort needed to be put forth in Africa and Eastern Europe. HIV and multi drug resistant TB infections would pose a challenge to effective TB control in these areas. A broader agenda for TB control should be adopted worldwide, including the enhancement of the DOTS strategy and the introduction of new evidence based technology (Dye 2005).

In their article “To control and beyond: moving towards eliminating the global tuberculosis threat”, Timothy F. Brewer and S. Jody Heymann argued from a similar point of view that Global TB control needed to be reassessed, because TB epidemiology at that time had changed substantially from the time when the DOTS strategy was first designed and evaluated. In regions with a high prevalence of HIV such as Sub-Saharan Africa, latent TB infection might have been a main risk factor for a large number of patients developing active TB annually (Brewer 2004). The impact of treating latent TB infection on annual TB incidence might have been greater than what was estimated; the original estimate had used data from the Netherlands in the 1950s to 1970s (Styblo 1990), and had led to the conclusion that diagnosis and treatment of smear-positive cases only was the most effective TB control strategy in resource-limited settings (Brewer 2004). Brewer and Heymann further argued that the detection rates of sputum smear and X-ray would have to be reviewed, especially when taking into account that an estimated 56% of all active TB cases (9.1 million people worldwide) at that time were smear-negative (Brewer 2004).

Improving TB treatment programs would be of great value as primary infections with multi drug resistant tuberculosis (MDR-TB), which caused problems in maintaining anti-tuberculosis

treatment regimens, rose in populations with high rates of treatment default (Brewer 2004). In this context, especially, it became obvious that the treatment of patients with active disease only (secondary prevention) would be obviously limited compared to other effective control strategies that could protect susceptible people from acquiring the disease (primary prevention). DOTS should therefore be accompanied by improvements in research for new vaccines, infection control practices, treatment regimens, diagnostic methods, and expanded use of chemoprophylaxis (Brewer 2004). Local epidemiologic conditions such as TB, HIV and MDR-TB prevalence as well as available resources should be considered for control program development. Reliable "surveillance data needs to be gathered so that the effectiveness of TB control and elimination strategies can be assessed [...] Field studies to optimize the use of existing tools in different epidemiologic settings also need to be undertaken" (Brewer 2004). In their expert review "Multidrug-resistant and extensively drug-resistant Mycobacterium tuberculosis: epidemiology and control" which was published recently, Matteelli et al. expressed a similar opinion and emphasized the importance of laboratory diagnostic capacity improvement: "The long-term vision for the full control of MDR-TB requires the scaling-up of culture and drug-susceptibility testing capacity, which is very limited in disease-endemic countries, and the expanded use of high-technology assays for rapid determination of resistance" (Matteelli 2007).

1.5 Multi drug resistant tuberculosis

As described in chapter 1.1 drug resistance in anti-tuberculosis treatment is a phenomenon that has been known since treatment possibilities became available. The mechanisms resulting in monotherapy and their role in the etiology of drug resistance have been investigated and well described (O'Brien 1994). Today WHO defines multi drug resistant tuberculosis (MDR-TB) as an in-vitro proven resistance on at least isoniazid and rifampicin. In an updated analysis of the Global Project on Anti-tuberculosis Drug Resistance Surveillance performed in 76 regions worldwide, the median prevalence of MDR-TB was shown to be 1.0% with up to 14.2% in specified areas in Russia, Eastern Europe, Israel and China (Aziz 2006). Calculations using multiple logistic regression based on the data compiled from these regions estimated 424,203 cases as the global prevalence of MDR-TB, i.e. 4.3% of all new and previously treated TB cases in 2004 (Zignol 2006).

The two-drug combination of rifampicin with isoniazid once had been a dream come true in the anti-tuberculosis treatment, as it had been the first drug combination that showed high effectiveness at low toxic concentrations. With these two drugs as a basis, combination therapy

could be reduced to less than one year (Kim 2005). The introduction of short-term treatments, with the addition of pyrazinamide to isoniazid and rifampicin, was an important step to make a shift of TB treatment from the inpatient to the outpatient setting possible. From a socio-economic point of view, this development was of great value, but soon problems with patient compliance and an increase in relapse cases led to a strong policy effort toward the DOTS program (chapter 1.4).

Different mechanisms for the development of drug resistance have been discussed and are still under investigation. A number of bacilli as high as about 10^{16} is likely to include a mutant resistant to two drugs at the same time thus increasing the probability of multi drug resistance by selection of resistant strains through unintentional monotherapy in cavitary disease (due to the failure of first-line drugs) (Parsons 2004). There are four groups of second-line drugs available (WHO 2006c):

1. injectable anti-tuberculosis agents (e.g. streptomycin, kanamycin),
2. fluoroquinolones (ofloxacillin),
3. oral bacteriostatic second-line anti-tuberculosis agents (e.g. ethionamide, PAS),
4. other anti-tuberculosis agents with unclear efficacy (e.g. clofazimine, clarithromycin).

The term "DOTS-Plus" was introduced for the first time in 1998 as an evaluation platform and tool to "produce sound policy recommendations" for the treatment of MDR-TB (WHO 2006b).

1.6 Mycobacteriology laboratory techniques

1.6.1 Sputum smear microscopy

The role of sputum smear microscopy has been clearly defined in the context of diagnosis and treatment follow-up of tuberculosis. Chest X-ray alone has been shown to have low sensitivity and specificity (i.e. it leads to a large number of over- and under-readings, even with high intra-individual inconsistency), and is therefore not recommended as a single diagnostic test (Koppaka 2004). Mycobacteriological tests are far more reliable and sputum smear microscopy is the primary diagnostic tool in high-prevalence settings. Sputum smear microscopy is easy, does not require special material, and is therefore quick and cheap to perform. Sputum smear sensitivity is reported with 50-80% in patients with pulmonary tuberculosis (American Thoracic Society 2000). The concentration and distribution of bacilli per milliliter specimen has been shown to influence sputum smear sensitivity (Hobby 1973). Minimum concentrations of 100 – 1000 bacilli

per ml are needed for an experienced microscopist to pick up a positive result (Wolinsky 1994, Toman 2004a). Several cross-reading experiments have been performed which showed that "the frequency of agreement between equally proficient microscopists may reach 93% […] under experimental conditions and with experienced laboratory technicians" (Toman 2004b).

Despite this, there are many technical and operational influences on sputum smear quality other than the microscopist's technical experience, such as improper specimen collection, exposition of sputum to sunlight, stain and smear preparation etc. (IUATLD 2000, Van Deun 1999, Toman 2004d). However, field studies in India proved that even in rural health institutions, the quality of sputum examination was adequate (Toman 2004b):

Over-reading, i.e. reporting culture-negative sputum as smear-positive, which would lead to unnecessary anti-tuberculosis treatment, was not a problem (1.9% at peripheral health centers compared to 1.6% at the reference laboratory). Under-reading, i.e. reporting culture-positive sputum as smear-negative, which would lead to a missed diagnosis, appeared more frequently than over-reading but still in a range comparable to the reference laboratory (23% at peripheral health centres compared to 26% at the reference laboratory) (Nagpaul 1968).

There are several methods used for sputum examination, the two most widely used being: Ziehl-Neelsen stain with conventional light microscopy, and fluorescence microscopy. Comparing these methods, there is only a very slight difference in favor of fluorescence microscopy in the positive yield and practically no difference in false-positive results (Holst 1959, Toman 2004b). A fluorescence microscope and its technical supply are more cost-intensive than conventional light microscopy, but the size of the fluorescence microscopic field is larger (0.34 mm² compared to 0.02 mm² for a conventional microscope), so sputum microscopy can be performed much faster. For these reasons, fluorescence microscopy is recommended, but only for large, technically well-equipped laboratories with a high number of specimens per day and well-trained personnel (Bennedsen 1966, Smithwick 1976, Toman 2004c).

1.6.2 Mycobacterial culture

The role of mycobacterial culture is changing and highly dependent on the context in which it is used. The growth detection limit on mycobacterial culture is only about 100 bacilli per ml sputum. Even though the sensitivity of mycobacterial culture is high, it is quite susceptible to technical deficiencies and as a consequence, might produce false-positive results, especially due to cross-contamination (Aber 1980, Frieden 1996, Burman 1997). In high-prevalence countries the limited frequency of false-positive results in sputum microscopy might therefore outweigh

the advantage of high detection rates in mycobacterial culture for diagnostic purpose (Levy 1989, Githui 1992, Van Deun 2004).

The role of mycobacterial culture as a diagnostic tool is highlighted vividly in the context of HIV. Within the group of HIV-positive patients an increasing number of patients with suspected pulmonary TB but smear-negative sputum were reported (Harries 1997, Raviglione 1997, Van Deun 2004) as well as changes of the chest X-ray findings (Greenberg 1994, Van Deun 2004). Based on these findings and considering the spread of the HIV pandemic, mycobacterial culture might become more important for diagnostic purposes in the future (Urbanczik 1985, Karstaedt 1998, Van Deun 2004).

Mycobacterial culture also permits identification of the strain of mycobacteria (M. tuberculosis vs. non-tuberculous mycobacteria) and detection of drug resistance through subsequent drug susceptibility testing (DST) (Van Deun 2004). For these reasons mycobacterial culture plays an important role not only in TB control for epidemiological purpose, but also in the follow up of anti-tuberculosis treatment.

Culture might be more reliable than smear microscopy in the assessment of sputum conversion after initial treatment and treatment failure. Mycobacteria that were killed successfully under anti-tuberculosis treatment might still be stained and detected in sputum microscopy while they cannot be grown on culture medium (Al-Moamary 1999). Reasons other than non-viability of bacilli under treatment for smear-positive sputum not growing on culture are exposure to sunlight or heat, prolonged storage, and contamination. Epidemiological studies on culture positivity as a predictive marker for disease progress and changes in infectiousness showed that smear-negative, culture-positive cases are less infectious, but "that the development of new smear-positive tuberculosis does not necessarily go through an early, smear-negative stage" (Stýblo 1967, Van Deun 2004).

Culture media for the growth of mycobacteria can be divided into two groups: solid and liquid media. Within the group of solid media, a further subdivision into agar-based and egg-based media is useful. Factors that facilitate the choice of the optimum medium, and that are based on general considerations for all cultivation procedures in microbiology are (WHO 1998):

1. economical and easy preparation from readily available ingredients;
2. inhibition of contaminant growth;
3. support of luxuriant growth of small numbers of bacilli; and
4. facility for preliminary differentiation of isolates on the basis of colony morphology.

Table 1 summarizes the advantages and disadvantages and some frequently used media within those groups (WHO 1998).

So far liquid media techniques have been limited to industrialized, low-prevalence countries with easy access to technical supplies, trained personnel, and good financial resources. The application of new diagnostic tests in resource-limited settings is a topic of current investigation that is discussed diversely. On one hand, a public health thinking dominated by microbiologists, emphasizing the quality of diagnosis, could be an important influence on TB control strategies. On the other hand, "the introduction of new diagnostic methods without laboratory capacity to properly evaluate and implement them will generate waste […] and delay the arrival of truly useful new technologies" (Perkins 2002).

Table 1: Media for mycobacterial cultures: an overview

media groups	examples	advantages	disadvantages
agar-based solid media	Middlebrook 7H10 and newer ± Middlebrook OADC enrichment*	optimal growth supplements for mycobacteria	expensive special ingredients / equipment required
	5% sheep- blood agar†	cheap easy to prepare, ingredients available in resource-limited settings inoculation possible at different temperatures short time to detection of mycobacteria	for a long time considered inadequate for mycobacterial cultures, ongoing investigation
egg-based solid media	Ogawa‡ Löwenstein-Jensen‡	easy to prepare without high risk of contamination cheap can be refrigerated for several weeks easy inoculation procedure allows preliminary identification by culture morphology	long time to detection of mycobacteria if contamination occurs, the culture is usually lost
liquid media	BACTEC 460TB System§ BACTEC™ MGIT 960 System§	high growth rate from all types of clinical specimens short time to detection of mycobacteria	expensive does not allow preliminary identification by culture morphology radiometric (only BACTEC 460TB System) requires high level of training / experience for inoculation and handling

Abbreviations: OADC, (oleic acid, albumin, dextrose, catalase); MGIT, mycobacteria growth indicator tube.
Sources: *www.bd.com/ds/technicalCenter/inserts/Middlebrook_7H10_Agar.pdf (accessed on 22.01.08);
† Drancourt 2007; ‡ WHO 1998; § Cruciani 2004.

1.6.3 Drug susceptibility testing

The most frequently used methods for DST are indirect methods from mycobacterial culture. Again the non-radiometric automated BACTEC™ Mycobacteria Growth Indicator Tube 960 system (MGIT) was shown to be of good value and was recommended as an alternative to replace the older radiometric assays for drug susceptibility testing (Johansen 2004). Direct methods from sputum such as nucleic acid amplification techniques are more expensive and demand a high level of performance proficiency in lab techniques and have therefore not yet been widely used (Perkins 2002).

Drug susceptibility testing is strongly recommended on at least isoniazid and rifampicin, because these two drugs are maintained in combination for four months of continuous therapy after the successful completion of an intensive treatment phase. The intensive treatment is usually with a four drug combination until sputum conversion (i.e. sputum testing negative for acid-fast bacilli on smear microscopy after previously testing positive) or persistent smear-negative sputum after two months of treatment (U.S. Department of Health and Human Services 1995b).

Table 2: CDC recommendations recognizing the improved technology available for use in mycobacteriology laboratories in the U.S.

promotion of rapid delivery of clinical specimens to the laboratory for direct fluorescence based sputum smear microscopy
prompt registration of patients with positive sputum smear microscopy results or clinically suspect for TB
parallel inoculation of liquid media and solid media
rapid identification of mycobacterial growth
BACTEC™ based susceptibility testing on first-line drugs
prompt reporting of results
regular quality control procedures and safe work guarantee

Abbreviations: CDC, United States Centers for Disease Control and Prevention; TB, tuberculosis.
Source: Tenover 1993

Laboratories are encouraged to “define standard turnaround times” (WHO 2006a). An idea of how to achieve certain turnaround times regarding the laboratory tests available in U.S. settings was discussed in a guest commentary published by a specialist group of the CDC in the Journal of Clinical Microbiology in 1993 (Table 2). The readiness of a laboratory to perform good

quality culturing of mycobacteria and DST was approved if direct sputum smear microscopy within 24 hours, identification of MTB within 10-14 days and DST within 15-30 days of specimen collection were ensured (Tenover 1993).

1.7 Evaluation background

1.7.1 Motivation

The Thai Ministry of Public Health - United States Centers for Disease Control and Prevention Collaboration (TUC) is a bilateral collaboration between the primary public health agencies in the U.S. (U.S. Centers for Disease Control and Prevention (CDC)) and in Thailand (Thai Ministry of Public Health). This organization is dedicated to strengthening the control of infectious diseases in Thailand and enhancing the knowledge about these diseases in both countries. Funding for activities comes primarily from the U.S. CDC, but the agencies work together to develop, implement, and evaluate projects. One of TUC's efforts is to build the capacity for mycobacterial culture and susceptibility testing for three reasons (Varma 2006):

First, the feasibility and effectiveness of mycobacterial culture as a tool for the routine diagnosis of TB compared to smear microscopy and chest X-ray has not yet been demonstrated in a high-burden, resource-limited setting. Most of the studies about the effectiveness of routine liquid mycobacterial culture were performed in wealthy, low TB burden countries. This knowledge is crucial for deciding whether Thailand should invest resources on expanding culture capacity throughout the country.

Second, the diagnosis of TB in HIV-epidemic regions has to be strengthened and there is the concern that smear microscopy is insufficient in diagnosing TB in HIV-positive patients.

Lastly, mycobacterial culture, identification and drug susceptibility testing are important for the surveillance of MDR-TB cases, and national drug resistance surveys thus far may have underestimated the true burden of MDR-TB in Thailand.

At the district level, TB patients are diagnosed and registered for treatment using chest X-ray and sputum smear microscopy. Sputum for culture is then sent to the closest reference laboratory. Drug susceptibility testing and identification are performed most frequently at two public laboratories: the National Tuberculosis Reference Laboratory (NTRL) in Bangkok and the Bangkok municipal laboratory (BMA City Lab). Against this background the Thailand TB Active Surveillance Network, a large demonstration project, was begun in the four provinces

Bangkok, Chiang Rai, Phuket and Ubon-ratchathani in 2004 (Figure 1) with five core activities being (Varma 2006, Varma 2007):

1. the identification of all cases of TB in public and private health facilities;
2. the collection of standardized epidemiologic data about all TB cases in these provinces, including those in the private sector;
3. the attempt to perform culture and DST on all pulmonary TB cases;
4. the provision of HIV counseling, testing, care, and treatment to TB patients; and
5. the use of electronic recording and reporting to promote rapid analysis of data.

In 2002, the NTRL and BMA City Lab had capacity to perform culture and DST on both liquid and solid media. For this project additional supplies, equipment, training, and staff to increase the capacity to perform culture, identification, and DST using liquid media were procured.

In the three provinces outside of Bangkok, capacity to perform culture was limited. In 2003 laboratory space was renovated and equipment and supplies purchased to perform automated, liquid media culture using the BACTEC™ MGIT 960 system (MGIT). Additional clinical microbiology staff were hired and trained. By October 2004, routine culture on solid media (Ogawa) at all network sites and identification and DST at the NTRL and BMA City Lab had been implemented.

Previous laboratory studies had shown that MGIT had better test performance concerning the recovery of mycobacteria and time to detection compared to solid media techniques (Hanna 1999, Chien 2000, Lu 2002, Lee 2003, Cruciani 2004; for more details refer to chapter 4.2). These advantages of liquid medium compared to solid medium might increase the importance of mycobacterial culture, identification and drug susceptibility testing in the context of a tuberculosis control program with regard to surveillance as well as clinical use.

Therefore, the next step was the implementation of MGIT at the three sites outside of Bangkok (Chiang Rai, Phuket, and Ubon-ratchathani). In the following evaluation the laboratory of the Office of Disease Prevention and Control Region 7 (ODPC 7^{th}) at Ubon-ratchathani was chosen as one example out of the three "outside of Bangkok sites" (compare Figure 1). At this laboratory, in April 2005, the conventional laboratory pathway growing mycobacteria on solitary Ogawa solid culture medium (Ogawa) was replaced by a new laboratory pathway growing mycobacteria on Löwenstein-Jensen solid culture medium (LJ) and in liquid culture medium (MGIT) in parallel.

Figure 1: Thailand TB Active Surveillance Network, laboratory sites in 2004

Source: adapted from Varma 2006.

