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**DIAGNOSIS OF TUBERCULOSIS
IN A HIGH BURDEN RESOURCE LIMITED SETTING**

by

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A thesis submitted to the Open University U.K

For the degree of Doctor of Philosophy in the field of Life Sciences

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Ho Chi Minh City, Viet Nam

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of LED 40X increased in comparison with Ziehl-Neelsen (ZN) reading in the early phases of the evaluation (2.6% and 2.7% vs 0% in the validation and implementation phases vs continuation phase, respectively), probably due to lack of experience among technicians, but no significant difference was found ($P>0.05$). In the last phase, no false positive result was recorded. Although the reading time of fluorescence-LED (FM-LED) and ZN reading were less than 2 minutes, ZN-light microscope (ZN-LM) reading time was shorter than that of FM-LED40X, especially in positive smears ($P=0.007$) in the early study phase. By the end of the study, FM-LED reading time was shorter than ZN-LM which was recorded in the early phase. With high experienced technicians there may be little or no additional benefit to case detection if a light microscope is replaced by a fluorescence microscope.

In general, MODS and LED microscopy are reliable methods for use in diagnosis of TB in resource limited settings due to their accuracy, reliability and low costs. Large scale operational projects should be conducted to evaluate the feasibility and cost-effectiveness of these methods in countries where these techniques are to be implemented.

Co-Authorship

- The author of this work, Dang Thi Minh Ha (DTMH), was responsible for conduct, supervision, data analysis and writing of all studies described in this thesis. DTMH also conceived and designed the MODS studies (chapter 4). The iLED evaluation (chapter 5) was designed as a multi-country study by FIND (PI: Catherina Boheme). For the MODS study, the author completed all laboratory work related to the MODS technique in the pilot phase conducted at the Hospital for Tropical Diseases. For the MODS Implementation phase at Pham Ngoc Thach Hospital, DTMH supervised 2 technicians performing MODS and all staff from the Microbiology Department and 3 District Tuberculosis Units conducting the iLED study.
- Do Dang Anh Thu, Bsc completed all laboratory work at the Hospital for Tropical Diseases related to smear microscopy and culture.
- Two technicians, Tran Van Quyet and Nguyen Thi Bich Tuyen, performed all procedures related to the MODS technique at Pham Ngoc Thach hospital under the supervision of DTMH. Phan Thi Hoang Anh, technician and Vo Sy Kiet, MSc were responsible for DNA extraction and spoligotyping, respectively, of all positive cultures collected from the MODS study at Pham Ngoc Thach Hospital.

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Abbreviations

| | |
|-------------------|--|
| µg | Microgram |
| µl | Microliter |
| ART | Antiretroviral therapy |
| C | Clofazimine |
| CRF | Case report Form |
| CSF | Cerebrospinal Fluid |
| ddNTP | Dideoxyribonucleotide triphosphate |
| DNA | Deoxyribose nucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| DOTS | Direct Observed Treatment Short course |
| DST | Drug susceptibility testing |
| DTU | District Tuberculosis Unit |
| EPTB | Extrapulmonary Tuberculosis |
| ETH, E | Ethambutol |
| FIND | Foundation for Innovative New Diagnostics |
| FIND | The Foundation for Innovative Diagnostics |
| FM | Fluorescence microscope |
| FM-iLED | Fluorescence Primostar iLED microscope |
| g | Gram |
| HCMC | Ho Chi Minh City |
| HIV | Human Immuno deficiency Virus |
| HTD | Hospital for Tropical Diseases |
| INH, H | Isoniazid |
| IUATLD | International Union Against Tuberculosis and Lung Disease |
| K | Kanamycin |
| KMnO ₄ | Potassium Permanganate |
| l | Litter |
| LED | Light Emitting Diodes |
| LJ | Lowenstein-Jensen |
| M | Moxifloxacin |
| MDR-TB | Multidrug Resistant Tuberculosis |
| MGIT | Mycobacteria Growth Indicator Tube |
| MODS | Microscopic Observation Drug Susceptibility Assay |
| NTP | National Tuberculosis Programme |
| OADC | Growth supplement including Oleic acid, Bovine albumin Dextrose, Catalase and Polyoxyethylen stearate (POES) |

| | |
|---------|--|
| P | Prothionamide |
| PANTA | Mixture of antimicrobial agents including Polymycin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin |
| PCR | Polymerase Chain Reaction |
| PCR | Polymerase Chain Reaction |
| PNT | Pham Ngoc Thach Hospital |
| PTB | Pulmonary Tuberculosis |
| PZA, Z | Pyrazinamide |
| RIF, R | Rifampicin |
| STR, S | Streptomycin |
| TB | Tuberculosis |
| TBM | Tuberculous meningitis |
| TDR | Programme for Research and Training in Tropical Diseases |
| WHO | World Health Organisation |
| XDR-TB | Extremely Drug Resistance Tuberculosis |
| ZN | Ziehl-Neelsen |
| ZN-iLED | Light Primostar iLED microscope |
| ZN-LM | Ziehl-Neelsen staining – standard light microscope |

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Chapter 1

INTRODUCTION

1.1 HISTORY OF TUBERCULOSIS

It is known that humans have suffered from tuberculosis (TB) since ancient times; DNA of the causative organisms, *Mycobacterium tuberculosis* (*M.tuberculosis*) has been isolated from 3000 year old Egyptian mummies (140). In addition, literature described tuberculosis in India and China as early as 3300 years ago and 2300 years ago, respectively (91). Around 460BC, Hippocrates reported that phthisis, an ancient term to describe tuberculosis, usually occurred at a young age and was always fatal. Its presentation was described by Clarissimus Galen as cough, fever, pus and malnutrition with ulceration in the respiratory system (91). These are also the classical symptoms of pulmonary tuberculosis recognized today: prolonged productive cough, fever, night-sweats and weight loss (232). The gradual wasting experienced by TB patients over a period of months or years led to the term 'consumption' for the disease. However, only when the TB epidemic reached its peak in Europe and North America during the 18th and 19th centuries, scientists started focusing on the pathogenesis of this disease (55); and Théophil Laennec was the first leader in this era. At this time, TB was perceived as a heritable disease in Northern Europe, and a disease infectious in nature in Southern Europe, Laennec found that in some lesions, a grey and semi-transparent matter is formed which then gradually turns yellow, opaque, and dense. When it softens and becomes liquid, it is expelled through the airways and leaves cavities (168). Following this discovery was the demonstration of

transmissibility of a specific organism from human to cattle and from cattle to rabbits by injection by Jean-Antoin Villemin in 1865 (168). It is important that both Theophil Laennec and Jean-Antoin Villemin did not identify the causative agent; they only described the phenomena they observed. Twenty years later (1882), Robert Koch confirmed that tuberculosis is an infectious disease which is caused by *M. tuberculosis*. One of his major contributions was the development of a method for staining tubercle bacillus which was named after him as the Koch's bacillus, although this term is rarely used today (23). In 1907, Clemens Von Pirquet developed the tuberculin skin test (205), which was recommended as an aid to diagnosis of tuberculosis in children (169). At the same time, the French bacteriologists Calmette and Guerin used specific culture media to reduce the virulence of *Mycobacterium bovis* (*M.bovis*) through serial passage to create Bacillus Calmette-Guerin (BCG) which was subsequently used as the BCG vaccine strain administered worldwide for TB prevention and control (218). Unfortunately, this vaccine is not completely effective and meta-analyses suggest the effectiveness ranges from 0 - 80% (154), with the reasons for observed discrepancies in various settings unknown. Suggestions have included variation in prior exposure to environmental *Mycobacteria*, the age of administration, or differences in the vaccine strain administered (5). It is still widely used today due to its effectiveness in preventing the most severe forms of TB in young children, especially TB meningitis and military TB (203, 218).

Besides understanding the disease, the search for a cure is also a priority. In the pre-antibiotic era, sanatoria (116) and surgery (260) were the only available options for treatment during the late 19th and early 20th centuries. However, bed-rest in sanatoria was lengthy, with many patients spending years in isolation with cure rates of

approximately 30%. The experience of a European sanatorium patient is most famously recreated in Thomas Mann's *The Magic Mountain*, which won the Nobel Prize for Literature in 1929 (142). Many treatments were tried in the desperate search for a cure and some, such as sanocrysin and gold therapy created widely reported rumors of success, only to be disproved in treatment trials (45, 95, 97). It was not until the discovery of streptomycin and Para-aminosalicylic acid (PAS) in the 1940's that effective chemotherapy was possible (87).

The purpose of TB treatment is to sterilize all tuberculous lesions in a short duration with low or ideally no relapse rate. Streptomycin was extracted from *Streptomyces griseus* in 1943 and was administered to critically ill TB patients one year later. Despite the seemingly miraculous recovery of the first TB meningitis patients given streptomycin (26), initial hopes that a cure had been found were dashed when it became apparent in the first cohorts that relapse rates were high (187, 199). It was realized that monotherapy led to relapse due to the rapid emergence of resistant organisms and PAS was added to the regimen and shown to reduce the risk of relapse (19). As new TB drugs were discovered they were added to the regimen in a landmark series of randomized controlled trials conducted by the British Medical Research Council and the International Union Against Tuberculosis and Lung Disease (IUATLD) (84). In the decades following the discovery of Streptomycin and PAS, several new antibiotics were discovered: Isoniazid (INH; 1952), Pyrazinamide (PZA; 1954), Cycloserine (1955), Ethambutol (EMB; 1962) and Rifampicin (RIF; 1963).

After the discovery of Rifampicin (RIF) in 1963, research into new TB drugs stagnated. No potent novel classes of anti-tuberculous agents was discovered in the

subsequent decades until the development of second and third generation fluoroquinolones in the 1980's (62, 177), although several modifications of existing drugs such as rifabutin and aminoglycosides were developed. The recognition of widespread emergence of resistance to the first line drugs has precipitated calls for action (143) and since the millennium, renewed efforts have been made to develop novel anti TB agents (48, 188). The TB Alliance (189) and STOP TB (167) have coordinated efforts to bring new TB drugs to phase 3 trials, beginning with the later generation quinolones: levofloxacin, gatifloxacin and moxifloxacin.

Phase 3 trials are now underway evaluating gatifloxacin or moxifloxacin for use as first line agents in a shortened four month regimen and several agents, including TMC207 (Tibotec – Johnson & Johnson) and PA-824 (Chiron Corporation-USA) are in phase 2 trials (111, 177, 227). Despite the progress made in the last decade, the pipeline for novel TB drugs is weak, with the probability that a significant number of candidate drugs will fail efficacy and safety evaluations, and the discovery of further new agents is needed to enable completely novel replacement regimens and prevent the widespread emergence of resistance to existing and new agents (111).

1.2 EPIDEMIOLOGY OF TUBERCULOSIS GLOBALLY

1.2.1 Prevalence and incidence

Tuberculosis is transmitted by aerosol and it is estimated by the World Health Organisation (WHO) that more than one-third of the world population is latently infected with the causative organism. TB is called the “disease of poverty” because it principally affects the poor and young adults in developing countries (176, 222). Despite many efforts and strategies of international and national organizations, the tuberculosis pandemic still causes more mortality than any other infectious disease. It is estimated that there were 9.4 million incident TB cases and 14 million prevalent cases causing 1.7 million deaths in 2009 according to WHO data from 196 countries and territories (accounting for 99.7% of the world population), resulting in estimated death rates of 1.3 million HIV negative TB cases and 0.4 million HIV positive TB cases. Importantly, most cases were from South and South-East-Asia (35%), Africa (30%) and Western Pacific region (20%) (244). Although there was an increase in the absolute number of incidence, prevalence and mortality as a result of population growth, the number of cases per 100,000 population is slightly decreasing by less than 1% each year from 1996 to 2007 (237). The speed of decrease is much slower in regions with accompanying severe HIV epidemics, and for the sub-Saharan African region overall, where the HIV burden is greatest, TB incidence per capita has increased marginally (60).

Children who are under 15 years old and living in close contact with an infectious source - a smear positive pulmonary TB case - are at high risk of TB infection, especially children under 4 years old presumably because of the immaturity of their

immune system. HIV infection and malnutrition are also key risk factors for TB infection in children. Children less than 4 years old have a higher risk of progressing from TB infection (latent TB) to active TB disease. Generally, it takes approximately 1 year for this process in adults or older children; but in children less than 4 years of age progression is often shorter, a few weeks or months. Furthermore, the immaturity of the immune system often fails to localise *M.tuberculosis* in the lungs and dissemination to other organs occurs more frequently. Therefore, extra pulmonary TB occurs more frequently in children than in adults; and children are more likely to suffer from severe and disseminated TB forms. This is well-documented in the literature; however, paediatric TB does not have a priority in TB control and prevention programmes, particularly in high burden countries. In comparison with adult cases, fewer children than adults have TB disease; children are usually not infectious due to sparse, paucibacillary disease and not coughing strongly enough to generate infectious airborne droplets. Identifying and treating cases which are an infectious source is the priority in resource-limited settings and it had been assumed that if adult TB cases are treated, childhood TB will also decline, therefore childhood TB has historically been neglected by the TB research community. In recent years, increasing calls have been made to prioritise a childhood TB research agenda (166) and the new WHO Stop TB Strategy, 2006 – 2015 was launched in 2006. This strategy aims to ensure all TB patients, including children, have equal access to international standard care. This document provides a recommended approach to diagnosing paediatric TB, treatment regimens, contact screening and management, recording and reporting and BCG vaccination for children under 15 years old in high burden countries (225). This document has increased focus on paediatric TB and the

development of systematic approaches to the management of pediatric TB has increased within national TB programs. It is estimated that of 9 million annual TB cases, approximately 11% were diagnosed in children less than 15 years of age. Especially, 75% of these estimated cases were from high burden countries (225).

1.2.2 Drug resistant TB

Drug resistant *M.tuberculosis* has the ability to exist in the presence of anti-TB drug that would normally kill it or inhibit its growth. Clinically, drug resistant TB is the disease caused by *M.tuberculosis* that is resistant to any drug, most commonly the first line antituberculosis drugs (Isoniazid, Rifampicin, Ethambutol, Streptomycin and Pyrazinamide). In prokaryotes, drug resistance usually occurs due to horizontal genetic transfer through transmissible genetic elements or plasmids (76, 184). However, this mechanism has never been documented in *M.tuberculosis* (73). The development of drug resistance in *M.tuberculosis* normally occurs through bacterial chromosomal mutations, including single nucleotide polymorphisms or homologous recombination. The resulting mutations may be insertions, deletions or point mutations. For example, resistance to RIF is associated with point mutations on *ropB* gene (most commonly in codons 516, 526 or 531) which encodes the beta subunit of the RNA polymerase. These mutations prevent RIF binding without preventing the transcription and elongation of bacterial RNA (262). Mutations occur spontaneously in *M.tuberculosis* at between 1 in 10^6 - 10^8 and are selected under drug pressure, usually due to poor adherence with therapy of the patients or suboptimal (dose or duration) therapy. Other mechanisms, such as drug efflux pumps, play a role in *M.tuberculosis* drug resistance or drug tolerance, but are incompletely understood

(52). The relative fitness cost of drug resistance mutations is also not yet clear but it is known that drug resistant strains retain virulence and transmit successfully within in the community (18). In the fourth report of WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance, data collected from 1994 – 2007 in 114 countries showed that any resistance among new cases and previously treated cases were 17% (95%CI: 13.6, 20.4) and 35% (95%CI: 24.1, 45.8), respectively. For all TB cases, the resistance to any TB drug was 20% (95%CI: 16.1, 23.9) (231).

1.2.3 Multidrug resistant TB (MDR) – extremely drug resistant TB (XDR)

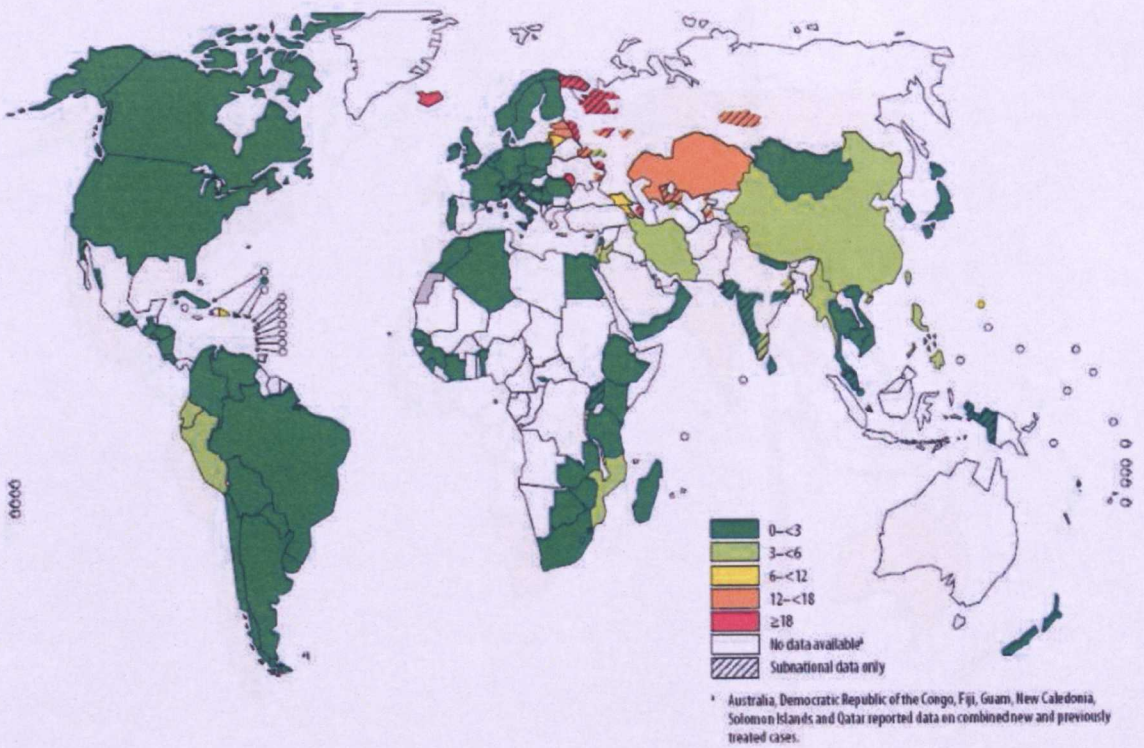
Multidrug-resistant TB (MDR-TB) is caused by *M.tuberculosis* that is resistant to at least rifampicin and isoniazid, the two most powerful drugs of the first line TB drugs which are used for TB treatment in DOTS (Direct Observation Treatment Short Course) strategy. Extensively drug-resistant TB (XDR-TB) is the result of infection with *M.tuberculosis* that is resistant to at least rifampicin and isoniazid in combination with resistance to a fluoroquinolone and one of the second-line anti TB injectable drugs (amikacin, kanamycin or capreomycin). Both of these forms do not respond to the standard first-line treatment regimen (6 or 8 months) recommended by WHO. Therefore, an appropriate treatment regimen is urgently needed to target these populations. The current standard regimens are not effective (**table 1.3**). A multi-country clinical trial conducted by the British Medical Research Council (MRC) is now being established in Ethiopia, South Africa and Viet Nam (110). The Standardised Treatment Regimen of Anti-TB drugs for patients with MDR-TB (STREAM trial – Registration code: ISRCTN78372190), aims to evaluate if a newly developed 9 month treatment regimen containing high dose fluoroquinolone and

clofazimine (4KCMEHZP/5MEZC) is better than the standard minimum 18-month MDR-TB treatment regimen approved by WHO. Recruitment is expected to start in mid-2011. If this trial has positive results, it will be a major advance towards the control of MDR-TB worldwide.

By 2008, there were 27 high MDR-TB burden countries, including Viet Nam, classified by WHO. These countries had at least 4000 new MDR-TB cases annually or 10% of newly registered TB cases with MDR-TB. The global distribution of MDR-TB is shown in **figure 1.1** Fifty eight countries had confirmed identifying at least one XDR-TB case by 2008 (245). However, this number had increased to 69 by the end of 2010 (252). Countries reporting XDR-TB cases are shown in **figure 1.2**.

It is estimated that there were approximately 440,000 MDR-TB cases detected globally in 2008, accounting for 3.6% of all new TB cases of that year and resulting in 150,000 deaths. Almost 50% of MDR-TB cases in the world are estimated to occur in China and India (245).

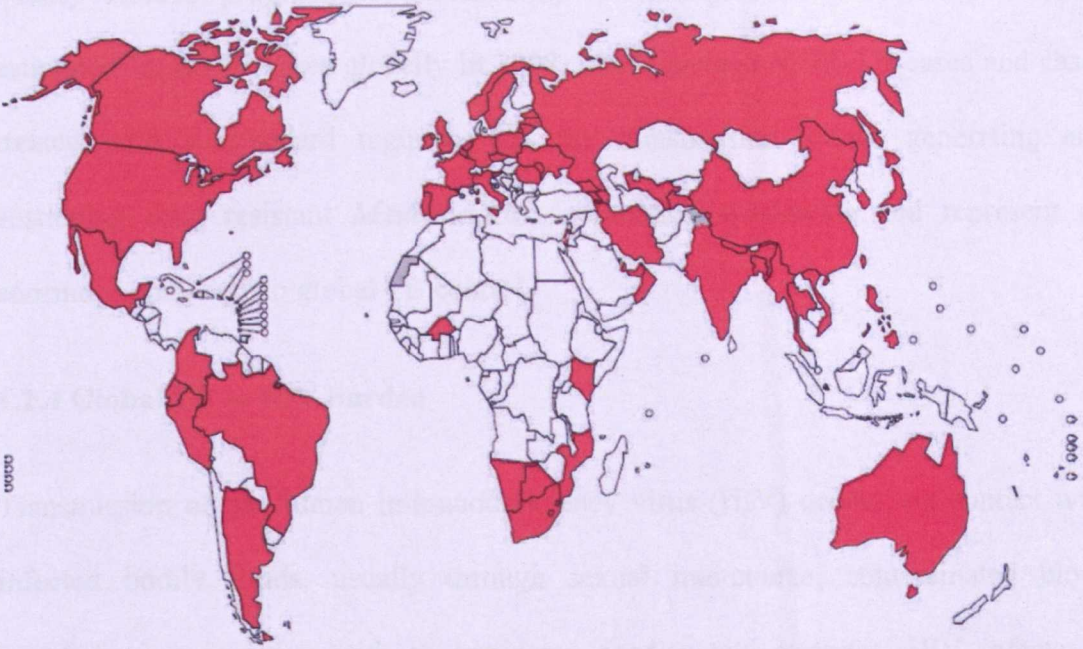
Figure 1.1 Distribution of proportion of MDR-TB among new TB cases, 1994 - 2009



Different colours depict different proportions of MDR-TB among new cases worldwide from 1994 – 2009.

Source: WHO. 2010. MDR-TB and XDR-TB (245)

Figure 1.2 Countries and territories reporting at least one XDR-TB case as of January 2010



Red regions indicate settings reporting at least one XDR-TB case.

Source: WHO. 2010. MDR-TB and XDR-TB (245)

A report from WHO showed that there were 5.8 million TB cases notified in 2009, generating an estimated global detection rate of 63% (increased by 2% in comparison with 2008) (244). Among notified TB cases, an estimated 250,000 cases were infected with multidrug resistant strains. Practically, only 12% (>30,000 cases) of these estimated cases were diagnosed and *notified* with MDR and approximately 82% (n=24,500/30,000) of *notified* MDR-TB cases were enrolled into treatment. Therefore, over 10% of notified MDR-TB cases were lost to follow-up by national TB programmes. Standards for treatment of MDR-TB differ widely between countries.

Low income countries have an opportunity to provide high quality treatment for their patients through the Green Light Committee (GLC) (245). By the end of 2009, only 78% (n=19,000/24,500) of patients on MDR-TB treatment received treatment in quality assured programmes provided by GLC, representing only 1% of total estimated MDR-TB cases globally in 2008. The untreated MDR-TB cases and cases treated with substandard regimens are the transmission source generating and sustaining drug resistant *M.tuberculosis* population worldwide and represent an enormous challenge to global TB control.

1.2.4 Global TB in HIV burden

Transmission of the human immunodeficiency virus (HIV) occurs via contact with infected bodily fluids, usually through sexual intercourse, contaminated blood transfusion or injection with contaminated needles and syringes. HIV infects T-lymphocytes, predominantly CD4+ cells, which play an important role in the cell mediated defense against *M.tuberculosis* of an individual. HIV infection destroys these T-lymphocytes, leading to a gradual decline in numbers or malfunctioning of surviving cells. Once the decline in T-lymphocytes exceeds the limit required for a healthy immune response, the cell-mediated immune response of HIV infected individuals is progressively impaired but the mechanisms are still not completely understood; the decline in T-cells exceeds the number directly infected and lysed by HIV virions. After infection with HIV (acute HIV infection), 40%-60% patients develop symptomatic illness in 2-4 weeks, usually presenting with a 'flu-like' syndrome (fever, rash), arthralgia and lymphadenopathy. Neurological syndromes may occur in some cases. Since serological tests normally first become positive after

4-12 weeks of infection, serological tests are usually negative at this stage and the general nature of symptoms means HIV infection is rarely identified accurately. The most accurate diagnosis of acute HIV infection is based on measurement of HIV RNA in plasma, which is rarely available in high burden HIV settings. In untreated adults, the chronic phase of HIV infection to the onset of HIV disease (Acquired immune deficiency syndrome - AIDS) is approximately 10 years, defined as the chronic stage. There may be few clinical symptoms during the chronic stage. However, in children or infants, this lag phase is shorter, usually from a few months to a few years. During this phase, lymph nodes serve as reservoirs for multiplying HIV, leading to the gradual enlargement of the lymphoid tissues bilaterally, which is diagnosed as persistent generalized lymphadenopathy (PGL). If untreated, all HIV-infected individuals will develop an HIV-related disease or AIDS. The rate of this progression depends on the type of HIV and host characteristics. In the AIDS stage, usually occurring when the CD4 count falls below or equal 200 cells/ml, the patient is more susceptible to opportunistic infections, including TB, pneumonia, fungal infections and herpes. Chronic diarrhoea with weight loss can also occur in this stage. Since the immune system of the host is less effective in preventing the growth and spread of *M.tuberculosis*, disseminated and extrapulmonary are more common in this group (221).

The interaction between HIV and TB has implications for TB control among HIV-infected patients. HIV infection causes immunodeficiency of the host, increasing susceptibility to TB infection and increasing risk of progression to active TB disease. It is known that HIV negative individuals with latent TB have 5% - 10% risk of developing active TB during their lifetime. However, in HIV positive individuals, this

risk is 50% during their lifetime or 10% per year (221). TB infection increases TNF α and MCP-1 levels that may activate HIV replication in lymphocytes, monocytes and macrophages. This vicious circle leads to progressive and severe status in TB co-infected HIV patients (46). HIV associated TB is a major challenge for TB control programmes and HIV/AIDS programmes.

HIV infection is a global pandemic. It was estimated that 33 million prevalent cases existed in 2007, of which 2.7 million were new infections (213). The most affected population is aged from 15 to 49, the age group who are the primary drivers of economic development of countries. It is estimated one-third of people living with HIV are co-infected with tuberculosis worldwide and the majority of them are from Sub-Saharan Africa (206). HIV is the main cause of TB treatment failure and TB is the main cause of death in patients living with HIV; up to half of all AIDS patients died from TB (206). A report from WHO showed that among an estimated 9.4 million new TB cases in 2008, 1.4 million were HIV positive, accounting for 15% of all incident cases. Almost 80% of HIV co-infected TB cases were from Africa and estimated 13% was from South East Asia region. Among an estimated 1.3 million deaths from TB of all forms worldwide in 2008, one estimate suggests 40% of them were new HIV/TB cases (236). The availability of HIV antiretroviral therapy (ART) has been massively scaled up during the last decade in resource limited settings. The aim of this treatment is to maintain HIV viral load in the body at a low or 'undetectable' level to prevent the progressive impairment of the immune system, dramatically slowing progression to AIDS, and reducing the probability of transmission. The WHO recommendations on ART were made after review of different levels of evidence from clinical trials, cohort data and expert opinions but

the long term consequences of ART scale-up are still unclear since therapy is life-long and issues such as sustainability, provision of second and third line regimens, and drug resistance amplification will need to be addressed. Further, concerns have been raised that reducing viral load in infective individuals may not result in the predicted decrease in transmission if sustained health results in more potential infective contacts over a lifetime (241).

1.2.5 Risk factors of infection

Tuberculosis is an infectious disease and the risk of infection is largely related to the source of infection, contact time and the host immune response. Exposure to pulmonary TB patients, especially smear positive TB cases will generate a higher risk of TB infection than extrapulmonary TB and smear negative TB patients, although significant transmission can occur from smear negative cases who may intermittently expectorate bacilli (15). In addition, close contact with an infectious source over a long time, such as household members or in healthcare facilities will also increase the risk. Especially, if an individual suffers from malnutrition, chronic disease, HIV infection or other immuno suppressive diseases, they are at high risk of TB infection and are likely to develop active TB disease (89, 170). However, not all individuals infected with *M.tuberculosis* will develop TB disease. The levels of the risk factors will contribute to the risk of development of the disease; therefore it is estimated that 5% - 10% of infected individuals will develop active TB with the highest risk in the first 2 years after exposure (denoted primary TB), a further 5% -10% will have latent TB and subsequently develop active disease over their life-times (denoted post-primary or reactivation TB) (37) and the remaining will maintain latent infection and

never develop active symptomatic TB. In HIV positive individuals, a lifetime risk of 50% of developing active TB has been recorded (221).

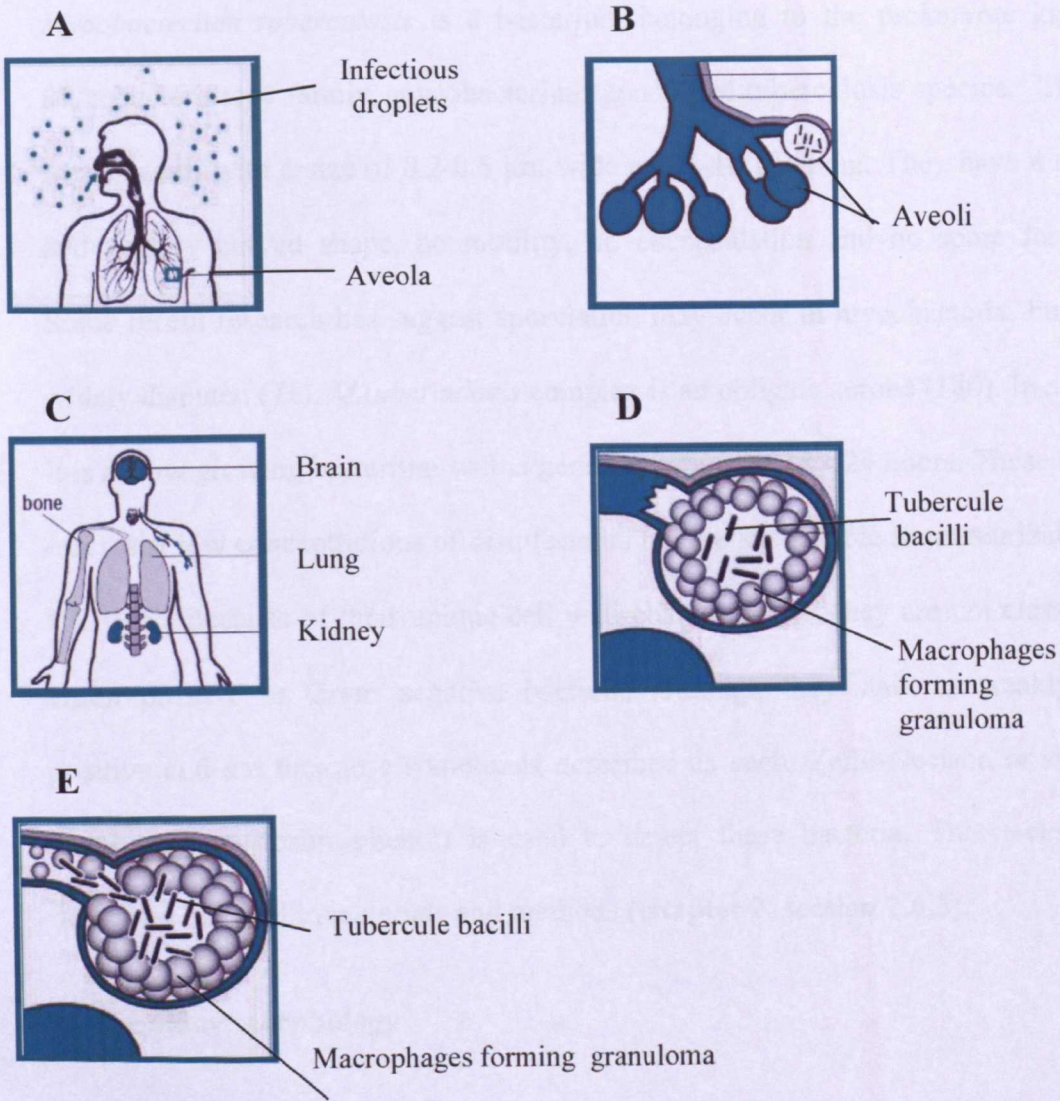
1.2.6 Transmission and pathogenesis

TB transmission occurs via infectious aerosol droplets containing *M.tuberculosis* which are released from TB patients, usually via coughing, circulate in the air and then are inhaled into the respiratory tract of other individuals through inhalation. Droplet size is the most important factor for transmission, all heavy droplets will settle down onto the floor after release except nuclei droplets which remain suspended in the air at a height of 3 metres for up to 24 hours in an internal environment and can be inhaled by other individuals. Generally, only droplets from 1 - 3 μ m in size will penetrate into the lungs to potentially establish infection. To minimize TB transmission, infection control procedures have been established which include administrative control, environmental control and personal protective equipment. Of which, administrative control is the most important measure because it controls infectious sources by isolation, early diagnosis and treatment for TB cases. Ideal infection control scenarios can be extremely difficult to apply in resource-limited settings.

Once inhaled, the larger infectious droplets (>5 μ m in size) are trapped and eliminated from the upper airway by the mucocilliary clearance on the surface of epithelial cells. This is the first-line of physical defense of the host to TB infection. Smaller infectious droplets have a higher chance to reach the alveoli where they can be surrounded and engulfed by alveolar macrophages, which constitute the second line of host defense. The interaction between macrophage and bacilli triggers a signaling cascade which

activates lymphocytes; and the accumulation of activated T lymphocytes and macrophages in alveoli may result in eradication of the infection or in the progressive formation of a granuloma, the third defensive system of the host, which contains the bacilli and limits the spread of infection to other sites. The anaerobic environment within the granuloma destroys macrophages and produces a solid necrosis in the center of granulomas. The necrotic environment results in a lack of oxygen and low pH with limited nutrients which inhibits further growth of mycobacteria. In individuals with an adequate immune response, this lesion becomes fibrosed and calcified; and finally the infection is controlled but not eradicated, resulting in 'latent' infection. However, in individuals with a less effective immune response, the bacilli may multiply and disseminate to other alveoli and organs where TB disease can become established. Mechanisms of dissemination are not well understood but it is thought the cells of the immune system act as 'trojan-horses' carrying the *M.tuberculosis* bacilli to the lymph nodes. Failure to contain the infection within the lungs may result in transient bacteraemia which 'seeds' other locations in the body and can result in extrapulmonary forms of disease, which account for approximately 20% of TB cases (100, 208). Disseminated forms occur more frequently in individuals with an impaired or immature immune system, such as HIV-infected individuals or children. **Figure 1.3** describes the pathogenesis of tuberculosis.

Figure 1.3 Pathogenesis of Tuberculosis.



Source: CDC-Self study modules on tuberculosis (39)

(A) Droplet nuclei containing tubercle bacilli are inhaled and travel to the alveoli. (B) Tubercles multiply in the alveoli. (C) A small number of bacilli enter the blood stream and spread through the body. (D) Macrophages surround the bacilli and form granulomas or shells to contain infection. (E) If the immune system becomes weaker, the shell loses integrity; bacilli escape, multiply rapidly and spread to other alveolar or organs.

1.3 MYCOBACTERIUM TUBERCULOSIS – THE BACTERIA

1.3.1 Morphology

Mycobacterium tuberculosis is a bacterium belonging to the prokaryote kingdom, Mycobacteriaceae family, mycobacterium genus and tuberculosis species. They are small bacilli with a size of 0.2-0.6 µm wide and 1-10 µm long. They have a rod-like and slightly curved shape, no motility, no encapsulation and no spore formation. Some recent research has suggest sporulation may occur in mycobacteria, but this is widely disputed (71). *M.tuberculosis* complex is an obligate aerobe (186). In addition, it is a slow growing bacterium with a generation time of 18 - 24 hours. These bacteria can resist low concentrations of disinfectants but are susceptible to pasteurization and UV light. Because of their unique cell wall characteristics, they are not classified as Gram positive or Gram negative bacteria, although they stain as weakly Gram positive and are therefore sometimes described as such. Zeihl-Neelson or Acid-fast staining (carbolfushin-phenol) is used to detect these bacteria. This technique is described in detail in materials and methods (**chapter 2, section 2.6.3**).

1.3.2 Colony morphology

When grown on solid media, such as Lowenstein Jensen media, mycobacteria often form colonies. *M. tuberculosis* often forms typical cream colored colonies with dried and rough nature (214). In contrast, Mycobacterium Other Than Tuberculosis (MOTT) displays yellow, orange or cream colonies with smooth and moist properties. The presumptive identification of these two classifications based on shape and

characteristics of colonies is used often as a screening test for further biochemical identification tests to identify these bacteria.

1.3.3 Cord formation

M.tuberculosis often shows characteristic serpentine cording in liquid culture media which is an aggregate of tight rope-like strings in which the long axis of the bacteria is paralleled with the long axis of the cord (**Figure 1.4**). Recently, the characteristic of cord formation has been evaluated for the presumptive identification of *M.tuberculosis* by microscopy (10, 135). The sensitivity of this method very much depended on the personal skills, training and time of the technician: with poor training, the sensitivity of cord formation for the identification of *M.tuberculosis* was approximately 60% while it was 75% for those with specific training (8). In addition, the number of bacilli also contributed to the formation of this characteristic (115). In McCarter's study (115), almost 90% of *M.tuberculosis* exhibited cording when the growth index (cfu/ml) was between 100 and 200. Another study showed that if the growth index was below 100, only 29% of *M. tuberculosis* cultures exhibited cording (135).

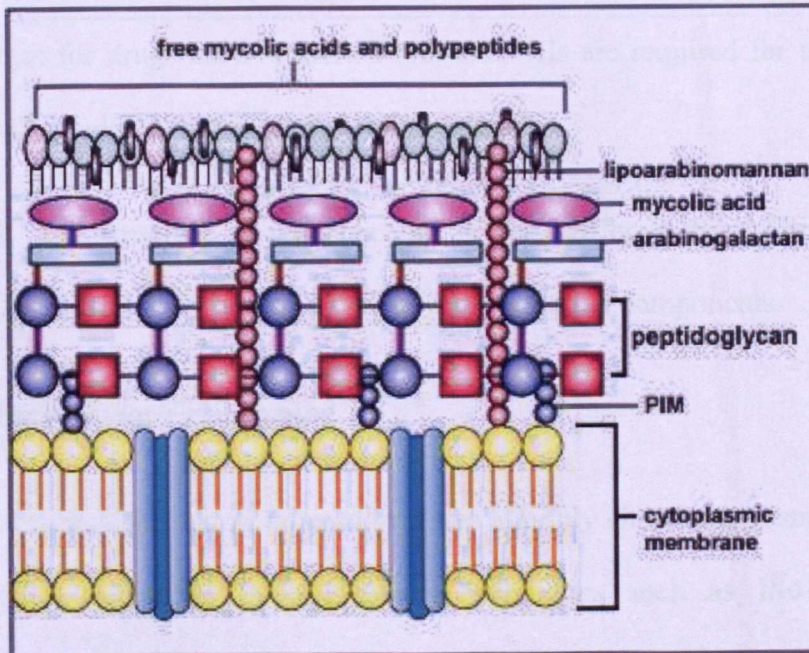
Figure 1.4 Cording characteristic of *M.tuberculosis*



Arrows indicate M.tuberculosis cording in liquid culture

1.3.4 Cell wall structure

Figure 1.5 Components of a mycobacterium cell wall



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The cell wall of Mycobacteria (**figure 1.5**) consists of three layers: inner layer, cell wall core and outer layer. The inner layer is a lipid bilayer which covers the cytoplasm and plays a vital role for the mycobacterium (53). The cell wall core is the main structure of the cell wall in which peptidoglycan cross-links to arabinogalactan (D-arabinose and D-galactose), and then this complex links to high molecular weight mycolic acid. This layer has been known to play an important role in pathogenicity of the bacteria (21). The outer layer is composed of extractable, noncovalent lipid, glycolipid (lipoarabinomannan and phosphatidylinositolmannosides (PIM) and protein which is essential for the host immune response. The lipid components make up almost 60% of the cell wall weight. The physical organizational structure of this cell wall forms the primary hydrophobic barrier that protects the bacteria from external enemies. Disrupting this basic structure enhances permeability of the cell wall and endangers the growth, viability and reproducibility of mycobacteria and is therefore a drug target for drugs such as INH. Porin channels are required for transportation of hydrophilic molecules through the cell membrane.

The cell wall structure of mycobacteria is very different from Gram positive and Gram negative bacteria in terms of lipid and glycolipid components.

1.3.5 *M.tuberculosis* genotyping

It was long thought that *M.tuberculosis* was a highly clonal organism due to the lack of diversity identified through typing techniques such as IS6110 RFLP and spoligotyping. However, these techniques were developed to identify polymorphism using strains from Europe and America (173) and therefore did not identify diversity between isolates from Africa and Asia, where most *M.tuberculosis* occurs. The advent

of whole genome sequencing and microarray techniques has allowed a more complete picture of variation within *M.tuberculosis* genomes and projects are now establishing the global diversity of *M.tuberculosis* strains (9).

M.tuberculosis does not carry plasmids or undergo lateral gene transfer therefore the principal sources of diversity within *M.tuberculosis* are homologous recombination and Single Nucleotide Polymorphisms (SNPs) arising spontaneously or through selection pressure (59). *Mycobacterium tuberculosis* strains have evolved via genomic deletions called large sequence polymorphism (LSP) or regions of difference (RD). Phylogenetic analysis of these gene deletions forms the basis of LSP genotyping method. Based on this analysis, there are six major lineages worldwide of *M.tuberculosis* (**figure 1.6**): Indo-Oceanic lineage, East Asian lineage, East African-Indian lineage, Euro-American lineage, West African lineage I and West African lineage II (69). The distribution of these lineages is associated with regions of origin (24, 162) (**figure 1.7**).

Insertion sequences, small mobile genetic elements which are widely distributed in the bacterial genome, are the target of Restriction Fragment Length Polymorphism (RFLP) for epidemiology research and detection of laboratory errors or cross-contamination (114). RFLP to detect IS6110 insertion elements has been the standard typing method for TB epidemiology since 1999. IS6110 insertion elements are scattered in the entire length of the genome and occur in different numbers and locations between strains (ranging from 1 to 26 copies). The patterns of IS6110 RFLP are stable over approximately 1-2 years with respect to time but quite polymorphic so they are useful for studies in transmission dynamics at local and population levels.

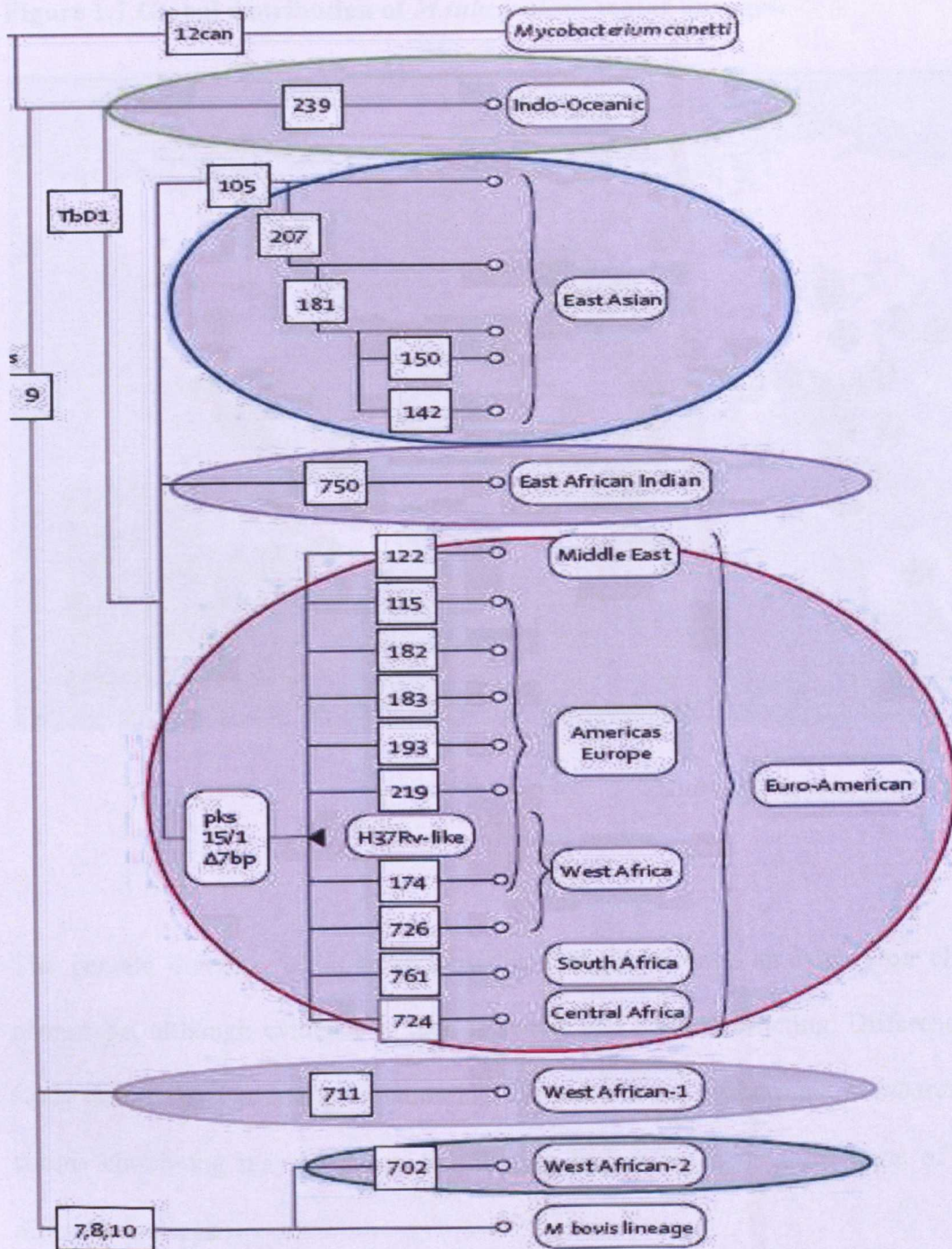
However, IS6110 RFLP has limited capacity in discrimination of strains with less than five IS6110 copies (which are common in South East Asia and the Western Pacific region), requires a large amount of chromosomal DNA, is time consuming, laborious and inter-laboratory comparisons are unreliable (102).

The Spoligotyping method detects the presence of spacers that separate direct repeats (DRs) in a specific locus of the *M.tuberculosis* genome. This method can discriminate strains with less than 5 copies of the IS6110 fragment but does not discriminate strains of the East-Asian/Beijing genotype, which are highly prevalent in Asia. Since it targets a single locus that presents less than 0.1% of the *M.tuberculosis* genome, its discriminatory is lower than IS6110 RFLP. However, since it is a simple technique and requires less labour and DNA material, it is a common method applied for epidemiology studies. The resolving capacity of Spoligotyping is increased if used in combination with IS6110 RFLP (102). In this thesis, Spoligotyping was used for detection of laboratory cross-contamination due to its advantages over RFLP IS6110 method.