1.7.2 Objectives

MGIT is a cost-intensive, fully automated, non-invasive system for the recovery of mycobacteria in liquid culture medium. From previous laboratory studies it is known to yield a higher and faster recovery of mycobacteria from sputum specimens than solid medium culture. Two objectives were chosen to assess whether the introduction of MGIT can be recommended in a high-burden, resource-limited setting despite its high costs and requirement of staff training and experience due to its complicated laboratory handling. The ODPC 7th laboratory in Ubon-ratchathani was chosen as an example of a tuberculosis control program in a resource-limited, high-burden setting.

The primary objective was to evaluate the new laboratory pathway at the Office of Disease Prevention and Control Region 7 (ODPC 7th). Similar to the approach of previous studies on liquid medium culture, MGIT was compared to LJ solid medium culture with regard to the recovery and the time to detection of mycobacteria from sputum specimens.

The secondary objective was to evaluate the changes of the role of mycobacterial culture in the context of disease surveillance (detection of infectious TB cases, identification of non-tuberculous mycobacteria (NTM) and detection of drug resistance) and for clinical use (availability of test results and procedure times) after the introduction of MGIT at the ODPC 7th.

2 Methods

For a sound understanding of the present evaluation's methodology it is important to bear in mind that the evaluation is based on a "two-step approach" toward a recommendation concerning the introduction of liquid medium culture for tuberculosis in a high-burden, resource-limited setting. This "two-step approach" was chosen with the intention of bypassing several difficulties that derived from retrospective compilation of the data.

The study population included sputum specimens from all patients treated in Ubon-ratchathani province who were diagnosed with TB by a clinician and/or started on anti-TB treatment (so called TUC patient) within the fiscal year 2004/05 (01-Oct-2004 to 31-Sep-2005). Sputum specimens were processed for mycobacterial culture and drug susceptibility testing. As the applied laboratory techniques for mycobacterial cultures changed after the introduction of MGIT to ODPC 7th laboratory on 22-Apr-2005, two groups of sputum specimens can be identified, i.e. one group of specimens that were processed following the laboratory pathway before the introduction of MGIT (group TUC 1) and one group of sputum specimens that were processed following the laboratory pathway after the introduction of MGIT (group TUC 2) (Figure 2).

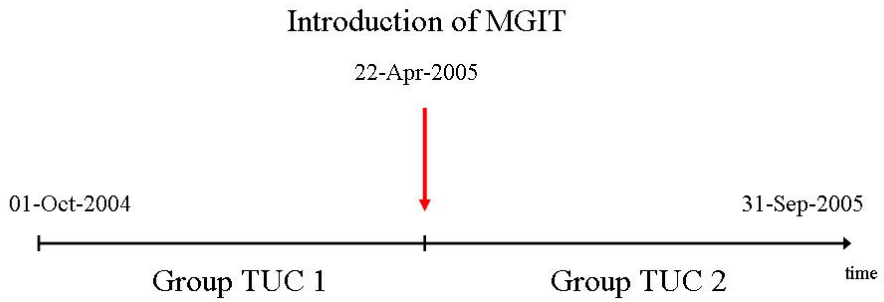

Figure 2: Time axis of evaluation period

Abbreviations: MGIT, BACTEC™ Mycobacteria Growth Indicator Tube 960, Becton Dickinson, Franklin Lakes, NJ, USA; TUC, Thai Ministry of Public Health-U.S. CDC Collaboration.

Looking at the time axis in Figure 2 it becomes clear that the sputum specimens in groups TUC 1 and TUC 2 were processed sequentially and not parallel in time, and that a direct comparison of the groups would result in a "before and after comparison".

Therefore, in a first step, resembling the primary objective defined in chapter 1.7.2, a direct comparison was performed for sputum specimens processed in liquid medium culture (MGIT) with solid medium culture (LJ) in parallel after the introduction of MGIT (i.e. within group TUC 2).

In a second step, resembling the secondary objective defined in chapter 1.7.2, effects on the role of mycobacterial culture in the context of disease surveillance and for clinical use were evaluated by using a purely descriptive comparison of group TUC 1 vs. group TUC 2, and interpreted carefully.

2.1 Inclusion criteria

Physicians had been trained and encouraged, but not required, to follow the following guidelines:

Patients who presented themselves for one or more of the symptoms listed below,

1. cough for longer than three weeks,
2. chest pain related to breathing or coughing,
3. weight loss,
4. fatigue,
5. malaise,
6. fever and/or
7. night sweats

were screened by Chest X-ray and sputum smear microscopy and in case of positive findings then classified as a patient with

1. newly diagnosed or suspected TB,
2. suspected latent TB,
3. relapse of former TB or
4. treatment failure of TB.

2.2 Laboratory pathways

The flow chart presented in Figure 3 summarizes all steps involved in the laboratory pathways of group TUC 1 and group TUC 2. It may serve the reader as a frequent reference at any time. A detailed description of the single steps and the applied laboratory techniques can be found in chapters 2.2.1 to 2.2.3.

2.2.1 Laboratory pathway of group TUC 1

Step 1: Sputum collection and transport to the ODPC 7th laboratory

Sputum was collected at either the regional hospital or the ODPC 7th laboratory in compliance with Thai MOPH and international DOTS standards, i.e. two "on-the-spot" collections and one "morning sputum" collection that the patients brought from home. Sputum smear microscopy was performed as described in detail in chapter 2.2.3.

Step 2: Mycobacterial cultures at the ODPC 7th laboratory

Sputum was homogenized and decontaminated following the Sodium hydroxide (NaOH) method (Della-Latta 2004) and inoculated in two bottles of 3% Ogawa solid medium. The bottles were incubated for 56 days. If after 56 days no growth was seen either both bottle, "no growth" was reported to the treatment site (hospital or ODPC 7th TB clinic).

Step 3: Transport of mycobacterial isolates to NTRL

If uncontaminated growth was detected in either one of the bottles, and the culture morphology and microscopy appeared to be typical of M. tuberculosis, the isolate was shipped for drug susceptibility testing to NTRL in Bangkok (identification tests as described in chapter 2.2.3 were encouraged to be performed prior to DST, but routine application and timing of these tests remained uncertain on investigation).

Step 4: Drug susceptibility testing

Drug susceptibility tests were performed using two methods in parallel: BACTEC™ MGIT 960 and proportion method. Para-nitrobenzoic acid (PNB) medium and culture morphology were applied as screening methods for the detection of non-tuberculous mycobacteria (NTM). A detailed description of the DST procedures is given in chapter 2.2.3.

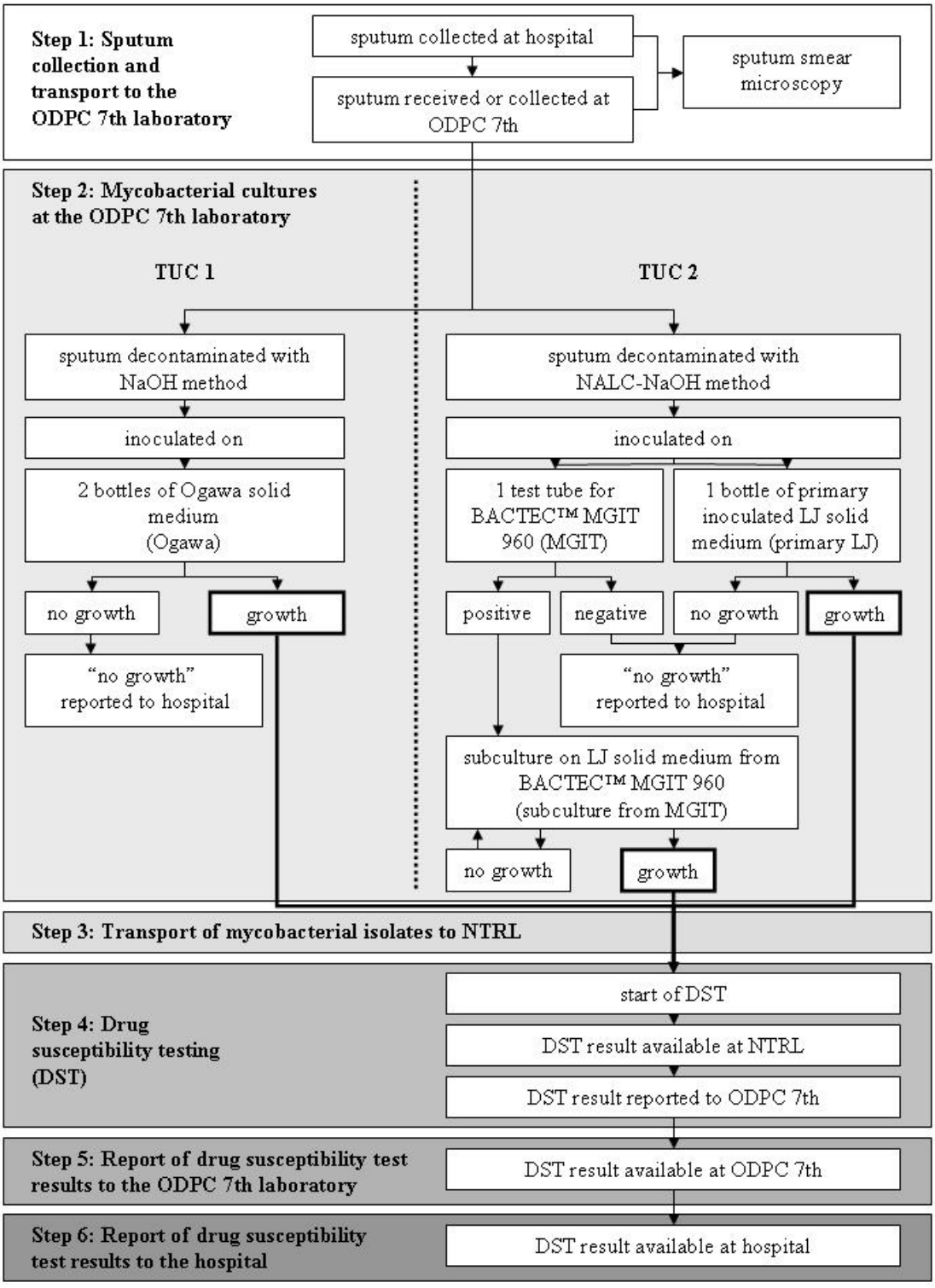

Figure 3: Laboratory pathways of groups TUC 1 and TUC 2

Abbreviations: DST, drug susceptibility testing; LJ, Löwenstein-Jensen medium; MGIT, BACTEC™ Mycobacteria Growth Indicator Tube 960, Becton Dickinson, Franklin Lakes, NJ, USA; NALC, N-acetyl cysteine; NaOH, sodium hydroxide; NTRL, National Tuberculosis Reference Laboratory, Bangkok; ODPC 7th, Office of Disease Prevention and Control Region 7 (Ubon-ratchathani); TUC, Thai Ministry of Public Health-U.S. CDC Collaboration.
Groups TUC 1 and TUC 2 are presented on the same flow chart level to visualize differences in single procedure steps. This might wrongly suggest a time parallelism of the groups. Once again, the sputum specimens processed in groups TUC 1 and TUC 2 were processed sequentially in time, i.e. before and after the introduction of MGIT to ODPC 7th laboratory (Figure 2).

Steps 5 and 6: Report of drug susceptibility test results to the ODPC 7th laboratory and to the hospital

After DST results were available at NTRL in Bangkok they were reported to ODPC 7th by mail. From there they were reported further to the treatment site (hospital or ODPC 7th TB clinic) by mail.

2.2.2 Laboratory pathway of group TUC 2

Differences in the pathway of group TUC 2 compared to the pathway of group TUC 1 were only present in

Step 2: Mycobacterial cultures at the ODPC 7th laboratory

The sputum was homogenized and decontaminated using the N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) method (Della-Latta 2004) and used for inoculation of one test tube for BACTEC™ MGIT 960 (MGIT) and one bottle of primary inoculated Löwenstein-Jensen medium (primary LJ).

For primary LJ, one bottle of Löwenstein-Jensen medium was inoculated with 0.1 ml (4 drops) of concentrated specimen and incubated at 37°C. "No growth" was reported if, after 56 days of weekly controls, growth of mycobacteria could not be detected.

For MGIT, a lyophilized vial of BBL MGIT PANTA (polymyxin B, amphotericin, nalidixic acid, trimethoprim and azlocillin) antimicrobial agent mixture was reconstituted with 15 ml of BACTEC™ MGIT growth supplement and after a period of five days used for culture. With a transfer pipette 0.8 ml of this mixture and 0.5 ml of concentrated specimen were inoculated in one test tube. The test tube was then scanned into the BACTEC™ MGIT 960 liquid culture system. The test result was reported as "negative" if within a period of 42 days growth could not be detected. If growth was indicated by the system, the test result was reported as "positive", and colonies were drawn from the tube. With the aid of a microscope, cord formation (serpentine cording on smear from cultures grown in liquid medium) was used as rapid and presumptive confirmation of M. tuberculosis (Attorri 2000) and contamination excluded. Then the specimen was transferred to a subculture on LJ solid medium (subculture from MGIT) without further decontamination and incubated at 37°C for a maximum of 56 days or until a mycobacterial isolate started growing.

2.2.3 Laboratory techniques shared in both pathways

Sputum smear microscopy for the detection of acid-fast bacilli (AFB)

The ODPC 7th laboratory and the 24 community hospital laboratories used Ziehl-Neelsen technique for staining of smears done directly from the sputum (Della-Latta 2004):

1. Smears were heat fixed on an electric warmer.
2. The heat fixed slides were flooded with carbol fuscin stain and, steamed on a heating rack for further 5 minutes and then rinsed with water.
3. After destaining with acid-alcohol for 2 minutes the slides were rinsed with water again and drained.
4. Counterstaining was done with methylene blue for 1 to 2 minutes.
5. The stains were rinsed with water, drained, air dried and examined with a 100 x oil immersion objective (x 1000 total magnification) using a light microscope.

The classification used for the documentation of sputum smear microscopy results using Ziehl-Neelsen technique is presented in Table 3.

Table 3: Classification of sputum smear microscopy results for the detection of acid-fast bacilli, Ziehl-Neelsen Stain (Della-Latta 2004)

bacterial density (number of bacteria / field)	report
0	no AFB seen
single bacteria seen	scanty (+ number)
1-9 / 100 field	1 +
1-9 / 10 fields	2 +
1-9 / field	3 +
>9 / field	4 +

Abbreviations: AFB, acid-fast bacilli.

Only one hospital (Sapphasiththiprasong Regional Hospital) performed fluorochrome stains (Della-Latta 2004):

1. The smear was flooded with the fluorochrome stain and stained for 15 minutes.
2. The smear was rinsed with chlorine-free water and the excess water drained from the slide.
3. The smear was flooded with 0.5% acid-alcohol and allowed to decolorize for 2 minutes.
4. The smear was rinsed again and the excess water drained from the slide.

5. The smear was flooded with the counterstain (potassium permanganate or acridine orange) and counterstained for 2 minutes.
6. The smear was rinsed with water a third time and the excess water drained from the slide.
7. The smear was allowed to air dry.
8. The smear was examined with a fluorescent microscope with a 25 x or 40 x objective (x 250 or x 450 total magnification).
9. The morphology was confirmed under oil immersion at a x 1,000 or x 450 magnification.

The classification used for the documentation of sputum smear microscopy results using fluorochrome stain is presented in Table 4.

Table 4: Classification of sputum smear microscopy results for the detection of acid-fast bacilli, fluorochrome stain (Della-Latta 2004)

bacterial density (number of bacteria / field)		report
x 250 magnification	x 450 magnification	
0	0	no AFB seen
1-9 / 10 fields	2-18 / 50 fields	1 +
1-9 / field	4-36 / 10 fields	2 +
10-90 / field	4-36 / field	3 +
>90 / field	>36 / field	4 +

Abbreviations: AFB, acid-fast bacilli.

Quality advice and evaluation concerning the correct selection of purulent sputum vs. saliva, smear thickness and size, air drying and fixation, staining and counterstaining and false-positive/negative readings were performed regularly at all sites. If sputum was sent from a hospital to the ODPC 7th laboratory, information on the patient and sputum smear microscopy results followed the specimen on a MDR-TB surveillance form and entered in ODPC 7th documentation.

For this analysis AFB stain results were classified as smear-positive (smear (+)), smear-negative (smear (-)) or unknown.

Identification tests

The ODPC 7th laboratory was encouraged to perform identification through culture morphology, microscopy and catalase, niacin production and nitrate reduction biochemical testing before shipping isolates to NTRL. On arrival at NTRL isolates were screened for culture morphology,

contamination and/or mixed growth. Isolates suspected of containing non-tuberculous mycobacteria (NTM) were subjected to further biochemical identification tests (catalase, niacin production, nitrate reduction, growth on thiophene-2-carboxylic acid hydrazide (TCH) medium, growth rate on LJ medium) and in very few cases probe hybridization (Gen Probe) was performed. Isolates likely to be M. tuberculosis complex (MTB) were screened for growth on para-nitrobenzoic acid (PNB) medium to exclude NTM only; no further biochemical tests were performed.

Drug susceptibility testing

Drug susceptibility testing was performed at the National Tuberculosis Reference Laboratory (NTRL) in Bangkok. Two indirect methods (i.e. the use of mycobacterial isolates from sputum specimens on solid medium) were used in parallel: The BACTEC™ MGIT 960 non-radiometric broth-based system (MGIT) and the proportion method on LJ medium.

For DST using MGIT, mycobacterial isolates growing on solid medium (Ogawa in group TUC 1 and primary LJ/subculture from MGIT in group TUC 2) were processed for DST to a suspension of >1.0 McFarland standard turbidity as a reference. After the adjustment of this suspension to a 0.5 McFarland standard, a 1:5 dilution organism suspension was created. Inoculation of a non-drug-containing MGIT 960 tube as growth control was effected with 0.5 ml of a 1:100 dilution from the organism suspension. Of the organism suspension 0.5 ml was then inoculated in test tubes containing the drugs at concentrations shown in Table 5.

Furthermore, a tryptic soy agar (TSA) with 5% sheep blood plate was inoculated with 0.1 ml of the organism suspension and checked for growth of contaminating bacteria other than M. tuberculosis complex (MTB) after incubation at 35-37°C for 48 hours.