Mycobacterial interspersed repetitive units (MIRU) is the specific name of variable number of tandem repeats (VNTR) typing when applied to *M.tuberculosis*. MIRU typing has become an important method in distinguishing *M.tuberculosis* strains based on the difference in the number of copies of tandem repeats at specific regions, or loci, of the *M.tuberculosis* genome. A total of 41 MIRU loci have been reported (183); however, most laboratories target only 12 loci for discrimination clinical isolates. The discriminatory power of MIRU-VNTR analysis is proportional to the number of loci evaluated. When 12 loci are used, the discriminatory rate of MIRU-

VNTR is lower than that of IS6110 RFLP genotyping for isolates with more than 5 copy-number IS6110 insertions. However, it provides a higher discriminatory power than IS6110 RFLP for isolates with low-copy-number IS6110 (114). MIRU-VNTR genotyping has been used in a number of molecular epidemiologic studies, as well as to elucidate the phylogenetic relationships of clinical isolates and cross-contamination.

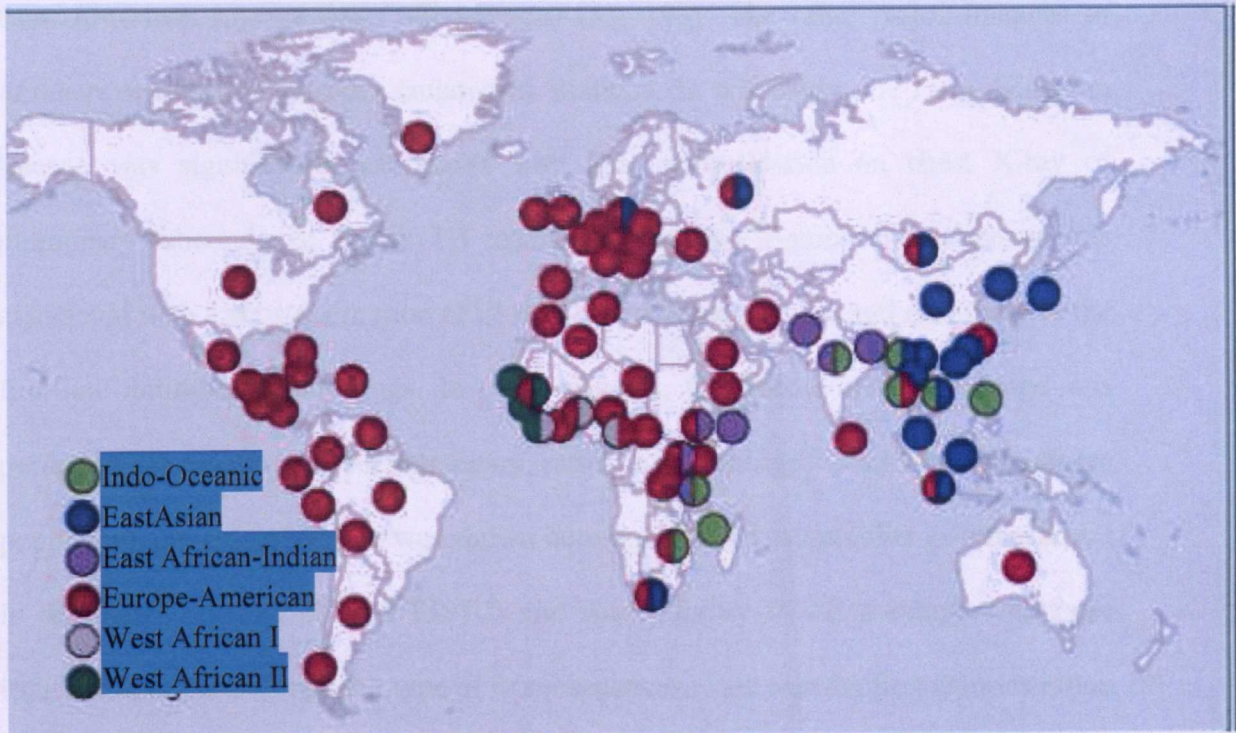
Figure 1.6 Global phylogeny of *Mycobacterium tuberculosis*



Gagneux et al. PNAS (68)

Lineage specific LSP marker and phylogeny. Numbers in rectangles refer to linear-specific large sequence polymorphism (LSP) markers or the names of regions of difference. The geographic regions associated with specific lineages are indicated.

Figure 1.7 Global distribution of *M.tuberculosis* major lineages.



Source: Gagneux et al. (69)

The genetic diversity of *M.tuberculosis* is thought to have an impact on clinical phenotype, although evidence to date is sparse and often conflicting. Differences in study design, host ethnicity variation and diversity in the background *M.tuberculosis* strains circulating may be some contributing factors accounting for some of these different findings.

Studies have been conducted locally in Vietnam to address this question. In one study conducted at Pham Ngoc Thach Hospital (PNT) and Hospital for Tropical Diseases (HTD), 397 strains isolated from HIV negative pulmonary TB and tuberculous

meningitis (TBM) patients in Viet Nam were classified into 3 lineages: East Asian/Beijing lineage (RD105 deletion), Indo-Oceanic lineage (RD239 deletion) and Euro-American lineage (*pks15/1* deletion) (35, 193). The other major lineages of *M.tuberculosis* have not been isolated in Vietnam. In this study, the Euro-American lineage was significantly associated with lung consolidation on chest X-ray of pulmonary tuberculosis; and in TB meningitis, the East Asian/Beijing lineage was associated with a shorter duration of illness, severe disease status and resistance to the first-line antituberculosis drugs. In these patients, the Euro-American lineage was preferentially associated with pulmonary, rather than meningeal TB in the Vietnamese population and an association was shown between a host susceptibility polymorphism in toll-like receptor 2 (TLR2 T597C) and susceptibility to TB meningitis in those infected with the Beijing genotype of *M.tuberculosis*. This was the first demonstration of interaction between a host polymorphism and a bacterial genotype in TB disease resulting in a specific disease phenotype. However, characterization of over 2000 *M.tuberculosis* complex isolates collected from London, UK showed no association between lineage and pulmonary or extrapulmonary sites of TB disease (24). East Asian/Beijing lineage had higher risk for relapse, but not treatment failure, than other lineages, especially among Asian-Pacific Islanders (27). A collection of *M.tuberculosis* strains from the Tuberculosis Trials Consortium study 28 was analyzed by Nahid *et al.* In this study, the authors showed that the Euro-American sublineage 724 was associated with severe disease and East Asian lineage was associated with low bacteriologic conversion after 8 weeks of treatment (138). In Gambia, the two most common lineages found were West African II and Europe-American. Pulmonary TB patients infected with West African II lineage were more

likely to have worse abnormality on chest X-ray, severe malnourishment and less likely to have a positive ESAT-6/CRP-10 ELISPOT result than the Europe-American lineage (56).

These findings provide strong evidence for genetic diversity which may result in phenotypic polymorphism of *M.tuberculosis* population. These findings may also help to explain geographical differences in clinical features and response to treatment and vaccination among TB patients. However, the complexity of interaction between environmental, host and pathogen factors and the relative influence of each on outcome will require large, detailed global studies to unravel completely and may never be possible.

1.4 DIAGNOSIS OF TB, DRUG RESISTANT TB AND MDR-TB

1.4.1 Diagnosis of TB

The diagnosis of TB disease is ideally based on microbiological confirmation. Unfortunately, current microbiological tests are not sensitive enough to detect all TB cases. Therefore, a clinical evaluation is used for diagnosis of smear negative TB cases. The clinical diagnosis is based on clinical and radiological symptoms consistent with tuberculosis, microbiological testing, evaluation of risk factors such as TB history, history of TB contact and exclusion of differential diagnoses.

Clinical diagnosis

The clinical symptoms of pulmonary tuberculosis are not specific. The most common symptom is productive cough, usually for more than two or three weeks at presentation. In addition, systematic signs including fever, night sweats and weight

loss are often present. Other local symptoms consistent with extra-pulmonary tuberculosis such as lymphadenopathy, pleural fluid and neurological signs may be recorded, especially in immune-deficient individuals (204).

Although the majority of pulmonary tuberculosis patients have cough when presenting to health care facilities, cough is not a sensitive and specific symptom for prediction of tuberculosis. A study from Peru showed that approximately 55% of TB suspects submitting sputum samples reported cough for < 2 weeks and 3% of them were smear positive (145). A cross-sectional study conducted in 3 hospitals from Tanzania reported that smear positive pulmonary TB rate was higher in patients with more than 2 weeks of cough compared to patients reporting cough for less than 2 weeks (141). Coughing is a reflex action in response to any stimuli in the airway and to keep the trachea clear of secretions, irritants, foreign particles and microbes. Therefore, any acute or chronic infectious and chronic non-infectious diseases in the sinuses, upper airways, lower airways and lungs may lead to acute or prolonged cough. As a result, in patients presenting with chronic cough, suspicion of tuberculosis will depend on the prevalence of tuberculosis in the community. Patients with chronic cough in high prevalence TB countries are likely to suffer from tuberculosis (164). Conversely, in low prevalence TB countries, they are more likely to have other diseases such as asthma, pneumonia, bronchitis or cancer.

WHO guidelines recommend patients with a cough of more than 2 weeks are screened for TB in high prevalence settings. If the threshold for TB screening is 2 weeks of cough, the number of TB suspects and laboratory workload greatly increases and the prevalence of true TB cases among those screened will decrease (204). A study from

India showed that the number of TB suspects and the number of sputum smear positive cases increased overall by 58% and 23%, respectively when the screening criteria were modified from cough lasting for 3 weeks or more to cough lasting for 2 weeks or more and 3 sputum samples were collected for examination (192). This would overburden existing laboratory capacity in many settings. Since the sensitivity of smear microscopy reached 90% to 95% from the first two sputum samples (216), it would be reasonable to access 2 sputum samples for microscopy instead of three samples to reduce workload in settings where both finance and human resource are limited.

Among systemic symptoms, loss of appetite, weight loss, weakness, night sweats and malaise are also common but are more difficult to quantify and may relate to co-existing diseases.

In HIV-infected individuals suspected of tuberculosis, clinical manifestations are rather different from HIV-negative individuals. A two year study conducted by the U.S. Center for Disease Control and Prevention (US CDC) from 2006 to 2008 in Cambodia, Thailand and Viet Nam found that fatigue (75%), fever (74%), weight loss (73%) and cough (71%) were the most common symptoms of HIV positive individuals suspected of tuberculosis presenting to outpatient clinics (29). In this study, cough was not a sensitive indicator. As a TB screening criteria, cough lasting for 2 weeks yielded a higher sensitivity than cough for 3 weeks, which was 33% and 22%, respectively. The authors also presented an algorithm for TB screening and diagnosis in HIV positive individuals, in which the combination of cough of any duration, fever of any duration or night sweats lasting for 3 weeks or more during the

previous 4 weeks had a sensitivity, specificity, negative predictive value and positive predictive value of 93%, 36%, 97% and 21%, respectively. Therefore, this algorithm would rule out tuberculosis in the majority of suspects and provide evidence for safe initiation of isoniazid preventive therapy (IPT) for HIV positive patients who had none of the three predictive symptoms. IPT is intended for use in HIV positive individuals infected with *M.tuberculosis* (latent TB) to eradicate the latent infection and therefore reduce the development of active TB in this population. There have been concerns that wide spread use of IPT may generate increased INH resistance. A meta analysis study reviewing all data published since 1951 showed that isoniazid resistance is not significantly associated with the provision of IPT; and IPT should be used after excluding the diagnosis of active TB (11). Importantly, IPT was found to have an important impact on reducing TB incidence among adults and children living with HIV (67, 74). In 2008, WHO has endorsed the use of IPT for people living with HIV, including children, after ruling out active TB (235); and a completed guideline for IPT therapy for people living with HIV in resource limited settings has recently been released by WHO in 2011 (251).

Extrapulmonary tuberculosis accounts for approximately 15 – 20% of TB cases in low HIV prevalence populations (204). However, in settings with high HIV prevalence, this figure is higher, for example 40% in South Africa (259) because *M.tuberculosis* bacteria disseminate to other organs due to host immune system impairment. Microbiological confirmation is rarely achieved because of low bacterial load in samples other than sputum.

Diagnosis of tuberculosis in children is even more challenging than HIV/TB coinfecting adults and extrapulmonary TB because of poor quality samples obtained for diagnosis. In children, TB is most common between 1 and 4 years of age. Children of this age are rarely able to articulate symptoms clearly and may simply cry or complain of discomfort. The physical manifestations tend to differ by age of onset. Therefore, disease history should be carefully considered. If a history of close contact or infrequent contact with a source case is detected, TB disease should be considered in the differential diagnosis. According to WHO guidelines on the management of tuberculosis in children, the commonest clinical symptoms are chronic cough for more than 3 weeks, fever for more than 2 weeks, weight loss and failure to thrive (225).

Radiological diagnosis

Chest-Xray (CXR) is valuable to assist TB diagnosis because of its high sensitivity. However, the low specificity of chest-Xray requires it to be used in combination with clinical assessment. Pulmonary TB is traditionally classified as primary or secondary/post-primary TB (reactivated TB). Primary TB is more common in children and usually accompanied with enlarged hilar or mediastinal lymphonodes (70% – 80%). Post primary TB, as a result of reactivation of dormant bacteria in tissues or macrophages, is more common in adults (148). The common CXR manifestations are nodular and cavitations. Pleural effusion and miliary TB are more often present in immunocompromised individuals suspected of tuberculosis.

CXR is an essential test to assist the evaluation of an individual suspected of TB but it should be interpreted in combination with clinical features due to the following

reasons: **First**, an abnormality identified on a CXR may be due to tuberculosis or a variety of other pulmonary diseases or conditions such as pneumonia or tumors. Therefore, patterns on CXR are not specific for the diagnosis of active pulmonary TB; but changes on a CXR in combination with suspicious clinical features may be an indication. **Second**, a normal CXR or lack of typical TB signs on CXR will make a diagnosis of TB unlikely in the majority of cases but in some cases may lead to TB being excluded incorrectly (false negative), especially in immunocompromised patients. **Third**, missed diagnosis on CXR may be due to proficiency of film readers such as failure to recognize hilar or mediastinal lymphadenopathy of primary disease in adults and tuberculoma (261). However, a repeat CXR may be helpful for comparison in some cases of clinically suspected tuberculosis. **Last** but not least, in cases without microbiological confirmation, CXR and clinical features in combination can be helpful in the decision to treat for TB.

Microbiological diagnosis: smear microscopy and culture

Microbiological diagnosis is the gold standard to confirm active TB in patients presenting with clinical symptoms suspected of tuberculosis. Clinical symptoms, CXR and microbiological methods (smear microscopy and culture) are the three conventional methods applied by national TB programmes (NTP) for the diagnosis of TB.

It is estimated that a sputum sample containing at least 5000 – 10,000 Acid-fast bacilli (AFB) per milliliter is likely to have a positive result on smear microscopy (216). A positive smear result is recorded if at least one AFB is found per 100 immersion fields

when observing a smear under a microscope. The WHO system for grading of smear results is described in **chapter 2, table 2.1**.

Smear microscopy is applied for TB diagnosis worldwide, especially in high burden countries, for early detection of infectious cases. Since it is a simple and rapid test, it is applied at all laboratory levels, including peripheral, intermediate and reference laboratories. Although this test has relatively low sensitivity, its specificity is very high, almost 100% when correctly performed, and thus it is thought to be very rare for patients to be diagnosed as false positive. The majority of false positive diagnoses are in cases with MOTT (Mycobacterium Other Than Tuberculosis), due to the inability of acid-fast staining to accurately distinguish some MOTT from *M.tuberculosis*, from cross contamination of slides during slide preparation or administrative errors in assigning sample codes or results. To increase TB case detection rates, culture methods can be used. The sensitivity of culture is higher than smear microscopy but it cannot be applied at all laboratory levels because infrastructure for biosafety is required for implementation, which is not usually available at peripheral and intermediate levels, often only at a single reference laboratory, if at all. Since most TB suspects in low and middle income countries present at local clinics for TB screening and since the specificity of smear microscopy is high, this test is reliable for use in the primary health care system for TB detection in high burden and low income countries. The priority is to detect the most infectious TB cases for the lowest cost in such settings and therefore, due to lack of resources, smear microscopy is often the only test applied. In contrast, in low burden settings or in high income countries, culture methods are applied for TB diagnosis because of high sensitivity and affordability of the test. Therefore, the application of available diagnostic methods highly depends on

the prevalence of disease and capacity of the setting. The simplest approach to increase case detection is to improve the efficacy of smear microscopy, sample processing method (to prepare homogenous smear) and fluorescent microscopy have been evaluated. Sample processing methods can increase the mean sensitivity of smear microscopy by approximately 9% (181) and fluorescent microscopy improves the sensitivity of conventional smear microscopy by approximately 10% with similar specificity (180).

The number of samples examined per patient for smear microscopy influences the cost effectiveness of the test. Before 2007, it had been recommended by WHO that a TB suspect should provide 3 sputum samples for smear microscopy ('spot, morning and spot'). However, in a systematic review Mass *et al*, concluded that the third sputum increased the TB detection rate by only about 2 – 5% (113). Therefore, if only 2 sputum samples are applied for microscopy, the case detection rate may not be affected remarkably but reduction of laboratory workload would be approximately 30%. In 2007, WHO modified recommendations from 3 sputum specimens (spot-morning-spot) to 2 sputum specimens, based on accumulated evidence that the third sputum specimen did not add sufficiently to the detection rate to justify a 33% increase in laboratory workload (228). In addition, in 2010, the GRADE process (The Grading of Recommendations, Assessment, Development and Evaluation) provided sufficient evidence for WHO to conclude that same-day-diagnosis approach (spot-spot sputa) is equivalent to the conventional approach (spot-morning-spot sputa), in terms of diagnostic accuracy for smear microscopy (sensitivity reduces approximately 3% but specificity is equivalent). WHO therefore endorsed same-day-diagnosis approach for TB screening. This approach would reduce patient-related cost and

patient loss during diagnostic pathway (247). This recommendation is under consideration but has not yet been implemented in Viet Nam NTP in 2011.

Culture methods have a higher sensitivity than microscopy. A sample containing 100 bacilli/ml can be positive by these methods. Therefore, culture helps to detect cases earlier, often before they become infectious. Culture also generates bacterial isolates for drug susceptibility testing and other tests. Solid culture methods (eg. Lowenstein Jensen – (LJ) media or Middlebrook 7H10 agar) have been used as the microbiological gold standard for the diagnosis of TB worldwide. Although culture provides high sensitivity and increases case detection in comparison to smear microscopy, its main disadvantage is long turnaround time, almost one month, due to the long generation time of *M.tuberculosis*. Culture also requires laboratories to be equipped with biological safety cabinet, centrifuge, incubator, and shaking machine which are usually only available in some intermediate and reference laboratories; and well-trained staff. Liquid culture methods such as the commercial automated system BACTEC MGIT 960 – (Becton Dickinson, USA) are approximately 10% more sensitive than solid culture (49) and provide shorter isolation time (10 – 14 days) than the conventional culture method. Liquid culture methods have been endorsed by WHO for use in routine TB diagnosis in low and middle income countries since 2007 (230) but costs of the technique limits its scale-up potential.

1.4.2 Detection of Drug resistance and MDR.

Drug susceptibility testing (DST) is performed to identify isolates that are resistant to antibiotics currently used for TB treatment. The most common method is the proportion method (indirect DST method) that provides a turnaround time of

approximately 42 days after inoculation from a positive culture. Four first line TB drugs are usually tested initially, including Isoniazid (0.2µg/ml), Rifampicin (40µg/ml), Streptomycin (4.0µg/ml) and Ethambutol (2µg/ml). Conventional DST is currently the gold standard for all novel DST methods. Pyrazinamide (PZA) DST is not recommended for routine testing because of the difficulty in standardization of drug susceptibility testing for this drug due to the sensitivity of pyrazinamide activity to pH (219).

1.4.3 Recent advances in diagnostic methods

In the last ten years, efforts to develop new TB diagnostic methods have been made globally and The Foundation for Innovative Diagnostics (FIND) played an important role in this process. With the initial grant of \$30 million from the Bill & Melinda Gates Foundation and a mission to improve the health for poor people, FIND was established in 2004. FIND has established collaborations with many companies and organizations including Becton Dickinson (BD), Eiken Chemical Co, LTD-Japan, Hain Lifescience GmbH, TAUNS-Japan, Carl Zeiss-Germany, Cepheid-USA to develop and/or evaluate new TB diagnostic technologies for example MGIT™ culture and DST techniques, loop-mediated isothermal amplification (LAMP) assay, Genotype®MTBDR, Genotype®MTBDR*plus*, MTB identification test - Capilia TB test, LED fluorescence microscope and Xpert MTB/RIF (63). By 2011, many of these tests have been endorsed by WHO for early detection of TB and MDR-TB in high burden countries (229, 238, 248). With the effort from FIND and other organisations, new diagnostic methods have been introduced to settings where urgently needed with reasonable prices. It is hoped that the implementation of new diagnostic methods

would improve TB case detection rate, shorten time to diagnosis and increase diagnostic accuracy. To date, MGIT culture has been adopted internationally but MGIT-DST is not widely applied due to high cost. Commercial Immunochromatographic tests for the detection of MPB64 antigen have been developed to differentiate *M.tuberculosis* isolates from non-tuberculous mycobacteria, such as SD TB Ag MPT64 Rapid (Standard Diagnostics, Seoul, South Korea) and Capilia TB test (Tanus, Namazu Japan). Genotype®MTBDR and Genotype®MTBDR*plus* are now used as screening tests for detection of MDR-TB in an international project funded by WHO and GLC. MDR-TB patients detected via this project are enrolled into MDR-TB treatment with second line drug regimens and GeneXpert are introduced in 11 out of 27 countries with high burden of MDR-TB/XDR-TB.

In the stream of development new diagnosis methods, WHO has developed a list of characteristics that make novel diagnostic tests appropriate for use in target settings, including ASSURED (Affordable by those at risk of infection, Sensitive, Specific, User-friendly, Rapid, Equipment-free and Deliverable to those in need) (207). The new tests that have been evaluated are classified into culture based methods and non-culture-based methods.

- a. **Culture -based methods:** MGIT culture, DST-MGIT, MODS, Thin layer Agar, Nitrate Reductase Assay

The Bactec commercial automated liquid culture system (BACTEC MGIT 960 – Becton Dickinson) is used to detect bacterial growth in tubes containing 7H9 broth, OADC and PANTA by detection of fluorescence emission from a substance coating the bottom of the culture tube which is quenched by dissolved oxygen in the broth.

Replicating microorganisms consume oxygen in the broth and allow fluorescence to be emitted and detected. Detection of growth is not specific to *M.tuberculosis* although growth of other organisms is suppressed by antibiotics and anti-fungal supplements in the media (13). Similarly, the commercial automated liquid drug susceptibility testing (BACTEC MGIT960 SIRE kit – Becton Dickinson) uses the same system to detect bacterial growth in tubes with or without antibiotic (14). The test is based on growth of the *M.tuberculosis* in a drug-containing tube in comparison with a drug-free tube. The instrument automatically interprets the results and reports the result as susceptible or resistant. For both systems, the result is available in approximately 4-13 days, which is much faster than traditional culture method (LJ culture) (214) and traditional drug susceptibility methods (proportional method) (14). In 2007, WHO endorsed the use of liquid culture and DST for early diagnosis of TB and drug resistant TB (230); however, its implementation was limited because of challenging cost in high burden countries.

Microscopic Observation Drug Susceptibility Assay (MODS): MODS is a liquid culture-based technique which was developed by a group of scientists in Peru for early detection of MDR-TB. The present thesis evaluates this technique in Viet Nam for diagnosis of TB and MDR-TB. Details are described in **chapter 3, section 1 (MODS introduction)**.

Thin layer Agar (TLA) on samples is a direct solid culture based method to detect *M.tuberculosis* under light microscope. This method provides time to positive from 9-14 days and allows the recognition of *M.tuberculosis* based on colony morphology and the characteristic of growing on 7H11 media but not 7H11 + PNB (para-

nitrobenzoic acid) media (4). A review reported that the pooled sensitivity and specificity of TLA were both 100% for detection of rifampicin and isoniazid resistance (124). However, this review was based on very few available data (3 publications) and therefore in 2009, the WHO Strategic and Technical Advisory group (STAG) experts concluded that there was insufficient evidence to recommend the use of TLA for rapid screening MDR-TB patients, but operational evaluation should proceed (249).

Nitrate reductase assay (NRA) is a non-commercial test which can be based on solid or liquid culture method to detect drug resistant isolates. The test can be applied to samples or positive cultures. The principle of this test is to detect the ability of *mycobacteria* to reduce nitrite into nitrate, by using nitrate reductase enzyme, which is detected by a colour reaction. A systematic review and meta-analysis of 15 studies revealed that the accuracy of NRA for detection of rifampicin and isoniazid resistance were higher than 94% and 92%, respectively when applied to isolates. However, this accuracy was decreased and ranged from 88% to 100% when applied directly to sputum. This test has turnaround time of approximately 10 days. This test has been endorsed by WHO in 2009 for screening MDR-TB suspects in low and middle income countries (238).

b. Non culture-based methods

➤ Fluorescence smear microscopy:

Fluorescence microscopy (FM) appears to have several advantages over light microscopy (LM) because it has a simple staining procedure, is easy to recognize

AFB on the dark background, has higher sensitivity than conventional staining method (Ziehl-Neelsen staining – ZN staining) and shorter reading time (180). Unfortunately, despite many apparent advantages against LM, FM has not been widely implemented, especially in low resource settings with a high burden of TB because of high price for FM and the lack of robustness and sustainability.

Primo Star iLED (iLED) is a new generation of fluorescence microscope, which was developed by Carl Zeiss, Inc company in agreement with the Foundation for Innovative New Diagnostics (FIND), using the ultra bright LED (light emitting diode) technology as a light source to detect bacilli on a fluorescent smear (6). Detailed characteristics and evaluation of this microscope is presented in **chapter 4**. In 2009, WHO has endorsed the application of LED fluorescence microscope to detect TB in all settings, including in both high and low-volume laboratories (238). This microscope is evaluated for use in Vietnam in **chapter 4**.

- Nucleic acid amplification tests: TB-LAMP tests, Molecular line probe assay (INNO-LiPA and Hain test) and GeneXpert

TB-LAMP test (TB-Loop mediated isothermal amplification) is a novel nucleic acid amplification method, which was developed by Eiken chemical Co.Ltd-Japan, to detect *M.tuberculosis complex*, *M.avium* and *M.intracellulerae* directly (*gyrB* gene) from sputum samples. The result is available in two hours and can be observed by naked eye (fluorescence signal, green colour). The detection limit of TB-LAMP is 50 target DNA copies (88). The performance of this test requires a customized extraction device, simple decontamination process and isothermal amplification. In 2007, the first clinical evaluation of TB-LAMP for the detection of *M.tuberculosis* was

conducted by FIND in microscopy centers in Peru, Bangladesh and Tanzania. The sensitivities of TB-LAMP against smear microscopy or LJ culture as the gold standard were 97.7% for smear positive/culture positive samples and 48.8% for smear negative/culture positive samples. The specificity of TB-LAMP was 99% (16). Since 2008, TB-LAMP has been evaluated in Viet Nam to evaluate its feasibility. TB-LAMP technology is expected to be presented to WHO scientific and Technical Advisory Group (WHO-STAG) in May 2011 (65). The main drawback to this technology is likely to be subjective interpretation of results by the reader, which may result in reduced specificity in wide scale application.

Molecular line probe assays (LPA) are PCR-based hybridization assays, marketed in strip formats and used for identification of *M.tuberculosis complex* and drug resistant isolates. There are currently two commercial LPA formats: INNO-LiPA assay (produced by Innogenetics-Belgium, including INNO-LiPA Mycobacteria and INNO-LiPARif.TB) and genotype assay (produced by Hain Lifescience-Germany, including Genotype Mycobacteria, Genotype MTBDR and Genotype MTBDR-*plus*). INNO-LiPA-Mycobacteria detects amplified 16S-23S rRNA spacer region to identify *M.tuberculosis complex* and other *Mycobacterium* species, *M.kansasii*, *M.xenopi*, *M.gordonae*, *M.genavense*, *M.simiae*, *M.marinum*, *M.ulcerans*, *M.celatum*, *MAIS*, *M.avium*, *M.intracellulare*, *M.scrofulaceum*, *M.haemophilum*, *M.chelonae complex*, *M.fortuitum complex* and *M.smegmatis* (85). INNO-LiPARif.TB detects rifampicin resistant isolates by amplification of the rifampicin resistant determining region (RRDR) of the *ropB* gene, followed by hybridization of the amplicon to membrane-immobilised probes for specific mutations or wild-type sequences. A probe to confirm the presence of *M.tuberculosis* is also included (86). Genotype mycobacteria

detects *M.tuberculosis* complex and some other Mycobacterium Other Than tuberculosis (MOTT) such as *M.avium*, *M.intracellulare*, *M.kansasii* and *M.malmoense* (78). Both Genotype MTBDR and Genotype MTBDR *plus* detects *M.tuberculosis complex* and its resistant patterns related to rifampicin and isoniazid. However, while Genotype MTBDR detects mutations in the *ropB* gene which confer rifampicin resistance and mutations in the *katG* gene which confer isoniazid resistance, Genotype MTBDR *plus* detects all of above resistant patterns plus mutations in promoter region of *inhA* gene which confer isoniazid resistance (127), leading to the increasing of the sensitivity of Genotype MTBDR *plus* in detection of INH resistance. Genotype MTBDR *plus* has now replaced Genotype MTBDR. A systematic review of INNO-LiPARif.TB by Morgan *et al.* in 2005 showed that on positive cultures the pooled sensitivity of this test was 95%. When applied on clinical samples, the sensitivity was lower and ranging from 80% to 100%. The specificity was 100% for both isolate and clinical sample (134). In 2008, a study to assess the performance and feasibility of MTBDR*plus* assay on smear positive and smear negative samples in high volume laboratory in Africa revealed that for smear positive sputum, the sensitivity and specificity were 98.9% and 99.4%, respectively for detection of rifampicin resistance and 94.2% and 99.7%, respectively for detection of isoniazid resistance. For smear negative sputum, 20% (n=4/20) uninterpretable results were recorded for rifampicin and 26% (n=5/19) were recorded for isoniazid (12). WHO has now endorsed the use of line probe assay for early diagnosis of patients infected with rifampicin and isoniazid resistant isolates in high burden countries. Therefore, WHO also recommended that this assay should only be applied on smear positive samples for the highest accuracy (233, 235). GenXpert MTB/Rif test is a one-

step automated nucleic acid amplification test developed by Cepheid (California, US) for detection of *M.tuberculosis* complex and rifampicin resistant *M.tuberculosis* directly from clinical samples (sputum). In this test, unprocessed sputum sample is treated with sample reagent and the three steps of molecular-based tests (sample preparation, amplification and detection) are automated within a cartridge based system. The result is available in less than two hours with minimal hands-on technical time (66). For pulmonary tuberculosis, when three samples per patient were tested for TB diagnosis, the sensitivity of GenXpert MTB/rif was 99.8% for smear positive/culture positive sample and 90.2% for smear negative/culture positive sample, the specificity was 98.1%. The sensitivity decreased by approximately 5% for each sample dropped. For detection of rifampicin resistance and MDR-TB, the sensitivities of GenXpert MTB/rif were 97.6% and 97.5%, respectively against phenotypic DST as the gold standard. The specificity was 98.1% (17). The sensitivity of this test was lower in non-respiratory specimens (urine, gastric fluid, tissue, pleural fluid, cerebrospinal fluid and stool samples) and differed markedly among sample types. The overall sensitivity and specificity were 77.3% and 98.2%, respectively. The highest sensitivity was recorded 100% for stool (n=2/2) and urine samples (n=5/5) and the lowest sensitivity was recorded for tissue sample (69%, n=20/29). However, the low sample size does not allow us to draw conclusions about the accuracy of the test on non-respiratory samples and further evaluation is required. In addition, the detection of rifampicin resistant isolates from these samples was not reliable in terms of sensitivity, according to Hillemann *et al* (81). Despite high accuracy of the test in sputum samples, high cost is the main challenge for implementation of this test worldwide. In 2010, GenXpert MTB/Rif has been endorsed by WHO for use as the

initial diagnostic test in individuals suspected of MDR-TB or HIV-associated TB in high burden countries (248). WHO also developed a roadmap for implementation of this test for target settings, including negotiation with Cepheid to achieve an agreement on reasonable and fixed price for 116 high burden and all low and middle income countries (246). Negotiated prices for eligible countries included GenXpert system-4 module with desktop computer (17,000 USD, 75% reduction relative to the standard market price), GenXpert system -4 module with laptop computer (17,500 USD), cartridge (16.86 USD) annual calibration (1,800 USD) and other services. The current running cost is 18 US\$/test for low-income countries. Evaluation and implementation of GenXpert in Viet Nam will commence in late 2011.

➤ Commercial serological tests

Immunological tests would seem to offer benefits for early detection of TB cases in resource-limited regions because of their speed and simplicity. Commercial serological tests are mainly based on the detection of the humoral (serological) antibodies in response to *M.tuberculosis* (172), interferon-gamma T cell based assay (99) or antigens (lipoarabinomannan-LAM) in samples other than sera (44, 92, 150).

Commercial serological tests- antibody detection

A systematic review from 68 studies evaluating commercial serological tests for diagnosis of pulmonary tuberculosis revealed that the accuracy of these tests is inconsistent; the sensitivity was estimated ranging from 10% to 90% and the specificity fluctuated between 47% and 100% against culture or smear as the gold standard. Interestingly, this review also stated that none of the reviewed assays

performed accurately enough to replace sputum smear microscopy (178). For diagnosis of extrapulmonary tuberculosis, the sensitivity and specificity of serological tests ranged from 0% to 100% and 59% to 100%, respectively against culture, smear or histology as the gold standard. The accuracy of serological tests in diagnosis of extrapulmonary tuberculosis was highly variable among investigators and specific forms of extrapulmonary tuberculosis. The estimated ranges of sensitivity of this test in diagnosis of lymphnode TB and pleural TB were 23% to 100% and 26% to 59% , respectively (179). In this review, 56 studies on tuberculous meningitis were screened but most of them were excluded from analysis due to low sample size (<25 TBM cases), inappropriate sample type (CSF samples instead of serum) and inappropriate gold standard (clinical gold standard instead of bacteriological/histological gold standard). Only one eligible TBM study in this review was recorded and the sensitivity and specificity of a commercial test (AMRAD ICT, TB03 Lot010110, French's Forest, NSW, Australia) were 48% and 82%, respectively (117). In September 2010, the WHO Strategic and Technical Advisory Group for Tuberculosis (STAG-TB) issued a policy statement recommending against the application of commercial TB serodiagnostic tests for diagnosis of active TB disease and detection of latent TB in low and middle-income countries. This was an unprecedented step as the first negative policy statement on a diagnostic test issued by WHO and precipitated by the finding that the market for such tests in India alone is worth over 15 million US dollars (136). There is now compelling evidence for lack of accuracy, with false results outnumbering true results for most tests (178).

Biomarker discovery programs are attempting to identify novel antigen/antibody targets for improved point-of-care serodiagnostic tests, but progress to date has not been encouraging.

M.tuberculosis Antigen Detection: LAM test

MTB LAM ELISA test (Chemogen-USA) is a commercialized test to detect the presence of lipoarabinomannan (LAM), a component of the bacilli cell wall, in clinical samples as a surrogate marker for TB detection. The result is available in 3 hours. Several studies have evaluated this test in settings with high burden of TB and HIV. Klaus Reither *et al.* clinically assessed the diagnostic value of this test in urine samples collected from patients suspected with pulmonary tuberculosis in Tanzania against smear microscopy/culture as the gold standard. The sensitivity and specificity were 50.7% and 87.8%, respectively. The sensitivity was higher in HIV positive patients (62.0%) than HIV negative patients (21%) (163). Another study in Africa evaluated this test on sputum and urine samples and showed similar sensitivity for urine LAM as that from the Tanzania study; sputum LAM had high sensitivity but extremely low specificity (86% and 15%, respectively) (57). Comparable results were obtained from CSF samples (152). In general, MTB LAM ELISA does not have any advantage over smear microscopy.

Detection of immunological response: Interferon gamma release assay

Interferon gamma release assays (IGRAs) have been developed for diagnosis of *M.tuberculosis* infection in terms of host immunological response *in vitro*. The principle of this test is to stimulate peripheral blood T cells with *M.tuberculosis*

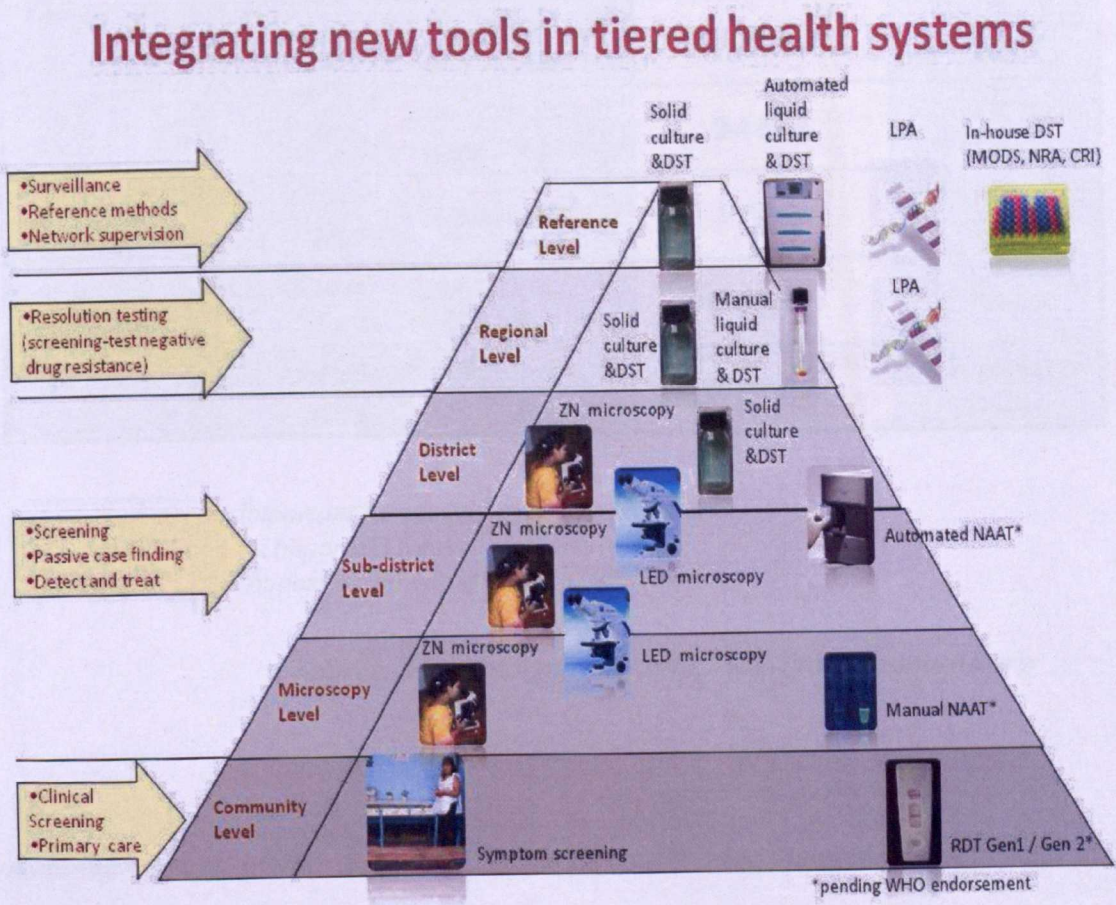
specific antigens (Early secretory antigenic target - ESAT-6 and culture filtrate protein – CFP-10). If memory T cells are present, they will be stimulated by specific TB antigens leading to the induction of interferon gamma (IFN- γ). Two commercial assays are available: QuantiFeron TB test assay (QFT) (Cellestis Ltd, Australia) that measure IFN- γ using ELISA (36) and the T-SPOTTB assay (Oxford Immunotec Ltd, UK) which is based on the counting of cells releasing IFN- γ with enzyme-linked immunospot technique (ELISPOT) (146). A systematic review evaluating the two commercial IGRAs in diagnosis of active TB disease reported that the pooled sensitivity of QFT and T-SPOT were 81% (95%CI: 78%, 83%) and 88% (95%CI: 86%, 90%), respectively. The sensitivity varies significantly between studies, ranging 58% to 100% for QFT and 50% to 100% for T-SPOT. The pooled specificity was 99.2% and 86.3% for QFT and T-SPOT, respectively. It is apparent that higher sensitivity was reciprocal to lower specificity and sensitivity is lower in high burden countries. Importantly, 3-5% indeterminate results were recorded for each test and the number of indeterminate results increased in immunosuppressed patients and children (32, 58, 112). Due to compelling evidence demonstrating the poor performance of commercial IGRAs in low and middle income countries, especially in settings with high TB/ and HIV burden, WHO released a policy to discourage the use of IGRAs in these settings in December 2010 (248).

1.4.4 Integration of novel diagnostic tests in the health system for diagnosis of tuberculosis in high burden countries.

In 2010, WHO has produced a framework for implementation of endorsed novel diagnostic methods in low and middle income countries. Below is the expected

implementation of new tools in the health system recommended by WHO (figure 1.8 and figure 1.9).

Figure 1.8 Integrating new tools in tiered health systems



Source: WHO: Framework for implementation of new TB diagnostics (243)

Figure 1.9 Recommended laboratory levels for application of diagnostic tests

| Year | Technology | Turnaround time | Sensitivity gain |
|------------------|---|------------------------|---------------------------|
| Before 2007 | ZN microscopy Solid Culture | 2-3 days 30-60 days | Baseline |
| 2007 | Liquid Culture / DST Rapid speciation | 15-30 days | +10% compared to LJ |
| 2008 | Line Probe Assay (1st line, Rif & INH) | 2-4 days | At this time for S+ only |
| 2009 | LED-based FM | 1-2 days | +10% compared to ZN |
| Conditional 2009 | In house DST (MODS, CRI, NRA) | 15-30 days | 1 st line only |
| Expected 2010 | Integrated NAAT (TB, Rif) | 90 minutes | +40% compared to ZN |

Red circle: Important for early diagnosis and care

Yellow circle: Important for smear negative TB

Pink circle: Important for rapid MDR/XDR detection

Source: FIND diagnostics: <http://www.finddiagnostic.org>

Although many novel diagnostic methods have been launched, evaluated and recommended by WHO, the implementation of these methods should carefully be considered based on infrastructure, finance, maintenance, personnel and technical issue at the site. GenXpert, for example, has been endorsed by WHO as the initial diagnostic test for MDR-TB suspects and immunocompromised patients suspected of TB. The majority of these cases are from high burden countries where financial resources are lacking. Even though the negotiated cost for a GenXpert system is 17,000 USD and cost of each test is markedly reduced to 18USD, the wide application

of this test is still challenging because the target population for this test is very impoverished. In addition, national TB programmes cannot implement this test in all district laboratories, as mentioned in the roadmap for implementation of GenXpert by WHO, due to financial shortage. Therefore, the application of novel tests highly depends on the capacity of the site. Another consideration is the sustainability of the test if the smear microscopy network is dismantled or if GenXpert can replace smear microscopy in wide scale implementation. Aside from cost, technical issues and maintenance, other foreseen challenges include storage conditions for cartridges and the likelihood that Genexpert will not be a suitable test for follow-up and monitoring of patients on treatment. It is suggested by the manufacturer that cartridges should be stored between 4⁰C - 30⁰C but the room temperature in high TB burden countries, including Viet Nam often exceeds this limit. Since GenXpert detects genetic material of *M.tuberculosis* present in samples, it cannot distinguish between living and dead bacilli. Therefore the classification of TB patient treatment outcome after 2, 5 and 8 months of treatment, which is currently based on the smear result, cannot be made using Genxpert. Although smear is also unable to distinguish dead bacilli, smear test is less sensitive and therefore false positives due to dead organisms are likely to be less frequent. A new classification is needed for follow-up patients if GenXpert is implemented in place of smear and further research is required on the programmatic integration of Genxpert.

Several funding agencies, such as TB REACH, have prioritized funding to support projects to increase implementation of GeneXpert, including in Viet Nam.

1.5 TB TREATMENT REGIMENS

1.5.1 Purpose of TB treatment

The objective of anti-tuberculous treatment is to achieve cure of the disease and prevent the emergence or amplification of drug resistance. Therefore, the treatment regimen should include multiple drugs to which the organisms are susceptible. With existing 1st line drugs (Isoniazid, Rifampicin, Pyrazinamide, Ethambutal and Streptomycin), a minimum of 6 months therapy is required to achieve high cure rates and prevent relapse. The treatment course includes two phases: intensive bactericidal phase and continuation (sterilization) phase. A combination of at least 3 drugs in the intensive phase and at least 2 drugs in the continuation phase is required to protect against drug resistance. By 2007, more than 99% of all notified TB cases reported to WHO were in DOTS (Direct Observation Treatment Short Course) programmes (244).

1.5.2 Direct Observation Treatment Short Course (DOTS strategy)

The DOTS strategy has been recommended for application in national TB programmes by WHO from 1993 to improve the efficacy of TB control and management. This strategy aims to ensure high cure rate and follow-up of TB patients during the treatment course. There are 5 components to the DOTS strategy which are critical for DOTS implementation: government commitment, case detection by passive-case finding, standardized short course therapy for all sputum smear-positive patients, regular drug supply and a monitoring system for programme supervision and evaluation (217). Based on DOTS implementation, two targets for TB control were

established – detection of at least 75% of estimated new smear positive cases and cure of at least 85% of detected cases by 2000. The number of TB patients under the DOTS programme has been scaled up rapidly since the Millennium. By 2009, 49 million patients were treated in DOTS programme, of whom 41 million people have been successfully treated and approximately 6 million lives saved through DOTS and stop TB strategy. The case detection rate reached 63% and the treatment success rate exceeded 85% worldwide in 2009 (244).

The private sector in which practitioners are not employed by the government, plays an important role in increasing the TB detection rate and the number of TB cases on TB treatment. Unfortunately, limited information about TB management in the private sector has been reported by WHO globally (83). In Viet Nam, it was documented that if all TB suspects presenting to private sectors in Ho Chi Minh City were sent to NTP for diagnosis, the case detection rate of new smear positive TB cases would be increased by 18% (157). Another study reported that at least 40% of TB cases in this city received TB treatment from private clinics (108). At national level, approximately 8% of TB cases in Viet Nam seek private clinics for TB treatment, mostly in southern Viet Nam (12%) and especially in urban areas (13%) (82). The treatment outcome of these patients depends on the collaboration level between private and public sectors. If TB diagnosis and treatment within private sectors are not quality controlled by the NTP (low quality of smear microscopy, use of poorly validated diagnostic tests, no DOTS and non-standard treatment regimen), low treatment success and high default rates are likely, 60% and 37%, respectively in one study (158) which is much lower than the targets recommended by NTP. However, if private clinics controlled by the NTP in terms of technical and management issues,

the treatment success can exceed the target (105) and private-public mix models should be scaled up worldwide.

1.5.3 TB drugs

Antituberculous treatment is usually based on five first line TB drugs approved by WHO, including Rifampicin (RIF), Isoniazid (INH), Streptomycin (STR), Ethambutol (EMB) and Pyrazinamide (PZA). The activity against TB and mechanism of these drugs are described in **table 1.1**

Table 1.1 The 1st line TB drugs and their mechanisms

| <i>1st line TB drug</i> | <i>Drug class</i> | <i>Activity against TB</i> | <i>Mechanism</i> |
|------------------------------------|--------------------------------------|---------------------------------------|---|
| RIF | Rifamycin | Bactericidal | Protein synthesis. |
| INH | Isonicotinic acid hydrazide | Bactericidal | Mycolic acid synthesis (cell wall). |
| STR | Amynglycoside | Bactericidal | Protein synthesis. |
| EMB | Unspecified | Bacteriostatic | Inhibition of cell wall synthesis. If used for a long time, EMB protects against further development of resistance. |
| | | Bactericidal | Only at the high end of dosing range. |
| PZA | Synthetic derivative of nicotinamide | Bactericidal for dormant tuberculosis | Unclear |

Source: Reference (91)

For MDR-TB, second line drugs (**table 1.2**), in addition to any 1st line drugs to which organism remains susceptible, are recommended by WHO. These include injectable drugs, fluoroquinolone, oral bacteriostatic second line drugs and drugs with an unclear role in treatment of drug resistant TB. The last group (group 5) is only prescribed if it is impossible to design adequate regimens using agents from group 1-4 and prescribed with consultation due to high toxicity and weak efficacy (239).

Table 1.2 TB regimens recommended for MDR-TB treatment

| Group | Drugs (abbreviations) |
|---|--|
| Group 1: First-line oral agents | <ul style="list-style-type: none">• pyrazinamide (Z)• ethambutol (E)• rifabutin (Rfb) |
| Group 2: Injectable agents | <ul style="list-style-type: none">• kanamycin (Km)• amikacin (Am)• capreomycin (Cm)• streptomycin (S) |
| Group 3: Fluoroquinolones | <ul style="list-style-type: none">• levofloxacin (Lfx)• moxifloxacin (Mfx)• ofloxacin (Ofx) |
| Group 4: Oral bacteriostatic second-line agents | <ul style="list-style-type: none">• para-aminosalicylic acid (PAS)• cycloserine (Cs)• terizidone (Trd)• ethionamide (Eto)• protionamide (Pto) |
| Group 5: Agents with unclear role in treatment of drug resistant-TB | <ul style="list-style-type: none">• clofazimine (Cfz)• linezolid (Lzd)• amoxicillin/clavulanate (Amx/Clv)• thioacetazone (Thz)• imipenem/cilastatin (Ipm/Cln)• high-dose isoniazid (high-dose H)^b• clarithromycin (Clr) |

Source: Reference (239)

1.5.4 Treatment regimens

The standardized regimens for TB treatment recommended by WHO apply to four patient groups, which are classified according to the bacteriology result and history of TB treatment. These categories are new smear-positive patients (Category I), relapse – treatment after default or treatment failure of Category I in settings with low rate of MDR-TB (Category II), new smear-negative pulmonary patients or less severe extrapulmonary TB forms (Category III), and treatment failure of cat I in settings with high rate of MDR-TB (Category IV). **Table 1.3** shows the TB treatment regimens recommended for corresponding diagnostic categories.

Table 1.3 Recommended treatment regimens for different diagnostic categories by WHO

| <i>TB patient diagnostic Category</i> | <i>Recommended TB treatment regimens in 2010*</i> | <i>Remark</i> |
|---------------------------------------|--|--|
| I | 2HRZE/4HR (preferred) 2(HRZE) ₃ /4(HR) ₃ (optional) | Applied for new PTB, EPTB (except TB of CNS, bone or joint) |
| II | 2HRZES/1HRZE/5HRE (preferred) 2(HRZES) ₃ /1(HRZE) ₃ /5(HRE) ₃ (optional) | Applied only if specific country data show low or medium MDR-TB rate in this population or data is unavailable |
| III | 2HRZE/4HR (preferred) 2HRZE/6HE (optional) 2(HRZE) ₃ /4(HR) ₃ (optional) | |
| IV | Designed standardized regimen based plus 2 nd line drugs | |

Source: Reference (239)

**Numbers preceding regimens indicate the length of treatment in months. Subscript numbers following regimens indicate the frequency of administration per week. If no subscription numbers are given, the regimen is daily.*

H (Isoniazid), R (Rifampicin), S (Streptomycin), E (Ethambutol) and Z (Pyrazinamide)

“Preferred” indicates a strong recommendation.

“Optional” expresses a conditional recommendation. It means “may” or “is not recommended. In this case, alternatives are often listed

PTB: Pulmonary TB, EPTB: Extrapulmonary TB, CNS: Central nervous system.

In 2009, WHO recommended some important changes in the previous treatment guidelines for the improvement of treatment outcome (239). **First**, 2HRZE/6HE regimen should be phased-out because this regimen uses rifampicin in only the first 2

months which significantly increased relapse, failure and acquired drug resistance after 8 months of treatment in comparison with 2HRZE/4HR in IUATLD 'study A' (120). **Second**, the retreatment regimen with the first line drugs (2HRZES/1HRZE/5HRE) is ineffective in MDR-TB patients because these patients are already infected with rifampicin and isoniazid resistant isolates. Therefore, while there is no ideal regimen for this group, it is important to detect MDR-TB as soon as possible and start an individual regimen based on the DST profile. However, this is not possible in many settings. **Third**, in settings where rapid molecular DST tests are available for MDR-TB detection, an effective regimen (consisting of effective drugs among the first line drugs and 5 groups of second line drugs) can be started 1-2 days after diagnosis. However, molecular DST tests do not accurately detect resistance to the remaining first line drugs, (STR, PZA and EMB). Therefore, the ideal regimen cannot be determined from molecular testing. In countries where molecular tests are not available, traditional DST methods are used; and treatment will be based on an empirical regimen until the DST results are available. **Fourth**, in new TB patients who are infected with INH resistant isolates, they are more likely to develop acquired MDR-TB. Therefore, adding Ethambutol to the continuation phase of treatment course (HRE) is recommended' in settings with a high prevalence of INH resistance', however, a 'high prevalence' is not defined and this recommendation is not evidence-based. Ethambutol has side effects, including ocular toxicity. Therefore, a study is needed to determine efficacy of the regimen and at what level of INH resistance it should be applied programmatically, if at all.

1.5.5 Treatment regimens currently applied in the Vietnamese NTP

There are 4 regimens currently used in Viet Nam (137):

Regimen I: 2HRZE/6HE (the first two months with HRZE daily then the last 6 month with HE daily) (only provided if DOTS is applied). This regimen is still used for treatment of new cases in Viet Nam even though it has been recommended to phase out by WHO since 2009. It is currently being phased out in a stepwise approach. Sputum examination is required at the end of month 2, 5 and 8.

Regimen I: 2S(E)RHZ/4RH (only provided if DOTS is applied). This treatment course is for new TB cases who have never received TB treatment or have undergone TB treatment course less than 1 month. Sputum examination is required at the end of month 2, 4 and 6.

Regimen II: 2SHRZE/1HRZE/5H₃ R₃ E₃. (the first 2 months with SHRZE daily, then the third month with HRZE daily and finally the last 5 months with HRE three times a week). This regimen is for patients who have relapsed or failed with regimen I, retreatment after default and some severe cases based on TB treatment histories. Sputum examination is required in the end of month 3, 5 and 7 (or 8).

Regimen III: 2HRZE/4HR or 2HRZ/4HR. Recommended for all pediatric TB cases in Vietnamese National guidelines. In severe cases, additional streptomycin can be prescribed. Sputum examination is required at the end of month 2, 4 and 6.

For smear negative TB cases, Sputum examination is required at the end of month 2 and 5 of treatment.

Anti tuberculous drugs and related doses are presented in the **table 1.4**:

Table 1.4 Antibiotics and related doses in TB treatment

| Antibiotics | Daily | Three times a week |
|---------------------|--|---------------------------|
| | Dose (mg/kg) | Dose (mg/kg) |
| Isoniazid | 5 (4-6) | 10 (8 -12) |
| Rifampicin | 10 (8-12) | 10 (8 -12) |
| Pyrazinamid | 25 (20-30) | 35 (30-40) |
| Ethambutol | Children 20 (15-25) Adults 15 (15-20) | 30 (25-35) |
| Streptomycin | 15 (12-18) | 15 (12-18) |

Source: Reference (137)

1.6 TUBERCULOSIS CONTROL PROGRAMME IN VIET NAM

1.6.1 Epidemiology

Viet Nam is a developing country in the South East Asia region with an estimated population of 86 million in 2009. It is classified by WHO as a high TB burden, high HIV burden and high MDR-TB burden country. The annual report from WHO showed that Viet Nam was ranked 13th among high TB burden countries in the world in 2009 with the following estimates and notifications (244) (**table 1.5**):

Table 1.5 Data on TB control activities of Viet Nam reported by WHO

| | |
|--|-------------------|
| <i>Population, 2009</i> | 85,789,573 |
| Estimated prevalence (all cases/100,000 population) | 333 |
| Estimated incidence (new sputum smear positive/100,000 population) | 200 |
| Estimated Incidence of HIV+/TB (new HIV+/TB/100,000 population) | 8.4 |
| Estimated case detection rate (%) of all forms | 54 |
| Estimated (%) new MDR-TB cases | 2.7 |
| Estimated (%) retreatment MDR-TB cases | 19 |
| All pediatric TB cases notified (<15 years) | 88 |

Source: Reference (244)

Although the detection rate has officially exceeded the WHO target of 70% of infectious cases since 2000, reaching 82% in 2007, the overall incidence rate has decreased only marginally each year, about 1% per year (237). It is likely that previous estimated TB incidence figures for Vietnam were an underestimate, as suggested by the 2006 - 2007 intensified prevalence survey (253): TB prevalence was found to be 1.6 times higher than the previous estimate; and the estimated case detection rate of all TB forms was re-estimated at 56% which is lower than the 70% target. This may account for the slow decline in incidence figures, despite apparently high case detection rates. The escalation of HIV infection and TB/HIV co-infection is likely to be a contributing factor for the high prevalence of TB in Viet Nam (202). Increasing case notification is now a priority of the national TB programme.

1.6.2 TB control network and laboratory TB network

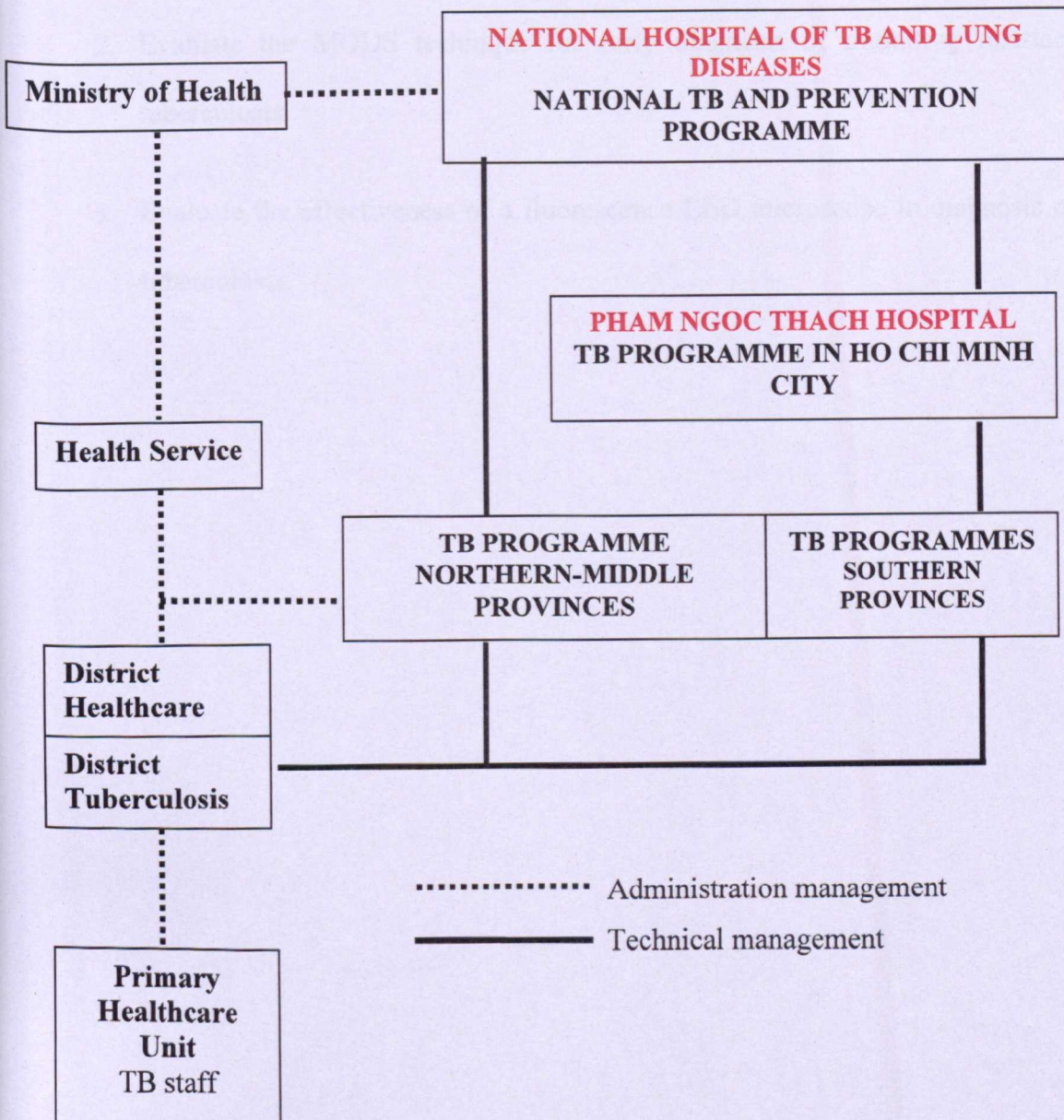
Viet Nam consists of 65 provinces, 685 districts and 10,934 communes. The TB control network (established in 1957) functions with 4 levels: national, provincial, district and commune level (**figure 1.10**). The district TB unit at the commune is the place where almost all TB suspects come for screening, diagnosis and treatment.

Since 2001 TB control and prevention activities in prisons and rehabilitation centers have been covered by the National TB programme (NTP). Aside from passive detection, active screening is also performed to increase TB case detection at these centers, inmates of which are at high risk of TB infection and development of active TB disease.

The tuberculosis laboratory network acts as “the spine” of the TB control programme. The laboratory network is organized according to 3 levels: peripheral (district) laboratory, provincial laboratory and national laboratory. There is an intermediate (regional) laboratory in the South of Viet Nam, hosted at Pham Ngoc Thach hospital, which is responsible for quality assurance and quality control of laboratory activities in the southern region. Smear is performed at all levels for TB diagnosis. Culture (LJ and MGIT), DST (proportional DST method) and other tests (molecular genotyping tests and identification tests) are performed at regional and national laboratories. In the south of Viet Nam, there are 5 laboratories in 5 provinces equipped for performing TB culture (Dong Nai, Dong Thap, Binh Thuan, PNT hospital and Can Tho) and one laboratory performing DST located at PNT hospital. At peripheral level, on average, each peripheral TB laboratory has one technician responsible for the activity of the laboratory. According to the national TB programme (NTP), there were 1,709,628

smears made in 2008 for TB diagnosis, of them 131,966 (7.7%) were positive smears. Personnel shortages and inconsistency lead to excess workload at almost half of the TB units (80).

Figure 1.10 Diagram of TB control network in Viet Nam



1.7 AIMS

1. Evaluate the MODS technique (Microscopic Observation Drug Susceptibility Assay) for early diagnosis of tuberculosis in 3 groups: pediatric TB, HIV-associated TB and TB meningitis.
2. Evaluate the MODS technique for early diagnosis of multidrug resistant tuberculosis.
3. Evaluate the effectiveness of a fluorescence LED microscope in diagnosis of tuberculosis.

Chapter 2

MATERIALS AND METHODS

In this project, the MODS study was conducted at the Hospital for Tropical Diseases and PNT hospital; the LED study was performed at District Tuberculosis Units in Ho Chi Minh City and at PNT hospital.

2.1 STUDY SETTINGS

The studies described in this thesis were conducted at 3 sites in Ho Chi Minh City, Viet Nam:

- Pham Ngoc Thach Hospital for Tuberculosis and Lung diseases (PNT hospital)
- Hospital for Tropical Diseases (HTD)
- Three District Tuberculosis Units (DTUs) in Ho Chi Minh City: DTU1, DTU5 and DTU8

The evaluation of MODS

The pilot evaluation of the MODS technique for diagnosing TBM described in **chapter 3, section 2** was conducted at HTD and the Oxford University Clinical Research Unit (OUCRU) laboratory based at HTD.

All other evaluations of the MODS technique described in **chapter 3** were conducted at PNT hospital.

The implementation phase of MODS for TBM diagnosis (**chapter 3 – section 2**), the evaluation of MODS in early diagnosis of tuberculosis in children (**chapter 3 – section 3**) and in HIV infected adults (**chapter 3 – section 4**), and the accuracy of MODS in detection of multidrug resistant tuberculosis (**chapter 3 – section 5**) were organized at TB ward, pediatric wards, TB/HIV wards and Out Patient Department of PNT hospital, respectively, from May to December 2008.

The evaluation of iLED microscope

Evaluation of iLED microscope on panel slides was performed at the Microbiology laboratory at PNT hospital. Patient recruitment, sample collection and smear preparation were performed at district tuberculosis units. The study was supervised by the microbiology laboratory at PNT hospital and conducted as part of a multi-country evaluation by FIND (South Africa, Russia, Peru, Lesotho, Ethiopia, Tanzania and Viet Nam).

2.1.1 Pham Ngoc Thach Hospital for Tuberculosis and Lung Diseases - The microbiology laboratory.

Pham Ngoc Thach hospital (PNT hospital) was founded in 1906 and served the French military at that time. In 1960, it was used as a TB hospital. Since 1989, it functions as a TB and Lung disease hospital. PNT hospital is a tertiary referral hospital for tuberculosis and lung disease in southern Viet Nam. This hospital has approximately 800 beds (320 beds for TB wards, of which 55 beds for TB/HIV patients; 60 beds for children with TB and lung diseases) working in 5 main sections: Emergency and Out Patient department (OPD), Tuberculosis and Intensive care unit

(ICU), non-TB, surgery and subclinics (microbiology, biochemistry and haematology, and radiology). In 2009, there were 321,482 patients visiting OPD, of which 20,267 were inpatients. TB patients occupied 45% of all cases. From 2005 to 2009, the number of TB/HIV patients admitting to the hospital decreased (15%, from 2313 to 2018) due to many HIV/TB projects in collaboration with international organizations conducted at peripheral TB units. The Paediatric Department received approximately 10,000 children in 2009, of which 1200 cases were hospitalized and 100 cases were diagnosed with tuberculous meningitis (TBM).

The microbiology laboratory at PNT hospital is a regional TB laboratory and is part of the NTP laboratory network. It plays a role as a reference laboratory for all TB laboratories in southern Viet Nam, including Ho Chi Minh City, in terms of technical support, technical assurance, supervision and training. The laboratory is responsible for the preparation of staining reagents and slide rechecking for all TB units in Ho Chi Minh City.

The microbiology laboratory is equipped with 2 Biosafety laboratory level 2+ (BSL 2+) laboratories for doing TB culture and DST. The laboratory has 65 staff, including one PhD.MD, one MSc.MD, two medical doctors, and the remaining staffs are BSc graduates and technicians. There are approximately 1000 samples of all types for TB diagnosis and around 30 samples for other microbiology diagnosis sent to the laboratory daily. The workload for smear reading is from 80 to 150 slides/technician/per day.

Any patient presenting to the Out Patient Department (OPD) at PNT hospital with clinical symptoms suggestive of tuberculosis is asked to produce 3 sputum samples

and a new chest X-ray for TB diagnosis. Currently, smear microscopy (direct or homogenous smear, light microscope or conventional fluorescence microscope), MGIT culture and proportional drug susceptibility testing methods (DST) are used as routine microbiological tests. With any positive smear sample or positive MGIT sample, the patient is diagnosed with tuberculosis and is eligible to receive TB treatment according to NTP guidelines. If all three samples are smear negative but chest X-ray suspected of tuberculosis, the patient will be asked to submit another three sputum samples after 2 weeks for further diagnosis or consultation as needed. In cases with moderate and severe general status, the patients are asked to admit to the hospital for treatment and follow-up. DST is not done for all TB patients because of limitation in finance and human resource. Only for TB patients in high risk groups (retreatment, default, failure and HIV/TB patients), DST are performed upon request of the treating clinician. From 2009, genotype MTBDR-*plus* (Hain Lifescience-Germany) is used to detect smear positive pulmonary TB patients infected with MDR isolates presenting to all District Tuberculosis Units in Ho Chi Minh City. This is a project of National TB programme (NTP) funded by WHO and Green Light Committee (GLC). All TB patients infected with rifampicin resistant isolates detected by this method are eligible to receive MDR-TB treatment regimen recommended by WHO and drugs was provided by WHO and GLC. Since early 2011, fluorescence LED microscope has been used as a routine diagnostic method at PNT hospital.

Aside from doing routine tests for TB diagnosis, PNT laboratory also participates in international projects for evaluation of new TB diagnosis tests (eg. MODS assay with OUCRU-Wellcome Trust, LAMP test with FIND and iLED microscope with FIND).

2.1.2 Hospital for Tropical Diseases – Oxford University Clinical Research Unit (OUCRU-HTD) – The OUCRU laboratory.

The Hospital for Tropical Diseases (HTD) was founded in 1862 and named “Cho Quan” hospital at that time. From 1989, it was named as HTD and has functioned as the tertiary referral hospital for patients with infectious diseases in southern Viet Nam. This hospital has approximately 600 beds divided into paediatric, adult and Viet Anh wards; and has laboratories for haematology, biochemistry, microbiology and radiology which support diagnosis and treatment.

OUCRU laboratory is located at HTD. This laboratory was developed for doing research on infectious diseases in collaboration with HTD, including tuberculosis, malaria, dengue, influenza and other respiratory infection, enteric, central nervous system infections, animal health and zoonoses, pharmacology and statistics, bioinformatics, modeling and mapping. To date, almost 1000 research papers have been published in the international literature.

In the pilot phase evaluation of MODS in diagnosis of tuberculous meningitis (**chapter 3 – Section 2**), samples were obtained from patients admitted to Viet-Anh ward and processed in the OUCRU BSL3 laboratory.

2.1.3 Tuberculosis Units in Ho Chi Minh City

Three district tuberculosis units (DTU) located within a radius of 8km from PNT hospital participated in this study: DTU1, DTU5 and DTU8. The DTU is the primary health care network of the NTP where almost all TB suspects come for TB screening and diagnosis. Routinely, a patient with suspected TB is asked to submit 3 sputum

samples (spot – morning – spot) to DTU for TB diagnosis by smear microscopy method. The smear results are available in two days. If any of the three sputum samples is positive, the patient is diagnosed with TB and will commence TB treatment following the DOTS strategy. Further sputum samples will be collected if results of the first three samples are not sufficient for determination. All smears done at DTU are stored at the site according to NTP guidelines. At the end of each month, all smears are sent to the microbiology laboratory at PNT hospital for blind rechecking using LQAS rechecking method (Lot Quality Assurance System) according to NTP guidelines.

The positive detection rates of the DTUs participating in LED study ranged from 10% to 15%. The workload of DTU5, DTU1 and DTU8 are approximately 10, 20 – 40 and more than 40 slides/person/day, respectively. DTU1 and DTU5 have one technician working full-time in the laboratory, whereas DTU8 has two technicians. These technicians are responsible for sample collection, registration, smear preparation, smear reading and reporting.

Patient recruitment, sputum collection and smear preparation, reading and reporting were performed on site at the DTU. The study was supervised by Dang Thi Minh Ha.

2.2 STUDY TIMING AND DURATION

- The enrollment of TBM study – pilot phase was conducted from June 1st to November 1st 2006.

- The enrollment of TBM study - implementation phase, paediatric TB, HIV-associated study and MDR-TB study were carried out from May to November 2008.
- The enrollment of the LED microscopy study was conducted from August 2008 to September 2009.

2.3 SCIENTIFIC AND ETHICAL APPROVAL

All protocols were approved by the Institutional Review Board (IRB) at Pham Ngoc Thach hospital or Hospital for Tropical Diseases, as applicable, and the Health Services of Ho Chi Minh City. Informed consent was not sought for the diagnostic studies because the studies were conducted on routine samples only and did not involve any intervention, additional samples or change in patient management. This patient consent waiver was approved by the IRB of Pham Ngoc Thach hospital or Hospital for Tropical Diseases in the protocol and is consistent with the guidelines on informed consent provided by the University of Oxford Tropical Ethical Review Board (OXTREC).

2.4 ENROLLMENT

2.4.1 Enrollment sites

MODS study

All patients with clinical symptoms suspected of tuberculosis meningitis presenting to HTD were enrolled into the pilot study of evaluation of MODS in diagnosis of tuberculous meningitis (TBM) – pilot phase (**chapter 3 – section 2**).

Patients suspected of TBM and pulmonary TB presenting to Pham Ngoc Thach hospital were enrolled into the study of evaluation of MODS in diagnosis of TBM – implementation phase (**chapter 3 – section 2**), paediatric TB (**chapter 3 – section 3**), HIV-associated TB (**chapter 3 – section 4**) and multidrug resistant TB (**chapter 3 – section 5**).

LED microscopy study

Adult patients suspected of pulmonary TB presenting to any of the three DTUs (DTU1, DTU5 and DTU8) were enrolled into the LED study (**chapter 4**).

2.4.2 Inclusion criteria

- Suspected of tuberculosis (all studies)
- ≥ 16 years of age for all adult studies
- Under 16 years of age for paediatric MODS study
- HIV-infected for the HIV/TB MODS study
- Not on TB treatment (new cases) or on TB treatment less than or equal 7 days (MODS study only).

2.4.3 Exclusion criteria

- Receiving TB treatment for more than 7 days before sample collection (MODS study only).
- Under 16 years of age (LED microscopy study and adult MODS studies).

2.4.4 Data collection

MODS STUDY

Data on socioeconomic and demographic features, TB history, TB contact history, HIV status and presenting clinical features were prospectively collected on a standard case report form (CRF). Samples were collected as per routine care as deemed appropriate by the treating physician. No additional samples were collected as part of this study. All specimen types from suspected cases were included in the study except blood.

LED MICROSCOPY STUDY

Data on socioeconomic and demographic features, TB history, TB contact history, HIV status and presenting clinical features were prospectively collected as part of routine care for TB management of NTP but not for study evaluation. All sputum samples collected for routine care at DTUs were used for evaluation of LED microscopy. No additional samples were collected as part of this study.

2.5 SAMPLE COLLECTION AND TRANSFER

2.5.1 Sample collection

- The number of samples collected from patients was determined by the treating clinicians.

- No additional samples were collected as part of this study.

- CSF samples were collected for TBM study and all other sample types (not including blood) were collected for the paediatric study and HIV-associated study. Only sputum samples were collected for the MDR study and the LED study.
- The samples were collected into sputum containers for sputum samples provided by NTP or sterile tubes for other body fluids provided by the study.

2.5.2 Sample transfer

MODS studies

- If the samples were collected during working hours, they were transferred immediately to the microbiology laboratory for processing.
- If the samples were collected after 4:00pm, they were kept in refrigerator overnight at the wards or they were sent to be stored at 4⁰C at the microbiology laboratory immediately.
- If samples were collected during the weekend (Saturday and Sunday), they were sent immediately to the microbiology laboratory and stored at 4⁰C until processed.

LED microscopy study

For the LED study, no samples were collected at DTUs after 4:00pm or during the weekend because the official hours of these units are from 7:00 to 16:00, from Monday to Friday.

2.6 LABORATORY METHODS

2.6.1 Sample processing

- Cerebral spinal fluid (CSF): If CSF samples were collected from patients admitted to PNT hospital, CSF (sterile sample) was processed in the MODS culture room which is separated from the sample preparation room and the culture room to minimize the risk of contamination with unsterile samples.
- CSF samples collected at HTD were processed in OUCRU BSL3 laboratory where smear microscopy, MGIT culture and LJ culture were also performed. Techniques involving culture manipulation were performed in a separate cabinet to sample inoculation to reduce the risk of cross-contamination.
- Other samples (not including blood) collected from PNT hospital were manipulated in the sample preparation room and the culture room because these samples require decontamination.

2.6.1.1 Cerebrospinal fluid (CSF)

The sterilin tubes containing CSF samples were centrifuged at 3000g, 4⁰C for 20 minutes (Eppendorf-5810R, Germany). After discarding the supernatant, 2ml pellet was used for smear, MGIT and MODS culture. Technicians responsible for each test were blinded to the other microbiological results and clinical data on the patient records.

2.6.1.2 Samples other than CSF

All samples, except for blood, were homogenised and decontaminated by Sputaprep (NaOH-NALC 2%) manufactured by Nam Khoa Company-Viet Nam prior to testing. The kit contains Mucoprep (NaOH 0.5M and Na₃Citrate 0.05M, NALC (N-Acetyl-L-Cysteine) and Phosphate buffer (PO₄ 10X - 0.67M). Homogenization and decontamination buffer (HDB) were prepared from Mucoprep and Phosphate buffer 1X was diluted from the provided concentration. In brief, 3 – 5ml sample was added to 3 – 5ml HDB contained in a 50ml falcon tube. The tube was shaken slightly by automated shaker and left at room temperature for 20minutes. After that, 35 – 39ml phosphate buffer 1X was added into the mixture. The mixture was shaken by hand and then centrifuged at 3000g, 4⁰C for 30 minutes. The supernatant was then discarded and 0.5ml pellet at the bottom was re-suspended with 2ml distilled water. The pH of 2.5ml pellet was 11.0. The deposit was then aliquoted into 3 parts for smear, MGIT culture and MODS. If the sample was from MODS/DST group, the deposit was divided into 3 aliquots for DST-MODS, MGIT culture and LJ culture; any growth from MGIT or LJ was used for indirect DST on LJ media (1% proportional method).

2.6.2 Microbiological tests for diagnosis of tuberculosis

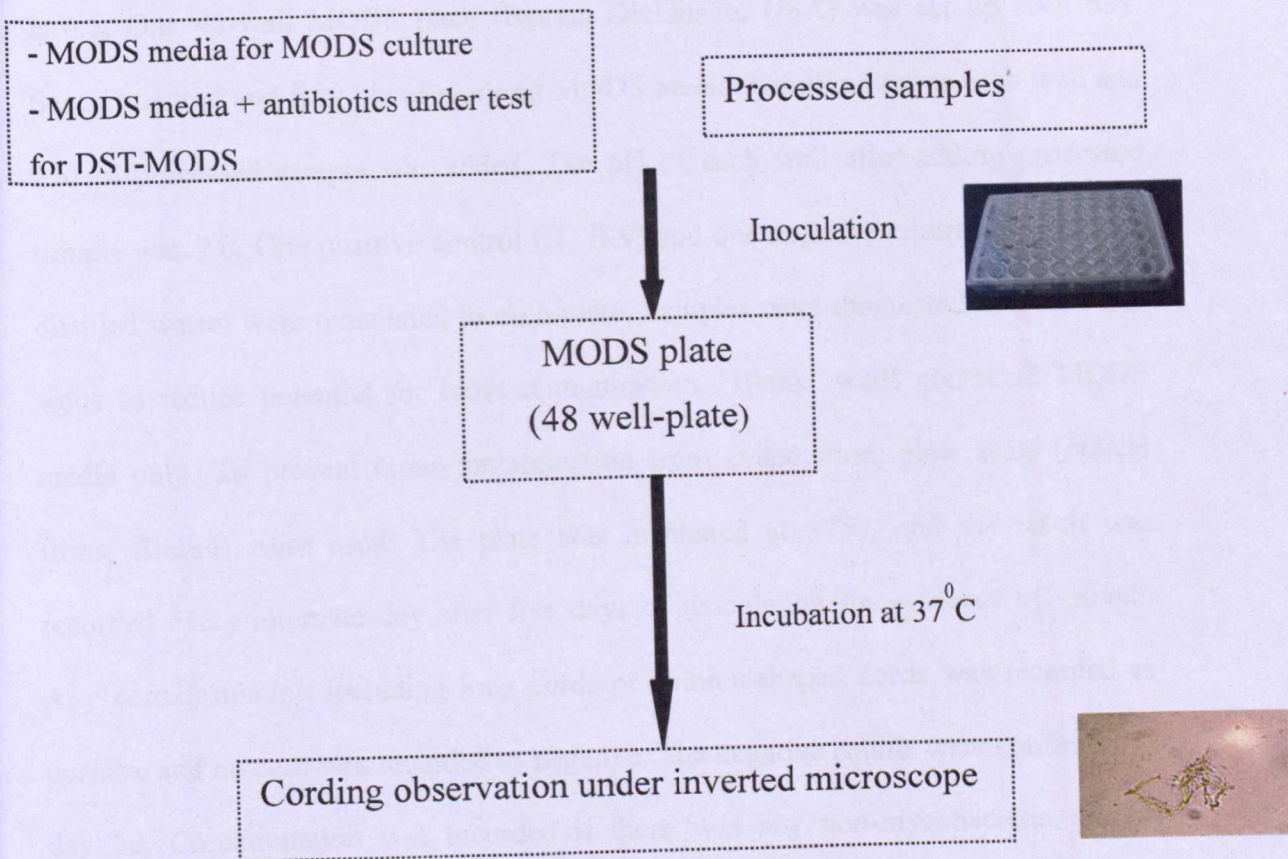
2.6.2.1 MODS culture

Principle: MODS detects the living Mycobacterium based on the two well-known characteristics of *M.tuberculosis*; that is the rate of growth in liquid medium is considerably quicker than that on solid medium, and the morphology of

M.tuberculosis in liquid culture as cording factors, strings or tangles observed under an inverted microscope.

Figure 2.1 Scheme of MODS culture/drug susceptibility testing

Procedure:



2.6.2.1.1 MODS culture with 250µl processed sample

The MODS culture was conducted in a biosafety cabinet class I which was placed in a separate room from the sample processing room, smear preparation room and MGIT culture room. The MODS method was performed as described in Park *et al.* (151) using the modification described by Caws *et al.* (34). Briefly, MODS media was

prepared with 7.9 g Middlebrook 7H9 broth (Difco, Sparks, MD), 4.13 ml glycerol and 1.67 g bactocasitone (Difco, USA) in 880 mls sterile distilled water. The media was autoclaved and stored in 22 ml aliquots at 4⁰C. Each new batch was tested for sterility by incubating one aliquot at 37⁰C for 1 week. Before use, OADC and PANTA (Becton Dickinson, USA) were added into each tube to final concentrations of 5.5% and 0.22% to make working MODS media. The pH of this working solution is 6.5. One 48-well MODS plate (Becton Dickinson, USA) was set up each day. Seven hundred and fifty µls of working MODS media was aliquoted to each well and 250 µl processed sample was added. The pH of each well after adding processed sample was 7.0. One positive control (H37Rv) and one negative control well (sterile distilled water) were inoculated to each plate. Samples were inoculated into alternate wells to reduce potential for cross-contamination. 'Blank' wells contained MODS media only. To prevent cross-contamination from evaporation, plate seals (optical flims, Biorad) were used. The plate was incubated at 37⁰C, and the result was recorded every alternate day after five days of inoculation for evidence of growth. Any cord formation including long cords or comma-shaped cords was recorded as positive and no cord was recorded as negative. The negative results were confirmed at day 30. Contamination was recorded if there was any non-mycobacteria growth (based on microscopy and color) and/or turbidity in any negative control well.

2.6.2.1.2 MODS culture with 100µl processed sample

The MODS media was prepared with 5.9 g Middlebrook 7H9 broth (Difco, Sparks, MD), 3.1ml glycerol and 1.25 g bactocasitone (Difco, USA). The procedure was exactly the same as described in "MODS culture with 250µl processed sample".

Before each use, 2.5ml OADC and 500µl PANTA were added to 22ml aliquot of MODS to maintain the concentrations of OADC and PANTA of 5.5% and 0.22%, respectively. After that 900µl of working MODS media and 100µl processed samples were added into each well. Setting up the MODS plates, incubation and reading were followed the MODS culture procedure of 800µl samples.

2.6.2.2 Smear microscopy

Principle: *M.tuberculosis* an Acid-fast bacilli (AFB) in terms of morphology and staining color when observed under the microscope. However, microscopy cannot distinguish *M.tuberculosis* from other AFBs such as Mycobacteria Other Than Tuberculosis (MOTT). Since *M.tuberculosis* represents the overwhelming burden of AFB disease in high burden settings it is assumed that smear positive cases are infected with *M.tuberculosis* and a positive Acid-fast smear is considered sufficient indication to initiate anti-tuberculosis chemotherapy in these countries. Resource limitations do not permit the culture confirmation and mycobacterial speciation of all sputum smear positive cases.

Procedures:

2.6.2.2.1 Smear preparation

a. Direct smear preparation:

A new, clean and unscratched slide was used for each sputum sample. A portion of fresh specimen blood-speckled or yellowish cheesy mucus was transferred to the slide using a bacteriological loop. The sample was smeared over an area of 1x2cm

according to the guidelines of WHO (216). The smear was then dried at room temperature and fixed over a Bunsen flame before staining.

b. Homogenous smear preparation

This procedure was done in a class I biological safety cabinet. Two drops (approximately 100µl) of processed sample were transferred onto the middle of a slide by a Pasteur pipette. The drop was then smeared continuously and evenly over an area of 1x2cm using a plastic bacteriology loop. Next, the smear was left at room temperature for 15 – 30 minutes to air dry, followed by flame-fixing before staining by either ZN or fluorescent method according to the requirement of each substudy and WHO standard protocol (214, 216).

c. Panel slide preparation (NALC – NaOH – Formaldehyde):

Sputum samples with high volume (≥ 3 ml), yellowish cheesy mucous and either smear positive (2+ or 3+) or smear negative results were collected for panel slide preparation. In brief, 3ml of each sample was transferred to a 50ml Erlenmeyer tube, and 150µl Formaldehyde 40% was added. After incubation at room temperature for 30 minutes, an equal volume of NALC – NaOH was poured into the Erlen. This Erlenmeyer was then incubated on a stirring machine at 60°C for 30 minutes. The processed sample was divided into 2 – 3 centrifuge tubes (50ml tubes). Distilled water was added into each tube to make a final volume of 40ml. This tube was centrifuged at 3,000 x g for 20 minutes at room temperature. After discarding the supernatant, 2-3ml of deposit was used for dilution step. Smear negative processed deposit was used to dilute smear positive processed samples to make a final positive solution with the

required AFB number. Two drops of the final mixture were added to a new slide to make a smear. After being dried at room temperature, the smear was then incubated at 37°C for two days for fixing. For each batch of panel slides, 15 – 20% of slides were selected randomly for ZN staining and checking. If the reading result was not as expected, the batch was discarded and the whole process for panel slide preparation was repeated.

2.6.2.2.2 Staining methods

A maximum of 12 slides were placed onto a staining rack over the sink with a distance of at least 1cm between each slide as recommended by WHO (216).

a. Ziehl-Neelsen staining(216)

➤ Reagents: The reagents for ZN methods were prepared according to WHO guidelines

- Fuchsin 0.3%
- Decolourising solution: 3% acid-alcohol
- Counterstaining solution: 0.3% Methylene blue

➤ ZN staining procedure

First, smears were flooded with 0.3% Fuchsin It is mandatory that the smear area is completely covered with Fuchsin so any AFB present on the slide will come into contact with the Fuchsin. Second, these smears were heated until steaming to ensure the lipid layer of the bacteria melts to allow intercalation of fuchsin. After this step, all bacteria present on the slide will be stained with fuchsin color (pink-red). Third, all

free stain was washed away by a gentle stream of running water. To decolorize fuchsin color of bacteria other than AFB, HCL 3 % was used. This step exploits the Acid-fast property of *M.tuberculosis*, which will remain stained with fuchsin while other non acid-fast bacteria are decolourised with acid. Next, other particles on the smear (fiber, cells and other bacteria and fungi) were stained with 0.3% Methylene (blue color) to enhance contrast with red AFB. Last, the slide was viewed under a light microscope, AFB appear as a pink-red slightly curve rod shape against a blue background (**figure 2.2**).

b. Fluorescence staining(216)

➤ Reagents:

- Auramine O, 0.1%
- Decolourising solution: 0.5% acid alcohol
- Quenching solution: 0.5% Potassium permanganate (KMnO₄)

➤ FM staining procedure

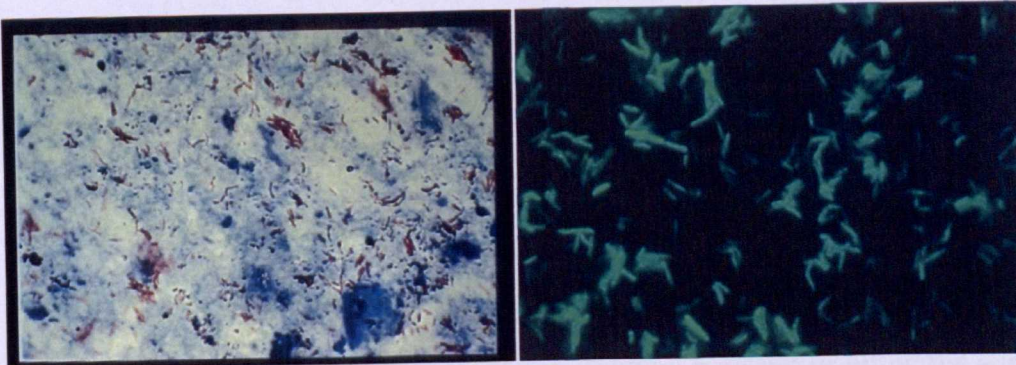
Slides were placed on a staining rack as described above in the ZN staining section. 0.1% auramine solution was then flooded onto these slides without heating. After incubating at room temperature for 20 minutes, auramine solution was gently washed away under running water and decolorization solution was applied (0.5% acid-alcohol for 3 minutes). Another running water wash step followed to make sure macroscopically visible stain had been washed away. Background staining was then carried out. Potassium permanganate 0.5% was flooded onto the slide for 1 minute. Finally, all excess stain on the slide was washed away with running water. The slide

was air-dried or dried on a hot plate. Critically, these slides must be kept away from strong light to prevent colour fading. These slides were then placed in boxes ready for reading. Under the microscope, AFB was recognized as bright-yellow slightly curved rod-shapes which was predominant on the dark background. This observation can be compared as stars on the night sky (**figure 2.2**).

2.6.2.2.3 Smear reading and recording

All smears were read under light microscope or fluorescence microscope according to the study protocol. Semi-quantitative results were recorded and reported following WHO guidelines (**table 2.1**) (216).

Figure 2.2 AFB observed under (a) light microscope (ZN staining) and (b) fluorescence microscope (Auramine staining)



(a) *ZN staining*

(b) *Auramine staining*

Table 2.1 Reading and recording results according to WHO guidelines for ZN staining and protocol of the LED study for fluorescence staining

| IUATLD/WHO SCALE (1000x field=HPF) Result | MICROSCOPY SYSTEM USED | | |
|---|--|--|--|
| | BRIGHTFIELD (1000x magnification; 1 length = 2cm = 100 HPF) | FLUORESCENCE (200-250x magnification; 1 length = 30 fields = 300 HPF) | FLUORESCENCE (400x magnification; 1 length = 40 fields = 200 HPF) |
| Negative | Zero AFB/1 length | Zero AFB/1 length | Zero AFB/1 length |
| Scanty (actual count) | 1-9 AFB/1 length or 100 HPF | 1-29 AFB/1 length | 1-19 AFB/1 length |
| 1+ | 10-99 AFB/1 length or 100 HPF (=1-9 AFB/10 fields) | 30-299 AFB/1 length | 20-199 AFB/1 length |
| 2+ | 1-10 AFB/1 HPF on average | 10-100 AFB/1 field on average | 5-50 AFB/1 field on average |
| 3+ | ≥10 AFB/1 HPF on average | ≥100 AFB/1 field on average | >50 AFB/1 field on average |

Source: Reference (216) and (64)

2.6.2.2.4 Slide storage at microscopy centers (peripheral laboratories) and transfer to Pham Ngoc Thach laboratory.

After the smear preparation procedure, all smears were put into slide boxes provided by FIND to prevent exposure to sunlight. After each reading, slides were put back into the boxes. All positive and negative smears (without marking result on the slide) were arranged in the boxes in the order of samples arriving to the laboratory according to LQAS guidelines.

At the end of each calendar month, all slide boxes were transferred to PNT laboratory for rechecking according to NTP standard operating procedures.

2.6.2.2.5 Smear rechecking and classification of errors

All smears were blindly rechecked by the microbiological laboratory at PNT hospital. The blind rechecking methods and numbers of slide to be rechecked depended on study and phase detailed in **chapter 4, section 4.3.1**.

Classification of errors (254):

MAJOR ERRORS

Major errors may indicate gross technical deficiencies, and include both High False Positive and High False Negative errors.

High False Positive: A negative smear that is misread as 1+ to 3+ positive.

High False Negative: A 1+ to 3+ positive smear that is misread as negative.

MINOR ERRORS:

Quantification Error: Difference of more than one grade in reading a positive slide between peripheral laboratory and reference laboratory.

Low False Positive: A negative smear that is misread as a low (1-9AFB/100fields) positive between peripheral laboratory and reference laboratory.

Low False Negative: A low (1-9AFB/100fields) positive smear that is misread as negative.

2.6.2.3 Bactec MGIT culture (Mycobacteria Growth Indicator Tube)

Principle: Mycobacteria are known to grow more rapidly in liquid than solid media (155). This commercial automated method (Becton Dickinson, New Jersey, USA) uses Middlebrook 7H9 broth for cultivation of mycobacteria. A fluorescent compound coats the bottom of each MGIT tube. The fluorescence is quenched by dissolved

oxygen in the media. Growth of micro-organisms in the tube will consume oxygen and therefore lessen oxygen concentration, leading to fluorescent emission, which can be detected by automatically by the MGIT machine or manually by observation under a UV light. A positive MGIT culture was then used for ZN-smear preparation. The observation of AFB by smear microscopy was considered confirmed positive MGIT culture for *M.tuberculosis*. Speciation of mycobacteria was not done to confirm *M.tuberculosis*, due to resource limitations.

Procedure: Processed samples were subjected to MGIT culture following the protocol of Becton Dickinson (13). In brief, 0.1ml PANTA, 0.5ml OADC and 0.5ml of each processed sample were added into a MGIT culture tube. The mixture was mixed by inversion by hand and then incubated at 37⁰C in the MGIT machine. Positive results were reported automatically by the MGIT system. Any positive MGIT culture was then subjected to ZN smear to determine acid-fastness, to confirm positive mycobacterial MGIT culture.

Figure 2.3 MGIT culture tubes



MGIT culture tubes. From left to right: tube 1 and tube 2: MGIT culture positive, tube 3: positive control of MGIT culture, tube 4: MGIT culture negative.

2.6.2.4 Lowenstein-Jensen (LJ) culture

Principle: LJ is the most widely used media for tuberculosis culture. It is an egg-based media and contains glycerol/pyruvate which favors the growth of *M.tuberculosis* complex/MOTT, respectively and malachite green which suppresses the growth of non-mycobacterial organisms. The LJ media is prepared in slants.

Procedure: Two LJ slopes were used for each sample. Two to three drops (0.1ml) of specimen suspension were inoculated on each slope. It is important to spread suspension evenly over the entire surface of the slope to ensure even distribution of inoculums. Screw-cap was loosened for incubation. The tube was then incubated at 37⁰C in a slanted position for 24- 48 hours and contamination was checked. After that, the tube was placed upright in racks for the remaining incubation time. The culture

tube was examined weekly for bacterial growth. However, if any mycobacterial growth was detected within 5 days of incubation, rapidly growing mycobacteria were suspected. Positive culture of *M.tuberculosis* is rough, crumbly, waxy, non – pigmented (cream coloured) and slow-growing. Colonies are detected from 3-5 weeks after inoculation. If no growth is detected after eight weeks, culture is recorded as negative. Quantitative results are recorded according WHO guidelines (214). The reporting of LJ cultures in this study is a qualitative result, ie. positive or negative.