Results were documented consistent with the BACTEC™ MGIT 960 interpretation as susceptible or resistant. BACTEC™ MGIT 960 interpreted the result as susceptible when the fluorescence in the drug-containing tube was less than that of the growth control tube and as resistant when the fluorescence in the drug-containing tube was equal to that of the growth control tube. In certain cases the machine reported X for error and no susceptibility interpretation was possible. If a strain of MTB was shown to be resistant to more than two primary anti-tuberculosis drugs or to both isoniazid (INH) and rifampicin, additional testing for susceptibility to secondary anti-tuberculosis drugs was undertaken at the concentrations shown in Table 5.

For DST using the proportion method, three drops of a 1.0 McFarland suspension diluted to concentrations of 10^{-2} and 10^{-4} were inoculated on control- and drug-containing LJ medium at drug concentrations of primary anti-tuberculosis drugs as shown in Table 5.

Table 5: Drug concentrations for susceptibility testing

drugs	concentration (µg/ml)	
	proportion method on LJ	BACTEC™ MGIT 960
first line drugs		
isoniazid (critical)*	0.2	0.1
isoniazid (high) †	1.0	n.a.
rifampicin	40.0	1.0
ethambutol	2.0	5.0
pyrazinamide	n.a.	100.0
streptomycin	4.0	1.0
second line drugs		
kanamycin	20.0	n.a.
ofloxacillin	2.0	n.a.
ethionamide	not done for routine work	not done for routine work
cycloserine	not done for routine work	not done for routine work

Abbreviations: LJ, Löwenstin-Jensen solid medium; MGIT, Mycobacteria Growth Indicator Tube 960; n.a., not applicable.
*"The critical concentration of a drug is the level of a drug that inhibits the growth of most cells within the population of a "wild" type strain of tubercle bacilli without appreciably affecting the growth of the resistant mutant cells that might be present" (American Thoracic Society 2000);
† "The additional higher concentration [...] can provide the physician with information about the level of drug resistance in deciding whether to continue therapy [...] either at the recommended dose or at an increased dose" (American Thoracic Society 2000).

The cultures were then incubated at 35-37°C under 5-10% CO_2 for four weeks. The result was documented as susceptible if no growth of MTB was detected on the drug-containing medium and growth of >200 colonies on the control. The result was reported as resistant if >1% growth of MTB was detected on the drug-containing medium compared to the growth of >200 colonies on the control (calculated as [number of colonies on the drug-containing medium / number of colonies on the control] x 100). If a strain of MTB was tested resistant to INH at the critical concentration of 0.2 µg/ml on LJ medium and tested susceptible to INH at the high concentration of 1.0 µg/ml on LJ medium, the result was reported as intermediately resistant to INH.

If a strain of MTB was shown to be resistant to more than two primary anti-tuberculosis drugs or to isoniazid as well as to rifampicin, then additional testing for susceptibility to secondary anti-tuberculosis drugs was undertaken at the concentrations shown in Table 5.

The results of drug susceptibility testing were reported to the laboratory of ODPC 7th by mail after testing using both the BACTEC™ MGIT 960 method and the proportion method, and after screening for non-tuberculous mycobacteria on PNB medium had been completed.

2.3 Approach to statistical analysis

2.3.1 End points

Primary objective ('MGIT plus subculture from MGIT' versus primary LJ within group TUC 2)

- recovery of mycobacteria (primary end point)
- growth and contamination rates
- time to detection (TTD)
- time to growth (TTG)

Secondary objective (group TUC 1 versus group TUC 2)

- growth and contamination rates
- yield of isolates for identification and drug susceptibility testing (YIID)
- yield of drug susceptibility test results (YDR)
- time to detection (TTD)
- time to growth (TTG)
- total turnaround time (TTAT)

Growth and contamination rates

The growth and contamination rates show the distribution of all possible culture results, i.e. "growth", "no growth" or "contaminated" for each culture medium that was used in the laboratory pathways of group TUC 1 (Ogawa) and group TUC 2 (LJ, MGIT and subculture from MGIT). Cultures were grown from sputum specimens of patients who already had been diagnosed with tuberculosis using chest X-rays and sputum smear microscopy. Due to contamination or aggressive decontamination not all cultures from smear-positive sputum would grow. Due to higher sensitivity of culture compared to sputum smear microscopy some cultures would grow even from smear-negative sputum.

The growth and contamination rates for cultures from smear-positive sputum were therefore calculated separately from those for cultures from smear-negative sputum, and test quality of a culture medium was considered higher the more growth from both smear-positive and smear-negative specimens was observed. The detection of growth from smear-negative sputum increased the number of TB patients who could be classified as infectious TB cases.

Furthermore, a higher growth rate of non-tuberculous mycobacteria (NTM) was reported for MGIT in previous studies (Hanna 1999), i.e. differences in the growth and contamination rates of different mycobacterial species on the compared culture media were expected. All results were therefore first calculated for all mycobacterial species, and then sub-specified MTB and NTM.

The general growth and contamination rates are an indicator for the "test quality" of each culture medium involved, but, as a single end point, they are not sufficient to test for significance of differences in the "test quality" of culture media in the context of this evaluation's approach to statistical analysis. This is due to the fact that only the sputum of patients who already had been diagnosed with TB (compare chapter 2.1) was used for this evaluation's investigations: the traditional concept of sensitivity and specificity as "test quality" criteria was not fulfilled. Only sensitivity could be calculated as a criterion for "test quality" - a term that therefore was replaced by the term "test performance" in order to prevent confusion with the traditional concept.

In addition, the use of 'MGIT plus subculture from MGIT' and primary LJ in parallel within group TUC 2 (compare Figure 3), made the introduction of the primary end points *recovery of mycobacteria* and *yield of isolates for identification and drug susceptibility testing* necessary.

The introduction of these unfamiliar end points might be confusing. For a better understanding, it might be helpful to emphasize some important differences between the laboratory pathways of groups TUC 1 and TUC 2 and to refer to Figure 3 again:

Whereas in group TUC 1 a single solid culture medium was used (Ogawa), in group TUC 2, a liquid culture medium (MGIT) and a solid culture medium (primary LJ) were used in parallel.

Whereas the infectiousness of a TB patient can be determined by growth of mycobacteria on solid culture medium as well as by the detection of growth in a MGIT liquid medium tube (BACTEC™ MGIT 960 flags tube as positive), DST could only be performed from mycobacterial isolates growing on solid culture medium in this evaluation's setting.

Therefore, whereas in group TUC 1 the infectiousness of a TB patient could be determined and a mycobacterial isolate grown on solid culture medium in a single step, in group TUC 2 – in the

case of MGIT - infectiousness of a TB patient could be detected in a first step before a subculture from MGIT was then performed on solid culture medium for identification and DST in a second step.

Whereas the end point *recovery of mycobacteria* was used to determine the test performance of the group-specific culture media techniques in detecting infectiousness of a patient diagnosed with TB (i.e. detection of mycobacterial growth on either *solid or liquid* culture medium) the end point *yield of isolates for identification and drug susceptibility testing* was used to determine the test performance of the group-specific culture media techniques in isolating mycobacterial cultures on *solid* culture medium that could be shipped to NTRL in Bangkok for identification and DST.

Recovery of mycobacteria (primary end point)

The recovery of mycobacteria was introduced as the primary end point to compare the test performance of culture media techniques used within the same laboratory pathway in detecting a TB patient's infectiousness; in other words, differences of test performance of culture media techniques that were performed from an identical sputum specimen pool (i.e. MGIT vs. subculture from MGIT vs. primary LJ within group TUC 2). The recovery of mycobacteria is defined as a rate that describes the relative number of mycobacteria that could be recovered by each culture medium technique compared to the total number of mycobacteria that were recovered by all culture media techniques in combination within group TUC 2.

As subcultures from MGIT were only performed if mycobacterial growth was detected by MGIT, the total number of mycobacteria that were recovered in the group TUC 2 laboratory pathway $[N+]_{TUC2}$ was calculated as shown in formula [1] with $[N+]_{MGIT \cap primaryLJ}$ being the number of mycobacteria recovered by both MGIT and primary LJ, $[N+]_{MGIT}$ being the number of mycobacteria recovered by MGIT only and $[N+]_{primaryLJ}$ being the number of mycobacteria recovered by primary LJ only.

[1] $[N+]_{TUC2} = [N+]_{MGIT \cap primaryLJ} + [N+]_{MGIT} + [N+]_{primaryLJ}$

In group TUC 1, only one single solid medium culture technique (Ogawa) was used for both detecting infectiousness in a patient diagnosed with TB and isolating mycobacterial cultures on solid medium that could be shipped to NTRL in Bangkok for identification and DST. Having defined *recovery of mycobacteria* as a rate that describes the relative number of mycobacteria that could be recovered by each culture medium technique compared to the total number of mycobacteria that were recovered by all culture media techniques in combination within the same laboratory pathway, it now becomes clear that in group TUC 1 the *recovery of mycobacteria* was 1 (100.0%), i.e. equal to the total number of mycobacteria that were recovered in the group TUC 1 laboratory pathway (on Ogawa).

Yield of isolates for identification and drug susceptibility testing

The yield of isolates for identification and drug susceptibility testing (YIID) was defined as the number of uncontaminated mycobacterial isolates that could be grown on any *solid* culture medium used in the group-specific laboratory pathway from the number of sputum specimens that were initially inoculated.

Within group TUC 1 using Ogawa solid medium only, the YIID was calculated as shown in formula [2] with $[N+]_{Ogawa}$ being the number of sputum specimens from which an uncontaminated mycobacterial culture could be grown on Ogawa solid medium and $[N_{total}]_{TUC1}$ being the total number of sputum specimens that were initially inoculated in group TUC 1.

$$[2]\ YIID_{TUC1} = \frac{[N+]_{Ogawa}}{[N_{total}]_{TUC1}}$$

Within group TUC 2 liquid medium (MGIT) had been introduced, and as a consequence a subculture on LJ solid medium (subculture from MGIT) was necessary that could be shipped to NTRL in Bangkok for identification and DST when mycobacterial growth had been detected by MGIT (compare Figure 3). The YIID within group TUC 2 was therefore calculated as shown in formula [3] with $[N+]_{primaryLJ \cap sub\_MGIT}$ being the number of sputum specimens from which an uncontaminated mycobacterial culture could be grown on primary LJ as well as on subculture from MGIT, $[N+]_{primaryLJ}$ being the number of sputum specimens from which an uncontaminated mycobacterial culture could be grown on primary LJ only, $[N+]_{sub\_MGIT}$ being the number of sputum specimens from which an uncontaminated mycobacterial culture could be

grown on subculture from MGIT only, and $[N_{total}]_{TUC2}$ being the total number of sputum specimens that were initially inoculated in group TUC 2.

$$[3]\ YIID_{TUC2} = \frac{[N+]_{primaryLJ \cap sub\_MGIT} + [N+]_{primaryLJ} + [N+]_{sub\_MGIT}}{[N_{total}]_{TUC2}}$$

Time to detection

Similar to the end points *recovery of mycobacteria* and *YIID* the end points *time to detection* (TTD) and *time to growth* (TTG) were introduced to evaluate on the one hand the test performance concerning the detection of infectiousness of a patient diagnosed with TB and on the other hand the test performance concerning the isolation of mycobacteria on solid medium cultures that could be sent to NTRL in Bangkok for identification and DST.

The *time to detection* (TTD) was defined as the time that elapsed from the day when the sputum specimen was inoculated to the day when uncontaminated growth of mycobacteria was detected *on/in either solid or liquid medium*. This end point was used as an indicator to compare MGIT vs. primary LJ for the detection of acid-fast bacilli in sputum (detection of infectiousness) within the pathway of group TUC 2 only. In group TUC 1, using Ogawa as a single solid culture medium technique, the TTD was equal to the TTG.

Time to growth

The *time to growth* (TTG) was defined as the time that elapsed from the day when the sputum specimen was first inoculated to the day when an uncontaminated mycobacterial culture was growing *on solid medium*. Within the pathway of group TUC 1 growth on solid medium was equal to growth on Ogawa. Within group TUC 2 the definition of the TTG was not as straightforward, as in fact there were two different possibilities to determine when growth *on solid medium* took place: The first of these possibilities was growth of mycobacteria on primary LJ, and the second was growth of mycobacteria on subculture from MGIT (compare Figure 3).

This end point was introduced to determine how the test performance of the culture media used in the two group-specific laboratory pathways was in the context of growing isolates from sputum specimens on solid culture medium that could be shipped to NTRL in Bangkok for identification and DST.

The TTG was first compared for the culture media techniques within group TUC 2 (i.e. ‘MGIT plus subculture from MGIT’ vs. primary LJ), and later for Ogawa solid culture medium technique in group TUC 1 vs. the combined culture media techniques in group TUC 2.

Yield of drug susceptibility test results

The yield of drug susceptibility test results (YDR) was defined as the number of drug susceptibility test results that were available from the number of sputum specimens that were initially inoculated in each laboratory pathway and was compared for group TUC 1 vs. group TUC 2.

Total turnaround time

Ideally, the total turnaround time (TTAT) would have been defined as the time that elapsed from the day when the sputum specimen was collected (step 1) to the day when the drug susceptibility test result was available at the hospital (step 6) (compare Figure 3). A feasibility analysis showed that the data availability on exact sputum collection dates (step 1) and the dates when DST results were available at ODPC 7th (step 5) and at the hospitals (step 6) was poor. The TTAT was therefore defined as the time that elapsed from the day when the sputum specimen was inoculated on culture medium (step 2) to the date when DST results were reported to ODPC 7th laboratory by the National Tuberculosis Reference Laboratory (NTRL) in Bangkok (step 4).

2.3.2 Data management and tests for significance

Data were compiled retrospectively from documentation at ODPC 7th and at NTRL in Bangkok. A database was created with Microsoft Office Access 2003 and transferred to SPSS 12.0 for Windows.

String variables (e.g. the distribution of culture results) were tested with κ-statistics for intra-group comparisons (primary objective) and the χ^2-test for inter-group comparisons (secondary objective).

Numeric variables (e.g. time periods) were tested using the Kaplan-Meier Survival Analysis and the Log Rank Test.

Missing values were not frequent in the analysis, especially in the case of the primary end point. Wherever the completeness and reliability of data were questionable, this was directly addressed in the calculations and the results interpreted carefully.

3 Results

3.1 Group characteristics

The patient collectives whose sputum specimen were processed in groups TUC 1 and TUC 2 were similar in size, age, sex and sputum smear microscopy results (Table 6). The similarity of the size, epidemiologic characteristics (age and gender), and the characteristics of the specimens that were processed (distribution of sputum smear results) indicate that over time, i.e. before and after the introduction of BACTEC™ MGIT 960 (MGIT), the epidemiologic setting of the tuberculosis control program in Ubon-ratchathani did not change significantly.

Table 6: Group characteristics

	groups		
	TUC 1	**TUC 2**	**p-value**
	n (%)	**n (%)**	
size	545	562	
age (mean years±SD)	53.3±17.3	51.8±17.6	0.524*
0-20	14 (2.6)	14 (2.5)	
21-40	120 (22.0)	142 (25.3)	
41-60	187 (34.3)	203 (36.1)	
61-80	172 (31.6)	163 (29.0)	
>80	24 (5.1)	22 (3.9)	
sex			0.486†
male	360 (66.1)	360 (64.1)	
female	185 (33.9)	202 (35.9)	
sputum smear microscopy results			0.673†
smear (+)	355 (65.1)	380 (67.6)	
smear (-)	162 (29.7)	154 (27.4)	
missing	28 (5.1)	28 (5.0)	

Abbreviations: n, absolute number; SD, standard deviation; TUC Thai Ministry of Public Health-U.S. CDC Collaboration.
*t-test, † χ^2-test.

3.2 'MGIT plus subculture from MGIT' versus primary LJ within group TUC 2

3.2.1 Growth and contamination rates

In group TUC 2, one test tube of MGIT and one bottle of primary inoculated LJ solid medium (primary LJ) per specimen were inoculated with the sputum of 562 patients. As shown in Figure 3, step 2, three culture techniques (primary LJ, MGIT and subculture from MGIT) were used in the laboratory pathway of group TUC 2. Table 7 lists the growth and contamination rates of each of these techniques. Twenty-eight specimens (5.0%) were excluded from the analysis, because their smear microscopy results were not documented.

Growth was detected more frequently in MGIT than on primary LJ from smear-positive as well as smear-negative sputum. As expected, this difference was most obvious for isolates from Non-tuberculous mycobacteria. Contamination was unexpectedly high on primary LJ and within the expected range in MGIT. Both of these observations will be discussed in chapter 4.2. Looking at the growth and contamination rates of subcultures from MGIT it should be mentioned that subcultures were grown from liquid culture concentrate instead of sputum specimens. It is therefore not surprising that its growth and contamination rates appear very favourable.

For the reasons discussed in chapter 2.3.1, statistical tests for significance of differences in test performance between the culture media techniques will be performed in the context of the *recovery of mycobacteria* (chapter 3.2.2).

3.2.2 Recovery of mycobacteria (primary end point)

A more detailed and specific picture of the test performance of 'MGIT plus subculture from MGIT' compared to that of primary LJ within group TUC 2 is given by the primary end point recovery of mycobacteria (Table 8) that was deducted from the growth and contamination rates. In chapter 2.3.1, the difference of whether mycobacterial cultures were performed to detect infectiousness by recovery of acid-fast bacilli from sputum only or were to be used in preparation for identification and subsequent drug susceptibility testing was explained.

Table 7: Growth and contamination rates of MGIT, subculture from MGIT and primary LJ, group TUC 2

	total number of sputum specimens	growth n (%)	no growth n (%)	contaminated n (%)	not documented n (%)
MGIT					
All mycobacterial species	534	402 (75.3)	94 (17.6)	34 (6.4)	4 (0.7)
smear (+)	380	345 (90.8)	20 (5.3)	12 (3.2)	3 (0.8)
smear (-)	154	57 (37.0)	74 (48.1)	22 (14.3)	1 (0.6)
M. tuberculosis complex	503	375 (74.6)	94 (18.7)	30 (6.0)	4 (0.8)
smear (+)	367	333 (90.7)	20 (5.4)	11 (3.0)	3 (0.8)
smear (-)	136	42 (30.9)	74 (54.4)	19 (14.0)	1 (0.7)
Non-tuberculous mycobacteria	31	27 (87.1)	0 (0.0)	4 (12.9)	0 (0.0)
subculture from MGIT					
All mycobacterial species	402	345 (85.5)	18 (4.5)	20 (5.0)	19 (4.7)
smear (+)	345	303 (87.8)	17 (4.9)	14 (4.1)	11 (3.2)
smear (-)	57	42 (73.7)	1 (1.8)	6 (10.5)	8 (14.0)
M. tuberculosis complex	375	326 (86.9)	17 (4.5)	18 (4.8)	14 (3.7)
smear (+)	333	295 (88.6)	17 (5.1)	12 (3.6)	9 (2.7)
smear (-)	42	31 (73.8)	0 (0.0)	6 (14.3)	5 (11.9)
Non-tuberculous mycobacteria	27	19 (70.4)	1 (3.7)	2 (7.4)	5 (18.5)
primary LJ					
All mycobacterial species	534	297 (55.6)	138 (25.8)	79 (14.8)	20 (3.7)
smear (+)	380	270 (71.1)	47 (12.4)	52 (13.7)	11 (2.9)
smear (-)	154	27 (17.5)	91 (59.1)	27 (17.5)	9 (5.8)
M. tuberculosis complex	503	287 (57.1)	129 (25.6)	70 (13.9)	17 (3.4)
smear (+)	367	264 (71.9)	46 (12.5)	48 (13.1)	9 (2.5)
smear (-)	136	23 (16.9)	83 (61.0)	22 (16.2)	8 (5.9)
Non-tuberculous mycobacteria	31	10 (32.3)	9 (29.0)	9 (29.0)	3 (9.7)

Abbreviations: LJ, Löwenstein-Jensen medium; MGIT, Mycobacteria Growth Indicator Tube 960; M., mycobacterium; n, absolute number.