Figure 2.4 LJ culture tubes



Colonies of Mycobacterium tuberculosis on Lowenstein-Jensen

Table 2.2 Semi-quantitative result of LJ culture – Recording

| Reading | Report |
|--------------------|---|
| No growth | Negative |
| 1-19 colonies | Positive: Record the number of colonies |
| 20 ~ 100 colonies | Positive: 1+ |
| 100 ~ 200 colonies | Positive: 2+ |
| 200 ~ 500 colonies | Positive: 3+ |
| >500 colonies | Positive: 4+ |
| Contamination | Contamination |

Note: Report the quantitative positive result based on the average colonies number of two slopes.

2.6.2.5 Identification test

Identification tests were performed on isolates suspected of being MOTT based on colony morphology of MODS culture, MGIT culture or LJ culture.

M. tuberculosis is identified on the basis of acid-fastness, and 4 biochemical tests:

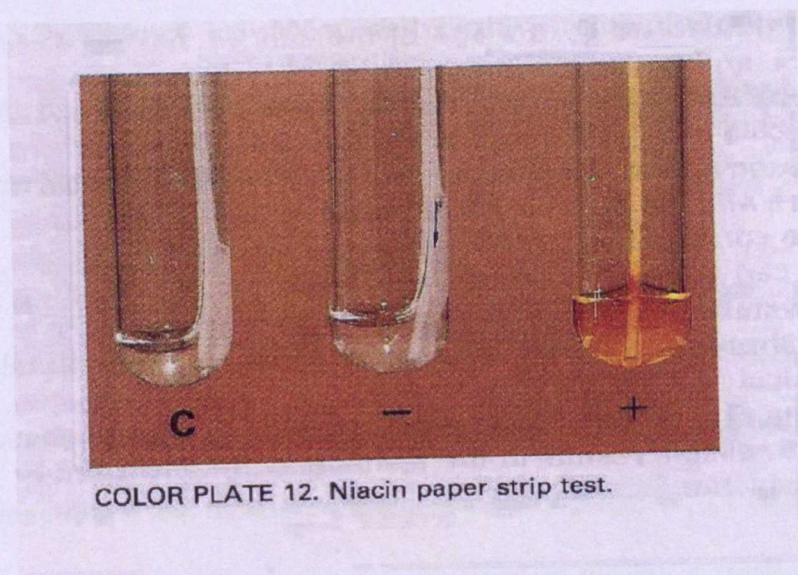
a. Niacin test

Principle: Niacin (also known as vitamin B3, nicotinic acid and vitamin PP) plays an important role in the oxidation-reduction reactions that occur during metabolic processes of all mycobacteria. Some species possess an enzyme that converts free niacin to niacin ribonucleotide. However, *M. tuberculosis* as well as some other

species lack this enzyme and accumulate niacin as a water soluble byproduct in the culture medium. Detection of accumulated niacin in egg-based media/liquid media is useful for identification. Niacin negative *M. tuberculosis* strains are very rare (214) and very few other mycobacterial species yield positive niacin tests.

Procedure: All cultures or subcultures of mycobacteria at 3-4 weeks in age on egg-based medium were used for this test. The test was performed with paper strip provided by BD (BD BBL Taxo TB Niacin Tests Reagents) or Nam Khoa Company (Nam Khoa TB Niacin Tests Reagents). In brief, 1ml of distilled water was added into a positive culture slant and make sure it covers the entire surface of the tube in 30 minutes. 0.5ml of the broth was then transferred to a sterile screw capped tube and a niacin paper was placed into the tube. Place the tube with tighten cap in approximately 30 minutes. The result was interpreted based on color change. If no color was observed, the negative result was record. If the strip turned yellow, the positive result was recorded.

Figure 2.5 Niacin reaction



Source: Reference (40)

b. Nitrate reductase assay

Principle: *M.tuberculosis* is classified as an obligate aerobe. However, in granulomas or culture tubes which may limit the availability of oxygen, *M.tuberculosis* enters a non-replicating persistent state 1 and state 2 (257). During this state, the reduction of nitrate to nitrite by the nitrate reductase enzyme encoded by *narGHJI* (258) occurs.

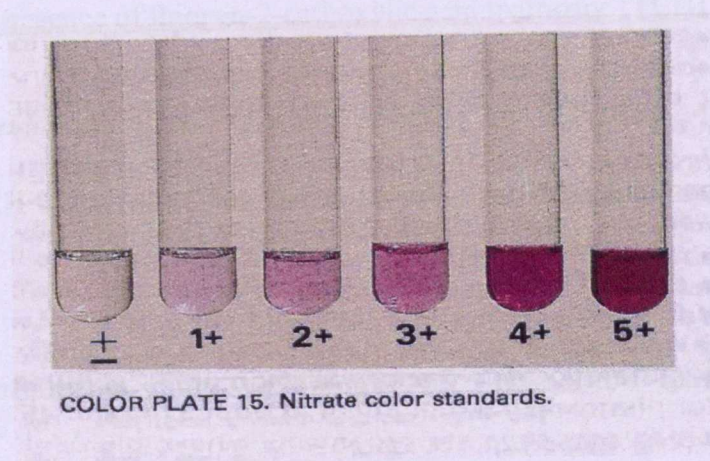
The nitrate reduction test, which measures the enzyme activity, is used to differentiate *M.tuberculosis* from other tubercle bacilli. The conversion of nitrate to nitrite is detected by nitrate reductase assay. *M.tuberculosis* exhibits strong nitrate reduction activity while *M.bovis* and *M.africanum* are usually negative. Only 20% of *M.africanum* and very rare strains of *M.bovis* give a positive test reaction.

Procedure: All cultures or subcultures of mycobacterium at 3-4 weeks in age on egg-based medium were used for this test. In brief, 0.2ml of distilled water was added to a screw-cap tube. After that, 2 loopfuls of colonies were transferred into the tube and emulsified in distilled water. The tube was then incubated at 37⁰C for 2 hours after adding 2ml NaNO₃ substrate and mixing well. Finally, 1 drop of Hydrochloric acid solution, 2 drops of Sulfanilamide solution (0.2%) and 2 drops of N-naphthylethylenediamine solution (0.1%) were added to the tube before reading the results.

If the pink color developed, a positive reaction was recorded. Otherwise, no nitrite was detected and a negative result was recorded. In some cases, the reduction has proceeded beyond nitrite and therefore the test resulted in negative. To determine if it is a true negative reaction (nitrate was not converted to nitrite), a small amount of

Zinc powder was added to negative tests. If nitrate is still present it will be reduced by the zinc powder and color will turn to red (true negative). If no color develops (no more nitrate present in the tube), nitrate was reduced beyond the nitrite and therefore the original reaction was positive.

Figure 2.6 Nitrate reductase reaction



Source: Reference (40)

c. Inhibition of growth by 500mg/l p-nitrobenzoic acid (PNB)

Principle: Inability to grow on medium containing p-Nitrobenzoic acid - 500mg/ml (PNB) is one of the key factors to differentiate *M.tuberculosis* from *Mycobacterium other than tuberculosis* (MOTT).

Procedure: All cultures or subcultures of mycobacterium at 3-4 weeks in age on egg-based medium were used for this test. In brief, bacterial suspension adjusted to McFarland turbidity standard No.1 was inoculated on to PNB-free LJ medium

(control) and PNB-containing LJ medium and the slopes were then incubated at 37°C in 28 days.

If confluent growth was found on both slopes, this isolate was MOTT. If abundant growth on the control tube but little or no growth on PNB medium, this isolate was *M.tuberculosis* complex strain. However, if no growth was detected on either slant, the test was repeated.

d. Growth in the presence of thiopen-2-carboxylic acid hydrazine (TCH)

Principle: *M.tuberculosis* is naturally resistant to Thiophen-2-Carboxylic acid hydrazide (TCH 2 µg/ ml). This test is valuable for differentiation *M.tuberculosis* from *M.bovis*.

Procedure: Performance of this test is similar to 1% proportional DST method

2.6.3 Microbiology tests for diagnosis of MDR-TB

2.6.3.1 Indirect drug-susceptibility testing – 1% proportional DST method

Principle: Drug susceptibility testing determines the susceptibility of a microbe to a certain microbial drug at a specific concentration.

Procedure: Phenotype indirect DST method was done at the Reference TB laboratory at PNT Hospital, which is accredited for DST by the reference TB laboratory of Western Pacific region. Drug-free LJ slants (control tubes) and drug-containing LJ slants (Isoniazid 0.2µg/ml, Streptomycin 4µg/ml, Rifampicin 40µg/ml and Ethambutol 2µg/ml) for traditional DST were prepared locally. The All positive LJ cultures with no less than 4 weeks old were prepared for indirect DST method and

H37Rv and *M.tuberculosis* complex strains with well characterised drug susceptibility profile were used as control strains. In brief, several loops of positive culture were transferred into a sterile flask containing 8-10 glass beads at 1mm size. It is important to have representatives of all colony types present in the culture tube. The flask containing *M.tuberculosis* colonies were vortexed for 30 seconds and then 2-4ml 7H9 Middlebrook were added. The mixture was shaken well to make a suspension. One to two mls of supernatant from a suspension were transferred to a 16.5 x 128mm sterile screw-capped tube to adjust for a density of McFarland #1 (called the P concentration) with sterile distilled water. A serial dilution of a suspension to the concentrations of 10^{-2} and 10^{-4} was made. Finally, suspensions with diluted concentrations of P, 10^{-2} and 10^{-4} were transferred to drug-free slants and drug-containing slants for traditional DST. Two hundred microlitres of each diluted concentration was transferred into two control tubes and drug-containing tubes. All of these tubes were placed in a slanted position with slightly loosened screw caps and incubated at 37°C for 24 – 48 hours. After that, all screw caps were tightened and all tubes were placed upright. They were continuously incubated at 37°C until checking.

The DST results were read on the 28th day and the 42nd day after inoculation. Resistance was determined based on the percentage of colonies observed on drug-containing slants over the number of colonies observed on control slants. If the percentage of resistance was less than 1%, the strain was recorded as a susceptible strain; otherwise it was recorded as a resistant strain. The DST was repeated when the colony count in the control tube was less than 200 or the percentage of resistance organisms appeared to approach 1%, by subjective assessment.

2.6.3.2 MODS for diagnosis of MDR-TB

Principle: The principle of drug susceptibility by MODS is similar to MODS culture. However, this procedure includes drug-free wells (control wells) and wells containing the drug of interest. The results of drug-containing wells are read on the same day that the drug-free control well becomes positive. If cording growth is observed in the control wells but not in the drug-containing well, a susceptible isolate is deemed to be present in this sample. If cording growth is observed in both the control wells and the drug-containing wells, a resistant isolate is present in the investigated sample.

Procedure: For each processed sample, 2 drug-free wells (control wells), 1 INH containing well and 1 RIF containing well were set up. In brief, the MODS media was prepared with 5.9g middle brook 7H9 broth (Difco, Sparks, MD), 3.1ml glycerol and 1.25g batocastione (Difco, USA) in 880ml distilled water. This media was autoclaved, filtered and stored in 4.5ml tubes at 4°C. Each new batch was tested for sterility by incubating one aliquot at 37°C for 1 week. Before use, 0.5ml OADC, 0.5ml processed samples and 100µl PANTA antibiotic were added into each 4.5ml tube to have a working MODS media. 900µl of the working MODS media was then transferred to each of four wells in a 48 well-plate as described above. Next, 100µl distilled water was added into the control wells. Finally, 100µl INH 4ug/ml (Sigma) or 100µl RIF 10µg/ml (Sigma) was added to the INH-containing well and RIF-containing well, respectively. The final concentrations of OADC and PANTA in each well were 10% and 20µl/ml, respectively. The drug concentrations in each well were 0.4µg/ml for INH and 1µg/ml for RIF. Daily, one susceptible clinical isolate (H37Rv), one INH-resistant clinical isolate and one RIF-resistant clinical isolate were

used as the control. A suspension of each isolate was made and its turbidity was measured against Mc Farland 0.5 (approximately 10^4 CFU/ml - Pasteur Institute, HCMC standard). After that, a 100 fold dilution (10^2 CFU/ml) was used.

The plate was incubated at 37°C , and the results were recorded on alternate days from day 5 to day 15 and twice a week from day 16 to 1 month. If there was any cord formation in both control wells, the drug containing wells were read. If cords were detected in only one control well, MODS-DST was recorded as uninterpretable for technical analysis. Any growth in the control and drug-containing wells was recorded as resistant. If growth was observed in control wells but not in the drug-containing wells, a susceptible result was recorded.

2.6.4 Subculture

Procedure: All positive cultures were subcultured on LJ media (Becton Dickinson) in duplicate for further investigation and incubated at 37°C for several weeks. These isolates were then used for standard biochemical identification tests, indirect susceptibility testing, DNA extraction and archiving.

2.6.5 Archiving

Procedure: Positive-LJ subculture was archived in 7H9 media supplemented with 20% glycerol (7H9A). One or two loops of isolates collected from LJ subculture were transferred into a cryotube containing 7H9A medium. These tubes were stored at -20°C for archiving.

2.6.6 Molecular tests

2.6.6.1 DNA extraction

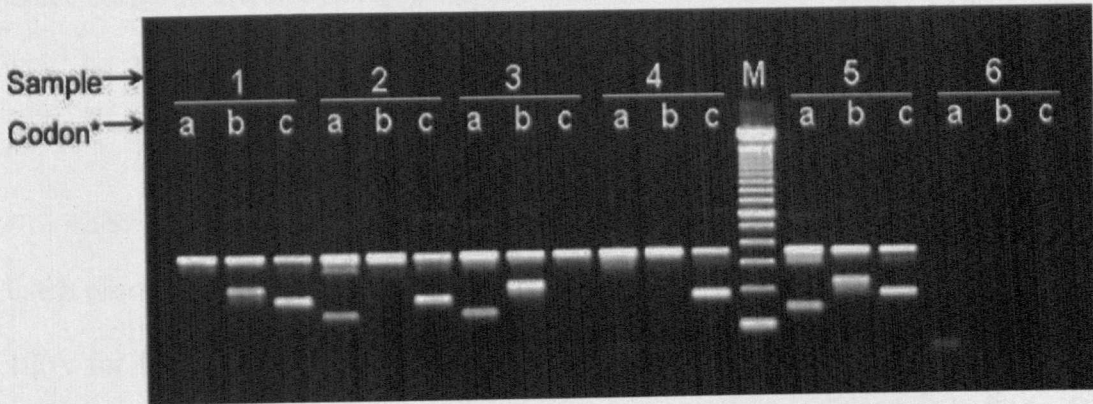
Procedure: DNA extraction was carried out for all positive cultures using the CTAB method (210). Mycobacteria were harvested from Lowenstein-Jensen slopes, placed into 400µl of 1X Tris-HCl EDTA (TE), and incubated at 80°C for 20 minutes to kill the mycobacteria. After that, 50µl of 10mg/ml lysozyme was added and the tube was incubated at 37°C overnight. Next, 75µl of 10%SDS/proteinase K solution was added and the tube was incubated at 55°C in 30 minutes. Then, 100µl of 5M NaCl and 100µl of prewarmed CTAB/NaCl solution (N-cetyl-N,N,N,-trimethylammoniumbromide and NaCl) were added and the tube was vortexed until opaque and incubated at 65°C for 15 minutes. DNA was extracted by the standard ethanol-chloroform extraction method. Seven hundred and fifty microlitres (750µl) of chloroform/isoamyl alcohol (24:1) was added to the tube and vortexed for 10 seconds, centrifuged at 11,000g for 8 minutes. The supernatant (aqueous phase) was transferred to a microcentrifuge tube and 0.6 volume of isopropanol was added. The mixture was incubated on ice for 10 minutes and centrifuged at room temperature for 15 minutes. After discarding the supernatant, the pellet was washed with 70% ethanol and centrifuged at room temperature for 5 minutes. The pellet was dried at room temperature and dissolved in 20µl of 1X TE buffer. The DNA was stored at 4°C for further use.

2.6.6.2 PCR for detection of mutations

a. Multiplex allele specific polymerase chain reaction (MAS-PCR) for detection of RIF resistance mutations.

This technique was performed following the method developed by Tho *et al* (190). Three separate PCR reactions were performed with the same outer primers (Proligo, Singapore) to amplify the 350bp fragment of rifampicin resistance determining region (RRDR) of *rpoB* gene, and one of three inner primers (Proligo, Singapore) targeted to wild type at hot spot codons 516, 526 and 531. The RRDR region was detected in all isolates, both wild type and rifampicin resistant isolates. Amplification of the second band occurred only in wild-type isolates with no mutation at the site targeted by the inner primer. Each PCR reaction contained 0.75 U Taq DNA polymerase (Bioline, Taunton, MA, USA), 0.2 mM of each dNTP (Roche, Basel, Switzerland), 125–400 nM of primers and 2 mM MgCl₂. Fifteen nanograms of genomic DNA was added. All of the reactions were amplified under the same thermocycling programme: 95°C for 1 min, followed by 35 cycles of 95°C for 10 s, 68°C for 20 s, 72°C for 20 s and a final step of 72°C for 5 min. A negative control (water) and a positive control for each of the three target codons were included with each batch processed. PCR products were analyzed by electrophoresis on 1% agarose gel at 150V for 30 minutes.

Figure 2.7 MAS-PCR for detection of RIF resistance mutations in the *rpoB* gene



Amplicon profiles in MAS-PCR of isolates carrying mutations at each site.

* a, codon 516; b, codon 526; c, codon 531.

Sample 1: mutation at codon 516.

Sample 2: mutation at codon 526.

Sample 3: mutation at codon 531.

Sample 4: codon 516 and 526 mutated.

Sample 5: wild type control sample.

Sample 6: negative control.

M = 100 bp ladder; MAS-PCR = multiplex allele specific polymerase chain reaction; bp = base pair.

b. MAS-PCR for detection of INH resistance mutations (191): Three primer sets (Proligo, Singapore) were used for multiplex PCR and sequencing. The first primer set targets *Hsp65* (HSP65F1 and HSP65R1) which is specific for *M.tuberculosis* and serves as control. The second primer pair targets the *inhA* promoter region (TB92 and *inhARmut*), with the reverse primers designed to detect mutation C-15T in this region. The third primer set (*katg2F* and P6) is located inside the *katG* gene where the forward primer will yield a PCR product if the isolate is wild type. Each PCR reaction contained buffer 1X, 1U Taq DNA polymerase (Bioline, Taunton, MA, USA), 0.2mM of each dNTP (Roche, Basel, Switzerland), 0.15 μ M – 0.3 μ M of each primer and 2mM MgCl₂.

Fifteen nanograms of genomic DNA were added. All of the reactions were amplified under the same thermocycling programme: 95°C for 2 min, followed by 10 cycles of 95°C for 20 s, 62°C for 1 min, 72°C for 20 s, then 25 cycles of 95°C for 20 s, 62°C for 20 s, 72°C for 40 s and a final step of 72°C for 5 min. A negative control (water) and a positive control for each of the three target codons were included with each batch processed. PCR products were analysed by electrophoresis on 1% agarose gel at 120V for 40 min.

2.6.6.3 Spoligotyping

Principle: This method is based on DNA polymorphism present at a certain chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *Mycobacterium tuberculosis* complex bacteria (104). In *M.tuberculosis complex*, the DR region consists of repeated sequences of 36 base pairs, which are interrupted by DNA spacers with each containing 35 to 41 base pairs in length. Forty-three spacers of known sequence, in total, were investigated in the DR region. Spoligotyping method is used to determine the presence or absence of 43 spacers by hybridization of PCR-amplified spacer DNA to a set of immobilized oligonucleotides, representing each of the unique spacer DNA sequences.

Procedure:

Spoligotyping was done according to the standard international Spoligotyping protocol (103, 104).

- a. Chromosomal DNA extraction: See section 2.6.6.1

b. Amplification of spacer DNA by PCR (104)

Since spacers are interspersed in the DR region, it is important to amplify the whole DR region by the primers DRa (5'-GGT TTT GGG TCT GAC GAC- 3', biotinylated at 5' end) and DRb (5'-CCG AGA GGG GAC GGA AAC-3') to amplify copies of spacers. The PCR reaction contained 20ng template DNA, 4µl primer DRa (20pmol-biotinylated) (Ocimum Biosolutions, Hyderabad, India), 4µl primer DRb (20pmol) (Ocimum Biosolutions,), 4µl dNTP mixture (2.5mM each dNTP)(Roche, Ho Chi Minh City, Vietnam), 5µl concentrated Super *Tth* buffer (10X)(Bioline, London, UK), 0.1µl Super *Tth* polymerase (5U/µl) (Bioline) and MQ water. The final volume of a PCR tube was 50µl.

c. Hybridization with PCR product and detection (104)

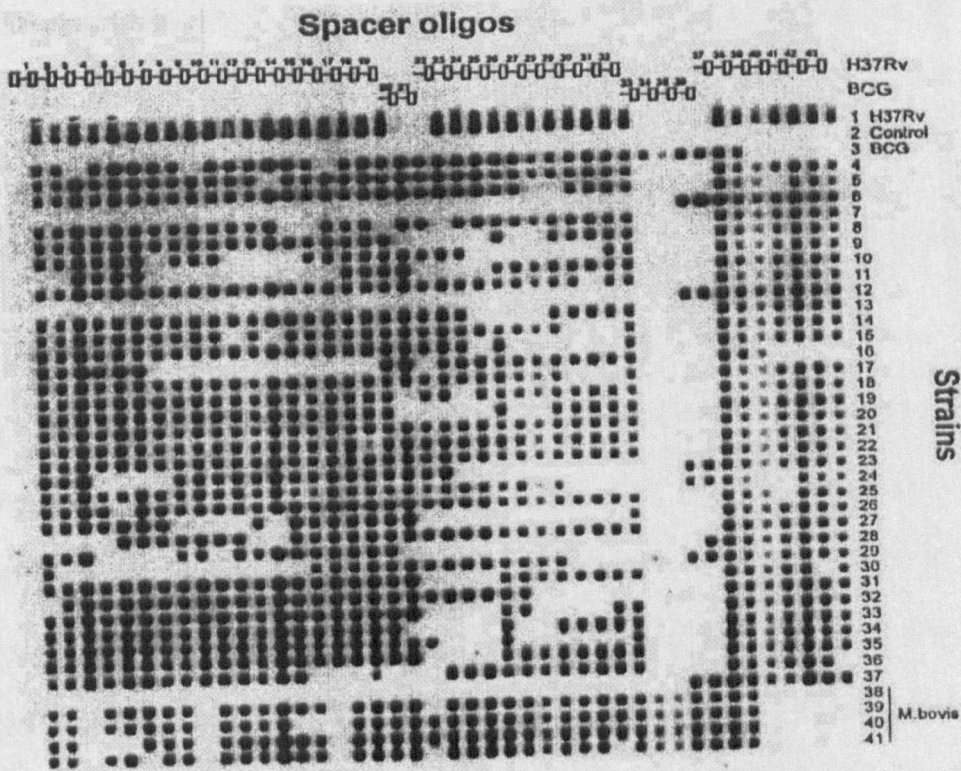
Biotin-labeled PCR products were hybridized with membrane bound immobilized spacer-oligos (Ocimum Biosolutions). After incubation with streptavidin-peroxidase (Roche) and ECL detection (Enhanced Chemo-Luminescence Detection solution) (GE Healthcare, London, UK), the presence of spacer on film (GE Healthcare) is visualized as black squares.

d. Result interpretation (104)

The presence of each spacer is indicated by the presence of black squares on the film; if the spacer is absent from the strain, the relevant square remains blank. The result is recorded as a binary readout indicating the presence (represented as 1) or absence (represented as 0) of the 43 spacers in sequence. This 43-digit binary number can be presented in a shorter format called the octal code, in which a 3-continuous-digit

binary number can be converted to a single digit octal number. These formats facilitate inter-laboratory comparison of genotypes (54). A shared-type (ST number) is described as a spoligotyping pattern common to at least two isolates. There are 1939 shared-types described in the fourth international spoligotyping database (25).

Figure 2.8 Spoligotyping



A typical spoligotyping result of M.tuberculosis H37Rv, M. bovis BCG P3 and 38 different clinical isolates. A membrane with 43 spacer oligonucleotides was used (vertical lines).

Source: Reference (104)

2.7 DEFINITION AND TB CLASSIFICATION FOR THE MODS STUDIES

2.7.1 Pulmonary tuberculosis

The definition of pulmonary TB was based on microbiological confirmation by either smear or MGIT, intention to treat, treatment management and outcome.

Tuberculosis was defined as “**confirmed TB**” if the patient had clinical symptoms consistent with TB (221, 225) and either smear or MGIT was positive in any sample, including samples which were collected before the enrollment started. These samples were not included in the sensitivity comparison but patients with prior samples positive in this illness episode by either smear or MGIT were classified in the “confirmed TB” group. A positive MODS culture was not considered as part of the definition of ‘confirmed TB’ because this was the test under evaluation.

The patient was defined as “**probable TB**” on ‘intention to treat’ if the patient had clinical symptoms consistent with TB (221, 225) but had no microbiological confirmation, received no alternative diagnosis and initiated TB treatment and transferred to a District Tuberculosis Unit for treatment and follow-up. Patients who satisfied the first two characteristics of “probable TB” but self-discharged prior to treatment were also classified in this group if the clinician intended to treat for TB. It was impossible to either rule-out or confirm TB in this group due to the lack of microbiological confirmation.

Patients were defined as “**TB unlikely**” if they recovered without TB treatment, had TB treatment but deteriorated or received an alternative diagnosis and treatment. It was impossible to ‘rule-out’ TB in these patients completely because clinical

deterioration on therapy may have been due to undetected drug-resistant TB. HIV screening is not a routine test for patients at PNT Hospital. HIV counseling and testing was offered if HIV was clinically suspected by the treating clinician, according to routine guidelines.

2.7.2 Tuberculous meningitis

The definition of TBM was based on microbiological confirmation by either smear or MGIT, intention to treat, treatment management and outcome.

- **“Confirmed TBM”** was defined as: patients with clinical symptoms consistent with TBM and either smear or MGIT positive from CSF collected in the study only. A positive MODS culture was not considered as part of the definition of ‘confirmed TBM’ because this was the test under evaluation.
- **“Probable TBM”** was defined as patients with clinical symptoms consistent with TBM but: no microbiological confirmation by either smear or MGIT for CSF samples collected in the study and at least one of the following characteristics, AFB found in samples other than CSF which were collected before or after enrollment, or radiological symptoms suspected of tuberculosis on chest X-ray or CT/MRI. In addition, these patients had received no alternative diagnosis, initiated TB treatment and were transferred to a District Tuberculosis Unit (DTU) for treatment and follow-up. Patients who satisfied the above characteristics of “probable TB” but self-discharged prior to treatment were also classified in this group if the clinician intended to treat for TBM.

- **“Possible TBM”** on intention to treat was defined as patients with a clinical presentation suspected of TBM and satisfying at least 4 of the following characteristics: TB history, more than 5 days of illness, increased CSF lymphocyte, CSF blood/glucose ratio <0.5 , altered consciousness, focal neurological signs or PCR positive on any CSF sample collected before the study. Patients who satisfied the above characteristics of “possible TBM” but self-discharged prior to treatment were also classified in this group if the clinician intended to treat for TB.
- **“Not TBM”** was defined as patients who did not fulfill the characteristics of the above groups, recovered without TB treatment or received an alternative diagnosis. This group also included patients receiving TB treatment before an alternative diagnosis was confirmed.

2.8 STATISTICS

The statistics of the MODS and iLED studies were performed under the supervision of Dr. Marcel Wolbers, Head of Bioinformatics, OUCRU.

2.8.1 The MODS study

The MODS study at Hospital for Tropical Diseases (Evaluation of MODS in TBM diagnosis – pilot phase)

The sensitivity, specificity, positive predictive value and negative predictive value for MODS were determined and compared for homogenous ZN smear and MGIT and LJ culture by Mc.Nemar’s test for patient analysis and Marginal regression model for sample analysis, using clinical diagnosis (instigation of anti-tuberculous chemotherapy) as the gold standard. Time to positive result for the three culture

methods was compared using Wilcoxon signed rank test. Samples were excluded where patient medical records were not available for analysis.

The MODS study at Pham Ngoc Thach Hospital (Evaluation of MODS in diagnosis of TBM – implementation phase, paediatric TB, HIV/TB and MDR-TB)

In the “per patient” analysis, reported confidence intervals for accuracy measures (sensitivities, specificities, positive and negative predictive values) were calculated according to the method of Pearson and Clopper (153). Comparisons of accuracies between tests were done using Mc.Nemar’s test.

In the ‘per sample’ analysis, a binary marginal generalized linear regression (GLM) models with an identity link function was used for all analyses. These models are very flexible, allow for the inclusion of covariates and account for the fact that results of multiple samples from the same patient or test results of different tests on the same sample may be dependent (153). Specifically, marginal regression model was used to calculate confidence intervals for accuracy measures, to compare the sensitivities of smear, MGIT, and MODS and to assess the impact of the duration of TB treatment on the sensitivity of MODS.

For the ‘per sample’ analysis, time-dependent sensitivity curves for MGIT and MODS were also calculated. A test result was considered as positive by time t if the respective test was positive overall and reached the positive value at most t days after sample collection. Time-dependent sensitivity curves were estimated with the Kaplan-Meier method and samples without a positive test result were formally regarded as censored on day “infinity”. Time-dependent sensitivities of MGIT and MODS by

days 7 and 14, respectively, were compared using a marginal regression model as described above. In addition, the time to positive MGIT and MODS, respectively, was compared in samples where both tests reached positivity with the Cox proportional hazards regression model.

Comparison of demographic and clinical features of patients between TB diagnoses (definite, probable, possible or unlikely) was done with Fisher's exact test for categorical data and the Kruskal-Wallis test for continuous data.

2.8.2 The iLED study

Reported confidence intervals for detection rates, sensitivity, specificity, false positive and false negative rates were calculated according to the method of Pearson and Clopper. Comparisons of these measurements between different time points were done using Fisher's exact test.

The time to result of ZN-iLED and FM-iLED 40X were compared by using the Wilcoxon Rank-sum test

For both studies, all reported confidence intervals are two-sided 95% confidence intervals and p-values ≤ 0.05 were regarded as statistically significant. All analyses and graphs were performed with Stata version 9 (Statacorp, Texas, USA).

Chapter 3

EVALUATION OF MODS

3.1 Section 1.

MODS INTRODUCTION

Microscopic Observation Drug Susceptibility Assay (MODS) is a non-commercial direct liquid based culture method, developed by a group of scientists in Peru for early detection of MDR-TB isolates (33). In this test, processed sample is inoculated into a 48-well plate containing 7H9 media with and without drug and then incubated at 37°C for at least 5 days. The principle of the test is to observe the presence or absence of serpentine cording in wells containing mycobacteria. It is a rapid, low cost and adaptable method in comparison to LJ and MGIT cultures. Based on this principle, MODS has been evaluated for early detection of TB. Previous studies have shown it sensitive, specific, economical and easy to perform for early diagnosis of TB and MDR-TB in adults and children in high burden countries (20, 33, 132, 133, 144). A systematic review showed that the pooled sensitivity and specificity of MODS were 98% and 99.4%, respectively for detection of rifampicin resistance and 97.7% and 95.8%, respectively for detection of isoniazid resistance (124). In 2009, MODS has been endorsed by WHO for rapid screening of patients suspected of MDR-TB in high burden countries as an “interim” solution (238). Equipment requirements are minimal with a level I Biological Safety Cabinet and an inverted microscope required, making it ideal for use in high TB burden settings.

In this thesis, MODS is evaluated in **chapters 3.2, 3.3, 3.4 and 3.5** for the diagnosis of TB in 3 patient groups, which are difficult to confirm by smear microscopy due to paucibacillary specimens: tuberculous meningitis, paediatric TB, HIV associated TB; and also for MDR-TB identification.

3.2 Section 2.

MODS IN DIAGNOSIS OF TUBERCULOUS MENINGITIS

3.2.1 INTRODUCTION

Tuberculosis meningitis (TBM) is the most severe form of tuberculosis disease caused by *Mycobacterium tuberculosis*. TBM causes death or severe long-term disability in as many as two thirds of treated TBM patients (197). Early diagnosis and appropriate TB treatment regimen improves treatment outcomes and reduces neurological sequelae in survivors.

Three options are currently available for the rapid diagnosis of TBM: diagnostic algorithms, nucleic acid amplification tests (NAAT) and microbiological tests (smear, culture). Diagnostic algorithms based on simple clinical and laboratory features can be sensitive and specific (196) but require further evaluation, especially in HIV-infected patients and children and may not be generalisable outside the setting in which they are developed since the spectrum of differential diagnoses will vary. Nucleic acid amplification techniques require highly trained technicians, capital equipment purchase and maintenance, specialized consumable supply chains, alongside rigorous quality control to guard against contamination. Rapid commercial NAAT tests are relatively expensive and are not more sensitive than smear microscopy (149, 194). Therefore, microbiological tests are commonly used for TB diagnosis, especially in resource-limited high burden countries. Among

microbiological tests, smear microscopy is widely used because of its simplicity, reproducibility, speed, low cost, high specificity, and the theoretical ability to delimit contagiousness if a standardised protocol of smear preparation is followed strictly. However, sensitivity of smear microscopy is rather low; detecting approximately 50% of active pulmonary TB cases and largely varies depending on bacterial load of clinical samples.

Microbiological diagnosis is more challenging in TBM cases because of the low number of bacilli in cerebrospinal fluid (CSF) samples. Larger CSF volumes (>6mls) and meticulous examination of slides (at least 30 minutes) increases the sensitivity of CSF smear up to 60% (98, 195). However, large volumes of CSF are rarely submitted to the laboratory, particularly from paediatric patients. In addition, it is often not feasible to devote enough time to examine a single slide in high-throughput routine laboratories. Although culture methods gain higher sensitivity than smear microscopy, the prolonged time to positive (about two weeks for liquid culture and approximately one month for solid culture) and high cost has limited their use in low income countries. Therefore, early diagnosis of TBM remains a challenging problem.

Direct susceptibility testing from CSF samples is unlikely to yield high sensitivity due to low numbers of bacilli in the specimens. Furthermore, it is often seen that CSF samples divided and tested by more than one method can be positive by one method but negative by another; this is thought to be due to the clumping of bacilli in aliquots of a sample (194). In the case of smear positive/culture negative samples, the smear technique may be detecting dead bacilli present in the sample, especially in patients already on TB therapy. In these cases, the patient will have shown clinical

improvement however the inability to differentiate viable bacilli remains a limitation of smear microscopy familiar to TB clinicians. Low bacterial load and clumping of bacilli are likely to lead to many uninterpretable and false negative results in direct susceptibility testing. This study has therefore evaluated MODS as a technique for rapid identification of *M.tuberculosis* in the CSF in comparison with smear, MGIT and LJ culture against a clinical gold standard.

3.2.2 METHOS

3.2.2.1 Enrollment

3.2.2.1.1 The pilot phase

Routine diagnostic CSF samples were collected from consecutive patients (age ≥ 18 years) with clinically suspected TBM presenting to HTD during the study period. All newly presenting patients were enrolled into this phase. Patients on TB treatment for at least one day were excluded from analysis. All patients were tested for antibodies to human immunodeficiency virus (HIV) as part of routine care at HTD. In some cases, more than one CSF was collected for diagnosis, as deemed appropriate by the treating physician. All samples included in this phase were taken as part of routine clinical care and the study did not involve any change to routine patient care and therefore specific informed consent was not sought for the laboratory evaluation. This is consistent with Oxtrec guidelines for patient consent waiver. In addition to routine biochemistry tests, all CSF samples were also examined by Gram-stain, and culture on blood, chocolate and sabouraud dextrose agar, to exclude bacterial meningitis and India ink stain to exclude cryptococcal meningitis.

This phase collected laboratory data and clinical diagnosis of enrolled patients.

3.2.2.1.2 The implementation phase

The implementation phase was conducted at PNT hospital. All eligible patients (**chapter 2, section 2.4**) presenting at the PNT hospital were enrolled into the study. Data on socioeconomic and demographic features, TB history, TB contact history, HIV status and presenting clinical features were prospectively collected on a standard case report form. HIV screening test is not routine at PNT hospital and is usually only performed if clinical suspicion of HIV is high. As for the pilot study, one routine diagnostic CSF sample was collected as per routine care as deemed appropriate by the treating physician and no additional samples were collected as part of this study.

3.2.2.2 Definition of TBM classification

The definition of TBM classification is described in **chapter 2, section 2.7**

3.2.2.3 Sample collection, sample processing and tests

The sample collection, sample processing and tests are described in materials and methods (**chapter 2, section 2.5 and section 2.6**)

The volume of CSF deposit used was different in the pilot and implementation phases. 100 μ l deposit of CSF was used for the pilot phase according to Park *et al* (151) and 250 μ l was used for the implementation phase. The CSF volume was increased in the implementation phase in an effort to increase the detection rate of the MODS technique because the detection rate has been shown to be related to CSF volume

(195) and the sensitivity of MODS in the pilot phase was slightly lower than MGIT with one difference between the techniques being the volume of inoculum. For paucibacillary samples, such as CSF, larger sample volumes have a higher probability of containing sufficient viable bacilli for detection.

Spoligotyping was done in the implementation phase for all cultures positive by MODS (n=37). CSF samples processed on the same day and inoculated on the same MODS plate were investigated for probable cross-contamination.

3.2.3 STATISTICAL METHODS

The statistical methods were described in **chapter 2, section 2.8**

In both the pilot and implementation phases of this study, the detection rates of MODS were also evaluated stratified by the HIV status of the patient.

Of note, in the implementation phase (study at PNT hospital), there were only 8/165 patients classified in the “Not TBM” group which is a small proportion of the study population. This was due to the fact that PNT hospital is a referral centre and patients being evaluated by lumbar puncture for TBM had a high clinical index of suspicion. It was possible to ‘rule-out’ TBM in only a small number of patients in whom another diagnosis was confirmed or who recovered without TB therapy. As we did not have a sufficient number in the ‘non-TBM’ population to make a valid comparison, the accuracy of smear, MGIT and MODS were compared in the “definite TBM” group and the “clinical diagnosis” group (definite, probable or possible TBM).

3.2.4 RESULTS

3.2.4.1 The pilot phase (at Hospital for Tropical Diseases)

3.2.4.1.1 Enrollment and TBM classification

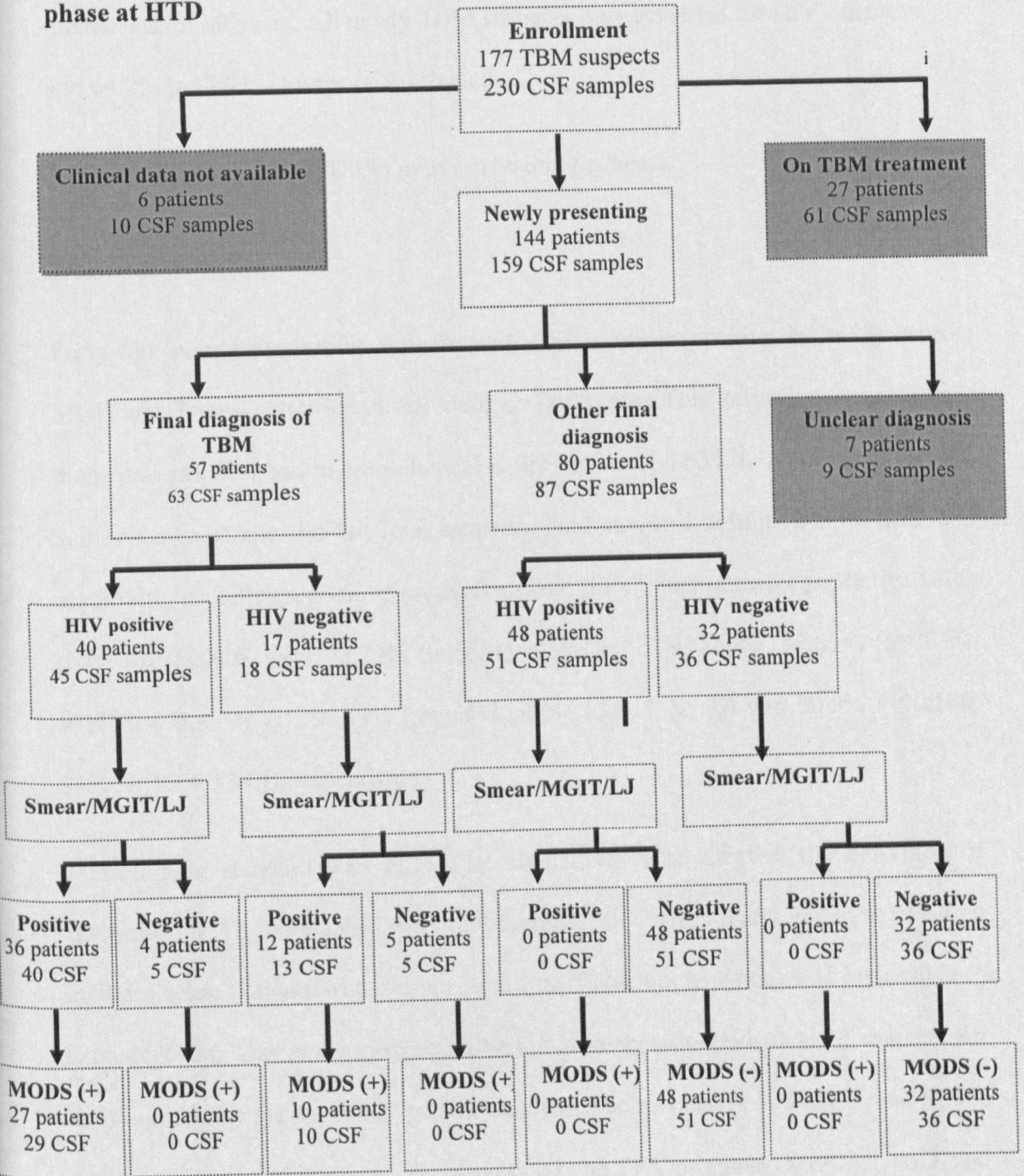
177 TBM suspects were enrolled into this phase. There were 6 patients for whom clinical data was not available and 7 patients with an unclear diagnosis, usually due to early self-discharge, who were excluded from the analysis. Finally, data of 137 newly presenting patients and 27 patients (61 CSF samples) who were on TB treatment at least one day were analysed separately (**figure 3.1**).

All CSF samples were sent to the OUCRU laboratory immediately or stored in the fridge until processing. The median (IQR) CSF volume of newly presenting patients and follow-up patients were 4ml (3-5ml) and 3ml (2-4ml), respectively. The characteristics of CSF samples from newly presenting patients are described in **table**

3.6

Among 137 newly presenting patients, 41.6% (n=57/137) patients were deemed to have TBM by clinical diagnosis, of which, 78.9% (n=45/57) were classified as definite TBM, 3.50% (n=2/57) as probable TBM and 17.56% (n=10/57) as possible TBM. The remaining newly presenting patients (58.4%, n=80/137) were in “not TBM” group.

Figure 3.1 Study flow chart of MODS evaluation for TBM diagnosis - the pilot phase at HTD



¹ This population is analysed separately

Seventy-nine percent (n=108/137) of the newly presenting patients were male with a median age of 30 years. All newly TBM suspects were screened for HIV infection and 64.2% (n=88/137) were HIV-infected.

3.2.4.1.2 Accuracy of MODS in newly presenting patients

By patient analysis

Forty-five newly presenting patients with CSF samples positive by either smear, MGIT or LJ were classified in the “definite TB” group. This definition includes only those patients who had microbiological confirmation of TBM by a reference method and is the most specific but least sensitive gold standard definition for TBM. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of MODS were 77.79% (n=35/45, 95%CI: [62.9, 88.8]), 97.83% (n=90/92, 95%CI: [92.4, 99.7]), 94.59% (n=35/37, 95%CI:[81.8, 99.3]) and 90.0% (n=90/100, 95%CI:[82.4, 95.0]), respectively.

Clinical gold standard was defined as all patients who satisfied the definition of “Definite TBM”, “Probable TBM” or “Possible TBM”. It is likely that this definition includes some patients who did not have true TBM, but in whom it is impossible to exclude TBM. This is the most sensitive but least specific gold standard measure for defining TBM patients. In ‘by patient’ analysis, the sensitivity of MODS was 64.9% against clinical gold standard. The specificity and PPV of smear, MODS, MGIT and LJ were 100%. The sensitivity and NPV of all four methods are described in **table 3.1**. No significant difference was observed between methods. The sensitivity of MODS against smear, MGIT and LJ culture as the gold standard were 90.0%

(n=27/30, 95%CI:[79.2, 100]), 82.5% (n=33/40, 95%CI:[65.2, 92.7]) and 77.5% (n=31/40, 95%CI:[61.5, 89.2]), respectively.

Table 3.1 Sensitivity and negative predictive value of smear, MODS, MGIT and LJ against clinical gold standard in the pilot study of MODS evaluation for TBM diagnosis.

| | Smear | MODS | MGIT | LJ |
|----------------------------------|--------------|---------------|--------------|--------------|
| Sensitivity | P1=0.49 | P2=0.79 | P3=1.00 | P4=0.34 |
| % | 52.6 | 64.9 | 70.2 | 70.2 |
| n=x/y | 30/57 | 37/57 | 40/57 | 40/57 |
| 95% CI | [40.1, 65.9] | [52.7, 77.3] | [58.0, 82.0] | [58.0, 82.0] |
| Negative predictive value | P1=0.74 | P2=0.88 | P3=0.69 | P4=0.64 |
| % | 74.8 | 80 | 82.5 | 82.47 |
| n=x/y | 80/107 | 80/100 | 80/97 | 80/97 |
| 95% CI | [65.4, 82.6] | [70.8, 87.33] | [73.4, 89.4] | [73.4, 89.4] |

Summary measure is n (%) for these two measurements.

P values (by Mc.Nemar's test) were used for comparison of all four methods.

P1: for comparison between Smear and MODS.

P2: for between MODS and MGIT.

P3: for between MGIT and LJ.

P4: for between LJ and Smear.

By sample analysis

Data from 150 CSF samples collected from 137 newly presenting patients were available for analysis. The sensitivity of MODS against clinical gold standard was 65.1% (n=41/63, 95%CI:[53.2, 76.8]), which was comparable to smear (52.4%, n=33/63, 95%CI:[39.7, 64.3], P=0.4), MGIT (73.0%, n=46/63, 95%CI:[62.0, 84.0], P=0.6) and LJ (68.3%, n=43/63, 95%CI:[56.4, 76.6]). The specificity and PPV of all four methods was 100%. The NPV of smear, MODS, MGIT and LJ was 74.4% (n=87/117), 79.8% (n=87/109), 83.7% (n=87/104) and 81.3% (n=87/107), respectively.

The agreements between MODS and MGIT, MODS and LJ and LJ and MGIT were 91.33% (kappa=0.789), 89.33% (kappa=0.735) and 90.0% (kappa=0.760), respectively.