In the first case, i.e. if cultures were performed for detecting infectiousness only, the liquid medium technique (MGIT) was far superior to the solid medium technique (primary LJ). Even considering the combination of MGIT with primary LJ to recover mycobacteria in order to detect infectiousness, the contribution of primary LJ was marginal (3.4%). However, twenty-five out of 380 smear-positive specimens (6.6%) were either culture-negative and/or contaminated and/or a result was not documented (two cases) in either culture system. In these cases, mycobacterial culture on primary LJ and MGIT did not provide any benefit over conventional sputum smear microscopy. Nevertheless, acid-fast bacilli were detected by MGIT and primary LJ in an additional sixty-one cases from smear-negative specimens.

In the second case, i.e. when cultures were not only performed to detect infectiousness, but were meant to be used for identification and subsequent drug susceptibility testing, the combination of both MGIT that required a subsequent subculture from MGIT on a solid medium ('MGIT plus subculture from MGIT') and primary LJ yielded a higher recovery of mycobacteria than either of the techniques alone.

Table 8: Recovery of mycobacteria by MGIT, subculture from MGIT and primary LJ, individually and in combination, group TUC 2

	total number of mycobacteria recovered in the group TUC 2 laboratory pathway	**MGIT**	**subculture from MGIT**	**primary LJ**	**primary LJ plus subculture from MGIT**
		n (%)	**n (%)**	**n (%)**	**n (%)**
All mycobacterial species	416	402 (96.6)	345 (82.9)	297 (71.4)	384 (92.3)
smear (+)	355	345 (97.2)	303 (85.4)	270 (76.1)	334 (94.1)
smear (-)	61	57 (93.4)	42 (68.9)	27 (44.3)	50 (82.0)
M. tuberculosis complex	388	375 (96.6)	326 (84.0)	287 (74.0)	362 (93.2)
smear (+)	343	333 (97.1)	295 (86.0)	264 (77.0)	325 (94.8)
smear (-)	45	42 (93.3)	31 (68.9)	23 (51.1)	37 (82.2)
Non-tuberculous mycobacteria	28	27 (96.4)	19 (67.9)	10 (35.7)	22 (78.6)

Abbreviations: TUC, Thai Ministry of Public Health-U.S. CDC Collaboration; LJ, Löwenstein-Jensen medium; MGIT, Mycobacteria Growth Indicator Tube 960; n, absolute number.

Table 9: Tests for significance of differences in recovery by MGIT, subculture from MGIT and primary LJ individually and in combination, group TUC 2

	p-values*					
	MGIT vs. primary LJ	**MGIT vs. subculture from MGIT**	**MGIT vs. combination of primary LJ and subculture from MGIT**	**subculture from MGIT vs. primary LJ**	**subculture from MGIT vs. combination of primary LJ and subculture from MGIT**	**primary LJ vs. combination of primary LJ and subculture from MGIT**
All mycobacterial species	<0.001	<0.001	0.006	<0.001	<0.001	<0.001
smear (+)	<0.001	<0.001	0.043	0.002	<0.001	<0.001
smear (-)	<0.001	0.001	0.054	0.006	0.093	<0.001
M. tuberculosis complex	<0.001	<0.001	0.033	0.001	<0.001	<0.001
smear (+)	<0.001	<0.001	0.123	0.002	<0.001	<0.001
smear (-)	<0.001	0.003	0.108	0.085	0.141	0.002
Non-tuberculous mycobacteria	<0.001	0.005	0.043	0.016	0.365	0.001

Abbreviations: TUC, Thai Ministry of Public Health-U.S. CDC Collaboration; LJ, Löwenstein-Jensen medium; MGIT, Mycobacteria Growth Indicator Tube 960.
*κ-statistics.

In tests for significance (κ-statistics) of differences in the recovery of all culture media techniques used in the group TUC 2 laboratory pathway (Table 9), it could be verified that MGIT as a single liquid culture medium technique achieved the better recovery of mycobacteria compared to primary LJ as a single solid culture medium technique. There were significant losses when subculturing isolates from positive MGIT tubes (Table 8 and 'MGIT vs. subculture from MGIT', Table 9). When both primary LJ and subculture from MGIT were combined with each other (as was de facto done in routine practice), a significant difference in the recovery of mycobacteria between the combined solid media techniques (primary LJ and subculture from MGIT) and MGIT liquid medium could not be detected ('MGIT vs. combination of primary LJ and subculture from MGIT', Table 9). In other words, losses due to contamination when subculturing isolates from MGIT tubes that were flagged positive could be compensated for by the inoculation of sputum specimens on both MGIT and primary LJ in parallel.

In tests for significance (κ-statistics) of differences in the recovery of the two solid culture media techniques (primary LJ and subculture from MGIT) used in the group TUC 2 laboratory pathway (Table 9), the recovery of mycobacterial isolates that could be shipped for identification and DST was higher for subcultures from MGIT, especially for isolates from smear-negative sputum and NTM, but again, the combination of both techniques achieved optimum recovery.

3.2.3 Time to detection and time to growth

The time to detection was much shorter in MGIT than on primary LJ (Table 10). The inverse Kaplan-Meier curve for all mycobacteria, smear-positive and smear-negative, shown in Figure 4 was chosen as a method for visualizing the differences in TTD between the two culture media techniques. The graph for MGIT quickly enters a steep logarithmic phase and then asymptotically reaches its maximum when the graph for primary LJ first enters its slow logarithmic phase. In other words, almost all culture results were available quickly within a quite distinct period of time (a couple of days) in MGIT when results on primary LJ just started to be available and their availability spread out over a broad period of time (a couple of weeks).

Table 10: Time to detection by mycobacterial species, MGIT vs. primary LJ, group TUC 2

	time to detection (days) (median; mean±SD [95% confidence interval for mean])		
species	**MGIT**	**primary LJ**	**p-value***
All mycobacteria	7.0 8.1±4.8 [7.6;8.6]	26.0 26.7±8.6 [25.7;27.7]	<0.001
smear (+)	7.0 7.7±4.6 [7.2;8.2]	26.0 26.3±8.2 [25.3;27.4]	<0.001
smear (-)	11.0 10.7±5.3 [9.3;12.1]	27.5 30.4±11.8 [25.6;35.1]	<0.001
M. tuberculosis complex	7.0 8.0±4.6 [7.5;8.4]	26.0 26.5±8.5; [25.4;27.5]	<0.001
smear (+)	7.0 7.7±4.5 [7.3;8.2]	26.0 26.2±8.1 [25.1;27.2]	<0.001
smear (-)	10.0 9.9±4.4 [8.5;11.2]	27.5 29.9±12.0 [24.6;35.2]	<0.001
Non-tuberculous mycobacteria	7.0 9.9±7.2 [7.0;12.7]	29.0 33.2±10.3 [25.8;40.6]	<0.001

Abbreviations: LJ, Löwenstein-Jensen medium; MGIT, Mycobacteria Growth Indicator Tube 960; M., mycobacterium.
*Log Rank Test.

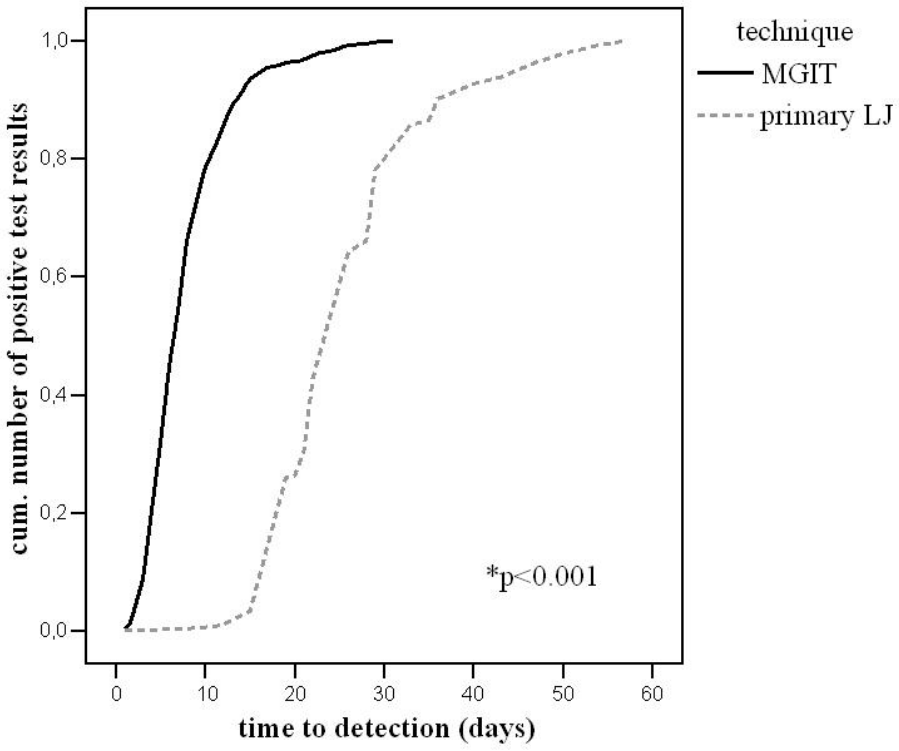

Figure 4: Time to detection, MGIT vs.primary LJ, group TUC 2

Abbreviations: cum., cumulative; LJ, Löwenstein-Jensen medium; MGIT, Mycobacteria Growth Indicator Tube 960; TUC Thai Ministry of Public Health-U.S. CDC Collaboration.
*Log Rank Test.

In the second case, i.e. as the culture was used for further procedures (i.e. for identification and drug susceptibility testing), mycobacteria had to be grown on solid medium (primary LJ or subculture from MGIT), and the time to the detection of growth on that solid medium was defined as time to growth (TTG).

For primary LJ, a single step was required (i.e. inoculation of two bottles of LJ medium with sputum) whereas subcultures from MGIT were grown as the second of two steps (i.e. inoculation of one bottle of LJ medium with concentrate from MGIT) if mycobacterial growth had been detected in MGIT (compare Figure 3).

From the above it is obvious that the time to growth (TTG) of primary LJ was equal to its time to detection (TTD), but that the TTD of MGIT described only the time that was needed for the first step on the way to a subculture from MGIT on solid medium.

Table 11: Time for subcultures from MGIT, group TUC 2

	time to growth (days) (median; mean±SD [95% confidence interval for mean])
species	**subculture from MGIT**
All mycobacteria	14.0 15.7±8.9 [14.8;16.7]
smear (+)	14.0 14.6±7.4 [13.7;15.5]
smear (-)	16.0 18.0±8.8 [15.3;20.8]
M. tuberculosis complex	14.0 15.1±8.6 [14.1;16.0]
smear (+)	13.5 14.2±7.0 [13.4;15.1]
smear (-)	14.0 15.8±8.4 [12.6;18.9]
Non-tuberculous mycobacteria	24.0 25.3±8.1 [21.4;29.2]

Abbreviations: MGIT, Mycobacteria Growth Indicator Tube 960; M., mycobacterium.

Table 12: Time to growth by mycobacterial species; 'MGIT plus subculture from MGIT' vs. primary LJ, group TUC 2

	time to growth (days) (median; mean±SD [95% confidence interval for mean])		
species	**'MGIT plus subculture from MGIT'**	**primary LJ**	**p-value***
All mycobacteria	22.0 24.6±8.5 [23.6;25.5]	26.0 26.7±8.6 [25.7;27.7]	0.003
smear (+)	22.0 23.7±7.8 [22.7;24.6]	26.0 26.3±8.2 [25.3;27.4]	<0.001
smear (-)	29.0 30.6±10.5 [27.3;34.0]	27.5 30.4±11.8 [25.6;35.1]	0.941
M. tuberculosis complex	22.0 23.8±7.7 [22.9;24.6]	26.0 26.5±8.5; [25.4;27.5]	<0.001
smear (+)	22.0 23.3±7.5 [22.4;24.2]	26.0 26.2±8.1 [25.1;27.2]	<0.001
smear (-)	27.0 27.6±9.1 [24.3;31.0]	27.5 29.9±12.0 [24.6;35.2]	0.461
Non-tuberculous mycobacteria	36.0 37.4±9.7 [32.7;42.1]	29.0 33.2±10.3 [25.8;40.6]	0.485

Abbreviations: LJ, Löwenstein-Jensen medium; MGIT, Mycobacteria Growth Indicator Tube 960; M., mycobacterium.
*Log Rank Test.

Table 11 shows how much time the second step to a subculture from MGIT took.

Putting both steps together for the preparation of subcultures from MGIT that could be used for further procedures, it now becomes clear that the difference in TTG between both culture media techniques (primary LJ vs. 'MGIT plus subculture from MGIT') was much less impressive (Table 12) compared to the difference in TTD between the solid and liquid media (primary LJ vs. MGIT, Table 10). A slightly shorter TTG was observed for smear-positive specimens, but this could be confirmed neither for smear-negative specimens nor for NTM.

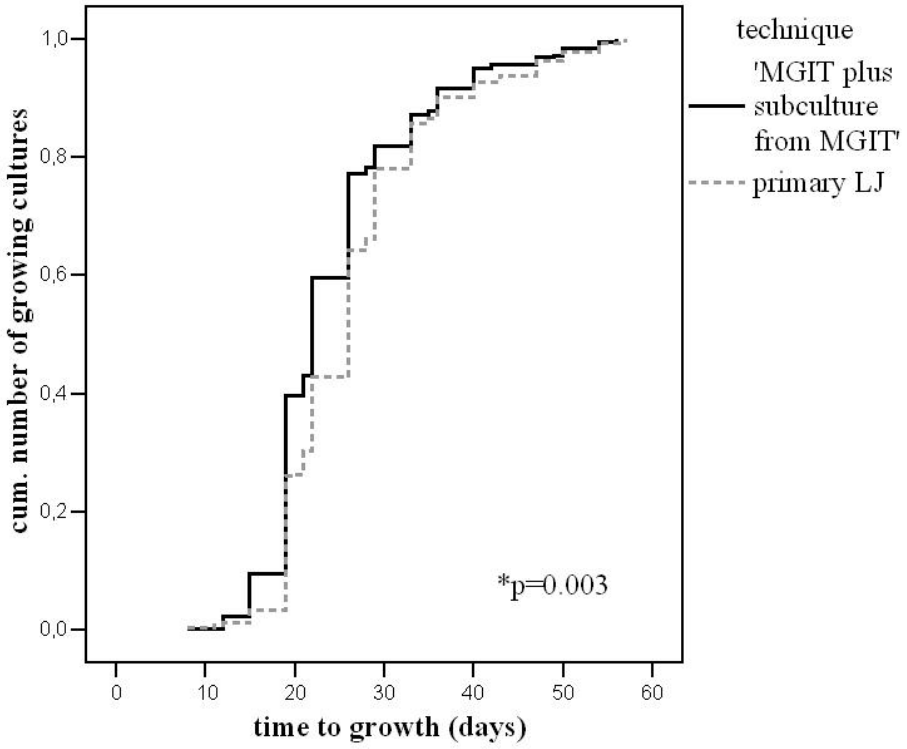

Figure 5: Time to growth, 'MGIT plus subculture from MGIT' vs. primary LJ, group TUC 2

Abbreviations: cum., cumulative; LJ, Löwenstein-Jensen medium; MGIT, Mycobacteria Growth Indicator Tube 960; TUC, Thai Ministry of Public Health-U.S. CDC Collaboration.
*Log Rank Test.

The inverse Kaplan-Meier curve for all mycobacteria, smear-positive and smear-negative, shown in Figure 5 was chosen as a method for visualizing the differences in TTG between primary LJ and 'MGIT plus subculture from MGIT'. The graphs run almost parallel and quite close to each other, and both logarithmic phases extend over a broad period of time (a couple of weeks).

Furthermore, the ODPC 7th laboratory reported that, if mycobacterial growth was detected on both solid media (i.e. on primary LJ and subculture from MGIT), the solid medium "growing first" was sent for drug susceptibility testing to Bangkok. Unfortunately, no documentation concerning which one of the two was sent in each specific case was available.

In conclusion, neither primary LJ nor 'MGIT plus subculture from MGIT' provided a clear advantage concerning the time to growth compared to the other.

3.3 Group TUC 1 versus group TUC 2

3.3.1 Growth and contamination rates

The growth and contamination rates of the Ogawa solid medium culture technique (group TUC 1) are presented in Table 13 and compared to those of the combined liquid plus solid culture media techniques (MGIT plus primary LJ group TUC 2).

In group TUC 1, Ogawa solid medium (two tubes per specimen) was inoculated with sputum from 545 patients. Twenty-eight specimens (5.1%) were excluded from the analysis because their smear microscopy results were not documented. In group TUC 2, one test tube of MGIT and one bottle of primary LJ per specimen were inoculated with the sputum of 562 patients. Twenty-eight specimens (5.0%) were excluded from the analysis because their smear microscopy results were not documented.

With the exception of NTM, the combined culture media techniques MGIT plus primary LJ (group TUC 2) detected growth of mycobacteria more frequently than the Ogawa solid medium technique (group TUC 1), from smear-negative specimens in particular.