By HIV status

Eighty-eight newly presenting patients were HIV infected, 40 of whom (45.45%, n=40/88) were clinically diagnosed with TBM. Although MODS, MGIT and LJ detected more positive samples or patients in the HIV- infected group than the HIV un-infected group, the difference did not reach statistical significance (**table 3.2**). The specificity of MODS, MGIT and LJ were 100% in both groups. Details of sensitivity of all 3 culture methods in relation to HIV status are shown in **table 3.2**

Table 3.2 Sensitivity of MODS, MGIT and LJ against clinical gold standard, stratified by HIV status, in the pilot study of MODS evaluation for TBM diagnosis.

| Sensitivity | By patient analysis | | | By sample analysis | | |
|---------------------|---------------------|--------------|----------------------------------|--------------------|--------------|--------------------------------|
| | HIV (+) | HIV(-) | P value ¹ [95% CI] | HIV (+) | HIV(-) | P value ² 95% CI |
| MODS culture | | | | | | |
| % | 67.5 | 58.8 | 0.82 | 68.9 | 55.6 | 0.82 |
| n=x/y | (27/40) | (10/17) | [-0.18, 0.24] | (31/45) | (10/18) | [-0.15, 0.25] |
| 95%CI | [53.5, 82.5] | [35.7, 82.3] | | [55.5, 82.5] | [42.7, 69.3] | |
| MGIT culture | | | | | | |
| % | 75.0 | 58.8 | 0.65 | 77.8 | 61.1 | 0.66 |
| n=x/y | (30/40) | (10/17) | [-0.15, 0.27] | (35/45) | (11/18) | [-0.14, 0.26] |
| 95%CI | [58.8, 87.3] | [32.9, 81.5] | | [62.9, 88.8] | [35.7, 82.7] | |
| LJ culture | | | | | | |
| % | 72.5 | 64.7 | 0.82 | 71.1 | 61.1 | 0.82 |
| n=x/y | (29/40) | (11/17) | [-0.18, 0.24] | (32/45) | (11/18) | [-0.17, 0.24] |
| 95%CI | [56.1, 85.4] | [38.3, 85.8] | | [55.7, 83.6] | [35.7, 82.7] | |

Summary measure is n (%) for these three methods

Time to positive

Time to positive or turnaround time was defined as the duration of time from sample inoculation to the presence of evidence of *M.tuberculosis* growth determined by each method. The time to positive of MODS was 6 days (IQR: 5 – 7days), which was faster than MGIT (15days, IQR: 13-24 days, P<<0.001) and LJ (24 days, IQR: 18-35 days,

¹ P values were calculated by Mc.Nemar's test

² P values were calculated by Marginal regression model

P<0.001). Interestingly, 90% (n=37/41) of the samples positive by MODS had a result available in 10 days after inoculation while this was true of only 10% (n=5/46) for MGIT.

3.2.4.1.3 Accuracy of MODS in diagnosis of “TBM patients already on TB treatment”

Of 61 CSF samples from 27 patients on TBM therapy for between 2 days and 9 months, 16 samples were positive by one of the four methods. The detection rate of MODS, smear, MGIT and LJ were 11.5% (n=7/61), 13.1% (n=8/61), 16.4% (n=10/61) and 14.8% (n=9/61), respectively.

The median time from starting TB treatment to sampling date was 28 days (7 – 56 days). The turnaround time of MODS, MGIT and LJ were 8 days (7-10 days), 15 days (14-17 days) and 21 days (21-23 days), respectively. In samples positive by both MODS and MGIT, MODS was faster than MGIT (P=0.04).

3.2.4.1.4 Diagnosis of patients without TBM diagnosis

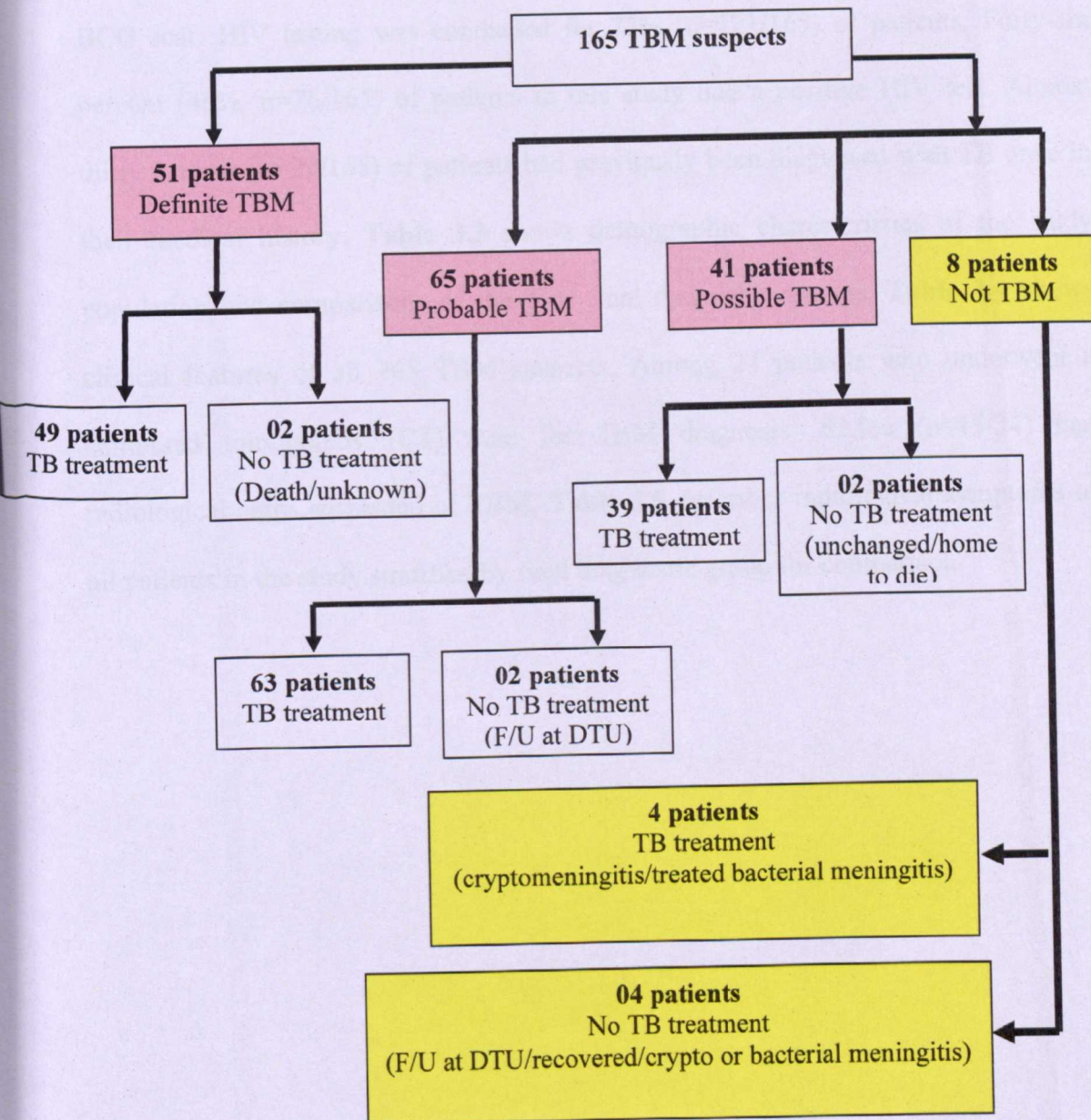
Among 80 patients not diagnosed with TBM, 35 had a final diagnosis of fungal meningitis, 3 viral meningitis, 16 bacterial meningitis, 3 toxoplasmosis, 2 herpes Simplex encephalitis, 7 viral encephalitis, 3 eosinophilic meningitis, 4 meningo encephalitis, 6 meningitis of indeterminate cause and 1 alcohol poisoning.

3.2.4.2 The implementation phase (at Pham Ngoc Thach hospital)

3.2.4.2.1 Enrollment and TBM classification

165 newly TB suspects were screened for TBM and all of them were eligible for the analysis (**Figure 3.2**). Of these, 30.9% (n=51/165) were classified as definite TBM, 39.4% (n=65/165) were probable TBM, 24.8% (n=41/165) were possible TBM and 4.9% (n=8/165) were in “not TBM” group. The probable group included 8 patients with positive sputum smears on samples collected before or during the study period and the possible group included 1 patient with PCR on CSF positive for tuberculosis done at PNT hospital. The validity of the PCR was not established and therefore the patient was classified as ‘possible’.

Figure 3.2 Flowchart of patient recruitment in the implementation phase of MODS evaluation for TBM diagnosis at PNT hospital.



Patients were grouped based on micro-confirmation (Smear or MGIT), TB treatment and outcome.

TBM: Tuberculous meningitis

F/U: Follow-up

DTU: District Tuberculosis Unit

3.2.4.2.2 Demographics and clinical features

Eighty percent (n=132/165) of the study population was male with a median age of 35. Over 53% (n=88/165) patients had evidence of BCG vaccination determined by BCG scar. HIV testing was conducted for 73% (n=121/165) of patients. Forty-six percent (46%, n=76/165) of patients in this study had a positive HIV test. Almost thirty percent (n=29/165) of patients had previously been diagnosed with TB once in their medical history. **Table 3.3** shows demographic characteristics of the study population and comparisons of the four final diagnostic groups. **Table 3.4** shows clinical features of all 165 TBM suspects. Among 24 patients who underwent a computed tomography (CT) scan for TBM diagnosis, 62.5% (n=15/24) had radiological signs suspected of TBM. **Table 3.5** describes radiological symptoms of all patients in the study stratified by final diagnostic group for comparison.

Table 3.3 Demographic characteristics of patients recruited in the implementation phase of MODS evaluation for TBM diagnosis at PNT hospital.

| Characteristic | Total population N = 165 | Definite TBM N = 51 | Probable TBM N = 65 | Possible TBM N = 41 | Not TBM N = 8 |
|--------------------------------------|-----------------------------|------------------------|------------------------|------------------------|------------------|
| Gender | P=0.53 | | | | |
| Male | 132 (80.0) | 44 (86.3) | 51 (78.5) | 31 (75.6) | 6 (75.0) |
| Age | <u>P=0.018³</u> | | | | |
| Median (year) | 35 | 31 | 40 | 35 | 31.5 |
| (IQR) | (27 – 50) | (27 – 41) | (32 - 59) | (24 - 49) | (28 – 43) |
| BCG vaccination^(*) | P=0.77 | | | | |
| Yes | 88 (53.3) | 31 (60.8) | 35 (53.9) | 19 (46.3) | 3 (37.5) |
| No | 50 (30.3) | 13 (25.5) | 19 (29.2) | 15 (36.6) | 3 (37.5) |
| Unknown | 27 (16.4) | 7 (13.7) | 11 (16.9) | 7 (17.1) | 2 (25.0) |
| TB history | P=0.98 | | | | |
| Yes | 29 (17.6) | 9 (17.7) | 11 (16.9) | 8 (19.5) | 1 (12.5) |
| No | 132 (80.0) | 40 (78.4) | 53 (81.5) | 32 (78.1) | 7 (87.5) |
| Unknown | 4 (2.4) | 2 (3.9) | 1 (1.5) | 1 (2.4) | 0 (0.0) |
| HIV testing | <u>P=0.01⁴</u> | | | | |
| Positive | 76 (46.0) | 33 (64.7) | 24 (36.9) | 15 (36.6) | 4 (50.0) |
| Negative | 45 (27.3) | 10 (19.6) | 18 (27.7) | 13 (31.7) | 4 (50.0) |
| Unknown | 44 (26.7) | 8 (15.7) | 23 (35.4) | 13 (31.7) | 0 (0.0) |

Summary measure is n(%) for all categorical characteristics. P value: Comparison for all four groups. (*)Presence of BCG scar

P values were calculated by Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables, for comparison of all four groups. If $P < 0.05$, P1, P2, P3 and P4 will be calculated.

P1: for comparison between definite TBM and probable TBM – P2: for between probable TBM and possible TBM, P3: for between possible TBM and not TBM, P4: for between not TBM and definite TBM, P5: for between probable TBM vs not TBM and P6: for definite TBM vs possible TBM.

³ For Age: P1=0.002, P2=0.04, P3=0.88, P4=0.77, P5=0.20 and P6=0.84

⁴ For HIV testing: P1=0.08, P2=0.14, P3=0.64, P4=0.69, P5=0.63 and P6=0.22

Table 3.4 Clinical features of 165 TBM suspects recruited in the implementation phase of MODS evaluation for TBM diagnosis at PNT hospital.

| Characteristic | Total population N = 165 | Definite TBM N = 51 | Probable TBM N = 65 | Possible TBM N=41 | Not TBM N = 8 |
|--|-----------------------------|------------------------|------------------------|----------------------|------------------|
| History of illness Median days (IQR) | P=0.97 14 (7-30) | 14 (7-30) | 14 (7-30) | 14 (7-30) | 14.5 (10-23) |
| Glasgow comma scale Median score (IQR) | P=0.26 14 (12-15) | 13(10-15) | 13(11-15) | 15 (13-15) | 14.5(12.5-15) |
| Fever | P=0.24 | | | | |
| Yes | 140 (84.9) | 39 (76.5) | 57 (87.7) | 37 (90.2) | 7 (87.5) |
| No | 25 (15.1) | 12 (23.5) | 8 (12.3) | 4 (9.8) | 1 (12.5) |
| Headache | P=0.75 | | | | |
| Yes | 148 (89.7) | 44 (86.3) | 60 (92.2) | 36 (87.8) | 8 (100.0) |
| No | 15 (9.1) | 6 (11.8) | 4 (6.2) | 5 (12.2) | 0 (0.0) |
| Unknown | 2 (1.2) | 1 (1.9) | 1 (5.6) | 0 (0.0) | 0 (0.0) |
| Nuchal rigidity | P=0.26 | | | | |
| Yes | 145 (87.9) | 48 (94.1) | 54 (83.1) | 36 (87.8) | 7 (87.5) |
| No | 19 (11.5) | 3 (5.9) | 11 (16.9) | 4 (9.8) | 1 (12.5) |
| Unknown | 1 (0.6) | 0 (0.0) | 0 (0.0) | 1 (2.4) | 0 (0.0) |
| Cranial nervous palsy | P=0.70 | | | | |
| Yes | 29 (17.6) | 9 (17.7) | 13 (20.0) | 7 (17.1) | 0 (0.0) |
| No | 136 (82.4) | 42 (82.3) | 52 (80.0) | 34 (82.9) | 8 (100.0) |
| Hemiplegia | P=0.3 | | | | |
| Yes | 20 (12.1) | 5 (9.8) | 12 (18.5) | 3 (7.3) | 0 (0.0) |
| No | 144 (87.3) | 45 (88.2) | 53 (81.5) | 38 (92.7) | 8 (100.0) |
| Unknown | 1 (0.6) | 1 (2.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Paraplegia | P=0.85 | | | | |
| Yes | 7 (4.3) | 3 (5.9) | 2 (3.1) | 2 (4.9) | 0 (0.0) |
| No | 158 (95.7) | 48 (94.1) | 63 (96.9) | 39 (95.1) | 8 (100.0) |

Summary measure is n (%) for all categorical characteristics

P values were calculated by Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables, for comparison of all four groups. If $P < 0.05$, P_1 , P_2 , P_3 and P_4 will be calculated. P_1 : for comparison between definite TBM and probable TBM – P_2 : for between probable TBM and possible TBM, P_3 : for between possible TBM and not TBM, P_4 : for between not TBM and definite TBM, P_5 : for between probable TBM vs not TBM and P_6 : for definite TBM vs possible TBM.

Table 3.5 Radiological diagnosis of 165 TBM suspects recruited in the implementation phase of MODS evaluation for TBM diagnosis at PNT hospital.

| Radiological tests | Total population N = 165 | Definite TB N = 51 | Probable TB N = 65 | Possible TB N=41 | Not TB N = 8 |
|------------------------------------|-------------------------------|-----------------------|-----------------------|---------------------|-----------------|
| Chest X-ray suspected of TB | <u>P<0.001⁵</u> | | | | |
| Yes | 109 (66.1) | 34 (66.7) | 64 (98.5) | 10 (24.4) | 1 (12.5) |
| No | 46 (27.9) | 13 (25.5) | 1 (1.5) | 26 (63.4) | 6 (75.0) |
| Unknown | 10 (6.0) | 4 (7.8) | 0 (0.0) | 5 (12.2) | 1 (12.5) |
| CT scan done | <u>P=0.02⁶</u> | | | | |
| Yes | 24 (14.6) | 7 (13.7) | 14 (21.5) | 1 (2.4) | 2 (25.0) |
| No | 141 (85.5) | 44 (86.3) | 51 (78.5) | 40 (97.6) | 6 (75.0) |
| CT scan suspected of TBM | P=0.06 | | | | |
| Yes | 15 (62.5) | 6 (85.7) | 9 (64.3) | 0 (0.0) | 0 (0.0) |
| No | 9 (37.5) | 1 (14.3) | 5 (35.7) | 1 (100.0) | 2 (100.0) |
| MRI done | P=0.74 | | | | |
| Yes | 4 (2.4) | 1 (2.0) | 1 (1.5) | 2 (4.9) | 0 (0.0) |
| No | 161 (97.6) | 51 (98.0) | 64 (98.5) | 39 (95.1) | 8 (100.0) |
| MRI suspected of TBM | P=1.00 | | | | |
| Yes | 2 (50.0) | 1 (100.0) | 0 (0.0) | 1 (50.0) | - |
| No | 2 (50.0) | 0 (0.0) | 1 (100.0) | 1 (50.0) | - |

Summary measure is n (%) for all categorical characteristics.

P values were calculated by Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables, for comparison of all four groups. If $P < 0.05$, P_1 , P_2 , P_3 and P_4 will be calculated. P_1 : for comparison between definite TBM and probable TBM – P_2 : for between probable TBM and possible TBM, P_3 : for between possible TBM and not TBM, P_4 : for between not TBM and definite TBM, P_5 : for between probable TBM vs not TBM and P_6 : for between definite vs possible TBM.

⁵ For chest X-ray suspected of TB: $P_1=0.16$, $P_2<0.01$, $P_3=0.54$, $P_4=0.08$, $P_5=0.03$ and $P_6=0.014$

⁶ For CT scan done: $P_1=0.36$, $P_2=0.01$, $P_3=0.03$, $P_4=0.49$, $P_5=1.00$ and $P_6=0.13$

3.2.4.3 Detection rates of MODS, MGIT and Smear.

In patients with “definite TBM diagnosis”

Of fifty-one patients with CSF samples positive by either smear or MGIT, thirty patients (58.8%, 95%CI [41.2, 72.4]) were positive by MODS. MGIT detected 92.2% (n=47/51, 95%CI [81.1, 97.8]) of definite cases while smear only detected 37.3% (n=19/51, 95%CI [24.1, 51.9]).

In patients with “Clinical TBM diagnosis”

a. Detection rates

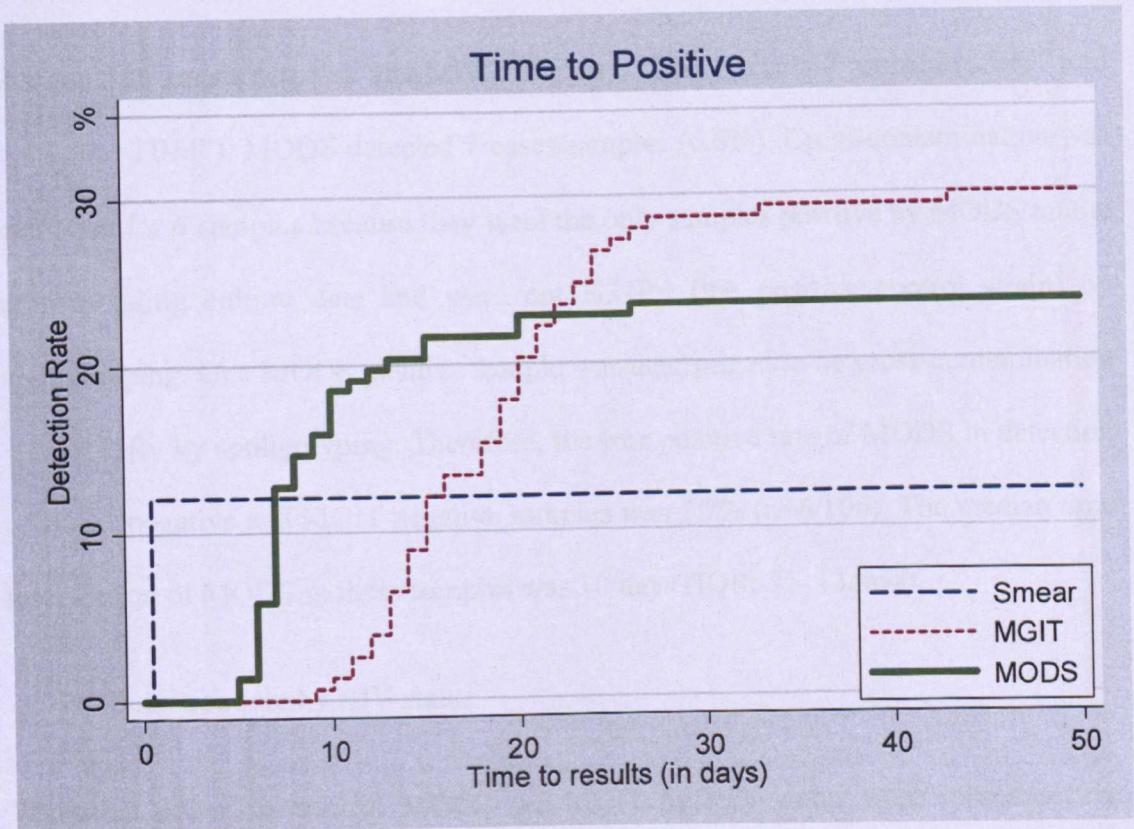
The “clinical diagnosis” group consisted of “definite TBM”, “probable TBM” and “possible TBM” patients combined. One hundred and fifty-seven patients were diagnosed with TBM by either microbiological confirmation or clinical symptoms and intention to treat. The detection rates of smear, MGIT and MODS were 12.1% (n=19/157, 95%CI [7.4, 18.3]), 29.9% (n=47/157, 95%CI [22.9, 37.8]) and 23.6% (n=37/157, 95%CI [17.2, 31.0]), respectively. The detection rate of MODS was higher than smear (P=0.0012, 95%CI of difference: [0.04, 0.20]) and comparable to MGIT (P=0.18, 95%CI of difference: [-0.12, 0.02]).

b. Time to positive

In samples positive by either MODS or MGIT (n=55/157), MODS detected mycobacterial growth after a median of 7 days (IQR: 7 – 10 days, n=37) while it took 18 days for MGIT (IQR: 14 – 22 days, n=47).

In samples positive by both MODS and MGIT, the turnaround time of MODS was faster than MGIT; median (IQR) times were 7 days (6 – 9days) vs 15 days (IQR: 13 – 18 days), respectively, $P < 0.001$. **Figure 3.3** shows time dependent detection rates of smear, MGIT and MODS in patients with clinical TBM diagnosis. MODS provided higher time dependent detection than MGIT until 21 days after inoculation.

Figure 3.3 Time dependent detection rate of Smear, MODS and MGIT in patients with clinical TBM diagnosis in the implementation phase at PNT hospital.



MODS had a higher time dependent detection than MGIT until 21 days after inoculation, by Kaplan-Meier graph.

The time to positive of MODS and MGIT were compared at 7 days and 14 days after inoculation. By day 7, no CSF sample was positive by MGIT while MODS detected mycobacteria in 54.1% (n=20/37) of all CSF samples positive by MODS. The shortest time to detection of MGIT and MODS were 9 days and 5 days, respectively. By day 14, MGIT only detected 29.8% (n=14/47) of all CSF samples positive by MGIT while 86.5% of all (n=32/37) MODS positive cases were diagnosed by MODS at that time. MODS (86.49%, n=32/37) provided higher detection rate than MGIT (29.79%, n=14/47) by day 14 (P<0.001, 95%CI of difference:[-0.17, -0.05]).

c. TBM patients with microbiological confirmation by MODS only

Among 106 smear negative and MGIT negative TBM patients (“probable TBM” and “possible TBM”), MODS detected 7 cases/samples (6.6%). Cross-contamination was excluded for 6 samples because they were the only samples positive by MODS on the corresponding culture date and were not H37Rv (the positive control strain) by spoligotyping. One MODS positive sample was determined to be cross-contamination with H37Rv by spoligotyping. Therefore, the true positive rate of MODS in detection of smear negative and MGIT negative samples was 5.7% (n=6/106). The median time to detection of MODS in these samples was 10 days (IQR: 7 – 13days).

➤ Detection rate by HIV status

When the detection rates of MODS and MGIT by HIV status were compared, we found that among 76 HIV infected-TBM suspects, MGIT detected 40.8% (n=31/76) and MODS detected 32.9% (n=25/76) of cases (P=0.18). In 45 HIV uninfected TBM suspects, MGIT detected 22.2% (n=10/45) and MODS detected 13.3% (n=6/45) of

cases ($p=0.21$). Our data also showed that MGIT and MODS detected significantly more cases in HIV infected TBM suspects than in HIV uninfected TBM suspects, ($P=0.0015$, 95%CI [0.19, 0.70] for MGIT and $P=0.009$, 95%CI [0.24, 0.78] for MODS).

In “Not TBM patients”

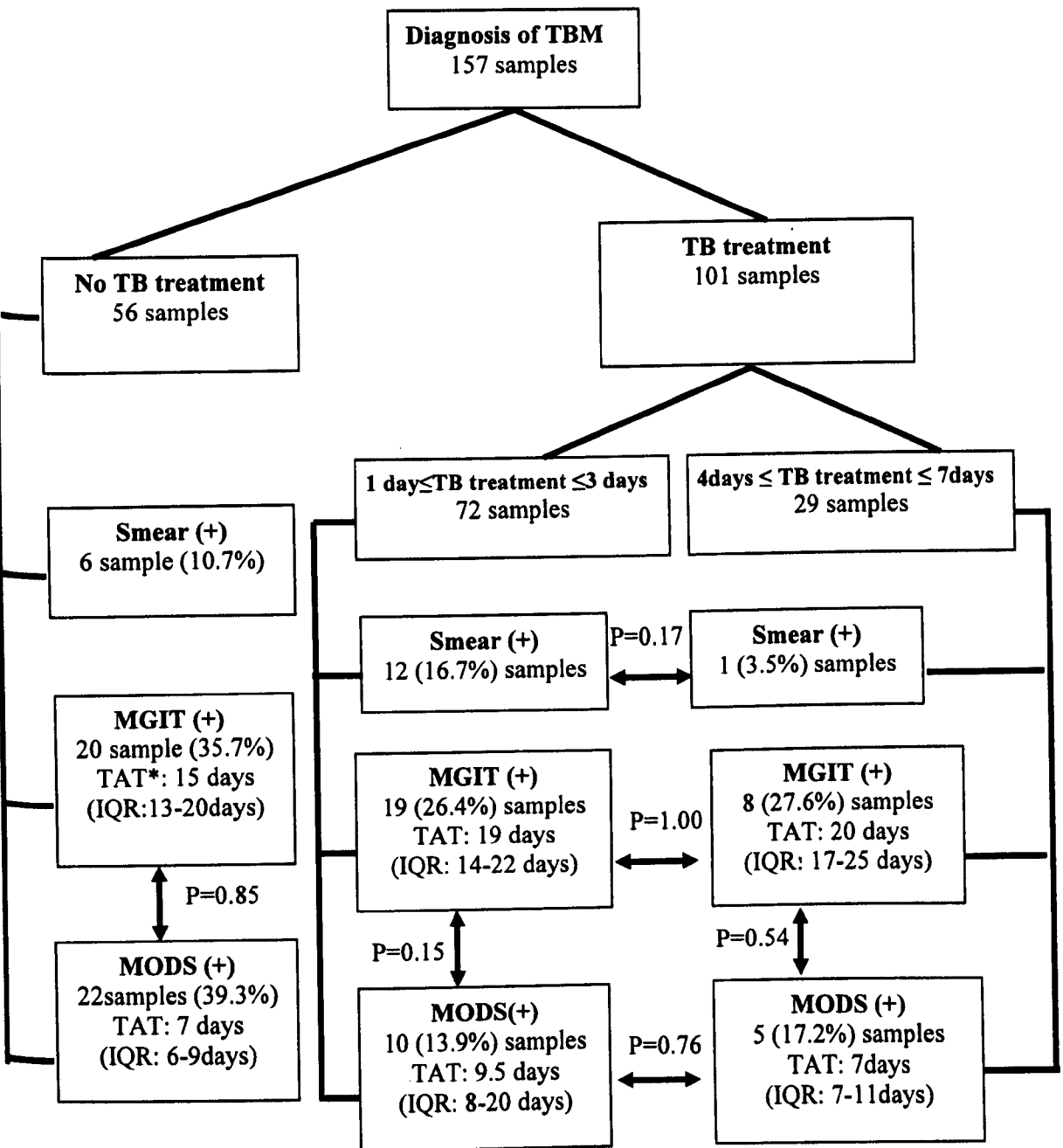
Eight out of 165 patients were classified in “not TBM” group and MODS was negative for all of these patients. The final diagnosis of these patients were *Cryptococcus meningitis* ($n=4$) and bacterial meningitis ($n=2$). The other two patients were suspected to suffer from unconfirmed viral infection. One patient recovered without TB treatment and the other had unknown diagnosis.

3.2.4.4 TB treatment-dependent detection

One hundred and fifty seven CSF samples from 157 patients with clinical TB diagnosis were analyzed. Thirty-six percent (35.7%, $n=56/157$) samples were from patients with no TB treatment before the lumbar puncture and 64.3% (101/157) of samples were collected from patients who were on TB treatment for a maximum of 7 days. Seventy-two samples ($n=72/101$, 71.3%) were collected from patients on TB treatment less than or equal to 3 days and 29/101 (28.7%) samples were from patients on TB treatment from 4 to 7 days (**figure 3.4**). In ‘no prior TB treatment’ group, although the detection rate of MODS was comparable to MGIT, 39.3% vs 35.7%, respectively, $P=0.85$, the turnaround time of MODS was shorter than that of MGIT (7 days vs 15 days, respectively). The findings were similar in the “on treatment” group. The detection rate of MODS was significantly decreased in samples from patients

already on treatment compared to no prior TB treatment; 14.9% (15/101) vs 39.3% (n=22/56), P=0.009, respectively. The detection rate of MGIT also tended to be lower in samples from patients receiving TB treatment prior to lumbar puncture but the result did not achieve statistical significance (26.7% vs 35.7%, P=0.39).

Figure 3.4 Detection rates of smear, MGIT and MODS in relation to TB treatment during the implementation phase of MODS evaluation for TBM diagnosis.



The positive rates of MGIT and MODS were similar and they were not related to treatment time. The positive rates were compared by Mc.Nemar's test.

** TAT: Turnaround time*

3.2.4.5 Cerebrospinal fluid characteristics

The characteristics of 165 CSF samples from the implementation phase (PNT hospital) were analysed. Sixty percent of (n=99/165) samples were not stored in the fridge because a fridge was not available at the time these sample were collected, 31.5% (n=52/165) were stored in the fridge and the remaining 14 samples had unknown storage condition. Among 52 samples stored in the fridge, 14 samples were stored for less than 3 hours, 31 samples were stored from 3-5 hours and 7 samples were stored overnight (>16 hours). The detection rates of MODS in “no fridge storage” and “fridge storage” were not significantly different (20% vs 26.9%, P=0.5). The same figure was observed for MGIT culture (27.3% vs 30.8%, P=0.7). The median storage time before sample processing was 2.5 hours.

Refrigeration principally reduces bacterial overgrowth in sputum samples and is therefore likely to be less important for ‘sterile’ CSF samples. This data provides reassuring evidence that, despite the tropical setting, the lack of a fridge for short-term storage of CSF samples prior to processing may not substantially decrease mycobacterial yield.

The median volume taken from a patient was 10ml but only approximately 3ml (median) was sent to the microbiological laboratory for *M.tuberculosis* testing. CSF appearance was clear for over 60% of samples. Characteristics of CSF collected in the implementation phase are detailed in **table 3.6**.

Table 3.6 Characteristics of CSF samples collected from newly presenting patients from the pilot and implementation phases of MODS evaluation for TBM diagnosis.

| CSF characteristics | Pilot phase N = 137 | Implementation phase N = 165 |
|--|------------------------|------------------------------------|
| CSF volume (ml) recorded in the lab | | |
| Median (IQR) | 4 (3-5) | 3 (2-5) |
| Mean (SD) | 4.6 (\pm 2.7) | 3.7 (\pm 1.9) |
| CSF appearance, n(%) | | |
| Clear | NA | 100 (60.6) |
| Bloody | NA | 9 (5.5) |
| Turbid | NA | 30 (18.2) |
| Yellow | NA | 26 (15.7) |
| CSF/blood glucose ratio | (n=111) | |
| Median (IQR) | 0.42 (0.29-0.54) | 0.4 (0.2-0.5) |
| Mean (SD) | 0.45 (\pm 0.5) | 0.4 (0.2) |
| CSF protein (g/l) | (n=118) | |
| Median (IQR) | 1.2 (0.8-1.6) | 0.4 (0.2-0.6) |
| Mean (SD) | 1.89 (\pm 5.33) | 0.4 (0.3) |
| Cell count, (cells/μl) | (n=113) | |
| Median (IQR) | 160 (38-510) | 31 (5-98) |
| Mean (SD) | 474 (\pm 996) | 89 (173) |
| Lymphocyte (%) | (n=105) | |
| Median (IQR) | 52 (23-80) | 100 (90-100) |
| Mean (SD) | 52 (\pm 31) | 92 (15) |
| HIV status, n(%) | | |
| HIV positive | 88 (64.23) | 76 (46) |

Characteristics of CSF samples collected in 2 phases. Summary measure is n (%) for all categorical characteristics. CSF collected in the pilot phase was more typical for TBM than in the implementation phase, in terms of CSF protein level and cell count.

3.2.4.6 Contamination and Spoligotyping in the implementation phase

The evaluation of contamination and cross-contamination were attempted for the implementation phase only due to resource limitation.

One hundred and sixty-five CSF samples were cultured by both MODS and MGIT. There were 55 samples positive by either MODS or MGIT. Of which, 29 samples were positive by both MODS and MGIT, 8 samples positive by MODS only and 18 samples positive by MGIT only. DNA extracts were available from 37 MODS positive cultures and were used for spoligotyping and evaluation of cross-contamination. Contamination was assessed in terms of fungi or other bacteria and *M.tuberculosis* cross contamination for the MODS assay.

In terms of fungal or bacterial contamination, there was 1.2% (n=2/165) samples contaminated with fungi in MODS culture. No fungal/bacterial contamination in MGIT was observed.

Cross-contamination was investigated for 29 samples positive by both MODS and MGIT and 8 samples positive by MODS only using spoligotyping. When investigating all samples cultured on identical days, on which 29 samples positive by both MODS and MGIT were inoculated, no sample cultured in the same plate or on the same day had the same spoligotype, so cross-contamination by MODS was excluded in this group. In 8 samples positive by MODS only, one sample was identified as H37Rv, and therefore was a false positive, cross-contaminated by the positive control strain. This patient had been classified in “possible TBM” group. The other 7 MODS positive isolates had genotypes different from those cultured in the

same plate or on the same day. Therefore, the cross-contamination rate of MODS was 0.6% (n=1/165).

The cross-contamination rate of MGIT was not addressed in this study due to limited resources.

3.2.5 DISCUSSION

MODS is a sensitive and rapid technique for the diagnosis of TBM, especially in HIV infected individuals suspected of TBM. Our data from both study phases (pilot phase at HTD and implementation phase at PNT) showed similar results:

First, although MGIT showed a slightly higher detection rate than MODS, the differences were not significant (71% vs 65%, $p=0.79$ from pilot data and 30% vs 24%, $p=0.18$ from implementation data). Interestingly, detection rates of both MODS and MGIT were significantly higher in the pilot study than in the implementation study (65% vs 24%, $p=0.0002$ for MODS and 71% vs 30%, $p=0.0011$ for MGIT).

Second, both MODS and MGIT detected more TBM cases among HIV positive cases than HIV negative cases; and this observation was significant in the implementation data (33% vs 13%, $p=0.009$ for MODS and 41% vs 22%, $p=0.0015$ for MGIT). The percentage of HIV positive/TBM patients detected by MODS and MGIT was comparable ($p=0.761$ for pilot study and $p=0.492$ for implementation study).

Third, MODS had a faster turnaround time than MGIT (7 days vs 15 days, $p<0.001$)

Fourth, the fungal contamination and cross-contamination rates of MODS were negligible in the implementation phase (1.2% and 0.6%, respectively).

Last, the detection rates of MODS and MGIT were reduced by TB treatment. Both MODS and MGIT detected more cases in non-TB treatment group compared to patients already receiving TB treatment. However, only MODS showed a significant difference (40% vs 15%, $p=0.009$).

A slightly lower sensitivity of MODS compared to MGIT in the pilot study may be due to a smaller volume of deposit inoculated into MODS culture than MGIT culture (100 μ l versus 500 μ l, respectively). However, when the volume of CSF deposit was increased to 250 μ l for MODS culture in the implementation phase, the detection rate of MODS was still slightly lower than MGIT even though the difference did not reach statistical significance. Interestingly, the detection rates of smear, MODS and MGIT in the implementation phase were much lower than the sensitivity rates in the pilot study, even though both studies focused on investigation of TBM suspects and CSF samples were evaluated. Some differences were observed between the two study settings related to patient population, characteristics of CSF and technicians responsible for the tests. First, most patients presenting to HTD were naïve to TB treatment or antibiotic treatment. Therefore, diagnosis based on clinical presentation or microbiological confirmation was not much different; smear detected about half of TBM cases and MGIT and MODS detected around 65-70% of cases. In contrast, 64.3% ($n=101/157$) patients admitting to PNT hospital were on TB treatment for at least one day. Our data showed that detection rate of MODS significantly decreased in the “on treatment” group in comparison to “no TB treatment”, as would be expected.

The finding of a previous study by Thwaites *et al.* also demonstrated the same phenomena (198). In this study, the sensitivity of direct CSF smear examination dropped from 52% to 2% in patients on 5-15 days of TB therapy. Therefore, unsurprisingly, TB treatment reduces the sensitivity/ detection rate of microbiological diagnostic tests for TBM. Since the majority of cases in the implementation phase were on TB therapy, clinical diagnosis was more challenging. It is possible the "probable" and "possible" TBM groups in the implementation phase were over diagnosed compared with the pilot phase, leading to lower detection rates of all techniques. However, clinicians at both sites are highly experienced in diagnosis of TBM and clinical TBM trials have been conducted involving both sites, leading to broad standardization of diagnostic criteria. A more restrictive classification for TBM would have improved comparability between sites but by definition would have only included patients with classical presentation and therefore probably overestimated sensitivity of the tests. This conundrum applies to any evaluation of novel TBM diagnostics. **Second**, characteristics of CSF collected from the pilot and implementation phases were different (**table 3.6**). CSF protein (g/l) and number of cells (cells/ml) recorded from CSF in the pilot study were higher and more typical for TBM than in the implementation phase. A retrospective study in Rio De Janeiro, Brazil, reviewing 13,000 medical records of patients whose CSF samples were sent to the laboratory by doctors for diagnosis of TBM reported that CSF culture is more likely to be positive in patients with a high level of CSF protein (1.5g/l in culture positive samples compared to 0.63g/l in culture negative samples) which suggests that the positive culture found in these patients may be associated with the presence of

high bacillary load in the CSF (156). In addition, this study also stated that more cases were detected in patients infected with HIV (73%) and this conclusion is similar to our findings. In the pilot phase, MODS and MGIT detected more TBM cases in the HIV positive population than in the HIV negative group but the data did not reach significance because of small sample size (40 HIV positive patients and 17 HIV negative cases). However, in the implementation phase, significant difference of detection rates of MGIT and MODS was observed. MODS and MGIT detected more TBM cases in HIV positive cases than in HIV negative cases (33% versus 13%, $p=0.009$ for MODS and 41% versus 22% for MGIT). This also demonstrates the difference between two study phases in terms of study population. This finding is inverse to pulmonary TB, where HIV positive cases are more likely to be sputum smear negative. **Third**, only three staff performed microbiological testing in the laboratory for the pilot study conducted at HTD. One staff member performed MODS, one MGIT culture and the third smear microscopy. Therefore, inter-technician variation related to technical issues due to many staff doing the same technique was controlled. This situation was totally different from the high-burden PNT laboratory, where staff work in groups as standard practice. Groups are responsible for a certain process such as registration, sample processing and culture. Although PNT staffs have a high proficiency level, many staff responsible for a chain of work may introduce errors. **Last**, in the pilot phase, 4ml CSF was sent for microbiological testing while only 3ml was tested in the implementation phase. The improvement of detection rate of microbiological tests correlated with CSF volume was demonstrated by Thwaites *et al.* (195).

Time to detection of *M. tuberculosis* by MODS was significantly faster than MGIT. While the median time for MODS was 7 days, the time to culture positive of MGIT was about 15 days. Theoretically, all cultures positive by MGIT are detected at approximately $10^5 - 10^6$ CFU/ml of *Mycobacteria* (13). Therefore, it takes time for *M.tuberculosis* to achieve sufficient concentration to be detected by MGIT. In contrast, MODS was developed to detect the presence of *Mycobacteria* by observation of cord formation under the inverted-microscope after several days of incubation. Since the cord morphology is magnified 400 times under the microscope, one can observe the cord formation from a small number of bacilli present in each well after a short time of culture. Therefore, MGIT has a longer turn-around time than MODS but MODS is more laborious than MGIT. Interestingly, median time to detection of *Mycobacteria* in sputa by MODS was 9 days (33), which is slower than that of *Mycobacteria* in CSF (7 days). Sample processing may be the cause of this observation. Since all sputa were digested and decontaminated before inoculation in MODS, digestion and decontamination solution will affect the viability of *Mycoabcteria* and residual decontaminant may impact the growth of bacteria. In contrast, CSF samples are sterile so CSF pellets are inoculated directly for culture without decontamination.

MODS is a reproducible and robust technique which is easily adopted by experienced microscopy technicians. The MODS technique in the pilot phase was the responsibility of a single staff member (Dang Thi Minh Ha) and in the implementation phase by two staff members. All three had a common background in smear microscopy technique and were well-trained on MODS. Although working in

different working environments, the results were accurate (comparison between MODS and MGIT) and contamination was controlled (<2%). Therefore, this technique can be scaled-up for use in TB laboratories which are equipped for doing TB culture.

We have shown MODS a promising method for early diagnosis of tuberculosis meningitis, because of these advantages. **First**, MODS can provide a comparable detection rate to MGIT which has been approved as a gold standard for TB diagnosis by WHO in 2007 (230). **Second**, MODS can detect the presence of *Mycobacteria* in a short time after incubation. In this study, the median time of MODS was 7 days, which is significantly faster than MGIT (15 days). **Third**, contamination is currently not a serious issue if the sample used in MODS is CSF and if care in manipulation is applied. The cross-contamination rate detected in this study was very low (0.6%). **Finally**, MODS is easy to set up and technicians with high experience on microscopy only need to participate in a training course in a short time (approximately 3 to 4 weeks) to reliably differentiate early *M.tuberculosis* cording.

3.3 Section 3

MODS IN DIAGNOSIS OF PAEDIATRIC TB

3.3.1 INTRODUCTION

It is estimated that 9.27 million new tuberculosis (TB) cases occurred worldwide in 2000, of which approximately 11% were in children. Importantly, 75% of these paediatric cases occur in 22 high-burden countries, including Viet Nam (225). It is estimated by WHO that 35% of paediatric TB is pulmonary TB and 65% are extrapulmonary TB and the ratio of pulmonary to extrapulmonary TB varies depending on age, genetic factors and immune response (220). Most of extrapulmonary TB patients are from infants and young children. The most severe forms are milliary TB and tuberculous meningitis (TBM).

PNT hospital admits approximately 500 children with suspected TB each year. In 2007, only 14.9% (59/395) of treated pediatric TB cases had microbiological confirmation (PNT hospital routine unpublished data). Almost 90% of confirmed cases had pulmonary TB. Confirmation of extrapulmonary TB in children is more challenging than pulmonary TB, as is the case for adults. This is consistent with reports from other settings (22, 160).

The confirmation of TB is based on demonstration of *M.tuberculosis* in a clinical sample. Culture and smear microscopy have been considered the gold standards for the diagnosis of TB in adults and children. Sputum microscopy and culture for TB diagnosis are widely used. However, young children and infants are usually unable to produce sputum, as the sputum is swallowed. When sputum samples cannot be

obtained, gastric aspirate samples or bronchial lavage samples are collected instead. Even though smear microscopy can achieve 50% - 60% sensitivity and culture can reach 70% - 80% sensitivity in diagnosis of TB in adults, less than 15% of pediatric cases are sputum smear positive and cultures yield 30% – 40% sensitivity (185). The sensitivity of microbiological diagnostic tests varies between settings. Factors affecting the sensitivity of the tests include sample type, sample volume, sample quality, sampling method and diagnostic method. A study from South Africa showed that in children aged from one month to 5 years, the bacterial confirmation (smear/culture) rate was 25%; smear and culture from induced sputum or gastric aspirate samples were positive in 87% and 65%, respectively (263). Similarity, in Peruvian children with a mean age of 5 years (ranging from 1 month to 16 years), LJ culture detected 54% of cases and smear microscopy detected 39% and 42% of cases, respectively for ZN staining alone and ZN and auramine staining in combination (129). Nucleic acid amplification tests have not proved sensitive for the diagnosis of TB in children (75). Diagnostic confirmation may be difficult because of non-specific clinical symptoms, coexisting malnutrition, atypical chest X-ray and paucibacillary or disseminated diseases in addition to low rates of bacterial confirmation. As result, the TB diagnosis relies mainly on case definition, chest X-ray and tuberculin skin test (225).

In 2007, there were approximately 60 suspected cases of TBM in children admitted to PNT Hospital. However, only 3/60 (5%) of these cases had microbiological confirmation. Overall, the problems of TB diagnosis in adults are exacerbated in children due to the difficulty of obtaining sufficient volumes of appropriate samples.

Available techniques are time consuming, have low sensitivity or require expensive capital equipment or reagents. Therefore, it is necessary to develop new diagnostic methods that can eliminate the disadvantages of the established techniques.

The previous study described in chapter 3 – section 1 - pilot phase of TBM MODS study has shown that MODS can diagnose approximately 65% of all clinically diagnosed adult TBM in 6 days (34) with a higher sensitivity than smear (50%) and a faster turnaround time than MGIT culture (12 days). A similar trend was shown in the implementation phase of the TBM-MODS study. The diagnosis of tuberculosis in children is frustrated by similar challenges as those experienced in adult TBM patients due to the paucibacillary samples and difficulty obtaining suitable sample volumes for testing. In this study, the effectiveness of MODS in diagnosis of tuberculosis in children presenting to PNT hospital was assessed.

3.3.2 METHOD

3.3.2.1 Enrollment

All children ≤ 16 years of age ful-filling the enrollment criteria (**chapter 2, section 2.4**) and presenting to the paediatric ward at PNT hospital during the study period were enrolled into the study.

Data on socioeconomic and demographic features, TB history, TB contact history, HIV status and presenting clinical features were prospectively collected on a standard case report form (CRF). Samples were collected as per routine care as deemed appropriate by the treating physician. No additional samples were collected as part of

this study. All specimen types from suspected cases were included in the study except blood.

3.3.2.2 Definition and TB classification

The definition and TB classification were described in **chapter 2, section 2.7**.

3.3.2.3 Sample collection, sample processing and tests

- As described in materials and methods (**chapter 2, section 2.5 and 2.6**).
- All samples except blood including sputum, cerebrospinal fluid (CSF), gastric fluid and pleural fluid were collected for this study.
- All processed samples were tested by homogenous smear, MGIT and MODS culture.