Estimating the role of Ogawa for the detection of infectiousness of a newly diagnosed TB case based on the data presented in Table 13, seventy-seven out of 355 smear-positive specimens (21.7%) did not grow or were contaminated on Ogawa medium, and in these cases, mycobacterial culture failed to detect mycobacteria that had already been detected by sputum smear microscopy. Only twenty-two additional infectious cases were detected from smear-negative specimens. The additional benefit of mycobacterial cultures of Ogawa over conventional sputum smear microscopy for the detection of acid-fast bacilli (infectiousness) was therefore questionable.

This was different for mycobacterial cultures in MGIT and on primary LJ, which failed to grow twenty-five (6.6%) of smear-positive specimens, but detected acid-fast bacilli in an additional sixty-one (39.6%) smear-negative specimens.

Table 13: Growth and contamination rates of Ogawa, group TUC 1 vs. MGIT plus primary LJ, group TUC 2

group TUC 1 (Ogawa)	number of sputum specimens	growth n (%)	no growth n (%)	contaminated n (%)	p-value (TUC 1 vs. TUC 2)*
All mycobacterial species	517	300 (58.0)	190 (36.8)	27 (5.2%)	<0.001
smear (+)	355	278 (78.3)	56 (15.8)	21 (5.9)	<0.001
smear (-)	162	22 (13.6)	134 (82.7)	6 (3.7)	<0.001
M. tuberculosis complex	505	288 (57.0)	190 (37.6)	27 (5.3)	<0.001
smear (+)	343	266 (77.6)	56 (16.3)	21 (6.1)	<0.001
smear (-)	162	22 (13.6)	134 (82.7)	6 (3.7)	<0.001
Non-tuberculous mycobacteria	12	12 (100.0)	0 (0.0)	0 (0.0)	0.264
group TUC 2 (MGIT plus primary LJ)					
All mycobacterial species	534	416 (77.9)	102 (19.1)	16 (3.0)	<0.001
smear (+)	380	355 (93.4)	20 (5.3)	5 (1.3)	<0.001
smear (-)	154	61 (39.6)	82 (53.2)	11 (7.1)	<0.001
M. tuberculosis complex	503	388 (77.1)	102 (20.3)	13 (2.6)	<0.001
smear (+)	367	343 (93.4)	20 (5.4)	4 (1.1)	<0.001
smear (-)	136	45 (33.1)	82 (60.3)	9 (6.6)	<0.001
Non-tuberculous mycobacteria	31	28 (90.3)	0 (0.0)	3 (9.7)	0.264

Abbreviations: Ogawa, Ogawa solid medium; M., mycobacterium; n, absolute number; MGIT, Mycobacteria Growth Indicator Tube 960.
*χ^2-test.

3.3.2 Yield of isolates for identification and drug susceptibility testing and yield of drug susceptibility test results

The yield of isolates for identification and drug susceptibility testing (YIID) in group TUC 1 was calculated from Table 13, that in group TUC 2 from Table 8.

In group TUC 1, a total of 300 cultures (grown on Ogawa) were sent for drug susceptibility testing (DST) at the National Tuberculosis Reference Laboratory (NTRL) in Bangkok, of which DST results (resistant or susceptible) were reported for 292 (97.3%).

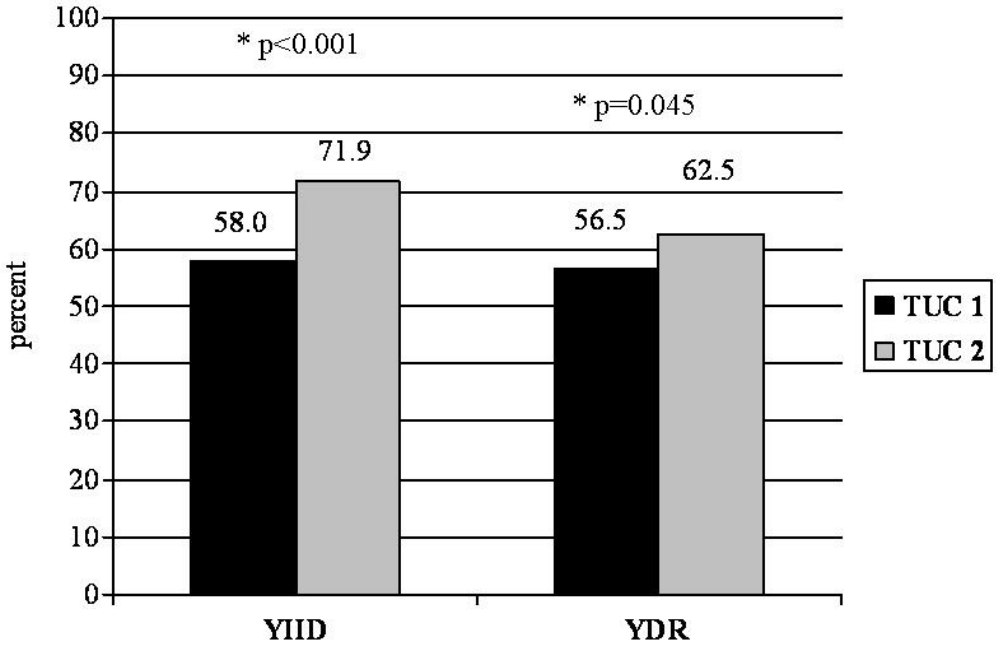

Figure 6: Yield of isolates for identification and drug susceptibility testing (YIID) and yield of drug susceptibility test results (YDR), group TUC 1 vs. group TUC 2

Abbreviations: TUC Thai Ministry of Public Health-U.S. CDC Collaboration; YDR, yield of drug susceptibility test results; YIID, yield of isolates for identification and drug susceptibility testing.
*χ^2-test.

In group TUC 2, a total of 384 cultures (grown on either primary LJ or subculture from MGIT) were sent for DST at NTRL in Bangkok, of which DST results (resistant or susceptible) were reported for 334 (87.0%).

The differences in the yield of isolates for identification and drug susceptibility testing (YIID) and the yield of drug susceptibility test results (YDR) are presented in Figure 6. Although the YIID increased by 13.9% in group TUC 2, the YDR increased by 6.0% only. One reason for the moderate increase in the yield of DST results in group TUC 2 compared to group TUC 1 may have been the high contamination rate on DST in group TUC 2 (13.0% vs. 2.9%, respectively, $p<0.001$; χ^2-test).

Another reason might have been, as expected, that more Non-tuberculous mycobacteria (NTM) would grow in liquid medium (MGIT) and therefore be excluded from DST prior to the transport to or upon arrival in Bangkok through biochemical identification or culture morphology. This was the case for seven cultures (2.3%) in group TUC 1 compared to fifteen cultures (3.8%) in group TUC 2. Furthermore, the expectation that the laboratory pathway of group TUC 2 would lead to a higher yield of cultures from NTM was confirmed by the bacteriology profiles of the specimens processed in group TUC 1 and group TUC 2 (Table 14). No difference was detected between the groups in the yield of cultures from MDR-TB.

Table 14: Bacteriology profiles, group TUC 1 vs. TUC 2

groups	number of cultures grown	MDR-TB n (%)	p-value*	NTM n (%)	p-value*
TUC 1	300	8 (2.7%)	0.697	12 (4.0%)	0.004
TUC 2	384	10 (2.6%)		31 (8.1%)	

Abbreviations: MDR-TB, multi drug resistant mycobacterium tuberculosis; n, absolute number; NTM, Non-tuberculous mycobacteria.
*χ^2-test.

3.3.3 Time to detection and time to growth

As explained in chapter 2.3.1, the TTD and TTG for the Ogawa solid medium culture technique were identical. This was different in group TUC 2, where it was shown that the TTD could be significantly reduced by the liquid medium culture technique MGIT. Table 15 and Table 16 show the results on TTD and TTG in group TUC 1 compared to those in group TUC 2.

While the TTD, in other words, the period until infectiousness could be detected in a patient diagnosed with TB, was significantly shorter for MGIT compared to Ogawa, no difference in this period could be seen between the two solid media techniques (Ogawa and primary LJ) (Table 15).

Table 15: Time to detection, group TUC 1 vs. group TUC 2

	time to growth (days) (median; mean±SD [95% confidence interval for mean])				
	TUC 2		TUC 1	*p-values	
species	MGIT	primary LJ	Ogawa	Ogawa vs. MGIT	Ogawa vs. primary LJ
All mycobacteria	7.0 8.1±4.8 [7.6;8.6]	26.0 26.7±8.6 [25.7;27.7]	26.0 26.8±8.2 [25.9;27.8]	<0.001	0.943
smear (+)	7.0 7.7±4.6 [7.2;8.2]	26.0 26.3±8.2 [25.3;27.4]	26.0 26.5±7.8 [25.6;27.4]	<0.001	0.904
smear (-)	11.0 10.7±5.3 [9.3;12.1]	27.5 30.4±11.8 [25.6;35.1]	26.0 31.4±11.7 [26.2;36.5]	<0.001	0.854
M. tuberculosis complex	7.0 8.0±4.6 [7.5;8.4]	26.0 26.5±8.5 [25.4;27.5]	26.0 26.5±7.6 [25.6;27.4]	<0.001	0.716
smear (+)	7.0 7.7±4.5 [7.3;8.2]	26.0 26.2±8.1 [25.1;27.2]	26.0 26.1±7.0 [25.2;26.9]	<0.001	0.548
smear (-)	10.0 9.9±4.4 [8.5;11.2]	27.5 29.9±12.0 [24.6;35.2]	26.0 31.4±11.7 [26.2;36.5]	<0.001	0.731
Non-tuberculous mycobacteria	7.0 9.9±7.2 [7.0;12.7]	29.0 33.2±10.3 [25.8;40.6]	31.0 35.4±15.5 [25.6;45.3]	<0.001	0.370

Abbreviations: Ogawa, Ogawa solid medium; M., mycobacterium; MGIT, Mycobacteria Growth Indicator Tube 960.
*Log Rank Test.

No clear advantage concerning the TTG, in other words, the time until a mycobacterial isolate on solid medium culture was ready to be shipped to NTRL in Bangkok for identification and DST, was detected for either 'MGIT plus subculture from MGIT' or primary LJ in group TUC 2 compared to Ogawa in group TUC 1.

Although a median decrease of four days could be attributed to 'MGIT plus subculture from MGIT' for smear-positive sputum from MTB, no difference was shown for smear-negative sputum from MTB or NTM. Furthermore, any advantage of a time difference of only four days is questionable, especially as it remained unclear which of the two isolates (i.e. the one on primary LJ or the one on the subculture from MGIT) was shipped to Bangkok.

Table 16: Time to growth, group TUC 1 vs. group TUC 2

	time to growth (days) (median; mean±SD [95% confidence interval for mean])				
	TUC 2		TUC 1	*p-values	
species	'MGIT plus subculture from MGIT'	primary LJ	Ogawa	Ogawa vs. 'MGIT plus subculture from MGIT'	Ogawa vs. primary LJ
All mycobacteria	22.0 24.6±8.5 [23.6;25.5]	26.0 26.7±8.6 [25.7;27.7]	26.0 26.8±8.2 [25.9;27.8]	0.001	0.943
smear (+)	22.0 23.7±7.8 [22.7;24.6]	26.0 26.3±8.2 [25.3;27.4]	26.0 26.5±7.8 [25.6;27.4]	0.001	0.904
smear (-)	29.0 30.6±10.5 [27.3;34.0]	27.5 30.4±11.8 [25.6;35.1]	26.0 31.4±11.7 [26.2;36.5]	0.614	0.854
M. tuberculosis complex	22.0 23.8±7.7 [22.9;24.6]	26.0 26.5±8.5 [25.4;27.5]	26.0 26.5±7.6 [25.6;27.4]	<0.001	0.716
smear (+)	22.0 23.3±7.5 [22.4;24.2]	26.0 26.2±8.1 [25.1;27.2]	26.0 26.1±7.0 [25.2;26.9]	<0.001	0.548
smear (-)	27.0 27.6±9.1 [24.3;31.0]	27.5 29.9±12.0 [24.6;35.2]	26.0 31.4±11.7 [26.2;36.5]	0.210	0.731
Non-tuberculous mycobacteria	36.0 37.4±9.7 [32.7;42.1]	29.0 33.2±10.3 [25.8;40.6]	31.0 35.4±15.5 [25.6;45.3]	0.591	0.370

Abbreviations: Ogawa, Ogawa solid medium; M., mycobacterium; MGIT, Mycobacteria Growth Indicator Tube 960.
*Log Rank Test.

3.3.4 Total turnaround time

Before the total turnaround time could be calculated, all procedural steps along the laboratory pathways of groups TUC 1 and TUC 2 (Figure 3) were described in detail. Data availability and reliability were tested in a feasibility analysis.

Step 1: Sputum collection and transport to the ODPC 7th laboratory

In total, the sputum of 1,107 patients was cultured at the ODPC 7th laboratory and - if mycobacteria could be grown on solid medium - sent to the National Tuberculosis Reference Laboratory in Bangkok (NTRL) for drug susceptibility testing. Out of these 1,107 sputum specimens, 876 (79.1%) were collected at a district hospital and transported to the ODPC 7th laboratory. 229 specimens (20.7%) were collected directly at the ODPC 7th laboratory, and for two specimens (0.1%), the collection site was not documented.

Within the group of sputum specimens collected at a district hospital, 435 (49.7%) were specimens from patients in group TUC 1 and 441 (50.3%) from patients in group TUC 2. During a feasibility test, information on the sputum collection date at the hospital was obtained using an investigation form and was ultimately available for only 304 specimens (34.8%), i.e. for 55/435 specimens (12.6%) in group TUC 1 and 249/441 specimens (56.5%) in group TUC 2. The date when the sputum specimen was received at the ODPC 7th laboratory was obtained from handwritten laboratory documentation. Inconsistencies between the data provided by the hospitals and the documentation at the ODPC 7th laboratory were observed. In conclusion, data reliability for the evaluation of this step was low, and an exact calculation of the time for the transport of sputum specimens from the hospital to the ODPC 7th laboratory was not possible.

Looking at Figure 3, it can be seen that no pathway difference was present in step 1 for groups TUC 1 and TUC 2. Based on the data on 240 specimens from both groups that could be compiled through the investigation forms and handwritten laboratory documentation, a rough estimation of the time for the transport of sputum specimens from the hospital to the ODPC 7th laboratory was made: On average, specimens collected at the district hospitals would arrive at the ODPC 7th laboratory the same day (mean 0.7±4.4). Cultures were not inoculated at the ODPC 7th laboratory every day but usually twice weekly. The time period after the sputum collected at a district hospital was inoculated on culture medium at the ODPC 7th laboratory should therefore not have exceeded four to five days.

Table 17 supports this estimation. It lists how many specimens were sent to the ODPC 7th laboratory from each hospital and how much time elapsed between sputum collection and culture

at the ODPC 7th laboratory. Bearing in mind that the recall concerning the exact dates of sputum collection at the hospital was only 34.8%, minimum and maximum time to inoculation was provided as well, and the wide range was a further sign that the data on which the evaluation of step 1 was based was unreliable.

Table 17: Time periods from sputum collection at the district hospital to inoculation on culture medium at the ODPC 7th laboratory

hospitals	number of specimens sent	% of total†	median time to inoculation (days)	minimum time to inoculation (days)	maximum time to inoculation (days)
SRH	315	36.0	4	1	17
Trakan Phuet Pon	31	3.5	3	2	8
Kut Khaopun	15	1.7	3	1	7
Muang Sam Sip	33	3.8	1	0	21
Warin Chamrap	101	11.5	2-3	0	7
Phibun Mangsahan	47	5.4	4	1	17
Tan Sum	7	0.8	5	5	5
Pho Sai	7	0.8	4	3	5
Samrong	16	1.8	2	1	10
Don Mot Daeng	2	0.2	n.d.	n.d.	n.d.
Sirindhorn	30	3.4	4	2	11
Si Mueang Mai	27	3.1	4	3	7
SMH	17	1.9	4	1	4
Romglao*	7	0.8	1	0	1
Rajavej*	37	4.2	2	1	7
Ubon Ruk*	12	1.4	5	2	9
Khong Chiam	7	0.8	0	0	0
Khuang Nai	35	4.0	4	2	8
Khemarat	35	4.0	7	1	13
Det Udom	23	2.6	4	3	5
Na Chaluai	16	1.8	8-9	3	18
Nam Yuen	32	3.7	n.d.	n.d.	n.d.
Buntharik	24	2.7	5	1	8
Total	876	100.0	3	0	21

Abbreviations: ODPC 7th, Office of Disease Prevention and Control Region 7 (Ubon-ratchathani); SRH, Sapphasiththiprasong Regional Hospital; n.d., not documented; SMH, Sapphasith Military Hospital
*Private Hospital; † values rounded.

Step 2: Mycobacterial cultures at the ODPC 7th laboratory

The median time for procedures at the ODPC 7th laboratory was defined as the period from the date the sputum was inoculated on culture medium to the date DST was requested for the mycobacterial culture grown from that sputum specimen (date of request). These dates were documented for 294/300 cultures (98.0%) in group TUC 1 and 372/384 cultures (96.9%) in group TUC 2. In group TUC 1, the median time for procedures at the ODPC 7th laboratory was 26.5 days (mean 28.8±9.3; 95% confidence interval [27.7;29.9] compared to 22 days (mean 25.0±9.2; 95% confidence interval [24.1;26.0] in group TUC 2 ($p<0.001$, Log Rank Test).

Step 3: Transport of mycobacterial cultures to NTRL

In total, 300 cultures from group TUC 1 and 384 cultures from group TUC 2 were sent to the National Tuberculosis Reference Laboratory (NTRL) in Bangkok for drug susceptibility testing (DST). The cultures were screened for contamination and suspected growth of Non-tuberculous mycobacteria (NTM) through culture morphology and smear microscopy before they were packed for transport. Documentation as to the number of cultures on which biochemical identification was performed prior to transport was not accessible.

Dates of request for DST and arrival dates at the NTRL were available for 294/300 cultures (98.0%) in group TUC 1 and 372/384 cultures (96.9%) in group TUC 2. Delays in requesting DST for cultures grown on solid media were unlikely, because the median times for procedures at the ODPC 7th laboratory of groups TUC 1 and TUC 2 were shown to be 26.5 and twenty-two days in chapter 2.2, step 2, which was very similar to the times to growth (TTGs) calculated in chapter 3.3.3.

The median transport time to Bangkok was seven days (mean 7.6 ± 3.6; 95% confidence interval [7.2;8.0]) for cultures sent in group TUC 1 and nine days (mean 9.3 ± 3.7; 95% confidence interval [8.9;9.7]) for cultures in group TUC 2, a difference of two days ($p<0.001$; Log Rank Test).

Step 4: Drug susceptibility testing

Differences in the yield of drug susceptibility results (YDR) between group TUC 1 and group TUC 2 were presented in chapter 3.3.2. The moderate increase in the YDR by only 6.0% in group TUC 2 was attributable partly to higher contamination rates during DST in group TUC 2 compared to group TUC 1.

In addition, contamination was detected on the cultures' arrival at the NTRL in Bangkok following the shipping process, and resulted in delays:

NTRL named fungus as the main source of contamination during the transport of mycobacterial cultures from the ODPC 7th laboratory to Bangkok. A high number of bacterial colonies other than mycobacteria growing on the culture were declaimed as a second, but less frequent, cause of contamination. In both cases, cultures needed to be re-ordered from the ODPC 7th laboratory.

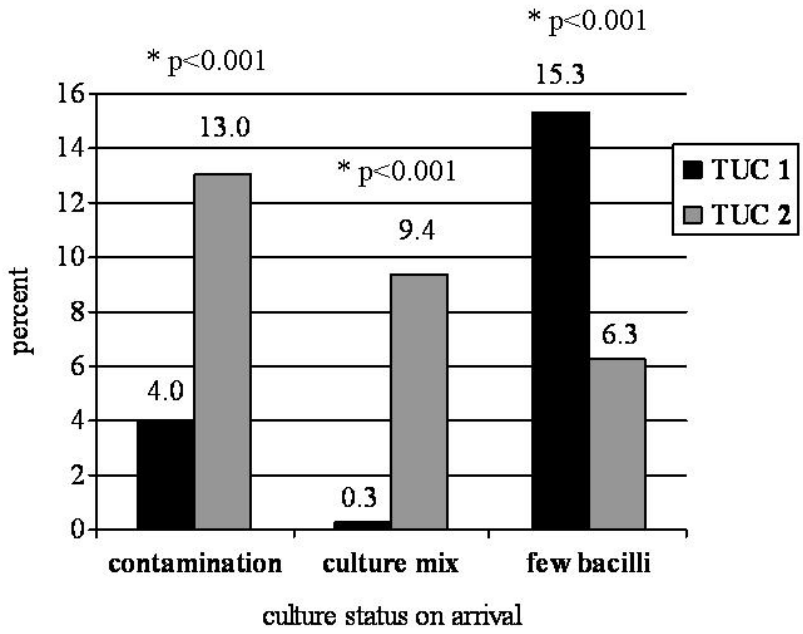

Figure 7: Problems concerning the status on arrival of mycobacterial isolates sent from the ODPC 7th laboratory to NTRL in Bangkok, group TUC 1 vs. TUC 2

TUC, Thai Ministry of Public Health-U.S. CDC Collaboration; contamination, isolate contaminated completely; culture mix, isolate contaminated partly, subculture possible; few bacilli, mycobacterial growth on isolate insufficient, subculture necessary; NTRL, National Tuberculosis Reference Laboratory, Bangkok.
*χ^2-test.

In the case of growth of only a few bacterial colonies other than mycobacteria in between colonies of mycobacteria (documented as "culture mix") or in the case of an insufficient number of mycobacterial colonies (documented as "few bacilli"), subcultures needed to be performed prior to DST. Documentation on "contamination", "few bacilli" and "culture mix" (i.e. the culture status on arrival) was available for 291/300 cultures (97.0%) in group TUC 1 and for 378/384 cultures (98.4%) in group TUC 2 (Figure 7).

In both groups, indirect drug susceptibility tests were performed using the BACTEC™ MGIT 960 method and the proportion method (chapter 2.2.3). All cultures were inoculated on PNB medium as a screening test for NTM. The NTRL in Bangkok stated that DST results using the proportion method and screening results for NTM on PNB medium were routinely read after thirty days. Data on the exact time required with this method were available for 278/300 cultures (92.7%) in group TUC 1 and 319/384 cultures (83.1%) in group TUC 2. A median time of twenty-eight days (mean 28.8 ± 5.2; 95% confidence interval [28.2;29.4]) for group TUC 1 and a median time of twenty-eight days (mean 27.9 ± 0.9; 95% confidence interval [27.8;28.0]) for group TUC 2 matched the data provided by the NTRL well (p=0.004, Log Rank Test).

DST results gained from the BACTEC™ MGIT 960 method were available more quickly, but the completeness of data available on the exact dates was poor, i.e. 131/300 cultures (43.7%) in group TUC 1 and 231/384 cultures (60.2%) in group TUC 2. The main reason for the incomplete documentation was that DST results obtained from the BACTEC™ MGIT 960 method were not reported prior to results from DST using the proportion method and screening for NTM on PNB medium. From the available documentation, the difference of the median time to DST results obtained from BACTEC™ MGIT 960 was calculated as twelve days in group TUC 1 (mean 11.4 ± 3.2; 95% confidence interval [10.8;11.9]) and ten days in group TUC 2 (mean 10.9 ± 4.0; 95% confidence interval [10.1;11.6]) (p=0.041, Log Rank Test).

Step 5: Report of drug susceptibility test results to the ODPC 7th laboratory

Drug susceptibility test results were completed and ready to be reported to the ODPC 7th laboratory after they were available from both the BACTEC™ MGIT 960 and proportion method and after the screening for NTM on PNB medium had been completed. These procedures were stated by NTRL and verified to require thirty days (compare *Step 4* above).

For evaluation purposes, it was important to find out if the DST results (contaminated DST results excluded) were reported to the ODPC 7th laboratory immediately when available, or, if not, how long the reporting delay was in each of the two groups.

The reporting delay was calculated as the time from the date when DST results should have been available on the above-mentioned conditions to the date when they were actually reported to the ODPC 7th laboratory. From data available for 282/292 completed DSTs in group TUC 1 (96.6%), the median reporting delay was thirty-nine days (mean 40.3 ± 23.2; 95% confidence interval [37.6;43.0]), and based on data on 295/334 completed DSTs in group

TUC 2 (88.3%) the median reporting delay was fifty-three days (mean 73.5 ± 52.7; 95% confidence interval [67.4;79.5]), a difference of fourteen days ($p<0.001$, Log Rank Test).

Administrative delay could therefore be identified as a major contributing factor to delay in reporting the results and was self-reported by the NTRL in 243 cases (81.0%) in group TUC 1 based on data provided on 294/300 cultures sent for DST (98.0%) – and in 275 cases (71.6%) in group TUC 2 based on data provided on 377/384 cultures sent for DST (98.2%) ($p=0.004$, χ^2-test).

If the self-report were reliable, this would mean that administrative delay was less frequent in group TUC 2, but, if it occurred, was more severe than in group TUC 1.

Step 6: Report of drug susceptibility test results to the hospital

Reporting took place by mail to the ODPC 7th laboratory and, from there, was forwarded by mail to the hospitals. The availability of data on times for reporting DST results to the hospitals was scarce. The response to an attempt to complete information on sputum collection dates and the dates when the DST results were known at the hospitals through investigation forms was poor; the desired information was completed in only 51/231 cases in group TUC 1 (22.1%) and 166/300 cases in group TUC 2 (55.3%).

Based on these data, the median time for the report of DST results to the hospital in group TUC 1 was forty days (mean 39.4 ± 28.7; 95% confidence interval [31.3;47.5]). In group TUC 2, this period was twenty-seven days (mean 23.7 ± 14.0; 95% confidence interval [21.1;26.3]).

As the data reliability on the reporting period was poor, it remains unclear how DST results were received and communicated on the district level. The use of DST results for clinical purposes and their effect on treatment changes during the observed period is questionable.

Putting all steps together

The total turnaround time (TTAT) was calculated along the laboratory pathway steps 2 to 5 (compare Figure 3 and chapter 2.3.1). The results are based on 282/292 (96.6%) mycobacterial isolates with completed DST in group TUC 1, and 322/334 (96.4%) mycobacterial isolates with completed DST in group TUC 2 (99.7%) (Table 18).

Table 18: Total turnaround time (days) for mycobacterial cultures and subsequent drug susceptibility tests, group TUC 1 vs. TUC 2

groups	total turnaround time (median)	mean ± SD	95% confidence interval*	p-value*
TUC 1	104	106.4 ± 24.8	[103.5;109.3]	<0.001
TUC 2	118	130.0 ± 58.0	[124.4;137.1]	

Abbreviations: SD, standard deviation; TUC, Thai Ministry of Public Health-U.S. CDC Collaboration.
*Log Rank Test.

After the introduction of MGIT at the ODPC 7th laboratory the TTAT in group TUC 2 was expected to become shorter than that in group TUC 1. Despite this expectation, the median TTAT of group TUC 2 was shown to be fourteen days longer than that of group TUC 1.

An overview of the median TTAT and the median time periods used for pathway steps 2 to 5 for group TUC 1 compared to group TUC 2 is given in Figure 8.

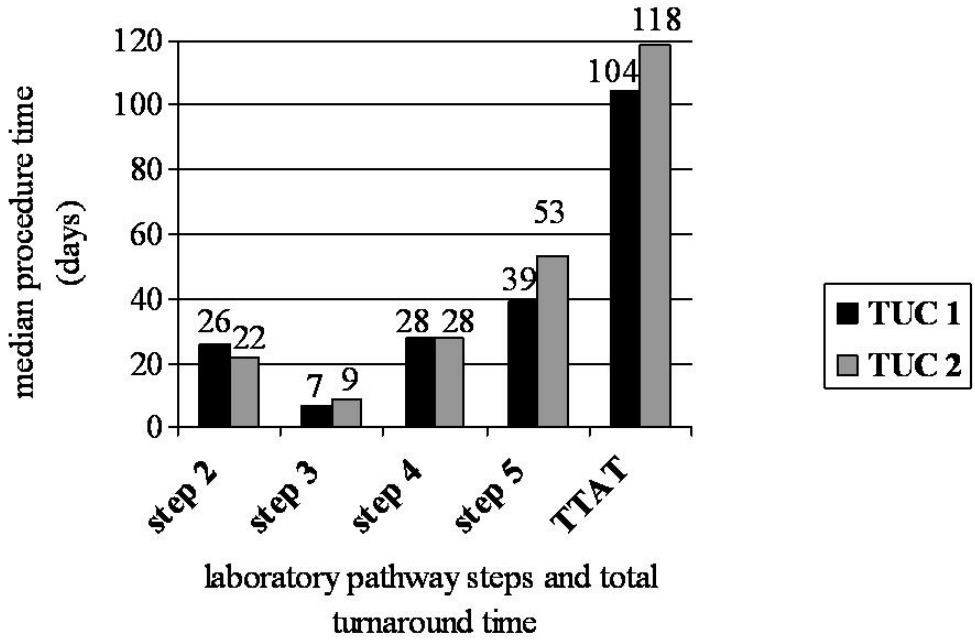

Figure 8: Overview of the median total turnaround time and the time periods used for laboratory pathway steps 2 to 5, group TUC 1 vs. TUC 2

Abbreviations: TTAT, total turnaround time; TUC Thai Ministry of Public Health-U.S. CDC Collaboration; step 2, mycobacterial cultures at the ODPC 7th laboratory; step 3, transport of mycobacterial cultures to NTRL; step 4, drug susceptibility testing; step 5, report of drug susceptibility test results to the ODPC 7th laboratory.

4 Discussion

4.1 Focus on the most important findings

The present results demonstrate that after the introduction of liquid media culture with the automated, non radiometric reading instrument BACTEC™ MGIT 960 (MGIT) to the Office of Disease Prevention and Control Region 7 laboratory in Ubon-ratchathani, the detection of infectiousness by mycobacterial culture in patients diagnosed with TB, the yield of mycobacterial isolates that could be used for identification and drug susceptibility testing (DST) and the yield of DST results did increase. In a comparison of the combined liquid and solid culture media techniques ('MGIT plus subculture from MGIT' vs. primary LJ) within the new laboratory pathway after the introduction of MGIT, this increase was mainly attributable to the high recovery of mycobacteria by the liquid culture medium technique MGIT.

Although the recovery of mycobacteria by MGIT was much higher than that by Löwenstein-Jensen medium (primary LJ), subcultures from MGIT on solid medium were needed for the identification of the mycobacterial species and drug susceptibility testing (DST) in Bangkok; and for this purpose the combination of both 'MGIT plus subculture from MGIT' with primary LJ resulted in the highest recovery of mycobacterial isolates on solid medium.

Furthermore, within the laboratory pathway after the introduction of MGIT, a decrease in the time needed to detect infectiousness by mycobacterial culture from sputum specimens from patients diagnosed with TB (time to detection) could be shown for MGIT compared to primary LJ. The time needed to grow mycobacterial isolates on solid medium subcultures from MGIT reduced the time saved through faster detection of mycobacterial growth by MGIT compared to primary LJ to a level that did not confer any advantage concerning the time to grow mycobacterial isolates on solid culture medium that could be shipped for identification and DST (time to growth) for 'MGIT plus subculture from MGIT' compared to primary LJ.

In a comparison between the combined liquid and solid media culture techniques ('MGIT plus subculture from MGIT' and primary LJ) used in the laboratory pathway after the introduction of MGIT versus the single solid medium culture technique (Ogawa) used in the laboratory pathway before the introduction of MGIT a similar picture was observed concerning the time to detection and time to growth. Whereas the time to detection could be decreased significantly after the introduction of MGIT, only a slight decrease of the time to growth was detected, not showing any obvious advantage in favor of the new pathway.

The total turnaround time from sputum collection to the report of a drug susceptibility test result for specimens processed after the introduction of MGIT was expected to decrease, but it increased instead. Administrative delay in reporting the results was identified to be responsible for this increase rather than technical difficulties related to the use of the new procedures.

4.2 Comparison of the results with recent research

In 1995, the Centers for Disease Control (CDC) recommended a combination of a broth-based culture technique (BACTEC™) with a primary solid medium culture technique for the recovery of mycobacteria from clinical specimens in the United States (Centers for Disease Control 1995). At about the same time, BACTEC™ MGIT 960 liquid culture system, a fully automated, non-radiometric system for growing mycobacteria, started to replace the previous, semi-automated radiometric version (BACTEC™ 460). To evaluate these changes with regard to reference standards concerning the liquid medium technique and the CDC recommendation mentioned above, a number of laboratory studies and meta-analyses were carried out. Similar to the ODPC 7th laboratory evaluation, typical end points in the cited studies were the recovery rate of mycobacteria by culture technique, contamination rates, and the time to detection (TTD) of mycobacterial growth for the media compared:

In 1999 Hanna et al. published a "Multicenter Evaluation of the BACTEC™ MGIT 960 System for Recovery of Mycobacteria" showing that BACTEC™ MGIT 960, when used in combination with a primary solid medium, demonstrated a performance comparable with BACTEC™ 460 for the detection of M. tuberculosis complex, while providing greater recovery of Non-tuberculous mycobacteria, especially M. avium complex (MAC) (Hanna 1999).

The study was carried out at six test sites - three health department laboratories, one university-based tertiary medical center, one private medical center, and one regional reference center, all of which were in the U.S.A. and in Germany. Prospectively, a total of 2,210 respiratory and 1,120 non-respiratory specimens were selected from 2,346 patients at random. For the comparison of the results reported by Hanna et al. with results from the evaluation at the ODPC 7th laboratory, there were three important differences:

1. the different epidemiologic backgrounds,
2. the large number of non-respiratory specimens used in the study by Hanna et al., and
3. the use of a "combined primary solid media technique" (i.e. LJ solid medium plus 7H11) in the study by Hanna et al.

Whereas a comparison with BACTEC™ 460 was not part of the ODPC 7th laboratory evaluation, the results concerning BACTEC™ MGIT 960 (MGIT) alone and in combination with primary solid medium were consistent with results reported by Hanna et al. in that MGIT yielded a higher recovery of mycobacteria compared to primary solid medium, and in that the combination of MGIT with primary solid medium yielded a higher recovery of mycobacteria than could be obtained with either of the techniques alone.

The contamination rate of MGIT reported by the test centers enrolled in the evaluation by Hanna et al. was between 1.8-14.6%, which was consistent with 6.4% calculated at the ODPC 7th laboratory. The contamination rate of primary LJ at the ODPC 7th laboratory was quite high (14.8%), but still good compared to the contamination rates on primary solid medium of 0.8-37.8% reported by Hanna et al. On the one hand, the comparison of these rates should be interpreted carefully, as the solid media used in the pathway of Hanna et al. were a combination of LJ medium with Middlebrook 7H11, and not LJ medium only. On the other hand, one could argue that the contamination rate detected at the ODPC 7th laboratory under these circumstances was excellent, considering the fact that a single bottle of primary LJ was inoculated within the group TUC 2 laboratory pathway.

In the Multicenter Evaluation by Hanna et al., the mean time to detection for all mycobacteria in MGIT was 11.8 days (14.4 days for isolates from M. tuberculosis complex and 10.0 days for isolates from MAC). A similar time to detection in MGIT was achieved at the ODPC 7th laboratory. The time to detection on LJ medium was not reported in the study by Hanna et al.

In the ODPC 7th laboratory evaluation, it was found that the recovery of Non-tuberculous mycobacteria by MGIT was higher than that recovered by primary LJ. As mentioned before, this phenomenon was also reported by Hanna et al. and attributable to a high recovery of MAC in particular (47.5%). In the case of the ODPC 7th laboratory evaluation, though, the number of isolates from NTM was very small compared to the number of isolates from M. tuberculosis complex, and, in contrast to the test sites enrolled in the study by Hanna et al., there were no resources to routinely use more exact techniques than indirect methods for identification of mycobacterial species. As a result, the high recovery rate of NTM by MGIT should be reported only as an observation that supports the findings by Hanna et al.

Studies carried out by Chien et al. at the Taiwan Provincial Chronic Disease Control Bureau, by Lu et al. at the Barnes-Jewish Hospital Microbiology Laboratory, St. Louis, MO, and by Lee et al. at the Tzu-Chi Hospital, Taiwan, all reported similar results showing a higher recovery rate

and faster time to detection for MGIT compared to LJ, and a positive effect of using the combination of MGIT with LJ (Chien 2000, Lee 2003, Lu 2002).

These three studies were carried out over periods ranging from 3-14 months and chose a similar approach to the evaluation of MGIT versus LJ as was chosen in the ODPC 7th laboratory evaluation. Specimens were collected consecutively, not randomly. In one of the studies, only sputum specimens (1,396 sputum specimens from 622 patients (Lee 2003)) were evaluated, whereas in the two other studies, clinical specimens other than sputum were included (365 clinical specimens including 24 aspirates and 35 probes of pleural effusion (Chien 2000); 6,062 clinical specimens of which 78.1% were from the respiratory tract (Lu 2002)).