3.3.3 STATISTICAL METHODS

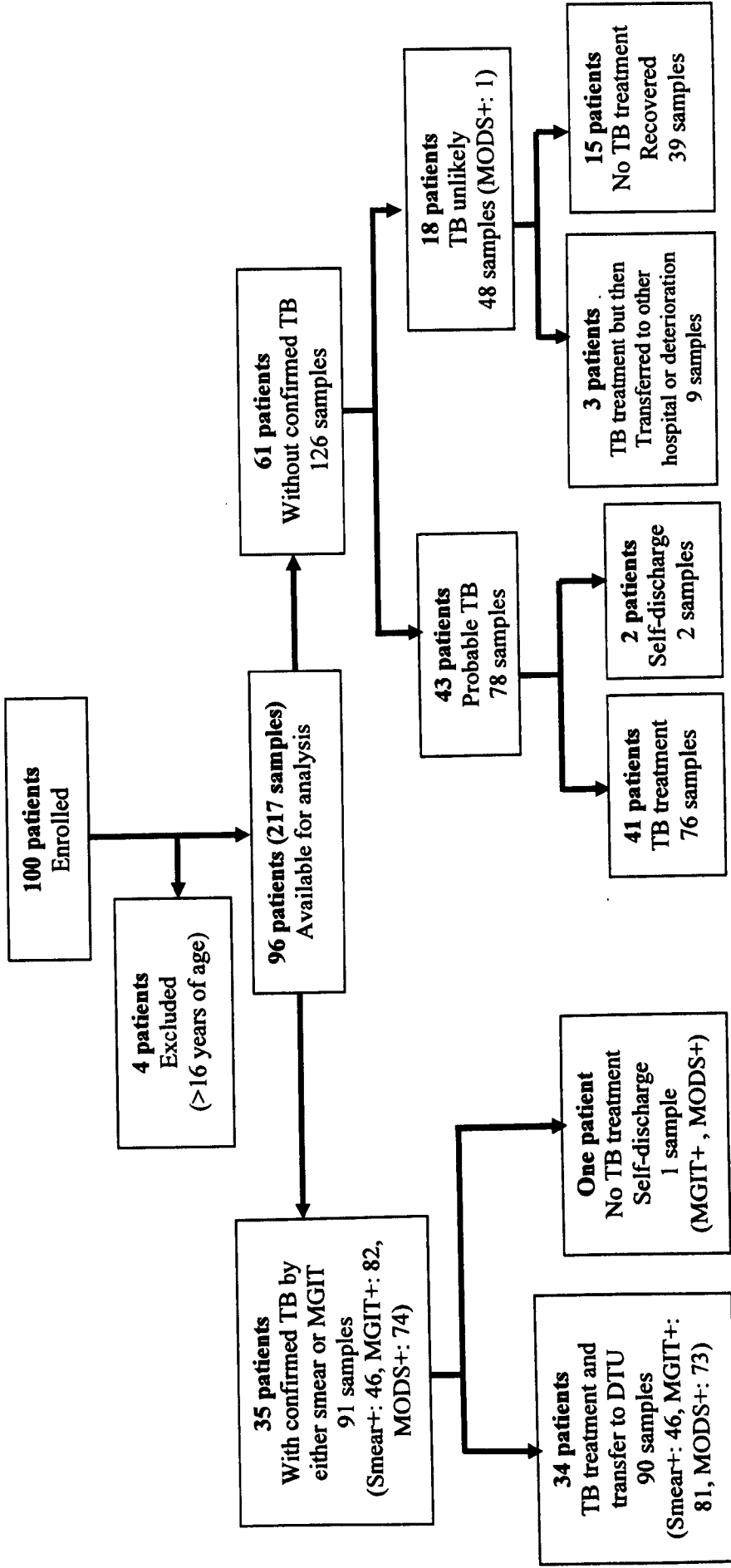
- Accuracy measures of the 3 tests were calculated for two different definitions of the 'gold standard' reference test: (1) microbiological confirmation (confirmed group) or (2) 'clinical diagnosis' (clinical gold standard including the confirmed and the probable group). In addition, data was analyzed on a 'per patient' or a 'per sample' basis.
- Details of statistical methods are described in **chapter 2, section 2.8**.

3.3.4 RESULTS

3.3.4.1 Patient recruitment and classification.

One hundred children suspected of TB were enrolled into the study. Data for 96 (96%) children were included in the analysis. Four patients were excluded from the study as they exceeded the age limit for the study population (equal or older than 16 years of age) (**Figure 3.5**). A total of 217 samples were collected including sputum (n=132), gastric fluid (n=50), CSF (n=32), and pleural fluid (n=3). Thirty five patients (36.5%, n=35/96) had microbiological confirmation by a method other than MODS. 43 patients (44.8%, n=43/96) were classified as 'probable TB' and 18 patients (18.8%, n=18/96) were classified as 'TB unlikely'. There were three out of 96 patients who self-discharged before a final treatment decision was made. Samples were collected within 4 days after admission and approximately 73% (70/96) of patients provided samples within the first two days.

Figure 3.5 Paediatric patient recruitment and assignment of patients to “confirmed”, “probable”, or “TB unlikely” groups.



3.3.4.2 Demographic characteristics and clinical presentation

General demographic characteristics of the study population are shown in **Table 3.7**. In brief, the male: female ratio was 1:1.3 and the median age was 9 (IQR=3-13). Almost 80% of suspects had evidence of BCG vaccination (neonatal BCG vaccination is routine in Viet Nam), only 5% had undergone previous TB treatment. Over 90% (n=88/96) of suspects were not screened for HIV infection. Only patients suspected of HIV were counseled for HIV testing, under routine practice on the ward. Seven out of eight (87.5%) of those tested were positive. A quarter (24/96) of the study population had a known TB contact according to parent interview and of those contacts 21/24 (87.5%) were a direct household member.

Table 3.7 Demographic characteristics of paediatric patients

| Characteristic | Total population N = 96 | Confirmed TB N = 35 | Probable TB N = 43 | TB unlikely N = 18 |
|--------------------------------------|----------------------------|------------------------|-----------------------|-----------------------|
| Gender | P=0.04 | P1=0.002 | P2=0.83 | P3=0.50 |
| Male | 41 (42.7) | 11 (31.4) | 22 (51.2) | 8 (44.4) |
| Age (year) Median (IQR) | P=0.005 | P1=0.004 | P2=0.34 | P3=0.07 |
| | 9 (3 – 13) | 13 (5 – 14) | 6 (2 – 11) | 8.5 (4 – 12) |
| BCG vaccination^(*) | P=0.31 | | | |
| Yes | 74 (77.1) | 27 (77.1) | 32 (74.4) | 15 (83.3) |
| No | 21 (21.9) | 8 (22.9) | 11 (25.6) | 2 (11.1) |
| Unknown | 1 (1.0) | 0 | 0 | 1 (5.56) |
| TB treatment history | P=0.86 | | | |
| Yes | 5 (5.2) | 2 (5.7) | 2 (4.7) | 1 (5.6) |
| No | 89 (92.7) | 33 (94.3) | 39 (90.70) | 17 (94.4) |
| Unknown | 2 (2.1) | 0 | 2 (4.6) | 0 |
| HIV status | P=0.34 | | | |
| Positive | 7 (7.3) | 2 (5.7) | 3 (7.0) | 2 (11.1) |
| Negative | 1 (1.0) | 0 | 0 | 1 (5.6) |
| Unknown | 88 (91.7) | 33 (94.3) | 40 (93.0) | 15 (8.3) |
| ARV therapy (HIV + only) | P=0.85 | | | |
| Yes | 2 (28.6) | 0 | 2 (66.7) | 0 |
| No | 5 (71.4) | 2 (100) | 1 (33.3) | 2 (100) |
| TB contact (Parent report) | P=1.0 | | | |
| Yes | 24 (25.00) | 9 (25.7) | 11 (25.6) | 4 (22.2) |
| Contact family member | 21/24 (87.5) | 7/9 (77.7) | 10/11(90.9) | 4/4 (100) |
| No | 72 (75.00) | 26 (74.3) | 32 (74.4) | 14 (77.8) |

Summary measure is n (%) for all categorical characteristics.

(*) Scar or parent report

P values were calculated by Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables, for comparison of all three groups. If $P < 0.05$, P1, P2, P3 was calculated.

P1: for comparison between confirmed TB and probable TB; P2: for between probable TB and TB unlikely and P3: for between TB unlikely and confirmed TB

Clinical symptoms of TB in this referral study population included cough (67.7%), fever (80.2%), weight loss (45.8%) and chest X-ray consistent with TB (75%) (**Table 3.8**). Other symptoms were haemoptysis, convulsion, unconsciousness, difficulty breathing and vomiting. The median history of illness was 20 days. The 'confirmed TB' patient group reported cough more frequently than 'probable' TB patients ($P=0.03$) and had a chest X-ray consistent with TB more often ($P=0.005$) than probable TB patients, probably representing more advanced disease in those with microbiological confirmation.

Table 3.8 Clinical features of 96 pediatric TB suspects

| Characteristic | Total population N = 96 | Confirmed TB N = 35 | Probable TB N = 43 | TB unlikely N = 18 |
|--|----------------------------|------------------------|-----------------------|-----------------------|
| History of illness Median days (IQR) | P=0.89 20 (14 – 30) | 15 (12 – 30) | 20 (14 – 30) | 24.5 (10 – 30) |
| Cough | P=0.025 65 (67.7) | P1=0.03 27 (77.1) | P2=0.03 23 (53.5) | P3=0.59 15 (83.3) |
| Fever | P=0.36 77 (80.2) | 27 (77.1) | 37 (86.1) | 13 (72.2) |
| Nightsweat | P=0.63 33 (34.4) | 11 (31.4) | 17 (39.5) | 5 (27.8) |
| Weightloss | P=0.37 44 (45.8) | 15 (42.9) | 18 (41.9) | 11 (61.1) |
| Failure to thrive | P=0.48 18 (18.7) | 5 (14.3) | 8 (18.6) | 5 (27.8) |
| Lymphadenopathy | P=0.38 14 (14.6) | 7 (20.0) | 4 (9.3) | 3 (16.7) |
| Chest X-ray suspected of TB | P=0.025 | P1=0.005 | P2=0.08 | P3=0.59 |
| Yes | 72 (75.0) | 31 (88.6) | 26 (60.5) | 15 (83.3) |
| No | 20 (20.8) | 3 (8.6) | 15 (34.9) | 2 (11.1) |
| Unknown | 4 (4.2) | 1 (2.9) | 2 (4.6) | 1 (5.6) |
| Radiological description (for chest X-ray suspected of TB only) | | | | |
| Cavity | 13 (18.1) | 10 (32.3) | 1 (3.9) | 2 (13.3) |
| Miliary | 3 (4.2) | 1 (3.2) | 1 (3.9) | 1 (6.7) |
| Infiltrate | 28 (38.9) | 7 (22.6) | 14 (53.9) | 7 (46.7) |
| Nodular lesion | 9 (12.5) | 5 (16.1) | 2 (7.7) | 2 (13.3) |
| Interstitial infiltration | 1 (1.4) | 1 (3.2) | 0 | 0 |
| Shadowing | 12 (16.7) | 5 (16.1) | 4 (15.4) | 3 (20.0) |
| Hillar enlargement | 4 (5.6) | 2 (6.5) | 2 (7.7) | 0 |
| No description | 2 (2.8) | 0 | 2 (7.7) | 0 |

Summary measure is n (%) for all categorical characteristics.

P values were calculated by Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables, for comparison of all three groups. If $P < 0.05$, P1, P2, P3 will be calculated.

P1: for comparison between confirmed TB and probable TB; P2: for between probable TB and TB unlikely and P3: for between TB unlikely and confirmed TB.

3.3.4.3 Accuracy of MODS

3.3.4.3.1 Clinically diagnosed TB as the gold standard

The clinical gold standard was defined as patients who satisfied characteristics of either “confirmed TB” group or “probable TB” group combined. Seventy-eight patients were diagnosed with TB by clinical features and/or microbiological confirmation and 18 patients were classified as ‘TB unlikely’.

When analysed by patient, the sensitivity of smear, MGIT and MODS were 28.2%, [95%CI: 18.6, 39.5] (n=22/78), 42.3%, [95%CI: 31.1, 54.0] (n=33/78) and 39.7%, [95%CI: 28.8, 51.4] (n=31/78), respectively. MODS was more sensitive than smear (P=0.011) [95%CI of difference: 2.3%, 20.7%] but comparable to MGIT (P=0.50; 95%CI of difference [-2.2%, 7.3%]). Specificity and positive predictive value (PPV) of smear and MGIT was nominally 100% because these tests were part of the definition for confirmed TB and therefore a true assessment of specificity of these tests was not part of this study. However, all patients with a positive smear or MGIT result had clinical symptoms consistent with TB and were considered to have TB by the treating clinician. Specificity and PPV of MODS were 94.4%, [95%CI: 72.7, 99.8] (n=17/18) and 96.8%, [95%CI: 83.7, 99.9], (n=31/32), respectively. Negative predictive value (NPV) of smear, MGIT and MODS was 24.3%, [95%CI: 15.0, 35.6] (n=18/74); 28.6%, [95%CI: 17.8, 41.3] (n=18/63) and 26.6%, [95%CI: 16.2, 39.0] (n=17/64), respectively.

When analysed by sample, the sensitivity of MODS, MGIT and smear was 43.8% [95%CI: 32.8, 54.7] (n=74/169), 48.5% [95%CI: 36.9, 60.0] (n=82/169) and 27.2%

[95%CI: 17.0, 37.4] (n=46/169), respectively. MODS was more sensitive than smear (P<0.001; 95% CI for difference [8.5%, 24.6%]) but less sensitive than MGIT (P=0.027; 95%CI of difference [-8.9%, -0.5%]). **Table 3.9** shows the sensitivity of the three methods against clinical diagnosis as the gold standard in terms of patient and sample.

Table 3.9 Sensitivity of MODS, smear and MGIT against clinical gold standard in 78 paediatric patients

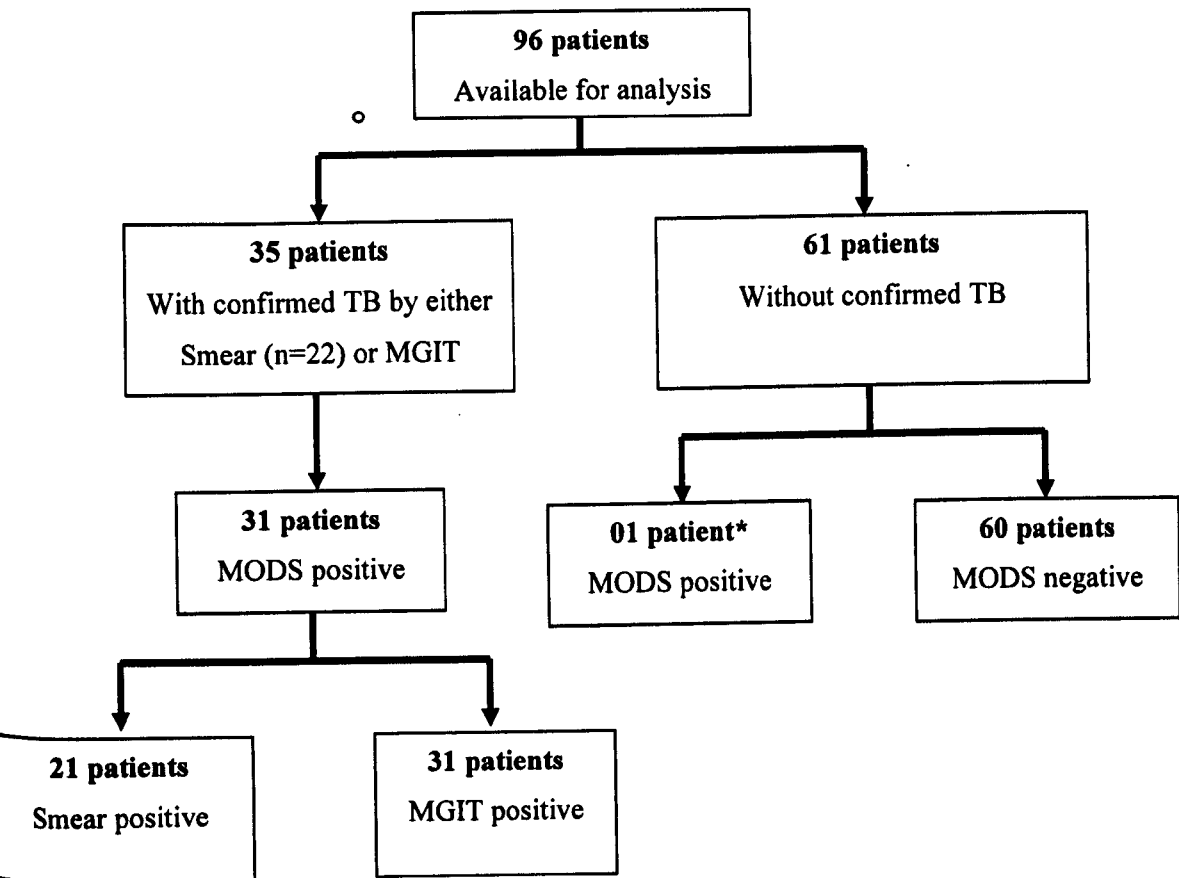
| | | MODS N (%) [95%CI] | SMEAR N (%) [95%CI] | MGIT N (%) [95%CI] | Comparison: P-value ⁷ , [95%CI of difference] | |
|----------------------|----------------------------|---------------------------|---------------------------|---------------------------|---|------------------------|
| | | | | | MODS vs SMEAR | MODS vs MGIT |
| By patient (N=78) | | 31 (39.7) [28.8,51.4] | 22 (28.2) [18.6,39.5] | 33 (42.3) [31.1,54.0] | 0.011 [2.3%,20.7%] | 0.5 [-2.2%,7.3%] |
| By sample (N=169) | | 74 (43.8) [32.8,54.7] | 46 (27.2) [17.0,37.4] | 82 (48.5) [36.9,60.0] | <0.001 [-2.5%,-8.5%] | 0.027 [-8.9%,-0.5%] |
| By Sample type | Sputum (N=101) | 63 (62.4) [48.1, 76.5] | 42 (41.6) [26.5, 56.6] | 68 (67.3) [52.8, 81.8] | <0.001 [9.6%,31.9%] | 0.051 [-0.9%,0.03%] |
| | Gastric fluid (N=35) | 10 (28.6) [5.1, 52.1] | 3 (8.6) [0.0, 20.5] | 10 (28.6) [6.1, 51.0] | 0.045 [-39.5%,-0.4%] | 1 [-8.2%,8.2%] |

⁷ P values were calculated by Mac.Nemar's test for patient analysis and by Marginal regression model for sample analysis.

3.3.4.3.2 Microbiological confirmation as the gold standard

Of 35 patients with confirmed TB, 31 (88.6%) had at least one sample positive by MODS (Figure 3.6). When analysed by patient, the sensitivity, specificity, PPV and NPV of MODS were 88.6% [95%CI: 73.0, 96.0] (n=31/35), 98.4% [95%CI: 91.0, 99.0] (60/61), 96.88% [95%CI: 83.0, 99.0] (n=31/32) and 93.8% [95%CI: 84.0, 98.0] (n=60/64), respectively against microbiological confirmation by MGIT/smear as the gold standard. The specificity of MODS was not 100% because there was one patient positive by MODS but negative by both smear and MGIT. Spoligotyping of the MODS positive isolate of this patient, showed it to be H37Rv, which was used as the positive control in the MODS technique and this result was therefore classified as false positive by cross-contamination. The final diagnosis of this patient was microbiologically confirmed *Staphylococcus aureus* infection.

Figure 3.6 MODS positive in relation to smear and MGIT, for 96 paediatric patients.



**This patient was deemed a false positive due to H37Rv (the positive control strain) identification by spoligotyping.*

When analysed by sample, MODS yielded a sensitivity of 81.3% [95%CI: 73.3, 89.2] (n=74/91), specificity of 99.2% [95%CI: 97.7, 100] (n=125/126), PPV of 98.6% [95%CI: 96.0, 100] (n=74/75) and NPV of 88.0% [95%CI: 81.7, 94.4] (n=125/142). Using stored aliquots of the original processed sample we recultured 8 samples by

MODS which had been positive by MGIT but negative by MODS. These samples remained culture negative by the MODS technique.

3.3.4.4 Effect of sample type

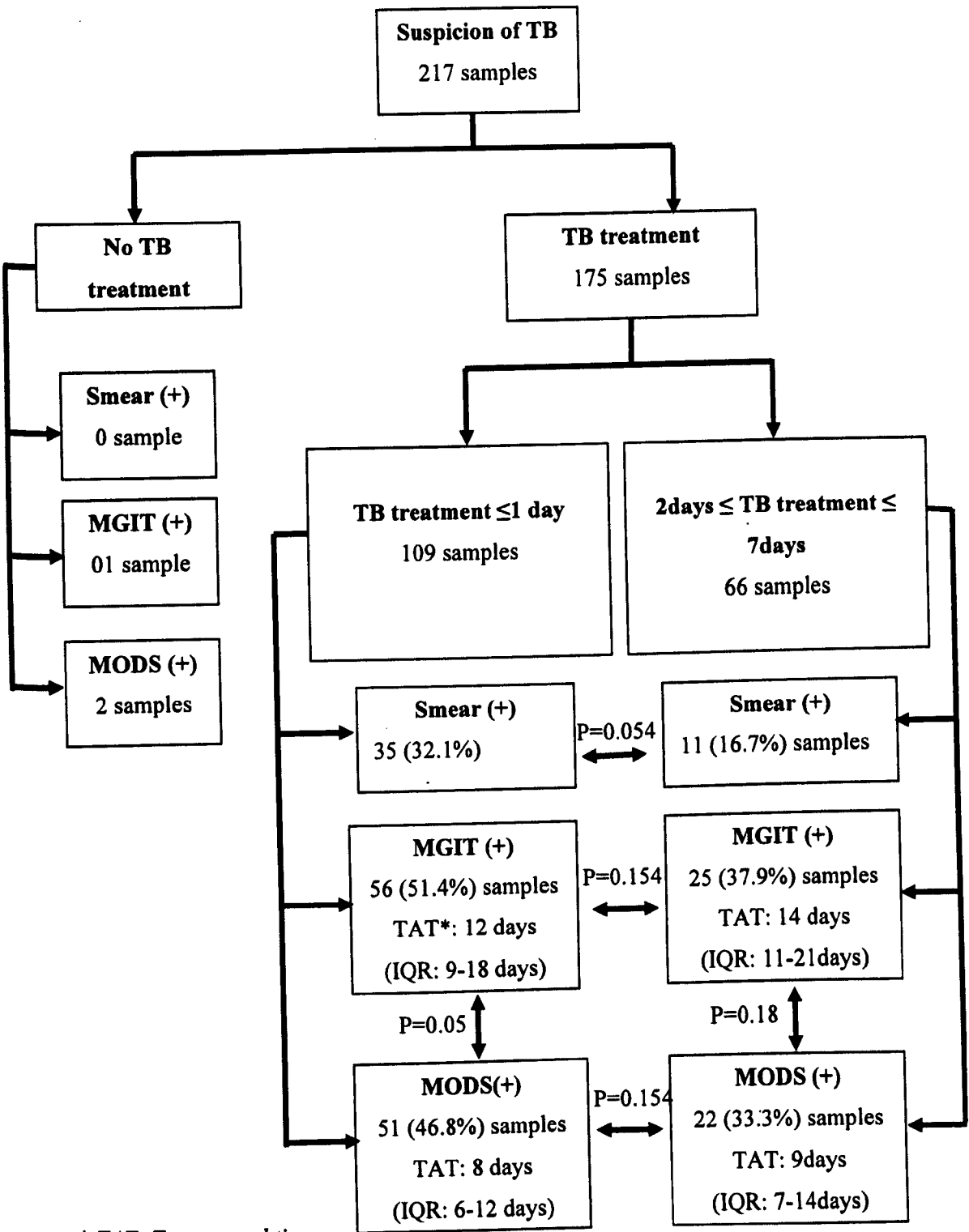
To investigate whether sample type had a strong impact on the sensitivity of the three methods, we investigated the number of each sample type collected from 78 patients with clinical picture of TB and analyzed the sensitivity of these methods in terms of sputum sample (n = 101), gastric fluid (n = 35), CSF (n=30) and pleural fluid (n=1) (table 4.4.3.1.1). Our data showed that in sputum, the sensitivity of MODS (62.4%, n=63/101) was significantly higher than smear (41.6%, n=42/101), $P < 0.001$; 95% CI for difference [9.6%, 31.9%]; and marginally lower than MGIT (67.3%, n=68/101) with $P = 0.051$; 95%CI of difference [-9.92%, 0.03%]. In gastric fluid, MODS and MGIT had the same sensitivity (28.6%, n=10/35); and they were more sensitive than smear ($P = 0.045$; 95% CI [0.4%, 39.5%]). In CSF samples, no positive result was detected by smear. MODS detected *M.tuberculosis* in one CSF sample and MGIT was positive with 4 CSF samples. There were insufficient samples to analyze the sensitivity of these methods in CSF (n=30) and pleural fluid (n=1).

3.3.4.5 Effect of anti-tuberculosis treatment

One hundred and seventy-five samples of all types from patients receiving TB treatment in this admission were collected on the day of TB treatment or within seven days of starting TB therapy. TB treatment for more than 1 day reduced the sensitivity of all 3 microbiological tests, but this difference did not reach significance; ($P = 0.054$;

95%CI of difference [-31.1%- 0.2%]) for smear, (P=0.154; 95%CI [-32.1%, 5.0%])
for MGIT and (P=0.154; 95%CI [-31.9%, 5.0%]) for MODS (**figure 3.7**).

Figure 3.7 Detection rates of Smear, MGIT and MODS in diagnosis of paediatric TB in relation to TB treatment.



* TAT: Turnaround time
P values were calculated by Marginal regression model for comparison of detection rates between MODS and MGIT.

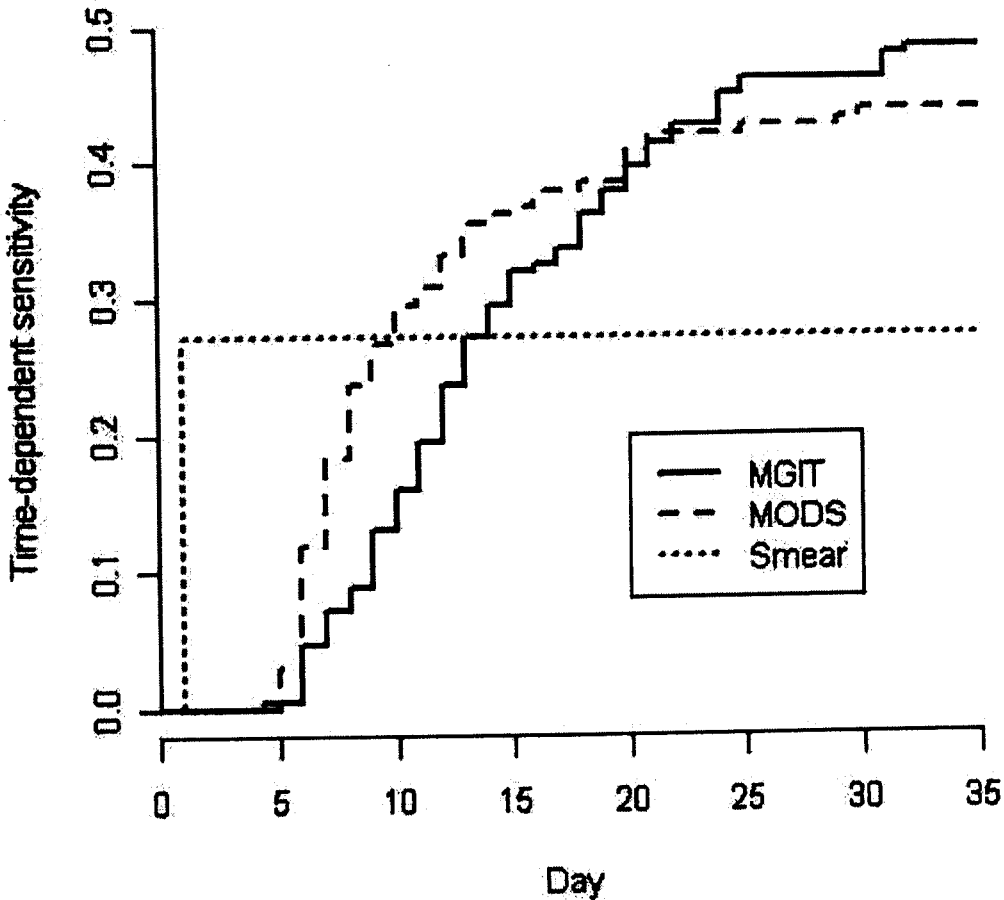
3.3.4.6 Time to detection

Time to detection was defined as the number of days from sample processing (day 1) to positive result available. Smear results were available one day after sample processing in accordance with routine practice due to the high volume of samples in this laboratory. The median time to detection by MODS was faster than MGIT, 8 days (IQR: 6 – 12) vs 13 days (IQR: 9 – 19), respectively, in samples positive by either MODS or MGIT. In the 73 samples which were both MGIT and MODS positive, MODS was faster for 64 (88%) samples and the median (IQR) time difference was 3 (1 to 6) days in favor of MODS ($p < 0.001$).

"Time-dependent sensitivity" analysis included only samples positive by both MGIT and MODS which reached positivity by day 7 or 14, respectively. The time-dependent sensitivity against clinical gold standard was significantly higher for MODS compared to MGIT on both day 7 (18.34% vs. 7.10% $P < 0.001$), and on day 14 (36.09% vs. 29.59%, $P = 0.04$) (**figure 3.8**).

Four CSF samples positive by MGIT had a median time to positive of 25 days (IQR: 23 – 25). One CSF sample was positive by MODS 6 days after inoculation.

Figure 3.8 Time-dependent sensitivity of smear, MODS and MGIT in diagnosis of paediatric TB.



In the 73 samples which were both MGIT and MODS positive, the time dependent sensitivities of MODS were higher than MGIT on both day 7 ($P < 0.001$) and day 14 ($P = 0.04$), by Marginal regression model.

3.3.4.7 Detection of smear negative TB

To evaluate if MODS is useful for the detection of smear negative cases, we evaluated the sensitivity of MGIT and MODS among 56 smear negative TB cases (confirmed

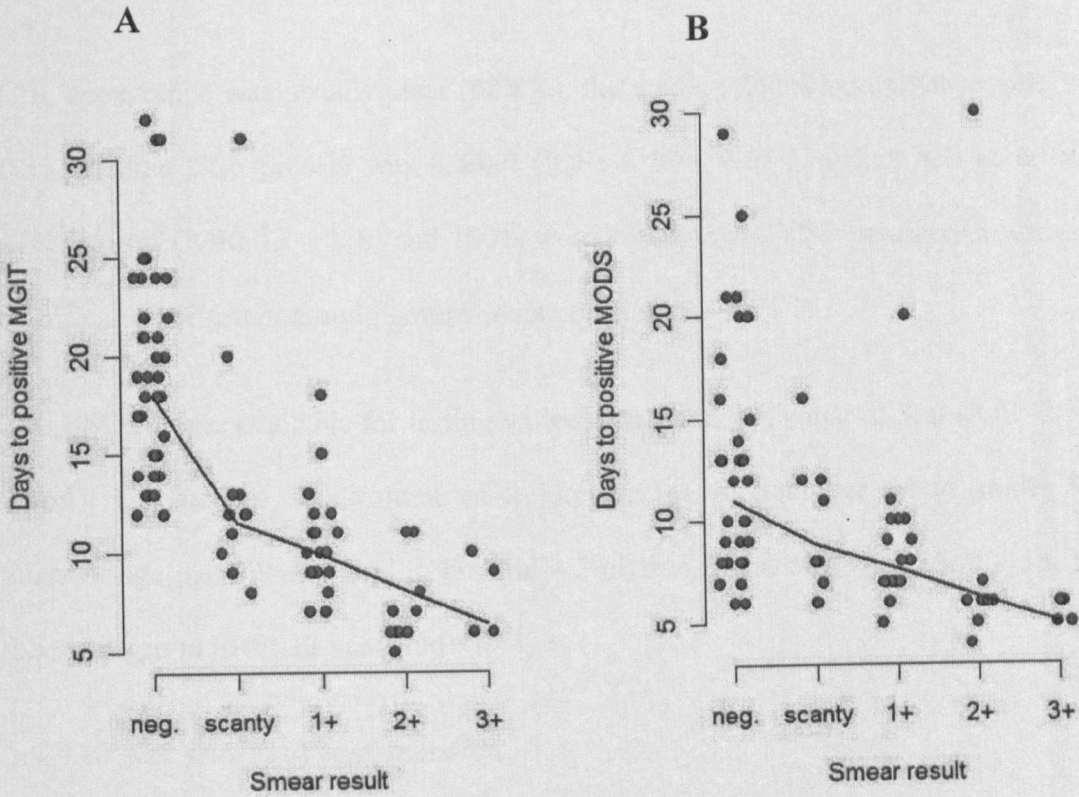
and probable) and 123 smear negative TB samples including either MODS or MGIT positive.

The sensitivity of MGIT and MODS by patient were comparable, ($P = 0.5$; 95% CI for difference [-3.1%, 10.2%]), which were 21.4% ($n = 12/56$) and 17.9 ($n = 10/56$), respectively. The sensitivities were 30.1%, [95%CI: 18.5, 41.6] ($n = 37/123$) and 25.2% [95%CI: 14.9, 35.5]($n = 31/123$) for MGIT and MODS, respectively, in terms of sample with $P=0.053$, 95% CI for difference [-0.05%, 9.80%]. The median time to positive of MODS for smear negative samples was considerably faster than MGIT, which was 12 days (IQR: 8 - 17 days) compared to 19 days (IQR: 15 – 22 days). In the 30 samples where both MGIT and MODS were positive, the median time difference was 6 days (IQR: 3 – 11days) in favor of MODS ($p=0.005$). Therefore, MODS appeared to have an advantage over MGIT in terms of time to detection.

3.3.4.8 Time to positive in relation to smear result

To investigate whether time to positive of MGIT and MODS is related to smear positive grading, we analyzed the association of time to detection of MGIT and MODS with smear grade. **Figure 3.9** demonstrates a negative correlation of the time to a positive test for both MGIT and MODS, respectively, compared to smear with both $P \leq 0.001$.

Figure 3.9 Time to MGIT positive and MODS positive in diagnosis of paediatric TB, in relation to smear grade.



Filled dots are samples positive by either (A) MGIT or (B) MODS, lines are scatter plot smoothers. Both MGIT and MODS had a negative Spearman rank correlation with smear grade ($P < 0.001$).

3.3.4.9 Cerebrospinal fluid

One CSF was collected from each patient with suspected TBM. Thirty-two out of ninety-six patients (33.4%) were suspected of TBM and had a lumbar puncture performed. Of these, 4 patients were classified as confirmed TBM. Among these 4

confirmed TBM patients, three of these patients had CSF positive by MGIT alone, one patient had CSF positive by both MODS and MGIT. Twenty six patients were defined as probable TBM (see chapter 2, section 2.7) and 2 patients were defined as 'not TBM' (see chapter 2, section 2.7).

CSF appearance was usually clear (68.8%), the median CSF: blood glucose ratio was 0.33, median CSF protein was 0.46g/l (IQR: 0.26 – 0.63), median cell count was 47cells/mm³ (IQR: 13 – 110) and 100% were lymphocytic. CSF parameters were not significantly different among groups (data not shown).

The CSF volume available for testing varied with a median value of 3ml (IQR: 2.5ml - 5ml). The median CSF volume of children in the younger age group (under five years of age) (n=19) was 3ml (IQR: 2ml – 3ml) and it was 5ml (IQR: 5ml – 5ml) in the older group (over 12 years old) (n=4).

3.3.4.10 Assessment of contamination

In terms of contamination with fungi, the original contamination rate of MGIT and MODS were 1.39% (n = 3/217) and 0.9% (n = 2/217), respectively. All MGIT contaminated samples were decontaminated again and reinoculated in MGIT medium. No contamination was observed after the second decontamination. Reprocessing for sample contaminated by MODS was not attempted because of the low total volume (1ml for each well).

Spoligotyping was done for all cultures positive by MODS (n=73). If MODS was contaminated while subculturing from MODS to LJ for DNA extraction (n=2) or

MODS was negative but MGIT positive (n=8), cultures positive by MGIT were used for spoligotyping.

In terms of cross-contamination with *M.tuberculosis*, one sample and four negative control wells on 4 different plates were contaminated with the positive control isolate (H37Rv) at the wells closest to the positive control well. **Figure 3.10** shows the positions of contamination observed in the MODS experiment. The average number of samples processed on each day having contamination was 8, ranging from 3 samples to 21 samples.

Figure 3.10 Position of contamination observed in MODS plates of MODS evaluation for diagnosis of paediatric TB

| | | | | | | | |
|---|----------------|---|---|---|---|---|---|
| C | F(2), Rv(4) | S | - | S | - | S | - |
| - | S, Rv | - | S | - | S | - | S |
| S | - | S | - | S | - | S | - |
| - | S | - | S | - | S | - | S |
| S | - | S | - | S | - | S | - |
| - | - | - | - | - | - | - | - |

C: Well containing positive control isolate (H37Rv),

S: Well containing sample.

F: Well was contaminated with fungi.

Rv: Well was contaminated with H37Rv.

Rv(4). Four plates were contaminated with H37Rv at this position.

One isolate that was culture positive by MGIT but negative by MODS was not transferred for genotyping because of contamination during archiving. Serial isolates or isolates from different samples from the same patient had identical spoligotypes except in 2 cases. One patient had different genotypes isolated from MGIT and MODS cultures. Spoligotyping was used to investigate the possibility of cross-contamination of the MODS culture. No other samples with the same spoligotype were processed for MODS on that day, therefore this may have been a cross-contamination in MGIT culture or a patient with dual infection. The patient had symptoms consistent with TB and was considered to have TB by the treating clinician and received TB treatment.

In another patient, a single MGIT culture was contaminated with *Mycobacterium fortuitum* on subculture. However, 3 MODS positive cultures and 2 MGIT cultures from this patient isolated *M.tuberculosis* with identical genotypes.

3.3.5 DISCUSSION

This study demonstrates that MODS is a suitable, rapid, sensitive and specific test for the diagnosis of paediatric TB.

With clinical diagnosis as the gold standard, MODS was more sensitive than smear ($P=0.011$) and the sensitivity was comparable to MGIT ($P=0.5$) on per patient analysis ($n=78$). However, on per sample analysis ($n=169$), MODS was more sensitive than smear ($P<0.001$) but less sensitive than MGIT ($P=0.027$). Larger sample size (169 samples compared to 78 patients) may account for this difference.

With microbiological diagnosis by MGIT/smear as the gold standard, MODS diagnosed 88% of TB cases, similar to a report from Peru in 2006 in which MODS detected 87% of pediatric TB cases (144). Although sensitivity of MODS was not statistically different from MGIT in terms of patient analysis, MGIT detected marginally more pediatric TB patients than MODS (33/35 patients vs 31/33 patients). One explanation for this difference may be the volume of sample inoculated in each well. It is known that sample volume is important in TB diagnosis for paucibacillary samples; an increased volume increases the likelihood of bacilli in the sample (195). We applied 0.5ml of decontaminated sample for MGIT culture while only 0.25ml (half volume) was used for MODS. In another study in adults, 0.5ml was used for MBBacT (Organon Teknika) and 0.72ml of decontaminated sputum was used for MODS, almost three times higher than our volume, although comparison between studies are difficult because the relative proportion of sample will depend on the resuspension volume of the processed deposit (132). In that study, the sensitivity of MODS was higher than MBBacT (97.8% vs 89.0%) and this may be due to a larger proportion of deposit being inoculated to MODS than MBBacT. In our laboratory, 48 well-plates are used instead of the 24 well-plates described in the standard MODS operating procedure (SOP of MODS)(90). This allows for faster plate reading, an important consideration in high-burden laboratories however this lowers the deposit volume inoculated which may have an impact on sensitivity. Combining a more concentrated deposit with a 24-well plate may potentially improve sensitivity without compromising speed.

Pediatric TB samples are paucibacillary and it is therefore important to consider which sample types yield highest sensitivity from the limited sample volumes that are inevitably available from children. Our study found that sputum and gastric fluid were appropriate for use to diagnose pediatric TB, in agreement with other groups who have also found gastric fluid to improve confirmed TB rates in sputum-negative pediatric TB (144). An alternative method of sampling, such as the string test (211, 212) may further improve diagnostic yield and should be considered for evaluation in this setting.

For patient samples for which both MODS and MGIT tests reached positivity, the time to positive of MODS was shorter than MGIT with a median time difference of 6 days. Technical issues affect the turnaround time of each method. In MGIT culture, 10^5 to 10^6 bacteria/ml are required for the machine to signal a positive result. In MODS culture, the result was read under an inverse microscope with 400x magnification. Therefore, a few bacteria in each well can be detected by MODS several days before MGIT. From this study, we also observed that the time to positive is inversely related to the number of bacilli in each sample as determined by the observed smear result classified as scanty, 1+, 2+ or 3+. Since the sensitivity of MODS and MGIT were similar in smear negative samples and MODS has a faster time to positive than MGIT, MODS is a candidate method for routine use in diagnosis of smear negative pediatric TB.

The specificity of MODS (98.4%) was slightly lower than smear (100%) and MGIT (100%) because we had one false positive sample due to cross-contamination with H37Rv. However, our analysis was biased against MODS since MGIT cultures were

not investigated for cross-contamination and may have occurred without detection. Our results were similar to the results of a study conducted in 2006 which recorded a specificity of 99.6% due to cross-contamination between samples (132). All MODS positive samples in this present study were identified as *M.tuberculosis* by spoligotyping and no sample with cross-sample contamination was detected. No mis-identification of Non-tuberculous mycobacteria (NTM) was recorded in this study but this needs further evaluation in populations with higher rates of NTM, such as patients co-infected with HIV.

Contamination is a common problem with all liquid culture and MODS is not an exception; careful manipulation and rigorous aseptic technique must be maintained to minimize contamination. Contamination with fungi was rare at only 1.4%. We have not detected any cross-contamination between samples. All cross-contaminated wells were sited at the positions closest to the positive control wells. A high concentration of H37Rv is inoculated into positive control wells ($>10^5$ bacteria/100ul), and therefore cross-contamination was more likely from the positive control than from paucibacillary samples.

Overall, MODS is a sensitive, quick and reliable culture test for use in the diagnosis of pediatric TB, allowing these vulnerable individuals to start TB treatment earlier. Costs of each test were not formally evaluated but MODS has lower reagent costs than the alternative rapid culture or molecular methods currently available. Further cost-effectiveness studies should be done to evaluate the specificity, cross-contamination, and feasibility of MODS in National TB Programs

Due to the lack of child-friendly diagnostic tools, it is difficult to confirm TB diagnosis in children and therefore it is challenging to determine the epidemiology and the efficacy of TB control strategies for this population. In the report of the 2011 STOP TB Childhood Tuberculosis subgroup meeting (50), Cuevas summarized that among 6699 publications on evaluations of diagnostic tests including fine needle aspiration, fluorescence microscopy, LED-fluorescence microscopy, MODS, BACTEC 960, fully automated BACTEC, line probe assays, LAM and gene Xpert, only 144 (2%) publications reported study results on children. Especially, only 4/144 (2.7%) studies evaluate the efficiency of fluorescence microscope, MODS and line probe assays in children. Aside from diagnostic tools as the major challenge in management of childhood TB, there are barriers to integration of childhood TB healthcare into public health programmes, implementation of WHO guidelines on childhood management and financial constraints. To overcome this problem, a call for action for childhood TB has been launched in 2011 (72) to appeal to national TB programmes, healthcare providers, policy makers, scientific organizations, social communities to include paediatric TB in their strategic plan development.

3.4 Section 4

MODS IN DIAGNOSIS OF HIV ASSOCIATED TUBERCULOSIS

3.4.1 INTRODUCTION

Of 9.4 million new cases of tuberculosis (TB) in 2008 1.4 million (15%) were in HIV-positive patients and 23% of all HIV deaths are attributable to TB according to WHO estimates (236, 237).

Viet Nam is a high TB burden country with steeply rising rates of HIV-TB co-infection (202); 8.1% of newly diagnosed TB patients are now HIV infected (236). These cases are the most urgently in need of diagnosis because they have the highest morbidity and mortality yet the diagnosis of TB among HIV infected individuals is difficult. Screening algorithms based on clinical symptoms alone show high sensitivity but low specificity (28, 29, 226). Microscopy smear while simple, specific and widely available in high burden settings, has particularly low sensitivity in HIV patients and cannot be used to rule out a diagnosis of TB (128, 182). Microbiological confirmation remains desirable and allows investigation of drug susceptibility profiles. Commercial rapid liquid culture techniques have been endorsed by WHO (230), show higher sensitivity and are more rapid than traditional solid media-based techniques such as Lowenstein-Jensen culture. However, their high cost and biosafety infrastructure requirements limit their applicability in many high burden settings. Rapid molecular line-probe assays, also endorsed for use in low-resource settings by WHO (233), allow simultaneous identification of *M. tuberculosis* and resistance to rifampicin or isoniazid but are currently only recommended for smear-positive

samples and positive cultures. In addition, they are expensive and require molecular expertise which is often not available in low-resource settings. Recently, GeneXpert has been approved by WHO for early diagnosis of TB and MDR-TB at peripheral levels (248). WHO also recommended that GeneXpert should be prioritized to detect TB and MDR-TB in high risk groups, including HIV infected individuals and patients suspected of infection with MDR-TB (246). However, rolling out this test may encounter challenges in terms of a low positive predictive value for MDR TB, costs, sustainability, monitoring during treatment, storage condition, maintenance, shelf-life of the cartridge, delivery and technical issues to prevent cross-contamination.

MODS has been shown effective in identification of TB in HIV patients (7, 161). The increasing number of HIV-positive pulmonary TB suspects presenting to PNT hospital has led to an urgent need for a rapid and sensitive test to detect TB for this population.

Here, we evaluated the MODS assay for TB diagnosis in HIV-infected patients presenting to PNT. As in the previous studies, we assessed the sensitivity, specificity, negative predictive value, positive predictive value, contamination rate and turnaround time of MODS against clinical gold standard and microbiological gold standard.

3.4.2 METHODS

3.4.2.1 Enrollment

- All HIV-positive individuals suspected of tuberculosis, who fulfilled enrollment criteria (**chapter 2, section 2.4**) and presented to the HIV/TB ward at PNT Hospital were enrolled into the study.
- Data on socioeconomic and demographic features, TB history, TB contact history, HIV status and presenting clinical features were prospectively collected on a standard case report form.

3.4.2.2 Definition and TB classification

- The definition and TB classification were described in **chapter 2, section 2.7**

3.4.2.3 Sample collection, sample processing and laboratory tests

- As described in materials and methods (**chapter 2, section 2.5 and 2.6**).
- Sputum samples were collected for this study.
- All processed samples were tested by homogenous smear, MGIT and MODS culture.

3.4.3 STATISTICAL METHODS

- Accuracy measures of the 3 tests were calculated for two different definitions of the 'gold standard' reference test: (1) microbiological confirmation (confirmed group) or (2) 'clinical diagnosis' (clinical gold standard including

the confirmed and the probable group). In addition, we analyzed data on a 'per patient' or a 'per sample' basis.

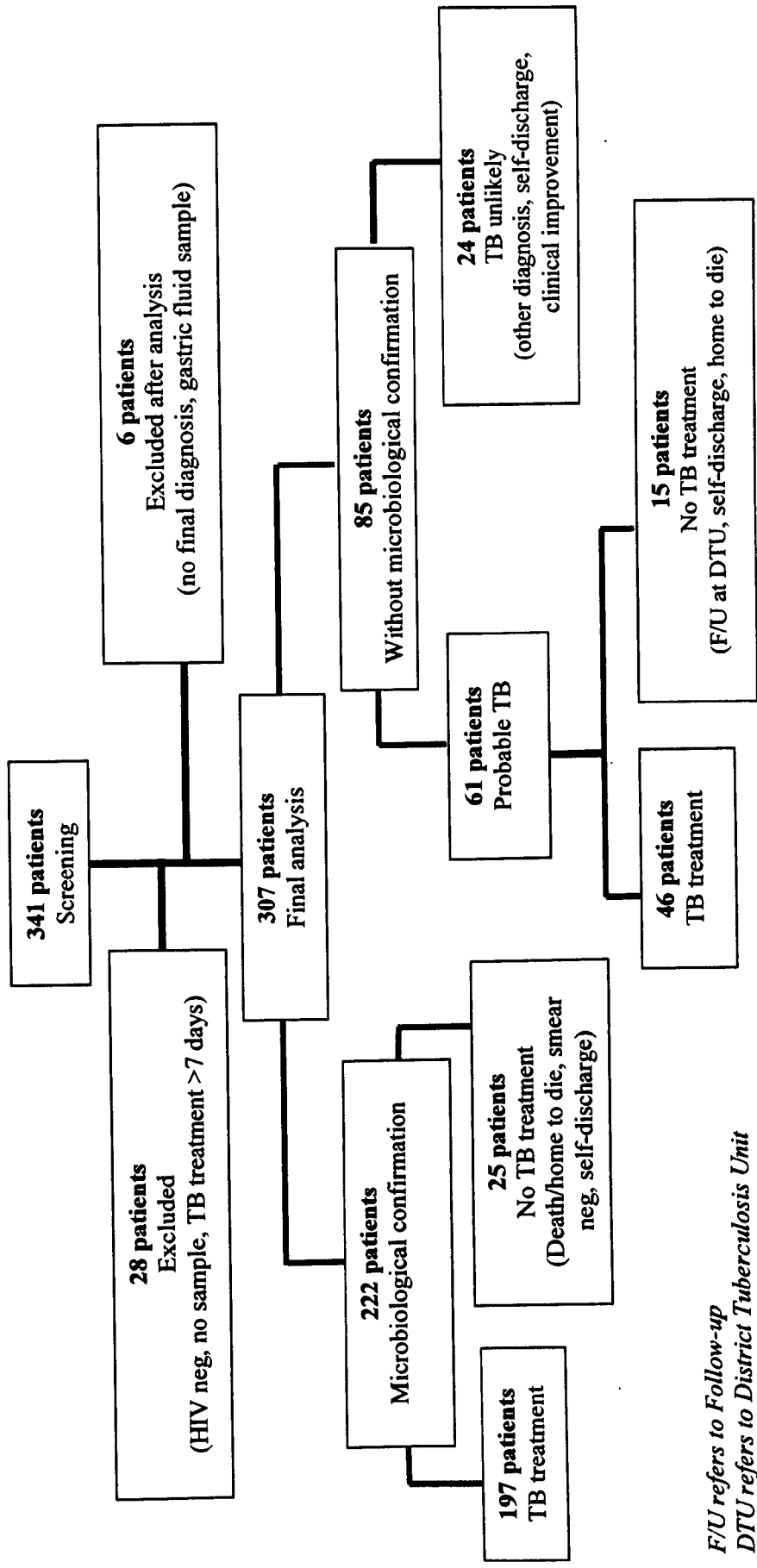
- Details of statistical methods were described in **chapter 2, section 2.8**. Comparisons between MODS, smear and MGIT in terms of accuracy, time to positive, TB treatment- dependent sensitivity and contamination were performed.

3.4.4 RESULTS

3.4.4.1 Enrollment

Three hundred and forty-one HIV positive individuals were screened for pulmonary tuberculosis (**Figure 3.11**). Of these, 8.2% (n=28/341) patients were excluded because they subsequently tested HIV negative (3 cases), no samples were collected (24 cases) or they had already received TB treatment for more than eight days (1 case). Thus, 313 patients were eligible for the analysis. However, six additional patients were excluded after clinical and laboratory analysis because insufficient information was collected prior to self-discharge of the patient (4 cases) and inappropriate sample (gastric fluid) was collected (2 cases). Thus, data from 307 patients were analyzed and reported in this study.

Figure 3.11 Flowchart of HIV positive TB suspect recruitment and groups of patient based on micro-confirmation (Smear or MGIT), TB treatment and outcome.



*F/U refers to Follow-up
DTU refers to District Tuberculosis Unit*

A total of 738 sputum samples were collected from these 307 patients. Two hundred and twenty-two (72%, n=222/307) patients had microbiological confirmation by a method other than MODS. This group also included 6 patients with microbiological confirmation by smear or MGIT based on samples collected prior to study enrolment. 61 patients (20%, n=61/307) were classified as 'probable TB' and 24 patients (8%, n=24/307) as 'TB unlikely'.

3.4.4.2 Demographics and clinical features

Over 90% (n=301/307) of the study population was male with a median age of 29. Almost 60% (n=182/307) of patients had evidence of BCG vaccination determined by BCG scar. Only 13% (n=42/307) were on antiretroviral therapy (ART). Twenty percent (n=61/307) of patients had previously been diagnosed with TB once in their medical history. **Table 3.10** shows demographic characteristics of the study population and comparisons of the three groups. **Table 3.11** shows clinical features of the 307 HIV-associated TB suspects. Cough, fever and weight loss were the most frequent symptoms with the majority of patients having a history of illness between 30-59 days. Lymphadenopathy was present in 43% of patients.

Table 3.10 Demographic characteristics of HIV positive patients enrolled into the study.

| Characteristic | Total population N = 307 | Micro- confirmation TB N = 222 | Probable TB N = 61 | TB unlikely N = 24 |
|--|------------------------------------|--|------------------------------|------------------------------|
| Gender | P=1.000 | | | |
| Male | 301 (98.1) | 217 (97.8) | 60 (98.4) | 24(100.0) |
| Age (year) | P=0.801 | | | |
| Median (IQR) | 29 (26 – 33) | 29 (26 – 33) | 30 (26 – 33) | 30 (27 – 35) |
| BCG vaccination^(*) | P=0.858 | | | |
| Yes | 182 (59.3) | 130 (58.7) | 39 (63.9) | 13 (54.2) |
| No | 117 (38.1) | 85 (38.3) | 21 (34.4) | 11 (45.8) |
| Unknown | 8 (2.6) | 7 (3.2) | 1 (1.7) | 0 |
| TB history | P<0.001 | P1=0.52 | P2=0.001 | P3<0.01 |
| Yes | 61 (19.9) | 36 (16.2) | 12 (19.7) | 13 (54.2) |
| No | 245 (79.8) | 186 (83.8) | 48 (78.7) | 11 (45.8) |
| Unknown | 1 (0.3) | 0 | 1 (1.7) | 0 |
| ARV therapy | P=0.116 | | | |
| Yes | 42 (13.7) | 29 (13.1) | 10 (16.4) | 3 (12.5) |
| No | 261 (85.0) | 192 (86.5) | 49 (80.3) | 20 (83.3) |
| Unknown | 4 (1.3) | 1 (0.5) | 2 (3.3) | 1 (4.2) |
| TB contact | P=0.758 | | | |
| Yes | 12 (3.9) | 10 (4.5) | 1 (1.6) | 1 (4.2) |
| No | 281 (91.5) | 203 (91.4) | 56 (91.8) | 22 (91.7) |
| Unknown | 14 (4.6) | 9 (40.1) | 4 (6.6) | 1 (4.1) |

Summary measure is n(%) for all categorical characteristics.

(*)Presence of BCG scar

P refers to the p-value of a (global) comparison of all three groups. P values were calculated by Kruskal-Wallis test for continuous variables and by Fisher's exact test for categorical variables. If $P < 0.05$, the pairwise comparisons P1, P2, P3 were also performed.

P1: confirmed TB vs probable TB.

P2: probable TB vs TB unlikely.

P3: TB unlikely vs confirmed TB.

Table 3.11 Clinical features of 307 TB/HIV suspects

| Characteristic | Total population N = 307 | Micro- confirmation TB N = 222 | Probable TB N = 61 | TB unlikely N = 24 |
|---------------------------|---|---|-----------------------------------|-------------------------------|
| History of illness | | | | |
| ≤ 29 days | 74 (24.1) | 52 (23.4) | 12 (19.7) | 10 (41.7) |
| 30 – 59 days | 185 (60.3) | 130 (58.6) | 42 (68.8) | 13 (54.2) |
| ≥ 60 days | 48 (15.6) | 40 (18.0) | 7 (11.5) | 1 (4.2) |
| | P=0.107 | | | |
| Cough | 297 (96.7) | 216 (97.3) | 58 (95.1) | 23 (95.8) |
| | P=0.465 | | | |
| Fever | 294 (95.8) | 216 (97.3) | 60 (98.4) | 18 (75.0) |
| | P<0.001 | P1=0.63 | P2<0.001 | P3<0.001 |
| Nightsweat | 245 (79.8) | 174 (78.4) | 54 (88.5) | 17 (70.8) |
| | P=0.106 | | | |
| Weightloss | 292 (95.1) | 212 (95.5) | 59 (96.7) | 21 (87.5) |
| | P=0.201 | | | |
| Lymphadenopathy | 138 (44.9) | 106 (47.8) | 24 (39.3) | 8 (33.3) |
| | P=0.031 | P1=0.08 | P2=0.89 | P3=0.25 |

Summary measure is n (%) for all categorical characteristics.

P refers to the p-value of a (global) comparison of all three groups. P values were calculated by Kruskal-Wallis test for continuous variables and by Fisher's exact test for categorical variables. If $P < 0.05$, the pairwise comparisons P1, P2, P3 were also performed.

P1: confirmed TB vs probable TB.

P2: probable TB vs TB unlikely.

P3: TB unlikely vs confirmed TB.

3.4.4.3 Accuracy of MODS

3.4.4.3.1 Accuracy against microbiological gold standard

Microbiological gold standard was defined as patients whose samples were Accuracy against microbiological confirmation as the gold standard positive by either smear or MGIT. MODS detected 87.4% of these cases with a specificity of 93%. The accuracy of MODS against microbiological gold standard, by patient and sample analysis, is detailed in table 3.12.

Table 3.12 Accuracy of MODS versus microbiological confirmation as the gold standard in 307 HIV/TB suspects.

| | By patients | By samples |
|----------------------------------|----------------|----------------|
| Sensitivity | | |
| % (n=x/y) | 87.4 (194/222) | 81.0 (431/523) |
| 95%CI | [82.3 – 95.1] | [76.3 – 85.7] |
| Specificity | | |
| % (n=x/y) | 92.9 (79/85) | 97.1 (200/206) |
| 95%CI | [85.3 – 97.4] | [94.8 – 99.3] |
| Positive predictive value | | |
| % (n=x/y) | 97.0 (194/200) | 98.6 (431/437) |
| 95%CI | [93.6 – 98.9] | [97.5 – 99.7] |
| Negative predictive value | | |
| % (n=x/y) | 73.8 (79/107) | 66.4 (200/301) |
| 95%CI | [64.5 – 81.9] | [58.5 – 74.4] |

3.4.4.3.2 Accuracy against clinical gold standard.

Clinical gold standard was defined as patients satisfying the definition of “microbiological confirmation” group (n=222 patients) or “Probable TB” group (n=61 patients). In total, 283 patients and 684 samples were classified as TB using the clinical gold standard. **Table 3.13** describes the sensitivity and negative predictive value of MODS, smear and MGIT against clinical gold standard by patient and by sample analysis. MODS was significantly more sensitive than smear (71% vs 57%, $p<0.001$ by patient analysis and 64% vs 54%, $p<0.001$ by sample analysis) but less sensitive than MGIT (75%, $p=0.03$ by patient analysis and 70%, $p<0.001$ by sample analysis). The specificity and positive predictive value of all methods were 100%.

Table 3.13 Sensitivities and negative predictive values of MODS, smear and MGIT methods versus the clinical gold standard in diagnosis of HIV/TB.

| | | MODS % (n=x/y) [95%CI] | SMEAR % (n=x/y) [95%CI] | MGIT % (n=x/y) [95%CI] | Comparison: P-value ⁸ , [95%CI of difference] | | |
|---------------------------|-------------------------|-----------------------------------|-----------------------------------|-----------------------------------|---|-------------------------|--|
| | | | | | MODS vs SMEAR | MODS vs MGIT | |
| Sensitivity | All subjects | | | | | | |
| | By patient (n=283) | 70.7 (200/283) [64.9, 75.9] | 56.9 (161/283) [50.9, 62.7] | 74.9 (212/283) [69.4, 79.8] | <0.001 [8.6%,18.9%] | 0.03 [-8.1%,-0.4%] | |
| | By sample (n=684) | 63.8 (437/684) [58.5, 69.2] | 53.9 (369/684) [48.2, 59.7] | 69.2 (473/684) [63.9, 74.4] | <0.001 6.9%,12.9%] | <0.001 [-7.7%,-2.8%] | |
| | Smear negative subjects | | | | | | |
| | By Patient (n=122) | 37.7 (46/122) [29.1, 46.9] | N/A | 45.1 (55/122) [36.1, 54.3] | N/A | 0.078 [-15.4%,0.7%] | |
| | By sample (n=315) | 29.2 (92/315) [22.4, 36.0] | N/A | 36.2 (114/315) [28.8, 43.6] | N/A | 0.003 [-11.5%,-2.4%] | |
| Negative Predictive Value | All subjects | | | | | | |
| | By patient (n=283) | 22.4 (24/107) [14.9, 31.5] | 16.4 (24/146) [10.8, 23.5] | 25.3 (24/95) [16.9, 35.2] | 0.323 [-12.7%,4.2%] | 0.711 [-7.9%,11.6%] | |
| | By sample (n=684) | 17.9 (54/301) [11.3, 24.6] | 14.6 (54/369) [9.1, 20.2] | 20.37 (54/265) [12.9, 27.8] | <0.001 [1.6%, 5.1%] | 0.002 [0.9, 3.9%] | |
| | Smear negative subjects | | | | | | |
| | By patient (n=122) | 24.0 (24/100) [16.0, 33.6] | N/A | 26.4 (24/91) [17.7, 36.7] | N/A | 0.770 [-8.6%, 11.7%] | |
| | By sample (n=315) | 19.5 (54/277) [12.3, 26.7] | N/A | 21.2 (54/255) [13.5, 28.9] | N/A | 0.009 [0.42, 2.9%] | |

⁸ P values were calculated by Mc.Nemar's test for patient analysis and by Marginal regression model for patient analysis

3.4.4.4 MODS in diagnosis of smear-negative, HIV-associated TB

One hundred and twenty-two patients with 315 samples were diagnosed with confirmed or probable TB but all their smear samples were negative. Of which, 15/122 (12%) patients were positive by MGIT only, 40/122 (33%) patients were positive by both MODS and MGIT, 6/122 (5%) patients were positive by MODS only, 6 patients were positive by either smear or culture in only samples collected before the enrollment and 55 patients were negative by all microbiology methods, including MODS, in samples collected before and after the study enrollment. MODS detected 75.4% (n=46/61) of culture positive-smear negative TB cases. Comparisons of the sensitivity and negative predictive value of MODS and MGIT are detailed in **table 3.13**. The sensitivity of MODS tended to be lower than MGIT in the 'by patient' analysis (38% vs 45%, p=0.078). Conversely, MGIT was significantly more sensitive than MODS (36% vs. 29%, p=0.003) in the "by sample" analysis. The specificity and positive predictive value of these tests in smear negative patients/samples were 100%.

Of 122 smear negative TB cases, 30 cases did not have TB therapy at Pham Ngoc Thach Hospital because of death, self-discharge or referral to District Tuberculosis Units to start TB treatment and follow-up. Ten out of these 30 cases (33%) did not receive TB treatment because the patient was discharged following a negative smear before the culture results were available.

3.4.4.5 TB treatment-dependent sensitivity

Six hundred and eighty four samples from 283 patients with a clinical TB diagnosis were analyzed. Fourteen percent (14%, n=97/684) of samples were from patients not

on TB therapy. Five-hundred and forty (n=540/587, 92%) samples were collected from patients on TB treatment ≤ 3 days and 47/587 (8%) samples were from patients on TB treatment >4 days. The sensitivity of MODS, Smear and MGIT against clinical gold standard in patients receiving TB treatment ≤ 3 days or ≥ 4 days was compared. The sensitivity of MODS and smear were significantly decreased among samples collected after 4 days of TB treatment compared to earlier samples (53% vs 70%, $p=0.035$ for MODS and 45% vs 61%, $p=0.034$ for Smear); The sensitivity of MGIT also tended to be lower for longer TB treatment duration but the result did not achieve statistical significance (60% vs 74%, $P=0.053$).

3.4.4.6 Time to positive

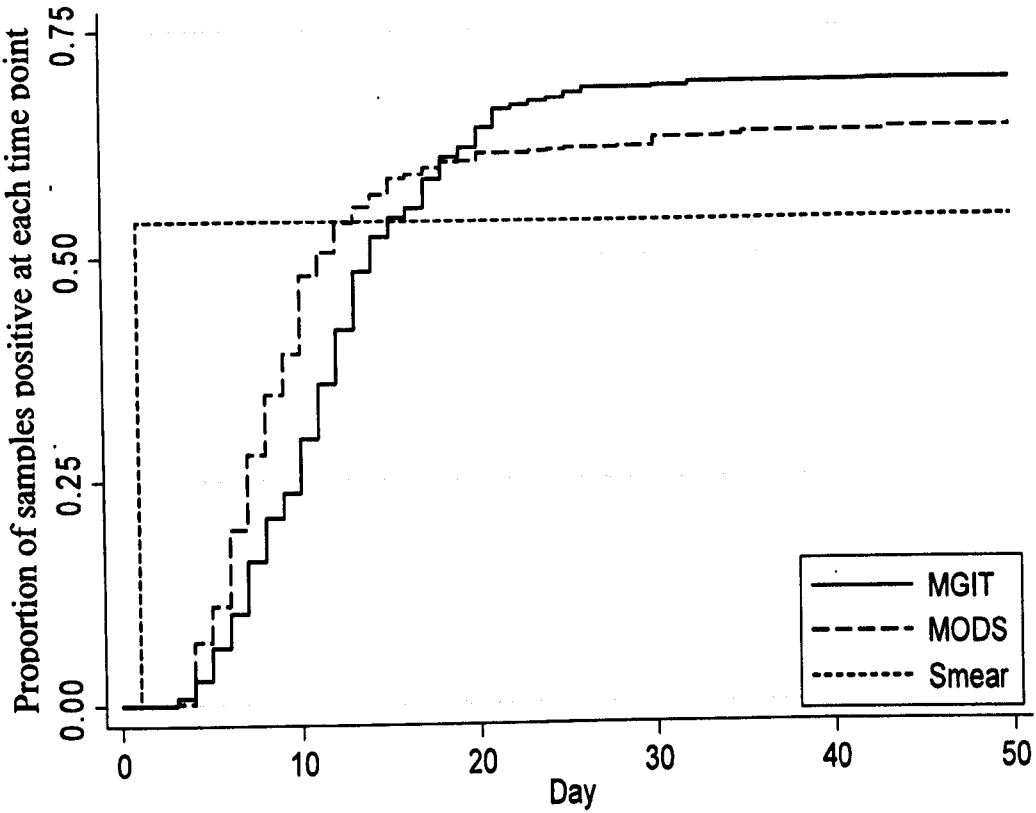
Time to positive was defined as the number of days from sample processing (day1) to positive result available. The results of smear were available on day 2 (routine procedure at PNT Hospital). In samples positive by either MODS (n=437/684) or MGIT (n=473/684), the median time to detection of MODS and MGIT were 8 days (IQR: 6 – 10days) and 11 days (IQR: 8 – 10 days), respectively. Among smear negative samples, the median time to detection of MODS and MGIT were 11 days (IQR: 9 – 16 days) and 17 days (IQR: 13 – 21 days).

3.4.4.7 Time-dependent sensitivity

Time-dependent sensitivity of MODS and MGIT are shown in **Figure 3.12**. In samples positive by both MODS and MGIT, MODS was faster than MGIT in 70% (n=289/418) samples with a median time difference of 2 days (IQR: 0 – 5 days, $P<0.01$). In smear negative samples, of 79 samples positive by both MODS and

MGIT, the MODS results were available 4 days earlier than MGIT (IQR: 0-7 days, $P < 0.01$). MODS also yielded a higher sensitivity than MGIT by day 7 (28% vs. 16%, $P < 0.001$) and day 14 (57% vs. 52%, $P = 0.009$) after inoculation.

Figure 3.12 Time-dependent sensitivity of MODS, smear and MGIT in diagnosis of HIV/TB



The sensitivities of MODS were higher than those of MGIT method by day 7 ($P < 0.001$) or by day 14 ($P = 0.001$), by Marginal regression model.

3.4.4.8 Assessment of contamination

In total, 738 samples were cultured by both MODS and MGIT. We assessed the contamination in terms of fungi or other bacteria and cross contamination between samples for the MODS assay.

In terms of fungal contamination, the original contamination rate of MODS in samples was 1.1% (n= 8/738) while it was 2.6% (n= 19/738) for MGIT. All MGIT contaminated samples were decontaminated again and re-inoculated in MGIT medium. The final fungal contamination rate of MGIT was 1.8% (13/738). Reprocessing for samples contaminated in MODS culture was not attempted because of low volume (total of 1ml for each well). Contamination with fungi was also observed in 8 negative control wells.

To assess cross-contamination of MODS with *Mycobacterium tuberculosis*, spacer oligonucleotide typing (spoligotyping) was applied to all available MODS isolates (n=437/478). Serial positive cultures from individual patients were compared for discrepancies in spoligotype. A positive MGIT culture (n=41) was used for comparison if the MODS culture yielded a negative spoligotype (n=21) or subculture was contaminated from MODS to LJ (n= 20). Among 21 MODS cultures with negative spoligotype, 2 cultures were *M.kansasii* and 19 cultures were *M.tuberculosis complex* identified by molecular line probe assay (INNO-LiPA Mycobacteria, Innogenetics, Ghent Belgium). 412/437 (94%) samples had defined spoligotypes while the remaining 25/437 did not because of negative MGIT culture (n=3), negative

spoligotype (n=3) and DNA not available (n=19). Spoligotypes were deemed possible cross-contamination if serial isolates from an individual patient were discrepant or an isolate was H37Rv (the positive control isolate).

Eight samples from 8 patients (1.1%, n=8/738) were positive by MODS with H37Rv, the positive control strain. An additional twenty-seven MODS isolates were identified as probable MODS cross-contamination due to multiple strains isolated from one patient. It is impossible to rule-out infection with multiple-strains in these patients, but the maximum cross-contamination rate of MODS with *M.tuberculosis* was 4.7% (n=35/738). All false-positive MODS cultures were in 'confirmed' or 'probable' TB groups.

3.4.5 DISCUSSION

We have shown MODS to be a sensitive and rapid method for diagnosis of TB in HIV-infected patients. Although MODS was slightly less sensitive than MGIT (71% vs 75%, P=0.03), MODS is faster than MGIT in samples positive by both methods with a 2 day difference (P<0.001). In smear negative TB cases, although MODS tended to be less sensitive than MGIT (38% vs 45%, P=0.078), MODS detected more cases than MGIT by day 7 (4.4% vs 0.6%, P=0.027) and day 14 (21% vs 12%, P<0.001). MODS detected 72.8% (40/55) culture-positive, smear-negative TB cases.

Therefore, MODS is an appropriate microbiological method for early detection of HIV-associated TB.

Delays in diagnosis result in poor outcomes, increased morbidity and ongoing transmission (28). MODS detected significantly more TB cases at day 7 (4.4% vs 0.6%) and day 14 (21% vs. 12%) than commercial rapid liquid culture, similar to findings comparing MODS and Lowenstein-Jensen in previous studies (7, 79, 132); this is crucial for early diagnosis of TB in immuno-compromised patients. Over 30% of the smear negative TB cases in our study did not receive TB treatment because the MGIT culture result was not available at discharge time. This underlies the need for a rapid diagnostic test in HIV/TB cases. Suspected TB cases who are smear negative are generally prescribed 7 to 14 days broad spectrum antibiotics to exclude other possible causes of community-acquired pneumonia before being re-tested for TB, in accordance with WHO policy (232).

Contamination is an issue with all microbiological techniques and evaluation of contamination is of importance for wide application of MODS. As for the MODS evaluations in pediatric and TBM, we have shown the fungal contamination rate to be low in our hands; 1.1% in this study. Probable cross-contamination of MODS was 4.7% which is within the expected contamination range of MGIT culture (3% to 8.5%) (42, 43, 106, 175). Cross-contamination is difficult to evaluate effectively in TB culture techniques because genotyping techniques have relatively low discriminatory power in endemic settings and it is difficult to rule-out TB infection in symptomatic patients in a high prevalence setting. The median cross contamination rate of TB laboratories is around 3% (3), but it can be much higher (159). An alternative typing technique, such as Restriction Fragment Length Polymorphism (RFLP) or Mycobacterial Interspersed Repetitive Units (MIRU) may have yielded further

resolution of suspected cross-contamination events, however, RFLP has low discriminatory power for isolates with less than 5 IS6110 copies, which represent around 40% of isolates in southern Vietnam (35) and MIRU was under assessment for discriminatory power in local strains in our laboratory at the time of this study.

In this study, only 14% of HIV-infected individuals suspected of tuberculosis and only 13% of TB confirmed-HIV patients were on ART at the time of TB diagnosis. This suggests further efforts are required to enhance integration of the TB and HIV programmes in HCMC. These efforts are ongoing. Since HIV fuels TB infection and increases the risk of development TB disease; and active TB disease accelerates HIV progression, this vicious circle increases the mortality rate of HIV-associated TB patients. Therefore, early ART and implementation of effective TB screening for HIV-infected individuals is crucial. It is known that early start of ART in combination with intensified TB case finding, infection control and isoniazid preventive therapy will reduce the burden of TB in HIV-infected people. Recently, WHO now recommends ARV for all HIV-infected individuals, who were screened for exclusion of active TB, as soon as possible, irrespective of CD4 cell count (degree of immunosuppression) to reduce the risk of TB infection and progressive to active TB (251). Monitoring and follow-up of patients after transfer to district tuberculosis units was not part of this study and we did not have information on ART or TB treatment outcome of these patients.

In conclusion, this study confirmed that MODS is an effective method for detecting *M. tuberculosis* in HIV infected patients, including sputum smear negative cases.

3.5 Section 5

MODS IN DIAGNOSIS OF MULTIDRUG-RESISTANT TUBERCULOSIS

3.5.1 INTRODUCTION

MDR-TB is caused by *M.tuberculosis* which is resistant to at least the two most powerful TB drugs, INH and RIF. In addition to the high costs, long duration of treatment and the lack of randomized controlled trials for optimal regimens, a major barrier to control of MDR-TB is the lack of laboratory diagnostic capacity in high-TB burden settings. Major initiatives are now under way to scale-up capacity for both *M.tuberculosis* culture and DST (70). According to WHO, of 27 high MDR-TB burden countries, only 22 settings had a National Reference Laboratory in 2008. Of 572 laboratories worldwide performing drug susceptibility testing (DST), only half participated in external quality assurance (245).

Recently, the documentation in over 50 countries of extensively drug-resistant tuberculosis (XDR-TB), defined as MDR-TB plus resistance to a fluoroquinolone and at least one second-line injectable agent (amikacin, kanamycin or capreomycin) (245) has further emphasized the need to scale up detection and treatment of MDR TB. In HIV co-infected individuals the classical smear diagnostic test has very low sensitivity and the need for enhanced culture has further urgency in settings with HIV co-epidemics; late diagnosis and treatment contributes to high mortality rates and on-going transmission in this population (226). Classical DST for *M.tuberculosis* on solid

media requires 6-8 weeks as the sputum sample must first be cultured and then regrown on drug-containing media. In 2007, WHO recommended the use of liquid culture and DST in low and middle-income countries as a step-wise approach to scale up TB and MDR-TB diagnosis and management (229, 234). Automated liquid culture allows fast turn-around times of 10-14 days when direct DST is performed but the automated equipment is expensive for most high-burden settings and cheaper alternative methods may be easier to implement. Molecular line probe assays (LPA) for rapid detection of MDR-TB, which are based on reverse hybridization technology have been endorsed by WHO in 2009 (238). With sensitivity and specificity of over 90% and 99%, respectively, against conventional DST method (233), LPA are an alternative rapid and accurate DST method but are only reliable in smear positive cases. Unfortunately, the cost and the requirement of relatively complicated technology and infrastructure in addition to well-trained staff have limited the use of LPA in high TB burden countries where the need is greatest. GenXpert, a novel molecular based technique, detects *M.tuberculosis* and *M.tuberculosis* carrying rifampicin resistance mutations directly from sputum samples. The test provides sensitivity and specificity of *M.tuberculosis* detection of 98% and 99%, respectively and correctly identifies 97.6% isolates with rifampicin resistance and 98% of rifampicin susceptible isolates compared with phenotypic DST (17). However, the cost remains relatively high for use in developing countries at the present time, with FIND negotiated pricing of 18USD per test cartridge (246). Therefore, no single test currently available provides all the characteristics of an ideal test for rapid diagnosis of MDR-TB, including rapid, low-cost, easy to perform, sensitive and specific.

Microscopic Observation Drug Susceptibility (MODS) assay is a direct rapid DST test which has been evaluated for MDR screening from clinical specimens. Previous studies have shown it an accurate, feasible and low-cost test that is promising for use in high burden countries for early diagnosis of MDR TB (61, 132, 133). Procedures for quality assurance of the test are still in development (130) and the accuracy of the test when adopted by non-expert groups needs to be confirmed (256). This technique is now endorsed by WHO for use in resource-limited settings as an interim solution to increase TB case detection (238). The MODS evaluation for early diagnosis of MDR-TB in new Vietnamese TB suspects was performed in this study.

3.5.2 METHODS

3.5.2.1 Enrollment

All patients suspected of tuberculosis, who were newly presenting to the Out Patient Department (OPD) at Pham Ngoc Thach Hospital from August to November 2008 were enrolled into the study. Details of recruitment criteria are described in **chapter 2, section 2.4**.

Data on demographic features and HIV status (if available) were prospectively collected on a CRF.

3.5.2.2 Sample processing, sample collection and tests

- Only the first sputum samples were collected for this study.

- Sample collection, transfer and testing are described in **chapter 2, section 2.5** and **section 2.6**. The samples were then sent for direct DST by MODS and indirect DST by 1% proportional method on LJ.
- All cultures positive by MGIT or LJ were subcultured on LJ (Becton Dickinson) for indirect DST, standard biochemical identification and archiving.
- All cultures positive by MODS (control wells) were subcultured on LJ in duplicate for DNA extraction and archiving.
- If DNA extraction from MODS isolates failed due to contamination or failure to grow on subculture, DNA extraction from MGIT or LJ was used.

Spoligotyping was performed for all cultures positive by MODS (n=329). If MODS was contaminated during subculture from MODS to LJ for DNA extraction (n=24) or MODS was negative but MGIT positive (n=36), cultures positive by MGIT were used for spoligotyping. Spoligotyping was used to support the screening of cross-contamination. Cross-contamination of MGIT was not addressed in this study due to resource limitations.

3.5.2.3 Definitions

- For performing DST-MODS, each processed sample was inoculated into two drug-free wells (control wells), one INH-containing well and one RIF-containing well.

- Any sample positive with at least one control well was recorded as a positive MODS culture.
- If two control wells were positive, results from drug-containing wells were recorded: If cording was detected in control wells but not in drug-containing wells, the strain was recorded as susceptible. If cording was detected in control wells and drug-containing wells, the strain was recorded as resistant.
- In samples for which only one control well was positive, the DST test was recorded as uninterpretable.
- MGIT or LJ culture was used as the gold standard for determining sensitivity and specificity of MODS culture; and DST-LJ was applied as the gold standard for sensitivity and specificity of DST-MODS.
- Cross-contamination was confirmed based on spoligotyping. Identification of H37Rv from any well except the positive control well was considered as a cross-contamination event.
- Cross contamination was suspected if only one control well was positive by DST-MODS and both MGIT and LJ culture were negative for that sample; or DST-MODS was positive with two controls but both MGIT and LJ culture negative. In these cases if spoligotyping showed the isolate was unique on the MODS plate processed that day, contamination was discounted.

3.5.3 STATISTICAL ANALYSIS

Detection rates for MODS, MGIT and LJ were summarized and compared between methods using McNemar's test. The accuracy of MODS for diagnosis of TB was then assessed using MGIT and LJ as the gold-standard reference test, i.e. a sample was defined as positive by the reference test if either MGIT or LJ (or both) was positive.

The accuracy of DST-MODS for drug-susceptibility testing was assessed in samples with a valid drug susceptibility test result by both DST-MODS and DST-LJ. The gold-standard reference test was the DST-LJ result. We also summarized agreement between DST-MODS and DST-LJ as raw agreement and by Cohen's kappa. Confidence intervals for accuracy measures (sensitivities, specificities, positive and negative predictive values) were calculated according to the method of Pearson and Clopper. Finally, we compared the time to a positive test for MGIT and MODS, respectively, in samples positive by both methods using the Wilcoxon signed rank test and visualized it using the empirical cumulative distribution.

Statistical analysis was done with Stata version 9 (Statacorp, Texas, USA).

3.5.4 RESULTS

3.5.4.1 Study population and demography

Seven-hundred and nine patients clinically suspected of tuberculosis were enrolled into the study. The median age was 39 years (IQR: 27 – 53 years). The male:female ratio was 1.7 (n=447/262). HIV status was unknown for the majority of the patients (99.0%, n=702/709) and only one patient was recorded as HIV positive.

3.5.4.2 Detection of TB

Accuracy of MODS culture for M.tuberculosis detection against MGIT culture or LJ culture as the gold standard.

In 709 sputum samples sent for TB diagnosis, the detection rate of MODS, MGIT and LJ were 50.5% (n=358/709, 95%CI: 46.7, 54.2), 51.6% (n=366/709, 95%CI: 47.9, 55.3) and 44.4% (n=315/709, 95%CI: 40.7, 48.1), respectively. No significant differences in detection rates were found between MODS and MGIT (P=0.80), MODS and LJ (P=0.2) or MGIT and LJ (P=0.1).

There were 373/709 (52.6%) samples positive by either MGIT or LJ culture. The sensitivity, specificity, positive predictive value and negative predictive value of MODS against MGIT/ LJ positive as the gold standard were 89.0% (n=332/373, 95%CI: 85.4, 91.9), 92.3% (n=310/336, 95%CI: 88.9, 94.9), 92.7% (n=332/358, 95%CI: 89.5, 95.2) and 88.3% (n=310/351, 95%CI: 84.5, 91.5), respectively.

3.5.4.3 Detection of drug-resistance

3.5.4.3.1 Drug-resistant isolates detected by 1% proportion method (DST-LJ - the gold standard method) (n=364)

Although there were 373 samples positive by either MGIT or LJ, 9 samples were identified as *M.fortuitum* or *M.Chelonae* which were identified by standard biochemical tests and therefore DST-LJ was not done for these samples. Of these 9 samples, seven samples were positive by MODS and could not be differentiated from *M.tuberculosis* complex by cording observation, one sample was MODS negative and

the last one was contaminated with fungi. As a result, 364/373 samples with DST results by 1% proportion method were available for analysis (table 3.14). The multidrug resistant isolates (MDR-TB) account for 3.8% (n=14/364) of cases. Isoniazid and rifampicin monoresistant isolates was detected in 3.8% (n=14/364) and 0.3% (n=1/364) of samples, respectively.

Table 3.14 Drug-resistant profiles of 364 samples with DST/LJ results available for analysis.

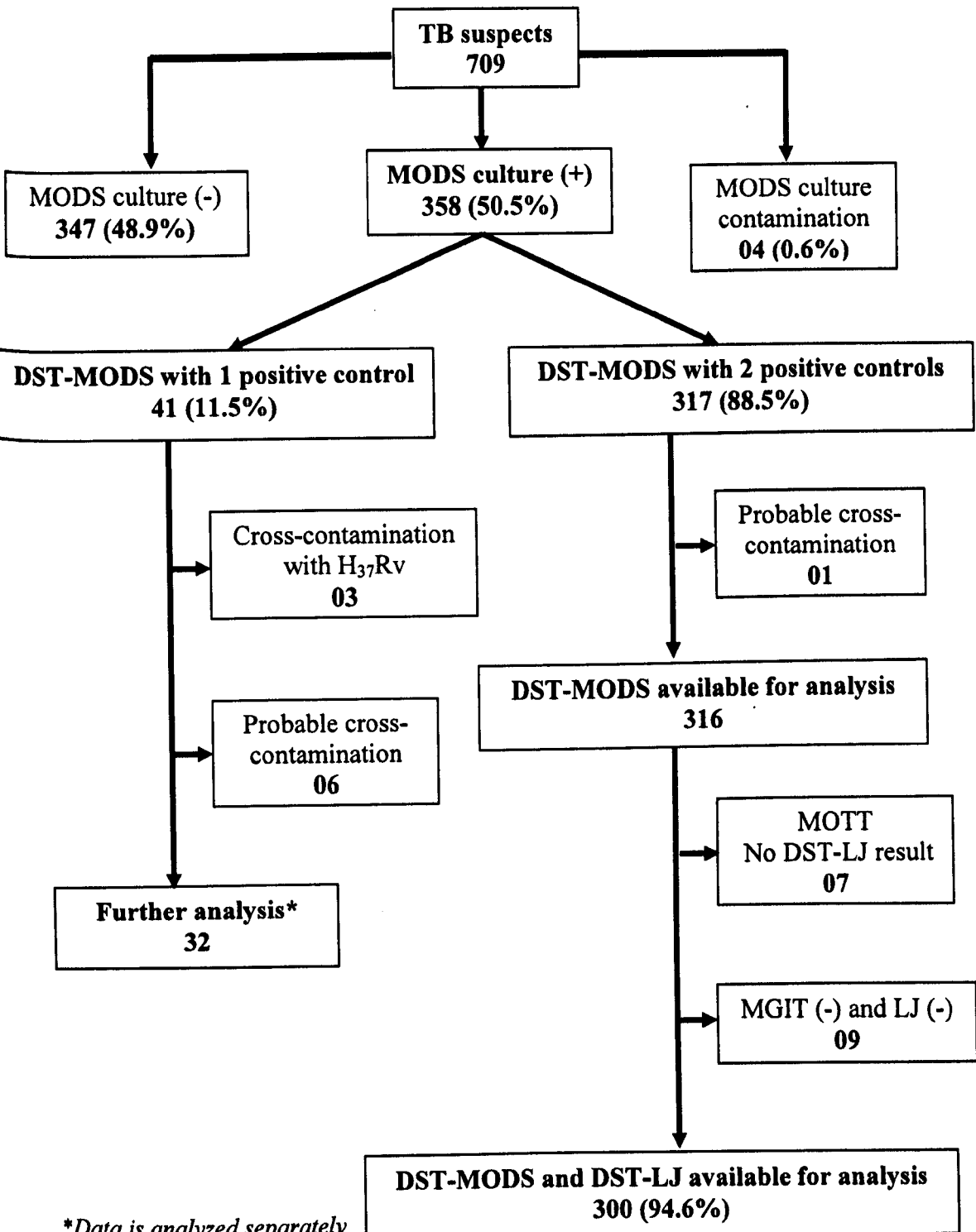
| DST results (n=364) | n | % |
|------------------------|-----|------|
| H | 14 | 3.8 |
| R | 1 | 0.3 |
| S | 59 | 16.2 |
| E | 1 | 0.3 |
| HS | 40 | 11.0 |
| HZ | 1 | 0.3 |
| SZ | 1 | 0.3 |
| RS | 1 | 0.3 |
| HRS | 5 | 1.4 |
| HSE | 2 | 0.6 |
| HRE | 1 | 0.3 |
| HSZ | 6 | 1.6 |
| HRSE | 2 | 0.6 |
| HRSZ | 3 | 0.9 |
| HRSEZ | 3 | 0.9 |
| Fully susceptible | 222 | 60.6 |
| Contamination | 2 | 0.6 |

H: Isoniazid (0.2µg/ml), R: Rifampicin (40µg/ml), S: Streptomycin (4µg/ml), E: Ethambutol (2µg/ml) and Z: Pyrazinamide (PZA - 200µg/ml). The MDR-TB rate (at least HR resistant) was 3.8%. Isoniazid monoresistance and Rifampicin monoresistance were 3.8% and 0.3%, respectively.

3.5.4.3.2 Study population for the evaluation of DST-MODS

Of the 358 samples positive by MODS culture, 88.5% (n=317/358) had two control wells (drug-free wells) positive by DST-MODS and 94.6% (n=300/317) of them were eligible for final DST analysis between DST-MODS and DST-LJ (**figure 3.13**). Five point four percent (5.4%, n=17/317) of samples were excluded from analysis because of probable cross-contamination by MODS (n=1) and no DST-LJ results (n=16). The remaining positive MODS samples (11.5%, n=41/358) only had one control well positive by MODS and were analyzed separately.

Figure 3.13 Recruitment flow chart of 709 TB suspects evaluation of MODS for detection of MDR-TB.



**Data is analyzed separately*

MOTT: Mycobacteria Other Than Tuberculosis

DST-MODS: Drug susceptibility testing done by MODS

DST-LJ: Drug susceptibility testing done by 1% proportional method on LJ media

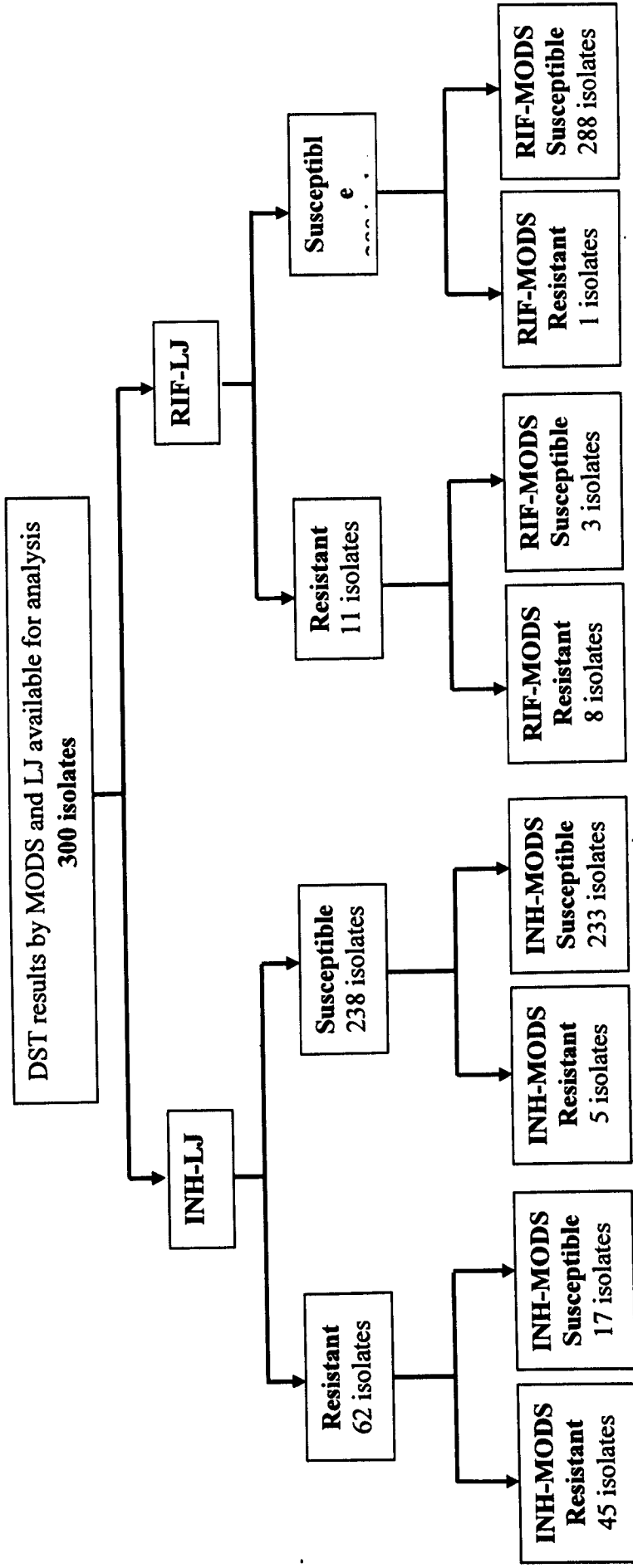
3.5.4.3.3 DST-MODS analysis

There were 300 isolates with DST results available by both MODS and traditional DST method. Accuracy of DST-MODS were evaluated based on these isolates

DST results by MODS

For 300 isolates with DST-MODS and DST-LJ available for analysis, DST-MODS detected INH, RIF and MDR resistant isolates at 16.7% (n=50/300), 3% (n=9/300) and 2.7% (n=8/300), respectively. DST results of 300 isolates by MODS in comparison to LJ for INH and RIF are described in **figure 3.14**.

Figure 3.14 Drug susceptibility testing results of 300 isolates by MODS and LJ for Rifampicin and Isoniazid in DST-MODS evaluation



RIF: Rifampicin, INH: Isoniazid, LJ: proportional DST method on LJ medium, MODS: microscopic observation drug susceptibility assay

DST-MODS against DST-LJ as the gold standard

Direct drug susceptibility testing results on MODS were compared with indirect DST on LJ as the gold standard for 300 samples. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of DST-MODS were 72.6% (n=45/62, 95%CI: 59.8, 83.1), 97.9% (n=233/238, 95%CI: 95.2, 99.3), 90.0% (n=45/50, 95%CI: 78.2, 96.7) and 93.2% (n=233/250, 95%CI: 89.3, 95.9), respectively for detection of INH resistant isolates; and 72.7% (n=8/11, 95%CI: 39.0, 93.9), 99.7% (n=288/289, 95%CI: 98.1, 99.9), 88.9% (n=8/9, 95%CI: 51.8, 99.7) and 98.8% (n=288/291, 95%CI: 97.0, 99.8), respectively for RIF resistant isolates. The agreement between DST-MODS and DST-LJ were 92.7% (n=278/300, 95%CI: 89.1, 95.3, kappa: 0.8, P<0.001) for detection of INH resistant isolates and 98.7% (n=296/300, 95%CI: 96.6, 99.6, kappa: 0.8, P<0.001) for RIF resistant isolates.

In terms of MDR-TB diagnosis, the sensitivity, specificity, PPV and NPV of DST-MODS against DST-LJ as the gold standard were 77.8% (n=7/9, 95%CI: 39.9, 97.1), 99.7% (n=290/291, 95%CI: 98.1, 99.9), 87.5% (n=7/8, 95%CI: 47.3, 99.6) and 99.3% (n=290/292, 95%CI: 97.5, 99.9%), respectively. The agreement between DST-MODS and DST-LJ was 99% (n=297/300, 95%CI: 97.1, 99.7, kappa: 0.8, P<0.001) for detection of MDR isolates.

3.5.4.4 MAS-PCR

In this study, MAS-PCR was used to confirm INH and RIF resistance in isolates classified as susceptible by MODS and resistant by LJ, or vice versa. MAS-PCR cannot

be used to 'rule-out' true resistance because approximately 5% and 20% of RIF and INH resistant isolates, respectively will not have mutations in the targeted gene sites (109).

Resolve discrepancy in resistant isolates detected by DST-MODS and DST-LJ

There was 25/300 isolates with discrepant DST results between MODS and LJ for INH, RIF or both INH and RIF (**table 3.15**). Of which, 3 samples were discrepant for RIF resistance, 22 samples were discrepant for INH resistance and 1 sample was discrepant for both INH and RIF resistance.