Contamination rates of 5.5-15.1% were reported for BACTEC™ MGIT 960, matching the findings in the ODPC 7th laboratory evaluation, and 4.1-10.1% for LJ medium, which was lower than the contamination rate documented for primary LJ at ODPC 7th laboratory (Chien 2000, Lee 2003).

In the ODPC 7th laboratory evaluation, the contamination rates for both primary LJ and MGIT were found to be especially high for smear-negative isolates and Non-tuberculosis mycobacteria, an observation that was not described in detail in any of the other studies. One possible explanation could be that the specimen quality of smear-negative sputum was worse than the quality of smear-positive sputum. Smear-negative sputum definitely contained fewer acid-fast bacilli and probably contained larger amounts of saliva that might have been a source of contamination by oral flora which were not killed during the decontamination process. Documentation regarding the assessment of specimen quality prior to inoculation would have helped to validate this suspicion but was unavailable.

Furthermore, the contamination rate of primary LJ was higher than expected, and especially high compared to the contamination rate of Ogawa, which was the solid medium of choice before the introduction of MGIT. Changes in the decontamination process after the introduction of MGIT and the inoculation of only one bottle of LJ medium, in contrast to two bottles of Ogawa, might have been possible reasons.

In general, the choice of sputum decontamination technique is always oriented toward the goal of effectively killing oral flora while avoiding unnecessarily diminishing the viability of mycobacteria. The sodium hydroxide (NaOH) method, especially when used in concentrations up to 4%, kills up to 60% of mycobacteria in the specimen. The N-acetyl cysteine sodium hydroxide (NALC-NaOH) method allows the NaOH concentration to be reduced to only 1%,

killing only up to 30% of mycobacteria in the specimen (WHO 1998). As a result, the less aggressive decontamination method that was used after the introduction of MGIT might be a plausible explanation for the higher contamination rate, but at the same time might have increased the recovery of mycobacteria.

In addition, sterilized NaOH solution keeps for several weeks, whereas NALC loses its activity quite rapidly and requires daily refreshing of the solution, each time an opportunity for contaminating the ingredients. It is therefore more sensitive to staff experience and hygienic precautions.

Contamination might also have occurred during the production of LJ medium, i.e. prior to inoculation, because this medium was produced at the ODPC 7th laboratory. In order to reduce the risk for contamination of LJ medium prior to inoculation, it was inspissated after it was filled in bottles, stored in the refrigerator and malachite green was added to the medium for the prevention of growth of organisms other than mycobacteria (WHO 1998). MGIT, instead, was ordered from the company and shipped to the ODPC 7th laboratory in vacuum seal. Again, no documentation was available on the medium quality, and the opportunity for staff interviews was missed.

The results reported for the recovery of mycobacteria by MGIT and LJ alone (86.5-100% and 59.7-87.2%, respectively) agreed with the findings in the ODPC 7th laboratory evaluation (96.6% and 71.4%, respectively). A higher recovery of Non-tuberculosis mycobacteria could be attributed to MGIT compared to LJ, and the combination of both media proved to yield optimum recovery of all mycobacteria. However, the studies reported eight to twenty-two sputum smear-positive specimens that were negative in both culture systems, and this was also the case for 25 specimens (6.6%) at the ODPC 7th laboratory. Unfortunately, none of the studies provided an exact number of all sputum smear specimens processed, so comparability is not given.

In the studies by Lee et al. and Chien et al., the mean time to growth for all mycobacteria was reported as 20.3-30.6 days for LJ and 10.7-11.6 days for MGIT. Growth was detected less quickly on cultures from smear-negative isolates. Similar findings can be reported from the ODPC 7th laboratory evaluation (chapter 3.2.3). The difference between the time to detection of M. tuberculosis complex (MTB) isolates vs. Non-tuberculous mycobacteria (NTM) isolates was investigated in the study by Chien et al. Whereas all species of NTM (MAC, M. fortuitum and M. kansasii) were growing faster than MTB in MGIT, MAC was growing more slowly than MTB on LJ. The evaluation carried out in northeast Thailand did not differentiate between the

specific NTM species, but the profile of the time to detection looked very similar to that reported for MAC by Chien et al.

The amount of NTM from all mycobacteria recovered by MGIT and LJ was 8.1% in the studies by Chien et al. and Lee et al. as well as in the ODPC 7th laboratory evaluation. The amount of NTM was much higher (MAC 37.8%, other NTM 38.8%) in the study by Lu et al. The interpretation of this difference is difficult. The epidemiological setting was very similar in the studies by Chien et al. (mycobacteriology laboratory of the Taiwan provincial chronic disease control bureau), Lee et al. (Taiwan teaching hospital, East Taiwan), and the ODPC 7th laboratory evaluation. The incidence rates of tuberculosis at the times of evaluation (112.5-113.4 per 100,000 in East Taiwan and 118 per 100,000 in the population covered by the Thailand TB active surveillance system) were very close. The epidemiological setting in the study by Lu et al. (microbiology laboratory at Barnes-Jewish hospital, St. Louis, MO), instead differed widely from the settings of the other two studies and the ODPC 7th laboratory evaluation. As a consequence, different bacteriological profiles of mycobacterial species might have been present in the high-burden setting compared to the low-burden setting of a laboratory in the U.S.A.

None of the studies/evaluations carried out in a high-burden setting routinely performed identification of mycobacteria by probe hybridization (Accu Probe, Gen Probe, San Diego, CA), as was done in the study by Lu et al. The question remains, was NTM under-reported due to identification through biochemistry and morphology only in the high-burden, resource-limited setting, or do the study results differ due to low prevalence of M. tuberculosis complex in the low-burden setting? It is questionable whether based on these studies investment in expensive routine probe hybridization techniques is justified for future research.

Finally, the results from the ODPC 7th laboratory were compared to a meta-analysis by Cruciani et al. (Cruciani 2004). This meta-analysis included ten studies that were carried out from January 1990 to June 2003. The recovery of mycobacteria from clinical specimens (primarily from the respiratory tract) was compared for BACTEC™ MGIT 960 versus BACTEC™ 460 versus primary solid medium, (of which, in nine out of ten studies, the solid medium was LJ medium) and for combinations of BACTEC™ MGIT 960 with solid medium versus BACTEC™ 460 with solid medium:

From 1,381 isolates (58.6% strains of M. tuberculosis and 41.4% Non-tuberculous mycobacteria) the mean recovery of all mycobacteria proved to be higher for liquid medium (81.0%) than for solid medium (67.0%). Through combination of liquid medium with solid medium, recovery

could be increased (87.0%). The combination of solid medium with BACTEC™ 460 could reach higher recovery of mycobacteria (89.0%) than the combination of solid medium with BACTEC™ MGIT 960, but this difference was not significant. A higher recovery of NTM by MGIT 960 was not detected in the meta-analysis.

Study results on contamination rates for BACTEC™ MGIT 960 were very heterogeneous ranging from 3.8-16.6%. Contamination rates for solid medium were reported as 12.3-13.4%.

Time to detection of mycobacterial growth was significantly shorter for BACTEC™ MGIT 960 (12.9 days) compared to both BACTEC™ 460 (15.0 days) and LJ (27.0 days). A longer TTD was reported for isolates from smear-negative specimens compared to those from smear-positive specimens of both media. All Non-tuberculous mycobacteria grew more slowly in MGIT except for MAC which grew more quickly in MGIT compared to M. tuberculosis complex.

In conclusion, it was suggested that the combination of BACTEC™ 460 with solid medium remained the gold standard for the recovery of mycobacteria. Valuable factors such as practicability (BACTEC™ MGIT 960 being a non radiometric assay) and faster time to detection still showed BACTEC™ MGIT 960 to be a promising alternative to BACTEC™ 460 (Cruciani 2004).

The results of the ODPC 7th laboratory evaluation were consistent with the results reported from the meta-analysis by Cruciani et al. Given the high level of evidence of meta-analyses, the results of the ODPC 7th laboratory evaluation are representative of all advantages concerning MGIT that have been reported from previous research. MGIT in combination with a primary solid medium for the detection of mycobacteria is effective in a high-burden, resource-limited setting.

4.3 Advantages and limitations of the ODPC 7th laboratory evaluation in the light of actual research

The ODPC 7th laboratory evaluation was motivated by the need of field studies for the optimization of existing tools in different epidemiologic settings (Brewer 2004) and it tried to integrate “operational methodological research into routine diagnostic laboratory activities” (Perkins 2002). It was carried out in the context of a large demonstration project of enhanced TB control in Thailand (The Thailand TB Active Surveillance Network), which incorporated strategies recommended as part of the new Global Plan to Stop TB, in particular the expansion of laboratory capacity for mycobacterial cultures and drug susceptibility testing (Varma 2007, chapter 1.7.1).

The two terms “field study” and “large demonstration project” mark the area of conflict from which imminent limitations of the evaluation’s methodology arose:

On the one hand, the term “field study” implied an investigation that considered the local, specific conditions in a certain environment. The laboratories at the project sites varied concerning resources, staff experience, data documentation forms, non-standardized operating procedures etc.

On the other hand the term “large demonstration project” implied an investigation that was carried out based on a highly standardized protocol and, ideally, carried out as a multi-center analysis. Test sites would have needed to be evaluated if they could comply with the standard operating procedures and could satisfy certain quality markers in advance. At present, much effort is made to evaluate test sites in resource-limited settings concerning their recruitment for multi-center research, but this level had not yet been achievable within the scope of the ODPC 7th laboratory evaluation.

In order to solve the area of conflict concerning the methodology, for the ODPC 7th laboratory evaluation one single laboratory site was chosen (Ubon-ratchathani) according to the characteristics of a “field study”. While compiling the data, detailed investigations of the operating procedures and of the data sources’ availability and reliability in combination with an intensive “on site presence” at the evaluation site (6 months) were preferred over a large, but inexact multi-center approach.

The primary objective, i.e. the comparison of the two culture procedure arms within the laboratory pathway of group TUC 2 (‘MGIT plus subculture from MGIT’ vs. primary LJ, Figure 3), was evaluated on a clear and well defined statistical level. Limitations compared to previous studies were:

1. the retrospective approach,
2. the use of consecutive specimens instead of randomization (which was only avoided in the large multicenter analysis by Hanna et al. and some of the studies included into the meta-analysis by Cruciani et al.), and
3. the lack of a definition for false-positive and false-negative results.

Despite these shortcomings the ODPC 7th laboratory evaluation generated results that were consistent with the results from recent studies concerning MGIT in combination with a primary solid medium for the detection of mycobacteria (chapter 4.2).

The ODPC 7th laboratory evaluation extends the scope of recent research as it links the "laboratory analysis" (primary objective) with the operational level in the context of a "control- and treatment program" (secondary objective). The introduction of the unique end points *yield of isolates for identification and drug susceptibility testing (YIID), yield of drug susceptibility test results (YDR)*, and *time to growth (TTG)* (compare chapter 2.3.1) made an inter-group "before and after comparison" possible on, at least, a descriptive/explorative level.

Definitely, a "before and after comparison" as such is statistically weak, but justified in a field setting with currently unsatisfactory documentation and lack of resources on the condition that the evaluation of "an existing tool in a specific epidemiologic setting with the intention to optimize its use in the future" (Brewer 2004) is very important.

In order to avoid inaccuracy of this inter-group comparison (TUC 1 vs. TUC 2), several precautions were taken:

Whereas recent studies had included various clinical specimens (sputum, bronchial aspirates and washings, some even lymph nodes, urine or blood) in the analysis, in the ODPC 7th laboratory evaluation only sputum specimens were taken. In previous studies, also more than one specimen per patient had been included, and, as a result, the focus had been on the specimen instead of the patient. This focus shift was avoided in the ODPC 7th laboratory evaluation by matching sputum specimens and patients and by including only one sputum specimen per patient. Only the sputum specimens of patients who fulfilled the inclusion criteria (chapter 2.1) were analyzed. Sputum specimens of which the sputum smear microscopy results were unknown were excluded from the analysis. Groups TUC 1 and TUC 2 were shown to be similar in the distributions of size, sex, age and sputum smear microscopy results (Table 6).

In the research article „Evaluating the potential impact of the new Global Plan to Stop TB: Thailand, 2004-2005" by Varma et al. (Varma 2007), difficulties concerning data collection as part of a surveillance and program-monitoring project had been named. Some of these difficulties also limited the above intentions:

1. the use of handwritten documentation;
2. the removal of duplicate records by comparing names and various demographic factors due to the lack of an unambiguous primary key; and
3. the time consuming matching of data from different sources without an unambiguous primary key that resulted in missing data or mismatch of information.

To handle these shortcomings adequately, the data was always checked for completeness, especially where mismatching was suspected on the ground of data inconsistencies.

4.4 Conclusion regarding the primary objective

'MGIT plus subculture from MGIT' versus primary LJ

For conclusions regarding the performance of 'MGIT plus subculture from MGIT'and primary LJ concerning the detection of mycobacterial growth it was important to define the purpose that the mycobacterial culture was required for. In the case of mycobacterial cultures at the ODPC 7th laboratory this purpose was twofold:

On the one hand, cultures were performed for detecting mycobacteria (detection of infectiousness of patients diagnosed with TB in the context of a TB control program) and only necessary on either MGIT and/or primary LJ medium. MGIT detected more mycobacteria than primary LJ in a much shorter time.

On the other hand, cultures were performed for isolating mycobacterial cultures that were used for identification of the mycobacterial species (M. tuberculosis or Non-tuberculous mycobacteria) and drug susceptibility testing (DST). As there were no resources for routine use of direct identification techniques such as hybridization (Gen Probe) and isolates were shipped for DST to Bangkok, growth of mycobacterial isolates was necessary on solid medium, i.e. on either primary LJ or a subculture from MGIT (Figure 3). The combination of both techniques proved to produce optimum test performance: losses due to contamination when subculturing isolates from MGIT tubes that were flagged positive could be compensated for by the inoculation of sputum specimen on both MGIT and primary LJ in parallel. MGIT with a subsequent subculture increased the number of isolates from smear-negative MTB and NTM (Table 8).

Whereas the time to detection of mycobacterial growth had been unbeatable for MGIT compared to primary LJ, no advantage was found for MGIT plus a subsequent subculture compared to primary LJ concerning the time that was needed to grow a mycobacterial isolate on solid culture medium (defined as time to growth, chapter 2.3.1) (Table 12). The time that could be saved through earlier detection of mycobacterial growth in MGIT was lost when growing subcultures from MGIT (Table 11).

When using MGIT in the setting of the Ubon-ratchathani regional tuberculosis control program, considering the fact that for the ODPC 7th laboratory evaluation only one sputum per TB patient

had been included in the analysis, more patients could be classified as infectious TB cases (acid-fast bacilli present in sputum) in a much shorter time than when using primary LJ.

MGIT with a subsequent subculture ('MGIT plus subculture from MGIT') also yielded the higher number of mycobacterial isolates from sputum on solid medium that could be used for identification and DST than primary LJ could yield. No clear advantage in time until these isolates were available for further procedures could be detected for 'MGIT plus subculture from MGIT' compared to primary LJ medium.

The quantity of mycobacterial isolates on solid medium (subculture from MGIT and primary LJ) increased by almost 10% when growing cultures on both 'MGIT plus subculture form MGIT' and primary LJ in parallel compared to either method alone. In other words, the opportunity for drug susceptibility testing increased by 9.6% when using liquid and solid medium in combination.

4.5 Conclusion regarding the secondary objective

Comparison of group TUC 1 vs. group TUC 2

As described in chapter 1.7, the motivation to build the capacity for mycobacterial culture and susceptibility testing was to demonstrate the effectiveness of mycobacterial culture as a tool for the routine diagnosis of TB compared to smear microscopy and chest X-ray in a high-burden, resource-limited setting. The present evaluation of BACTEC™ MGIT 960's introduction to the ODPC 7th laboratory in Ubon-ratchathani was not designed to prove this effectiveness as such, but to prove that in a resource-limited, high-burden setting mycobacterial culture procedure and DST quality could be improved successfully, and that therefore this quite expensive investment was justified.

The evaluation at the ODPC 7th laboratory in Ubon-ratchathani served as a feasibility study that could not only measure quality improvement in the mycobacteriology laboratory but also uncover pitfalls within the laboratory pathways that have not yet been solved and will need to be smoothed out prior to further research.

The introduction of MGIT at the ODPC 7th laboratory in the context of disease surveillance

In the comparison of the liquid medium MGIT with the solid medium LJ it has been shown that many more mycobacteria were detected from sputum in liquid medium than were on solid medium. Liquid and solid medium in combination detected mycobacteria in 416/562 sputum specimens (74.0%) in group TUC 2, whereas Ogawa as single solid medium in group TUC 1

detected mycobacteria in only 300/545 sputum specimens (55.0%). In the context of disease surveillance, this means that after the introduction of MGIT 19.0% more patients could be classified as infectious TB cases than before the introduction of MGIT. While mycobacterial culture was expected to be more sensitive than sputum smear microscopy, less mycobacteria were detected on Ogawa than on sputum smear microscopy (58.0% growth on Ogawa compared to 65.1% smear-positive microscopy results; Table 6 and Table 13). After the introduction of MGIT, sputum smear microscopy sensitivity increased by 6.4% when inoculating sputum specimens on/in both solid (primary LJ) and liquid (MGIT) culture media in parallel (74.0% growth in MGIT and/or on primary LJ compared to 67.6% smear-positive microscopy results; Table 6 and Table 8).

The importance of mycobacterial cultures for the surveillance of multi drug resistant tuberculosis (MDR-TB) cases was a further motivation for enhancing mycobacterial culture capacity in northeast Thailand. The national drug resistance surveys, until now, may have underestimated the true burden of MDR-TB in Thailand (compare chapter 1.7.1).

The results of the ODPC 7th laboratory evaluation do not suggest that after the introduction of MGIT more MDR-TB cases were detected (Table 14), although the overall availability of drug susceptibility test results increased by 6.0% in group TUC 2 compared to group TUC 1 (Figure 6). Similar results were reported from a very recent study carried out in a clinical setting in Romania. More strains with altered drug susceptibility could be isolated in liquid than on solid medium. An in-depth comparison with this study's results was limited, because the full text article was published in Romanian language only (Grigoriu 2007).

After the introduction of MGIT, more isolates from sputum specimens from patients that had been diagnosed and treated for tuberculosis were finally identified as Non-tuberculous mycobacteria compared to the cohort before the introduction of MGIT (Table 14).

These results alone are not sufficient to either agree or disagree with the above assumptions, hard surveillance data should be collected after the revision and/or formulation of standard operating procedures for drug susceptibility testing and identification tests before a definitive conclusion can be reached.

The introduction of MGIT to ODPC 7th laboratory for clinical use

The yield of mycobacterial isolates, that could be sent for identification and DST, increased by 13.9% after the introduction of MGIT. Taking into account that sputum specimens and patients were matched, this means that for patients diagnosed with tuberculosis the chance of a clinically

useful DST result increased by 13.9% for patients diagnosed after the introduction of MGIT (group TUC 2) compared to patients diagnosed before the introduction of MGIT (group TUC 1). Unfortunately, the ultimate yield of DST results was only 6.0% higher in group TUC 2 compared to group TUC 1 (Figure 6). This discrepancy was partly due to the larger number of isolates from Non-tuberculous mycobacteria generated in the group TUC 2 laboratory pathway (Table 14), but mainly to a much higher contamination rate during DST procedures (chapter 3.3.2). Furthermore, the mycobacterial isolates' status on arrival to NTRL in Bangkok in group TUC 2 was worse compared to that in group TUC 1 (step 4, chapter 2.2). It is uncertain if this high contamination rate was a single, unfortunate incident during the evaluation period (confounder) or secondary to changes in the laboratory procedures, such as decontamination techniques etc. (as discussed in chapter 4.2).

In chapter 1.6.3 a laboratory's readiness to perform good quality cultivation of mycobacteria and DST was defined as the ability to perform direct sputum smear microscopy within twenty-four hours, identification of M. tuberculosis within ten to fourteen days and drug susceptibility testing within fifteen to thirty days (Tenover 1993).

For the sputum specimens included in the ODPC 7th laboratory evaluation, smear microscopy was generally performed within twenty-four hours at the treatment site. The median time to detection of mycobacteria was seven days in MGIT and twenty-six days on both LJ and Ogawa (Table 10 and Table 16). Considering the median four to five days that elapsed from sputum collection to culture inoculation (Table 17), the recommended ten to fourteen days within which identification of M. tuberculosis was to be completed could only be achieved when using MGIT and microscopic cord formation (serpentine cording on smear from cultures grown in liquid medium) as rapid and presumptive confirmation of M. tuberculosis from isolates detected in liquid medium (Attorri 2000).

Following the DOTS recommendations, an intensive treatment phase made up of a combination of four anti-tuberculosis drugs (isoniazid, rifampicin, pyrazinamide and ethambutol), after two months, is cut down to a combination of two drugs (isoniazid and rifampicin) during a four months continuous treatment phase. Prior to this modification sputum must test negative for acid-fast bacilli on smear microscopy. In addition, early identification of Non-tuberculous mycobacteria can prevent a prolonged and unjustified anti-tuberculosis treatment with the risk of dangerous drug-related side effects and/or the risk for developing new drug resistances. It therefore makes sense to define a laboratory's readiness for drug susceptibility testing as its

ability to guarantee DST results before the drug combination's modification (i.e. within at least two months). The ODPC 7th laboratory – even after the introduction of MGIT – was not ready:

DST results were available after 104 days for patients treated in group TUC 1. In group TUC 2 the results were available - even later - after 118 days (Table 18). To find out, why the total turnaround time (TTAT) was so long that the clinical utility of DST results must be questionable in both groups, the total turnaround time was broken down into all procedure steps involved from the day of sputum collection to the day when the DST result was available at the treatment site (chapter 2.2).

While breaking down the laboratory pathway into the single steps it was observed that data were reported incompletely on the district level, i.e. in step 1 and step 6 (Figure 3). Presumably, documentation either did not exist or was not kept long enough (the recall on investigation forms was even smaller for specimens of patients in group TUC 1 compared to those in group TUC 2, step 1 and step 6, chapter 2.2). Therefore, drug susceptibility testing did not seem to have great influence on clinical management of patients at the treatment sites in northeast Thailand at the time of the ODPC 7th laboratory evaluation.

4.6 Can liquid medium (MGIT) be recommended in a high-burden, resource-limited setting?

In the ODPC7th laboratory evaluation in Ubon-ratchathani, northeast Thailand, MGIT proved to yield similar test performance compared to previous laboratory analyses which compared liquid medium (MGIT) with solid medium for cultivation of mycobacteria.

Compared to the laboratory pathway before the introduction of MGIT, more patients could be classified as infectious TB cases more quickly in the context of a TB control program. After subculturing mycobacterial isolates from MGIT on solid medium ('MGIT plus subculture from MGIT') the yield of isolates that could be processed further for identification and drug susceptibility testing did increase.

The higher yield of isolates for identification and drug susceptibility testing was shown when combining liquid medium plus subculture ('MGIT plus subculture from MGIT') with solid medium (LJ) in particular, but the procedure times for cultivation comparing both methods did not differ significantly. Therefore, liquid medium should not replace solid medium, but instead is recommended for cultivating mycobacteria in combination with solid medium.

After the introduction of MGIT, drug susceptibility test performance did not improve as remarkably as culture performance: While the availability of DST results increased, the detection of multi drug resistant M. tuberculosis was unchanged. Furthermore, considering the high contamination rate during drug susceptibility testing and the long procedure times for identification and DST after the introduction of MGIT at the ODPC 7th laboratory, an advantage of MGIT's introduction in a high-burden, resource-limited setting is debatable.

In conclusion, MGIT in combination with LJ medium can undoubtedly be recommended for culture procedures to detect infectious TB cases as well as to obtain mycobacterial isolates for further identification and drug susceptibility testing in the context of a TB control program in a high-burden, resource-limited setting. Concerning the recommendation of MGIT for drug susceptibility testing, more investigation is needed in the future.

Last but not least, a detailed cost analysis that was not included in the ODPC 7th laboratory evaluation might reveal additional findings that influence the recommendation of MGIT from a public health financing policy perspective.

4.7 Recommendations for future investigations

The findings from the detailed analysis of steps 2 to 5 uncovered pitfalls on the way to drug susceptibility results that might have resulted in time delays and require further investigation in the future:

The long time for subcultures from MGIT

When the introduction of MGIT was discussed, according to experts' opinion the time for subcultures from MGIT should be a matter of days. This opinion was not confirmed by the actual results. An initiative has already been started for a prospective analysis of the times for subcultures at the test sites involved in the Thailand TB active surveillance network (Figure 1), the results are pending.

Transport of mycobacterial isolates to Bangkok

Soon after this evaluation had been finished, i.e. with the beginning of the next patient cohort (October 2005), ODPC 7th laboratory started to perform drug susceptibility testing in Ubon-ratchathani. Time delay due to the transport of mycobacterial cultures to Bangkok will not be an issue in the future.

The mycobacterial isolates' status on arrival at NTRL in Bangkok

Concerning the mycobacterial isolates' status on arrival at NTRL in Bangkok (Figure 7), contamination and culture mix occurred more frequently in group TUC 2 whereas an insufficient number of mycobacterial colonies on the culture (few bacilli) was observed more often in group TUC 1. The underlying mechanisms for these observations were discussed in detail in chapter 4.2.

Contamination during the shipping process will no longer result in delays in the future once ODPC 7th laboratory starts to perform DST on site. In those cases where DST cannot yet be performed on site and shipping of cultures is still required, more investigation and a proper documentation on this issue will be necessary. The data on the status of cultures on arrival in this evaluation were based on self-report by NTRL and therefore do not meet a high level of reliability.

Report of drug susceptibility test results

The longest delays were caused when reporting DST results. A plausible reason for these delays could not be identified, and, finally, administration delay was self-reported in staff interviews. While administration is the most unfortunate pitfall to occur, it, fortunately, is the easiest to address in the future.

One very effective and cheap tool for improving the communication between test and treatment sites and for bypassing slow official government mail is the internet. During the observation period of this evaluation, TUC staff started to make DST results available in the ODPC 7th webpage's intranet. Patient confidentiality and network extension have since then been discussed.

The issue whether DST results should be reported immediately when available using BACTEC™ MGIT 960 or after the completion of DST using the proportion method on LJ medium and PNB screening (step 4, chapter 2.2) remains unsolved. While the ODPC 7th laboratory evaluation was carried out, standard operating procedures (SOPs) for identification and drug susceptibility testing had not yet been formulated, but should be available by now.

Identification tests

A re-evaluation of the pitfalls described above and the writing of standard operating procedures should be preferred over the introduction of cost-intensive direct identification techniques (such as for example Gen Probe). If drug susceptibility testing continued to be done at NTRL, the

laboratory sites where mycobacterial cultures are grown should perform identification through macroscopic culture morphology, smear microscopy, and biochemistry prior to shipping. In doing so repetitive identification tests should be avoided, and overall screening by using PNB medium should become obsolete.

Data collection

Lastly, a common, unambiguous primary key that is used in all data sources within the Thai Ministry of Public Health - United States Centers for Disease Control and Prevention Collaboration (TUC) is required for all further investigation. With this primary key only, reliable and complete matching of clinical data and laboratory data will be possible in the future. This insight is not a novelty but well known to some TUC staff in Ubon who work hard in promoting the computer based data collection with Access.

5 Summary

Increased mycobacterial yield by "BACTEC™ Mycobacteria Growth Indicator Tube 960" (MGIT), a fully automated, non-invasive, liquid culture-based system compared to solid medium had been shown in a number of studies. These had primarily been performed in low-burden, highly resourced settings. The objective of the present evaluation was to see if the use of MGIT can also be recommended in a high-burden, resource-limited setting.

Evaluation data were compiled retrospectively for sputum specimens from patients treated in Ubon-ratchathani province that were diagnosed with TB by a clinician and/or started on anti-TB treatment in the period 01-Oct-2004 to 31-Sep-2005. Whereas before the introduction of MGIT specimens had only been inoculated on Ogawa solid medium, after the introduction of MGIT specimens were inoculated both in MGIT and on Löwenstein-Jensen (LJ) solid medium in parallel.

The liquid medium MGIT was compared with the solid medium LJ concerning recovery of mycobacteria and time to detection. As mycobacterial isolates on solid medium were shipped for drug susceptibility testing (DST) to Bangkok, subcultures were needed from specimens flagged positive in MGIT. Therefore, yield of specimens for identification and DST (YIID) and the time it took for sufficient growth of an isolate that could be used for DST (time to growth) were introduced as additional end points for the comparison of 'MGIT plus subculture from MGIT' with LJ (post introduction of MGIT), and, in a second step, compared to Ogawa solid medium (pre introduction of MGIT).

1,107 sputum specimens were included in the analysis of which 545 (49.2%) before the introduction of MGIT and 562 (50.8%) after the introduction of MGIT.

The recovery of mycobacteria was higher for MGIT (96.6%) than for primary LJ (71.4%) ($p<0.001$). The optimum recovery of mycobacteria on solid medium for DST was obtained by the combination of 'MGIT plus subculture from MGIT' with primary LJ (92.3%) compared to either method alone (82.9% vs. 71.4%, respectively; $p<0.001$).

The yield of mycobacterial isolates was higher for the combined culture techniques (primary LJ, MGIT and subculture from MGIT) (71.9%) than for Ogawa medium (58.0%) ($p<0.001$), with the exception of isolates from NTM ($p=0.264$). The time to detection of mycobacteria was very short in MGIT (7 days) compared to primary LJ (26 days; $p<0.001$) and Ogawa (26 days; $p<0.001$) whereas no difference in the time to detection was observed when comparing the two solid

media culture techniques (primary LJ and Ogawa) with each other (p=0.943). Growth of mycobacteria on subcultures from MGIT took 14 days. The time it took for sufficient growth of an isolate that could be used for identification and DST depended on the species' growth characteristics, undermining any comparison between the combined culture techniques (primary LJ, MGIT and subculture from MGIT) compared to Ogawa medium (p-values ranging from 0.001 - 0.943 for different mycobacterial species). The availability of DST results (total turnaround time) took 118 days after compared to 104 days before the introduction of MGIT ($p<0.001$).

The results of this evaluation confirm the superiority of MGIT for the yield of mycobacterial cultures compared to solid medium only in a resource-limited, high-burden setting. However, the combination of MGIT with LJ medium is recommended, because losses due to contamination when subculturing isolates from MGIT tubes that were flagged positive can be compensated for by the inoculation of sputum specimen on both MGIT and primary LJ in parallel. Not only did growth of mycobacteria on subcultures from MGIT for DST take longer than expected, but also availability of DST results took longer after the introduction of MGIT.

Finally, the clinical utility of potentially rapid (less than two months) DST results secondary to rapid and increased yield of mycobacteria was undermined by the logistics of transport delays and administrative factors. Further investigation is needed concerning the role of MGIT liquid culture medium in drug susceptibility testing.

Zusammenfassung

Die vermehrte Anzucht von Mykobakterien durch die Verwendung von „BACTEC™ Mycobacteria Growth Indicator Tube 960 (MGIT)“, einem voll automatisierten, nicht invasiven Testsystem zur Anzucht von Mykobakterien in Flüssigmedium im Vergleich zu Festmedium war bereits in vorangegangenen Studien gezeigt worden. Diese waren jedoch vor allem in Gebieten niedriger Prävalenz und hinreichender Ressourcen durchgeführt worden. Ziel der vorliegenden Evaluation war der Nachweis, dass MGIT auch für den Gebrauch in einem Gebiet hoher Prävalenz und begrenzter Ressourcen empfohlen werden kann.

Zur Evaluation wurden Daten für Sputen von Patienten, die im Zeitraum vom 01. Oktober 2004 bis zum 31. September 2005 in der Provinz Ubon-ratchathani die Diagnose Tuberkulose erhalten und/oder mit einer antituberkulotischen Behandlung begonnen hatten, retrospektiv zusammengestellt. Während vor der Einführung von MGIT im April 2005 die Anzucht von Mykobakterien lediglich auf dem Festmedium Ogawa vorgenommen worden war, erfolgte sie nach der Einführung von MGIT parallel in sowohl MGIT als auch auf dem Festmedium Löwenstein-Jensen (LJ).

Das Flüssigmedium MGIT wurde mit dem Festmedium Löwenstein-Jensen bezüglich der relativen Anzucht von Mykobakterien und der Anzuchtszeit verglichen. Zur Resistenztestung wurden mykobakterielle Isolate auf Festmedium nach Bangkok geschickt, somit musste im Falle eines initial in MGIT positiv getesteten Sputums eine Subkultur angelegt werden. Die Ausbeute an mykobakteriellen Isolaten für die Resistenztestung sowie der Zeitraum für deren Fertigung wurden als weitere Endpunkte für den Vergleich zwischen MGIT mit anschließender Subkultur und LJ eingeführt und in einem zweiten Analyseschritt dem Festmedium Ogawa (vor der Einführung von MGIT) gegenübergestellt.

Insgesamt wurden 1107 Sputen in die Analysen aufgenommen, davon 545 (49.2%) vor der Einführung von MGIT, und 562 (50.8%) nach der Einführung von MGIT.

Die relative Anzucht von Mykobakterien im Flüssigmedium MGIT war höher (96.6%) als auf dem Festmedium LJ (71.4%) ($p<0.001$). Die optimale relative Anzucht von Mykobakterien zur Resistenztestung wurde bei paralleler Anzucht auf sowohl LJ als auch in MGIT mit anschließender Subkultur (92.3%) gegenüber der jeweils alleinigen Technik (82.9% bzw. 71.4%) erzielt ($p<0.001$).

Die Ausbeute von mykobakteriellen Isolaten zur Resistenztestung war bei paralleler Anzucht in MGIT und auf LJ (71.9%) höher als bei Anzucht auf solitärem Ogawa (58.0%) ($p<0.001$), ausgenommen Isolate von nicht-tuberkulösen Mykobakterien ($p=0.264$). Der Zeitraum bis zum Nachweis mykobakteriellen Wachstums war sehr kurz bei Verwendung des Flüssigmediums MGIT (7 Tage) verglichen mit den Festmedien LJ (26 Tage; $p<0.001$) und Ogawa (26 Tage; $p<0.001$), wohingegen kein Unterschied dieses Zeitraums im Vergleich der beiden Festmedien untereinander festgestellt werden konnte ($p=0.943$). Die Anfertigung einer Subkultur von in MGIT angezüchteten Isolaten dauerte 14 Tage. Der Vergleich des Zeitraums bis zum Wachstum einer für die Resistenztestung nutzbaren mykobakteriellen Kultur zwischen den vor- und nach der Einführung von MGIT verwendeten Anzuchtstechniken war durch große Schwankungen der p-Werte (0.001-0.943) für die unterschiedlichen mykobakteriellen Spezies erschwert. Der Gesamtzeitraum bis zum Vorliegen der Resistenzergebnisse nach der Einführung von MGIT betrug 118 Tage verglichen mit 104 Tagen vor der Einführung von MGIT ($p<0.001$).

In der vorliegenden Evaluation konnte die Überlegenheit von MGIT bei der Anzucht von Mykobakterien gegenüber alleinigem Festmedium in einem Gebiet hoher Prävalenz und begrenzter Ressourcen nachgewiesen werden. Die parallele Anzucht auf Löwenstein-Jensen Medium zur Optimierung dieses Verfahrens wird jedoch empfohlen, weil hierdurch Verluste von initial in MGIT positiv getesteten Sputen bei der Anlage der Subkulturen (sei es durch fehlende Anzucht oder durch Kontamination) kompensiert werden können. Der Zeitvorteil zum Nachweis von Mykobakterien bei Verwendung des Flüssigmediums wurde relativiert durch die Beobachtung, dass die Anfertigung einer Subkultur zur Resistenztestung länger dauerte als erwartet. Auch der Gesamtzeitraum bis zum Vorliegen eines Resistenzergebnisses verlängerte sich nach der Einführung von MGIT.

Schließlich war die klinische Nutzbarkeit von potentiell schnell verfügbaren Resistenzprofilen (innerhalb von weniger als zwei Monaten) infolge einer schnelleren und vermehrten Anzucht von Mykobakterien aufgrund logistischer Probleme bei Transport und administrativen Prozessen äußerst fragwürdig. Zur Klärung der Bedeutung des Flüssigmediums MGIT im Rahmen der Resistenztestung sind daher weitere Untersuchungen notwendig.

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Lebenslauf

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.

Erklärung an Eides Statt

Hiermit erkläre ich, Friedrich Borchers, an Eides Statt, dass die vorgelegte Dissertation von mir selbst und ohne die unzulässige Hilfe Dritter verfasst wurde, auch in Teilen keine Kopie anderer Arbeiten darstellt und die benutzten Hilfsmittel sowie Literatur vollständig angegeben sind.

Friedrich Borchers Berlin, den 23.09.2008