Table 3.15 Discrepant DST results between MODS and LJ resolved by MAS-PCR

| Isolate NO | DST-MODS | | DST-LJ | | MAS-PCR (mutation point) | | Remark |
|------------|------------|--------------|-------------|--------------|--------------------------|------------------------|---|
| | RIF 1µg/ml | INH 0.4µg/ml | RIF 40µg/ml | INH 0.2µg/ml | RIF resistant mutation | INH resistant mutation | |
| 1 | R | R | S | S | S | R (<i>katG</i>) | MDR by MODS |
| 2 | S | | R | | — | | RIF-MODS resistant on day 20. MDR by LJ. <i>ropB</i> sequencing: non sense mutation (H445C, CAC>TGC) |
| 3 | S | | R | | S | S | |
| 4 | S | | R | | R (codon 531) | | RIF-MODS resistant on day 20. MDR by LJ |
| 5 | | R | | S | | S | |
| 6 | | R | | S | | S | |
| 7 | | R | | S | | S | |
| 8 | | R | | S | | S | |
| 9 | | S | | R | | R (<i>katG</i>) | INH-MODS resistant on day 30 |
| 10 | | S | | R | | S | INH-MODS resistant on day 30 |
| 11 | | S | | R | | R (<i>katG</i>) | INH-MODS resistant on day 40 |
| 12 | | S | | R | | R (<i>inhA</i>) | |
| 13 | | S | | R | | R (<i>inhA</i>) | |
| 14 | | S | | R | | R (<i>inhA</i>) | |
| 15 | | S | | R | | R (<i>katG</i>) | |
| 16 | | S | | R | | R (<i>inhA</i>) | |
| 17 | | S | | R | | R (<i>katG</i>) | INH-MODS resistant on day 30 |
| 18 | | S | | R | | R (<i>inhA</i>) | |
| 19 | | S | | R | | R (<i>inhA</i>) | |
| 20 | | S | | R | | S | |
| 21 | | S | | R | | R (<i>inhA</i>) | |
| 22 | | S | | R | | R (<i>inhA</i>) | |
| 23 | | S | | R | | R (<i>katG</i>) | |
| 24 | | S | | R | | S | |
| 25 | | S | | R | | R (<i>katG</i>) | |

25/300 isolates (8.3%) had discrepant DST results between MODS and LJ for INH or RIF. R: resistant, S: susceptible, blank (-): no PCR product. Blank in black: concordance results between MODS and LJ therefore MAS-PCR was not attempted.

Among 4 isolates with RIF results discrepant between DST-MODS and DST-LJ (**table 3.15**), repeated DST-MODS for RIF 1µg/ml, MAS-PCR and *ropB* sequencing confirmed RIF resistance for one isolate (**isolate 4, table 3.15**) and RIF susceptible for 3 isolates (**isolate 1,2,3**) (**table 3.15**).

The sensitivity of MODS against LJ in detection of MDR-TB was 77.8% (n=7/9). Two MDR isolates determined by LJ but not by MODS (both isolates were INH resistant and RIF susceptible by MODS, (**table 3.15, isolate 2,4**) were resolved by MAS-PCR. Of these two isolates, one isolate was INH susceptible and RIF resistant by MAS-PCR. The other isolate was INH resistant by MAS-PCR. No RIF result by MAS-PCR for this isolate because of no hybridization.

The specificity of DST-MODS in detection of MDR-TB was 99.7% (n=290/291). There was one MDR isolate determined by DST-MODS but this isolate was INH and RIF susceptible by DST-LJ (**table 3.15, isolate 1**). MAS-PCR detected INH resistance mutation (*katG*) for this isolate but no mutation on *ropB* target gene was found.

Of 62 isolates with INH resistance by DST-LJ, 17 isolates were INH-susceptible by MODS (**table 3.15 and table 3.16**). Of these 17 isolates, mutations on *katG* and *inhA* promoter genes were detected in 35.3% (n=6/17) and 47.1% (n=8/17) of isolates, respectively. Wild type was found by MAS-PCR for 17.6% (n=3/17) of cases. Therefore, we concluded that 14/17 isolates were truly INH resistant due to the confirmed presence of resistance mutations. For the remaining 3 isolates we were unable

to confirm/exclude resistance since INH resistance conferring mutations may be present outside the MAS-PCR target sites.

Table 3.16 Analysis of MAS-PCR in 62 isolates with INH resistant by proportional DST method, in relation to DST/MODS results

| | | MAS-PCR | | | | | |
|----------|-------------|-------------|-------------|----------------------|----|------------|-------|
| DST/MODS | | <i>KatG</i> | <i>inhA</i> | <i>KatG and inhA</i> | WT | Indefinite | Total |
| | Resistant | 33 | 03 | 01 | 07 | 01 | 45 |
| | Susceptible | 06 | 08 | 00 | 03 | 00 | 17 |
| | All strains | 39 | 11 | 01 | 10 | 01 | 62 |

Of 17 isolates with INH susceptible by DST/MODS, 8 isolates (47.1%) had mutation on inhA promoter gene and 6 isolates (35.3%) carried mutation on katG gene.

Twenty-two isolates with INH results discrepant between DST-MODS and DST-LJ were repeated by DST-MODS for INH at a lower concentration (0.1µg/ml instead of 0.4µg/ml) because it was hypothesised the 0.4µg/ml concentration may be missing low-level INH resistant isolates since a recent meta-analysis (124) suggests better accuracy of the 0.1µg/ml INH concentration for MODS. Eighteen (81.8%, n=18/22) samples were culture negative on MODS (recruited smear results were negative for 13 samples, scanty for one sample and 1+ for 4 samples). Therefore, DST results for these samples were not

available. The results of the remaining 4 isolates were completely accordant with those of DST-LJ and MAS-PCR; two of them converted from resistant to susceptible and the other two converted from susceptible to resistant. The latter carries mutation on *inhA* promoter gene detected by MAS-PCR.

3.5.4.5 Analysis of samples with one positive control by DST-MODS (DST-MODS results were recorded as uninterpretable).

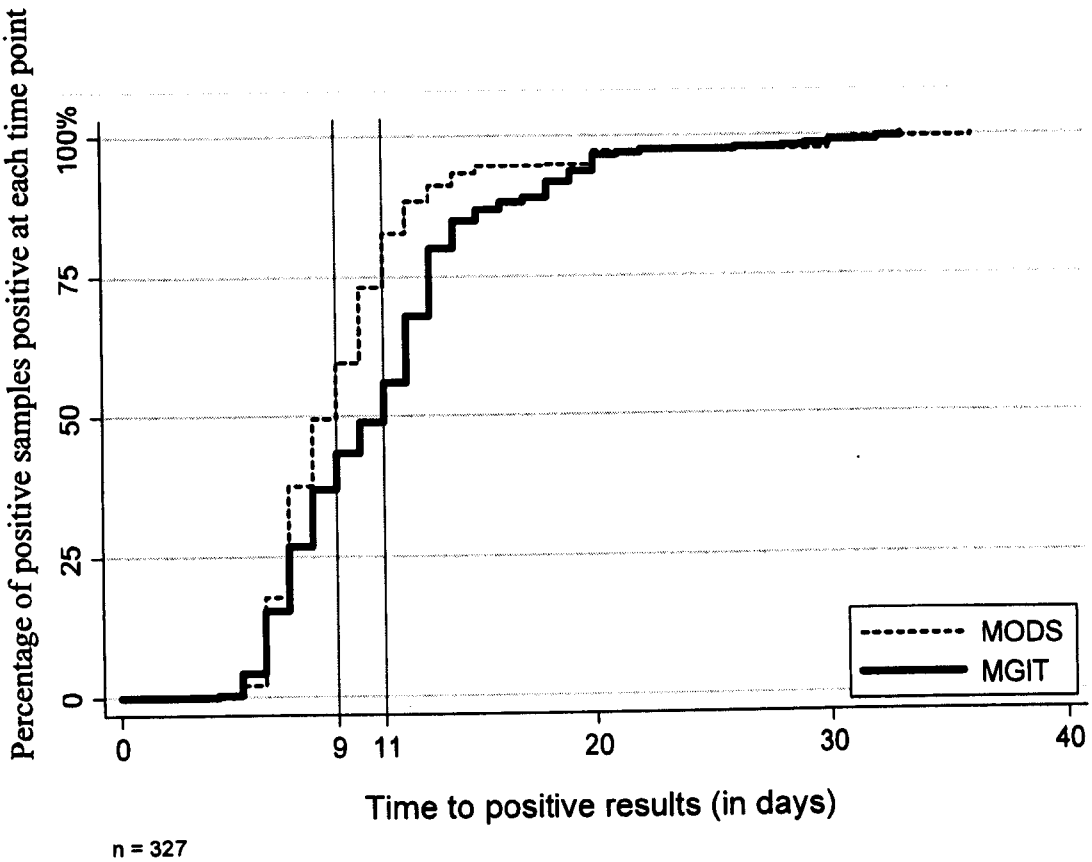
Thirty-two DST-MODS samples with one positive control (**figure 3.13**) were analyzed separately. Of which, 21.9% (n=7/32) were negative by both MGIT and LJ culture and 78.1% (n=25/32) were positive by either MGIT or LJ culture. Data from routine testing showed that 96% of these samples (n=24/25) were negative by direct smear and only one sample had positive smear (scanty result).

3.5.4.6 Time to detection

Time to detection (positive) for TB diagnosis

Time to positive was recorded as the duration of time from sample inoculation to positive result available in days. For 327 samples positive by both MODS and MGIT, the turn-around time of MODS was 9 days (IQR: 7-11 days) while it was 11 days (IQR: 7-13 days) for MGIT. The time to positive of MODS was significantly faster than that of MGIT (P<0.001) (**figure 3.15**).

Figure 3.15 Time to positive of MODS culture and MGIT culture in DST-MODS study.



In 327 samples positive by both MODS and MGIT, the turnaround time of MODS and MGIT were 9 days (IQR: 7-11 days) and 11 days (IQR: 7-13 days), $P < 0.001$ by Cox proportional hazards regression model.

Time to DST result available

Time to DST result available was defined as the period of time from sample inoculation to DST result available in days. For DST-MODS, this duration was exactly the same as the time to positive by MODS culture (median = 9 days). For DST-LJ, this time was the sum of time to positive of MGIT or LJ culture (a median of 11 days) and doing DST-LJ

(42 days). Therefore, the median total turn-around time of DST-LJ was 51 days which was much slower than DST-MODS.

3.5.4.7 Contamination

Contamination with fungi and cross-contamination were analyzed for all 709 samples.

Contamination of MODS culture: The initial fungal contamination rate was 0.6% (n=4/709). No re-decontamination and re-culture on MODS were attempted for contaminated MODS because of the limited volume of MODS culture (1ml). Three samples were cross-contaminated with H₃₇R_v, the positive control isolate used in this study and seven samples were suspected of cross-contamination between samples, generating a maximum possible cross-contamination rate of 1.4% (n=10/709).

Contamination of MGIT and LJ: The final fungal contamination rate of MGIT and LJ were 0.7% (n=5/709) and 0.6% (n=4/709), respectively. Four samples were contaminated for both MGIT and LJ. Cross-contamination by MGIT and LJ was not determined. However, there was one isolate found to be RIF susceptible by DST-MODS and synonymous mutation on *ropB* gene by sequencing but resistant by DST-LJ (**table 3.15, isolate 2**).

3.5.5 DISCUSSION

Our data showed that MODS is a sensitive and rapid method for diagnosis of TB and MDR-TB. Although TB detection rate of MODS (50.5%) was not different from MGIT (51.6%, p=0.8) and LJ (44.4%, p=0.2), MODS was faster than MGIT at 9 days vs 11 days.

For MDR detection, the turnaround time strongly favored DST-MODS (9 days) over DST-LJ (53 days). In terms of detection of INH or RIF resistance, the agreement between DST-MODS and DST-LJ were 92.7% for detection of INH resistant isolates and 98.7% for RIF resistant isolates. The sensitivity and specificity of DST-MODS against DST-LJ (the gold standard) were 72.6% and 97.9%, respectively for INH resistance and 72.7% and 99.7%, respectively for RIF resistance. The agreement, sensitivity and specificity of DST-MODS and DST-LJ for detection of MDR-TB isolates were 99%, 78.7% and 99.7%, respectively.

The sensitivities in detection of INH and RIF resistance in our study were lower than those from the study of Moore *et al* (72.6% vs 84.6% for INH and 72.7% vs 100% for RIF) (132) although both studies used the same INH concentration (0.4µg/ml) and RIF concentration (1µg/ml). These concentrations have been recommended by the MODS guidelines from the Peru group (90). However, a recent meta-analysis published after completion of this study concluded that the sensitivity was higher with a concentration of 0.1µg/ml without loss of specificity (124). To address this issue, we attempted to repeat DST-MODS and performed MAS-PCR for 26 isolates with discrepant results between DST-MODS and DST-LJ for INH and RIF.

For INH discrepant isolates, we found that 8/17 (47%) isolates susceptible by DST-MODS but resistant by DST-LJ carried mutation on *inhA* promoter gene (**table 3.15 and table 3.16**). Previous study has shown that *inhA* promoter mutation was associated with phenotypic INH resistance at low INH concentration (0.2µg/ml) (77). We attempted to repeat DST-MODS for INH at 0.1µg/ml concentration for all of these 8 processed samples but only 2 samples were re-identified as INH resistant. The remaining samples

were negative by MODS culture due to low bacterial load. This is a limitation of this technique. Recently, Mello *et al* found that the sensitivity of DST-MODS for detection of INH resistant isolates increased to 96.7% if INH 0.1µg/ml was used for the MODS assay (118); and similar conclusion was reported from a meta analysis (124). This review supported the use of INH 0.1µg/ml for DST-MODS and our data also supports the conclusion that the use of 0.4µg/ml reduces sensitivity in comparison with conventional DST. The clinical applicability of these concentrations has not been determined.

After resolving discrepant results between DST-MODS and DST-LJ for RIF for 4 isolates(**table 3.15**) by repeated DST-MODS, MAS-PCR and *ropB* sequencing, the final sensitivity of DST-MODS for detection of RIF was 77.8% (n=7/9). However, this sensitivity is still lower than previous studies (118, 132) although the number of RIF resistant isolates in this study was small leading to wide 95% confidence intervals on the sensitivity estimate (39-93%) and the number of false negative results for RIF resistance was only 3.

In 25 isolates with discrepant DST results between DST-MODS and DST-LJ for INH or RIF (**table 3.15**), 20 isolates were susceptible by DST-MODS for either INH 0.4µg/ml or RIF 1µg/ml but resistant by DST-LJ. One probable explanation is that the bacterial load present in processed samples was not equally aliquoted into each of 4 wells of DST-MODS due to the clumping characteristic of *M.tuberculosis*. More bacteria may be present in control wells than in drug-containing wells because processed samples were aliquoted into control wells first and then the drug-containing wells; and therefore cording formation was detected earlier in control wells than in drug-containing wells if

there were resistant isolates present in that sample. As a result, at the reading time, growth was seen in control wells but not in drug-containing wells and this isolate was determined as a susceptible isolate by DST-MODS. Clumping may be the main factor leading to one positive control of 32 samples in our study.

It is clear that INH 0.4µg/ml is not appropriate for performing direct DST-MODS and INH 0.1µg/ml is a more appropriate concentration for implementation. In addition, samples with low bacterial load (smear negative, smear scanty and smear 1+) are more likely to result in inconsistent results by direct DST-MODS due to unequal aliquoting.

The only equipment needed to perform the MODS assay are an inverted microscope, MODS plate and consumables, biological safety cabinet and incubator. The technical competence required is aseptic technique and microscopy skills; NTPs applying smear already have a workforce of experienced microscopists. A commercial MODS plate (TB MODS kitTM) has been developed by Hardy Diagnostics, USA in collaboration with PATH and is under evaluation MODS is appropriate for screening for MDRTB in high burden countries where such tests are urgently needed.

MODS meets many criteria for an MDR TB diagnostic test applicable for high-burden settings such as rapid, low-cost, accurate and can be performed without the need for biological safety level 3 laboratories (if the plate is not opened after inoculation). Therefore, MODS is an alternative method for rapid MDR-TB detection in these settings. Recently, wide application of MODS in resource-constrained settings has been endorsed by WHO (238). However, an international standard operating procedure and a

quality assurance system accredited by WHO should be developed to standardize and maintain accuracy.

3.6 Section 6

SAMPLE VOLUME FOR MODS ASSAY

3.6.1 INTRODUCTION

It has been known that sample volume affects the sensitivity and detection rate of a microbiological test, especially volume of processed sample used for testing. The importance of sample volume collected from patients has been confirmed in bacteriological diagnosis for TBM by Thwaites *et al* (195). In previous MODS studies, 100µl of processed sputum sample was inoculated into each well of a 24 well-plate and the sensitivity of MODS was higher than 90% (133, 151). In my project, the sensitivity of MODS in diagnosis of TBM was evaluated. When 100µl of CSF was used for MODS culture (**chapter 3 section 2, pilot study at HTD**), although the sensitivity of MODS was marginally lower than MGIT, the difference was not significant (64.9% vs 70.2%, P=0.8). With the hope that increasing sample volume would increase the sensitivity or detection rate of MODS, 250µl processed sample was used for the MODS study at PNT hospital (TBM-MODS, paediatric-MODS and HIV-MODS). The results showed that the sensitivities of MODS against clinical gold standard were comparable to those of MGIT in TBM-MODS study (23.6% vs 29.9%, respectively, P=0.18) and in paediatric-MODS study (39.7% vs 42.3%, respectively, P=0.5). However, the sensitivity of MODS was lower than that of MGIT in HIV-MODS study (70.7% vs 74.9%, respectively, P=0.03). Interestingly, our DST-MODS data showed that with 100µl processed sputum sample, the detection rates of MODS

and MGIT were comparable (50.5% vs 51.6%, respectively, $P=0.8$). From our data, it is not clear if the concept that “increasing sample volume would increase the sensitivity of MODS” is true for sputum samples. The aim of this chapter is to compare the sensitivity of MODS in diagnosis of pulmonary TB using different processed sample volumes (100 μ l vs 250 μ l).

3.6.2 METHOD

- One hundred smear positive routine sputum samples were consecutively collected for this study. These samples were part of the DST-MODS study conducted at PNT hospital at that time. Samples were collected at district tuberculosis units for smear microscopy and then transferred to PNT hospital for DST-MODS and volume evaluation. The average volume of each sputum sample collected was 2ml. One millilitre of each processed sample was used for DST-MODS. The remaining volume (1ml) was used for volume evaluation.
- 48-well plates were used for volume evaluation.
- Different processed sputum volumes were used. If 100 μ l and 250 μ l samples were used, termed 100 μ l MODS and 250 μ l MODS, respectively.
- Detection rates, time to detection and contamination with fungi were compared between methods.
- Details of laboratory methods were described in **chapter 2, section 2.6.**

3.6.3 STATISTICAL ANALYSIS

- The detection rates and reported confidence intervals were calculated according to the method of Pearson and Clopper. Comparisons of accuracies between tests were done using McNemar's test.
- The time to positive of MODS between methods was compared in samples for which both tests reached positivity with the Cox proportional hazards regression model.
- All analyses were done with Stata version 9 (Statacorp, Texas, USA).

3.6.4 RESULTS

3.6.4.1 Detection rate

In 100 smear positive sputum samples collected for this study, the detection rate of 250µl MODS and 100µl MODS were 79% (n=79/100, 95%CI: 69.7%, 86.5%) and 91% (n=91/100, 95%CI: 83.6%, 95.8%), respectively. The detection rate of 250µlMODS was comparable to 100µl MODS (P=0.49, 95%CI:-0.1, 0.06).

3.6.4.2 Time to detection

Time to positive 250µl MODS and 100µl MODS were 10 days (7-15days) and 10 days (7-14 days), respectively.

3.6.4.3 Evaluation of contamination

No fungal contamination was observed in 250µl MODS and 100µl MODS.

Evaluation of cross-contamination was not attempted due to resource limitation.

3.6.5 DISCUSSION

There was no evidence that increasing sample volume did not proportionally increase the detection rate of MODS. Although the detection rate of 250µl MODS and 100µl MODS were comparable (79% vs 91%, respectively, $P=0.49$), the higher detection rate was in favour of 100µl MODS.

In this study, the total volume of sputum sample collected from a patient was unchanged but the total volume of a processed sputum sample used for 3 MODS experiments was increased (2ml compared to 1ml if one MODS experiment was used). Performance of many experiments at the same time would increase the need of processed sample volume; leading to over dilution of these processed samples. This would result in a reduction in the number of bacilli aliquoted into each experiment and therefore reduce the sensitivity and detection rate of the test. In addition, clumping characteristic of *M.tuberculosis* also increases the challenges in generating equal aliquots for these experiments. Therefore, increasing total sample volume collected from a patient is more important. WHO recommended that for culture, a sputum specimen should have ideally a volume of 3-5ml with good quality (214). Smaller quantity is acceptable but not less than 2ml and good quality.

The increase in processed sample:broth ratio will change pH of final MODS culture and probably affect bacterial growth. With the sample:broth ratio of 1:10 (100µl MODS) and 1:3 (250µl MODS), the detection rates of MODS were comparable although 100µl MODS had a marginally higher detection rate. A recent study from

India suggested that the sensitivity of MODS was probably related to high sample:broth ratio and at the ratio of 1:5, the sensitivity of MODS was improved compared to that at 1:10 ratio (122). However, more studies should be conducted to confirm this theory.

3.7 Section 7

SUMMARY OF FINDINGS FROM THE MODS STUDY

3.7.1 MODS IN DIAGNOSIS OF TB

The accuracy of MODS in diagnosis of TB and MDR-TB has been evaluated in this project.

Compared to homogenous smear microscopy, **MODS had a higher sensitivity** against clinical gold standard in detection of TB for **all patient groups** (12.1% vs 23.6%, respectively, $P=0.0012$ for TBM; 28.2% vs 39.7%, respectively, $P=0.011$ for paediatric TB and 57% vs 71%, respectively, $P<0.001$ for HIV/TB). The pooled sensitivity of MODS was 13% higher than that of smear microscopy ($P=0.0092$)

Compared to MGIT, **MODS had a similar sensitivity** against clinical gold standard in detection of TB in **TBM** (29.9% vs 23.6%, respectively, $P=0.18$ for data from PNT hospital), **paediatric TB** (42.3% vs 39.7%, respectively, $P=0.5$) and in **adults pulmonary TB with unknown HIV status (DST-MODS)** (51.6% vs 50.5%, respectively, $P=0.8$). However, in **HIV-infected patients** suspected of pulmonary TB, **the sensitivity of MODS was lower than that of MGIT** (70.7% vs 74.9%, respectively, $P=0.03$). In patients suspected of TBM, both MODS and MGIT detected more cases in HIV positive group than in HIV negative group although no significant difference was detected (67.5% vs 58.8%, respectively, $P=0.8$ for MODS culture and

75% vs 58.8%, respectively, $P=0.6$). The pooled sensitivity across all studies of MODS was comparable to MGIT (53.0% vs 57.8%, respectively, $P=0.39$).

In general, although the sensitivity of MODS and MGIT were not always significantly different, the sensitivity of MODS was consistently marginally lower than that of MGIT across all studies.

The specificity of MODS was 100% for TBM-MODS, 94.4% for paediatric-MODS and 100% for HIV-MODS.

Time to positive of MODS was faster than MGIT in all sample types. For CSF sample, the turnaround time of MODS and MGIT was 7 days and 18 days, respectively. For other sample types, mainly sputum samples, the turnaround time of MODS and MGIT was 8 days (IQR: 6-12 days) and 13 days (IQR:8-19 days), respectively.

In smear negative TB patients, the sensitivity of MODS and MGIT was comparable (17.8% vs 21.4%, respectively, $P=0.5$ for paediatric study and 38.0% vs 45.0%, respectively, $P=0.078$ for HIV positive patients). However, median time to detection of MODS was faster than MGIT, 12days (IQR: 8-17 days) vs 19 days (IQR: 5-22 days), respectively.

3.7.2 MODS IN DIAGNOSIS OF MDR-TB

The sensitivity and specificity of DST-MODS were 72.6% ($n=45/62$) and 97.9% ($n=233/238$), respectively for detection of INH resistant isolates, 72.7% ($n=8/11$) and 99.7% ($n=288/289$), respectively for RIF resistant isolates and 77.8% ($n=7/9$) and

99.7% (n=290/291), respectively for MDR isolates. The agreement between DST-MODS and DST-LJ was 92.7% (kappa: 0.8, P<0.001) for detection of INH resistance, 98.7% (kappa: 0.8, P<0.001) for RIF resistance and 99.0% (kappa: 0.8, P<0.001) for MDR. PPV and NPV were 90.0% and 93.2%, respectively, for detection of INH resistance, 88.9% and 98.8%, respectively, for RIF resistance and 87.5% and 99.3% for MDR-TB. The low sensitivity of DST-MODS in our study was probably due to low bacterial load samples and high INH concentration (0.4µg/ml). The turnaround time of DST-MODS was 9 days and it was 53 days for DST-LJ.

Overall, these studies showed that MODS is an accurate and reliable method for diagnosis of TB and MDR-TB in high burden countries. Large scale operational research should be conducted to evaluate the implementation of MODS.

EVALUATION OF A NEW GENERATION OF FLUORESCENT MICROSCOPE (LED-based fluorescence microscope) IN TB DETECTION

4.1 INTRODUCTION

4.1.1 Conventional smear microscopy

Global Millennium Health Development goals for TB are to reduce TB prevalence and mortality by 50% relative to 1990 levels and to eliminate TB by 2050 (224). To achieve these targets, early diagnosis and treatment of new cases are crucial. In most countries, under National TB Programs, TB cases are primarily detected by acid-fast bacilli identification in sputum by conventional light microscope because it is specific, rapid, inexpensive and straightforward. However, the most important disadvantage of smear microscopy is poor sensitivity (estimated to be around 50% of active pulmonary cases) (234). Therefore, rapid, highly sensitive and inexpensive tests are urgently needed to improve case detection under the Stop TB strategy (234).

Since smear microscopy has been implemented in national TB programs worldwide, the easiest way to increase case detection in developing countries is to improve smear microscopy. Collaborative global efforts have been established to address this issue. According to a series of systematic reviews by the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases

(TDR) in 2005, there were three potential ways to improve sputum microscopy: sputum processing methods, number and quality of sputum samples examined per patient and fluorescence microscopy (223).

Sputum processing methods are used to concentrate the bacterial load in a sample to gain higher detection rates. Detection rates can be increased by either applying sputum processing in areas where only direct smear is currently performed, or by developing improved and simpler sputum processing methodologies. However, existing methods require laboratories equipped for safe sample processing and well-trained staff. In addition, sample processing increases the workload for staff whose workload may already exceeded the upper limit recommended by WHO. Therefore, sputum processing methods may not be a feasible priority in high burden countries which lack both financial and personnel resources.

Sputum smear microscopy utilising Ziehl-Neelsen (ZN) is currently the cornerstone of diagnosis applied in the National TB Program in Viet Nam despite its well-known disadvantages (121). The TB reference laboratory at PNT hospital receives about 900 to 1000 clinical samples of all types per day for TB detection; generating overwhelming work for laboratory staff. Approximately 100 slides are read by each technician daily, of which almost 30% are fluorescence smears. At peripheral sites (District TB Units), according to an internal report from the TB Control Programme in Ho Chi Minh City, 49,965 suspects were screened for TB in 2008, generating 207,590 slides for examination (80). This is a high burden of work for staff working at laboratories, especially in peripheral TB units where smear microscopy is the only microbiological method for use in TB diagnosis. To promote quality assurance of smear microscopy, the National TB Programme has a programme including annually

training and retraining for technicians, supervision and supply of high quality microscopes. However, recommendations on daily work-load per technician should be considered to prevent visual fatigue leading to a deterioration of reading quality. WHO suggests that at least 2-3 smears need to be read by a microscopist per day to maintain proficiency and each technician should examine a maximum of 20 smears per day to avoid visual fatigue and deterioration of quality (215, 254). However, staff at over 30% of TB laboratory units in HCMC have to examine over 20 smears per day due to a lack of or poor continuity of human resources. Furthermore, staff shortages combined with increasing numbers of requests for sputum microscopy are creating unmanageable workloads with negative effects on TB case finding. The poor sensitivity of light microscopy even under optimal conditions further aggravates this situation. Therefore, in the absence of a viable alternative diagnostic technique, reducing workload and increasing the sensitivity of smear microscopy as well as diminishing smear reading time are urgent priorities for the National TB Program. Recently, replacing light microscopy with fluorescence microscopy has been considered and may be one of the immediate options to improve the situation in high burden countries (64).

4.1.2 Fluorescence microscopy

Fluorescence microscopy (FM) appears to have several advantages over light microscopy (LM). First, the fluorescent staining procedure is simpler than that of ZN staining because no heating step is required. Second, AFBs are stained with auramine and appear as a bright yellow colour which is dominant on a dark background as 'stars on the night sky' and is easily recognized. Third, an increase in sensitivity has been reported for FM. A meta-analysis has shown that FM is 10% more sensitive than

LM in detection of AFB from specimens (180). Fourth, 40X objective can be used in FM instead of 100X in LM leading to shorter reading time by reducing the number of fields examined for a single slide. It has been estimated that using FM can save 75% of reading time in comparison with LM (180).

Unfortunately, despite many apparent advantages against LM, FM has not been widely implemented, especially in low resource settings with a high burden of TB. The major obstacles are the high price for FM and the lack of robustness and sustainability. Conventional FM use expensive and very fragile gas discharge lamps (such as Xenon- or Mercury-lamps) with high power consumption and a short lifespan of only 100-200 hours (6). The lifespan is further shortened by fluctuating power supply or turning them on and off frequently. Furthermore, heat production and the need to work in a dark room have prevented FM from being applied in many high TB prevalence areas.

4.1.3 ILED microscope

In response to a call from WHO in 2005 in an expert consultation meeting (223), progress has been made to develop a simpler FM which overcomes the disadvantages of the conventional FM. Carl Zeiss, Inc company (www.zeiss.com) has developed the Primo Star iLED (iLED) microscope, functioning as both light and fluorescence microscope, in agreement with the Foundation for Innovative New Diagnostics (FIND) (65) which has a high specification and lower price. The iLED microscope refers to iLED fluorescence microscope, unless demonstration as ZN-iLED for iLED light microscope, and was evaluated in this chapter.

iLED is a fluorescence microscope using the ultra bright LED (light emitting diode) technology as a light source to detect bacilli on a fluorescent smear (6). The lifespan of LED is at least 10 years. After connection to a power source, the microscope is ready to use immediately, without the warm-up and cool-down steps as required with mercury vapour lamps. In addition, it consumes only one-tenth the power of a 50 watt mercury-vapour lamp. The first generation of this microscope has undergone an initial performance evaluation using conventional FM and LJ culture as the gold standard, which showed promising results in the development phase: The sensitivity and specificity of the Primo Star iLED prototype on direct smear was equivalent to the results of standard FM, which is 71.6% vs 67.4%, respectively for sensitivity and 96.4% vs 96.1%, respectively for specificity. Furthermore, feedback from 4 reference laboratory settings revealed that in reading condition with or without a dark room, the contrast and brightness of iLED are still better than available standard FM. Interestingly, the time saved using the Primo Star iLED prototype was 45-75% compared to LM and was at least equivalent to standard FM (64). This microscope is therefore being evaluated and developed further. Data from FIND/WHO/TDR has shown that compared to LM, Primostar iLED is more sensitive and specific, faster in reading time and more flexible (64) There is no need to work in the dark room, a drawback of the conventional FM. Therefore, demonstration projects were initiated for the iLED microscope.

In recent years, aside from Primo Star iLED microscope, some commercial LED alternatives are available. CyScope® (Partec, Gorlitz, Germany), like Primo Star iLED, uses a LED source for both light and fluorescence illumination. The other products are attachments which are used to transform an existing light source from a light microscope into a fluorescence illumination so that all lenses can be used with both

light sources. These alternatives are Lumin™ (LW Scientific, Lawrenceville, GA, USA), ParaLens™ (QBC™ Diagnostics, Philipsburg, PA, USA) and FluoLED™ (Fraen Corporation Srl, Settimo Milanese, Italy). All of these competitors are battery powered but vary in weight and cost. Therefore, depending on purpose of use and financial resources, users can choose the most appropriate product for their application (126).

Pham Ngoc Thach hospital is one of the eight sites in the world (India, Viet Nam, South Africa, Russia, Peru, Lesotho, Ethiopia and Tanzania) participating in the FIND demonstration phase evaluation of the iLED microscope led overall by Catherina Boheme. The study aimed to determine the effectiveness of the iLED microscope in TB detection at peripheral tuberculosis units and also evaluated the performance and appraisal of the microscope by the operating laboratory technicians.

The work described here was conducted in collaboration with FIND diagnostics. Dang Thi Minh Ha was a principal investigator for the Pham Ngoc Thach hospital site and was responsible for the supervision, conduct and analysis of the work described here.

4.2 AIMS

1. To assess the sensitivity and specificity of Primostar iLED in TB diagnosis at microscopy centers with and without prior experience with fluorescence microscopy.
2. To determine the false positive and false negative rate of LED fluorescence reading compared to ZN baseline results and compared to results from the supervisory site.

3. To determine the development of false positivity and negativity rates of LED fluorescence reading over time (with increasing experience).
4. To determine if AFB fading affects quantitative reading smear results and factors leading to AFB fading.
5. To assess the impact of this implementation on daily workload and case detection rates.
6. To evaluate the appraisal of Primostar iLED by lab technicians.

4.3 METHODOLOGY

4.3.1 Enrollment

Phases involved in the enrollment period:

Phase 1: Pilot study. Determination of sensitivity and specificity of Primostar iLED using panel slides. This was done by one FM experienced technician at PNT laboratory to compare conventional LM, conventional FM 40X, FM-iLED 40X magnification and FM-iLED 20X magnification together.

Phase 2. Operational study. Programmatic implementation of Primostar iLED at three District Tuberculosis Units (DTU1, DTU5 and DTU8) in Ho Chi Minh City

Phase 2.1. Establishing ZN baseline

Phase 2.2. FM and iLED training

Phase 2.3. Validation

Phase 2.4. Implementation

Phase 2.5. Continuation

4.3.1.1 Phase 1: Pilot study

Determination of sensitivity and specificity using Primostar iLED compared to conventional light and fluorescence microscope using evaluation slide panels prepared from real sputum. This phase was done by one FM-experienced technician at PNT laboratory.

A slide set from real sputa was generated by Pham Ngoc Thach laboratory as requested from FIND. A set included 110 ZN stained smears and 330 auramine stained smears. All of these smears were read by conventional ZN microscope (110 slides), conventional fluorescent microscope 40X (110 slides), iLED 40X (110 slides) and iLED 20X (110 slides). Of each 110 slide set, 35 slides were smear negative (31.8%), 15 slides were scanty (13.6%), 45 slides were 1+ (41.0%) and 15 slides were high positive (2+ or 3+; 13.6%). Slides were put into standard slide boxes and stored in an air conditioned room. All slides were labeled with ID numbers, for which smear results were documented in a confidential Excel summary sheet which was stored at FIND.

Prior to participating in this study laboratory technicians had to familiarize themselves with the Primostar iLED using Auramine-stained slides that had been stored from routine work at PNT hospital.

One FM-experienced technician conducted blinded reading for comparison between Primostar iLED, standard light microscope and fluorescence microscope by examination of the 110 slide set stained with ZN or Auramine/KMnO₄. Each day, this technician read 15 - 16 ZN slides by light microscope (Olympus, model CX 21), 15 -

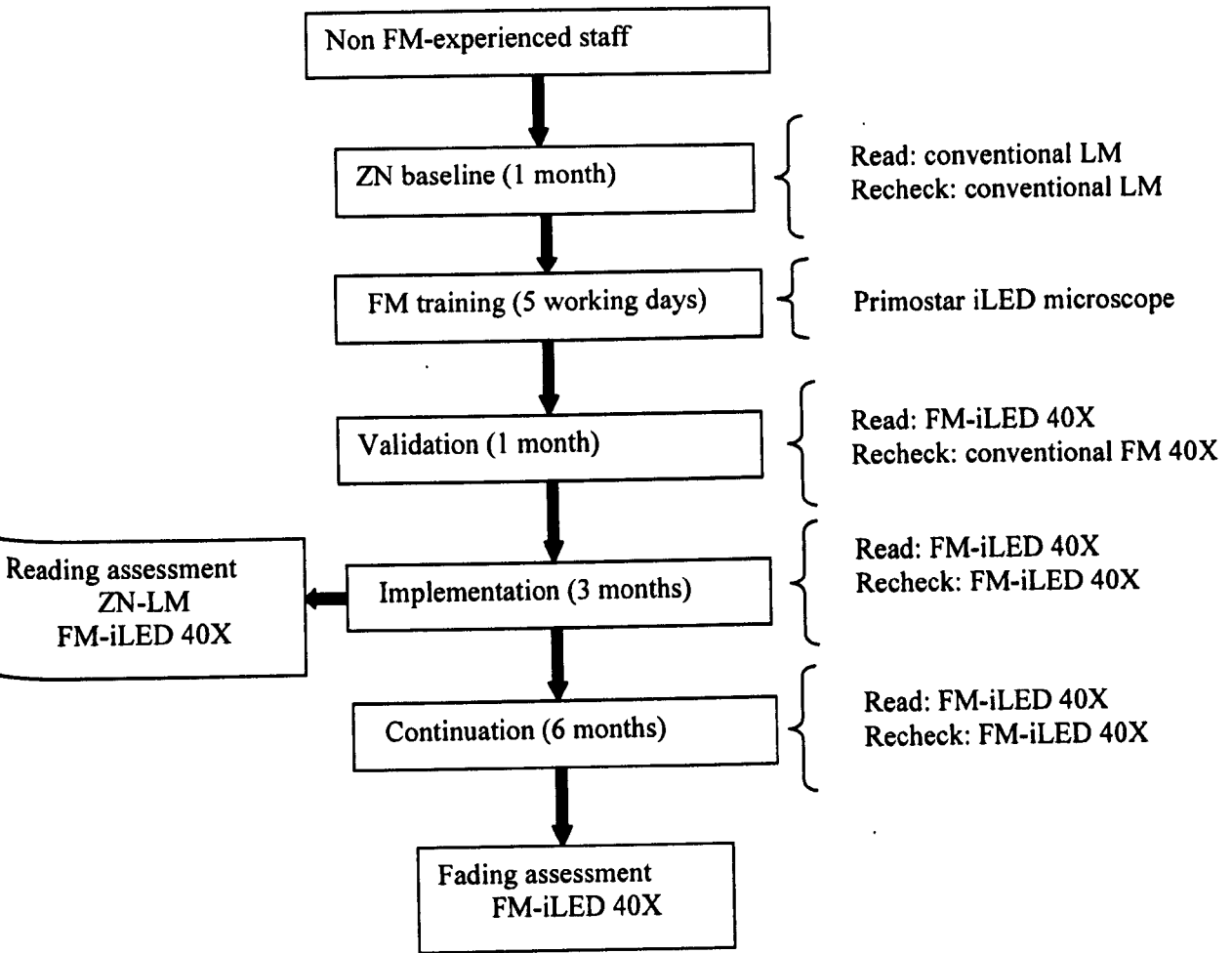
16 auramine slides by conventional FM (Olympus, model BX 51) and 15 – 16 auramine slides by iLED 40X. The order of reading by conventional bright microscope, conventional FM and iLED 40X were alternated every day to exclude bias of fatigue at the end of the day. A set of auramine slides was also read by iLED 20X for comparison with the iLED 40X. The whole process was completed in 3 weeks. We assessed the sensitivity, specificity, positive predictive value, negative predictive value, false positive and false negative rates, and identical rate and corresponding rates (see section 4.3.2 for definition) of these methods in comparison with phase 1 panel slide reference results. The time to result was also addressed in this study; and the time to identical or corresponding results was evaluated.

4.3.1.2 Phase 2: Operational study

Programmatic implementation of Primostar iLED in three routine microscopy centers in Ho Chi Minh City without prior experience in fluorescence microscopy, to determine operational performance and as well as laboratory staff appraisal.

The scheme of phase 2 is shown in **figure 4.1**.

Figure 4.1 Scheme of phase 2 – Operational study



LM: Light microscope
FM: Fluorescence microscope
ZN: Ziehl- Neelsen staining method

4.3.1.2.1 Establishing ZN baseline phase (1 month)

In order to be able to compare false positive and negative rates of LED fluorescence to the respective rates of ZN reading, it was necessary to establish a baseline under study conditions. Using data available from the routine quality assurance program would possibly result in a bias because the performance is likely to be better under study conditions. In this phase, all incoming sputum samples were examined by ZN microscopy according to program normal operating procedures and kept in slide boxes provided by FIND. All slides were rechecked by a ZN-experienced technician at PNT laboratory and discrepant results were rechecked a second time by another ZN-experienced technician from this laboratory according to the National TB programme guidelines.

4.3.1.2.2 FM and iLED training (5 working days)

Pham Ngoc Thach Hospital was responsible for training laboratory staff from 3 microscopy centers. The training focused on smear preparation, auramine staining method, FM reading and recording results. Slide storing and transfer to PNTH for rechecking was also emphasized in the course. This training took place over 5 days and all training materials were provided by FIND. All trainees passing the final test (maximum 3 slides with major errors [see definition in **chapter 2, section 2.6.2.2.5**] over 10 slides tested from the slide panel) had permission to start the validation phase immediately after the course.

4.3.1.2.3 Validation phase (1 month)

The validation phase lasted for one month, during which auramine slides were prepared from all incoming sputum samples at 3 participating sites and were

examined by using Primostar iLED with 40X magnification for screening at the sites. All slides were rechecked daily by the supervisory site using conventional fluorescence microscopy 40X. Results were sent back to the microscopy center the following day and treatment was started on the basis of the conventional fluorescence microscopy result. At the end of this phase all staff at the sites participating to the study underwent proficiency testing.

If the validation phase and proficiency panel met the following targets the sites were ready to enter the implementation phase:

- 1) >95% accordance between results of the demonstration site and supervisory site for fluorescence reading.
- 2) Quality of Auramine stains acceptable in 100% of slides.
- 3) ≤ 2 false results in the proficiency testing panel.

Sites that failed to meet performance targets received additional training and underwent proficiency testing until targets were met.

4.3.1.2.4 Implementation phase (3 months)

All routine sputum samples underwent routine microscopic examination with the Primostar iLED according to program normal operating procedures, and patients were managed based on Primostar iLED results for a period of 3 months. All slides were stored in slide boxes which were provided by FIND. Quality control (QC) and assurance (QA) followed the standards of the National TB Programme. All smears were rechecked without re-staining at PNT laboratory by iLED 40X microscope. In the first two months of this phase, all positive smears with scanty and 1+ results; and 20% of negative smears were rechecked. In the last month of this phase, rechecking

followed the LQAS guidelines; generating 14 slides which were rechecked for DTU1 and 8 slides each for DTU5 and DTU8. The rechecking process allowed determination of the sensitivity, specificity, false positive / false negative rates and detection rates over time (with increasing experience). These rates were then compared to the rates of the ZN baseline. Rechecking was performed by experienced staffs only and all discrepant slides were re-examined by another experienced technician at PNT laboratory. The prevalence of positive slides during implementation was compared with preceding quarters.

4.3.1.2.5 Continuation phase (6 months)

All routine sputum samples were examined by iLED 40X and the rechecking procedure followed the LQAS guideline. In addition, to examine if fading affected the reading accuracy of technicians, AFB fading in relation to sample processing method and storage conditions were also addressed during this phase. After staining and after each reading, all slides were put into the standard storage boxes to prevent exposure to sunlight. The average percentage of AFB faded in a reading field was recorded for each slide. This assessment was subjective and dependent on the proficiency skill of reference technicians to distinguish AFB colour of recent reading time to their experience. Semi-quantitative results were also recorded in the fading assessment. Results were recorded according to WHO scale and were compared with previous scale.

4.3.1.2.6 Reading result in relation to AFB fading and storage time

Slide collection was carried out in June, July and August of 2009 and rechecking was finished in November 2009.

In brief, routine slides from peripheral sites were kept at the sites until the end of the calendar month at room temperature ($28^{\circ}\text{C} - 32^{\circ}\text{C}$, approximately 70% - 80% humidity and without any air-conditioning in the room). After that, all of these slides were transferred to PNT hospital for LQAS procedure. These slides were stored in an air-conditioned room ($22^{\circ}\text{C} - 25^{\circ}\text{C}$, approx. 60% humidity) during the working day and at room temperature overnight (the normal storage conditions at PNT laboratory). Slide collection and rechecking schedule was carried out as described in **table 4.1**.

Table 4.1 Slide collection and rechecking schedule in fading assessment related to storage time

| | June 2009 | July 2009 | August 2009 |
|--|--|--|--|
| Total slides collected | 15 smears ^{+ve} 15 smears ^{-ve} | 15 smears ^{+ve} 15 smears ^{-ve} | 15 smears ^{+ve} 15 smears ^{-ve} |
| Number of slides in the 1 st rechecking (after 1 month, mainly slides from LQAS system) | 4 smears ^{+ve} | 5 smears ^{+ve} | 6 smears ^{+ve} |
| Number of slides in the 2 nd rechecking (rechecked in October 2009) | 30 slides | 30 slides | 30 slides |
| Number of slides in the 3 rd rechecking (rechecked in November 2009) | 30 slides | 30 slides | 30 slides |

In total, 45 positive smears and 45 negative smears were collected for this assessment. The first rechecking was done mainly for positive smears in the LQAS collection of the continuation phase. The second rechecking and the third rechecking were done in October and November, respectively for all 90 slides. Time difference (in days) between the two reading times of the same slide, reading result and AFB colour were recorded. The percentage of faded AFB on each slide was recorded at each time of reading. The quantitative results were compared to those from the previous reading.

4.3.1.2.7 AFB fading in relation to sample processing methods and storage condition.

One hundred and eighty smear positive sputum samples were subjected to smear preparation and Auramine staining and were divided into 3 groups:

- Group 1: Sixty samples were processed with NALC-NaOH to generate homogenous smears.
- Group 2: Sixty samples were processed with NALC-NaOH and Formaldehyde (method for panel slide preparation).
- Group 3: Sixty samples were subjected to direct smear preparation (without any processing method).

Thirty slides from each group were stored at room temperature and the remaining slides were stored in an air conditioned room during the working day. All slides were rechecked 3 times by iLED 40X at an average of one month intervals. The percentage of faded AFB on each slide was recorded on each occasion of reading.

4.3.1.2.8 Reading assessment

This assessment was performed during the Implementation phase to compare reading accuracy between ZN- light microscope (ZN-LM) and FM-iLED. This assessment was applied to all six technicians participating in this study, including 4 technicians from demonstration sites and 2 technicians from PNT laboratory. Each staff member read 1 ZN panel slide set (25 slides) and 1 FM panel slide set (25 slides) by using conventional light microscope and FM-iLED40X, respectively, with time taken recorded. Each ZN set consisted of 15 smear negative and 10 smear positive slides. Each Auramine set included 15 – 16 smear negative and 9 – 10 smear positive slides.

The sensitivity, specificity, false positive and false negative rates, reading time and focusing time (median time, in seconds) for each technician were used for comparison between technicians and between the two reading methods. Reading time, recorded by a supervisor, was defined as the time from starting to examine the first field of a slide to the time when the semi-quantitative result was available. If there was any discrepancy between the first reading results and the reference results, time to the results available of the second reading was recorded for evaluation. Focusing time was defined as the time taken for adjusting the microscope to starting to read the slide.

4.3.2 Definitions

- “Sensitivity” of a test is the number of slides read as positive by that test over the total slides read as positive by a gold standard method.
- “Specificity” of a test is the number of slides read as negative by that test over the total slides read as negative by a gold standard method.
- “Positive predictive value” is the number of slides read as positive by both evaluated test and gold standard method over the total positive slides read by that evaluated test.
- “Negative predictive value” is the number of slides read as negative by both evaluated test and gold standard method over the total negative slides read by that evaluated test.
- “False positive rate” is the number of slides read as negative by a gold standard method but positive by an evaluated test over the total number of slides observed.

- “False negative rate” is the number of slides read as positive by a gold standard method but negative by an evaluated test over the total number of slides observed.
- The ‘identical rate’ was defined as the number of slides having the same semi-quantitative results (according to IUALTD/WHO scale) over the total number of slides read.
- The ‘corresponding rate’ was defined as the number of slides with the same binary result (positive or negative, ignoring the positive scale) over the total slides examined.
- Time to result was defined as the duration of time from the start of reading to the result available.
- Time to identical or corresponding results is the duration of time from the start of reading to the results being available for slides with identical results or slides with corresponding results. This time did not include the time for microscope adjustment.

4.3.3 Quality assurance

Light microscopes (Olympus, CX21) with 3-4 years of use and conventional fluorescent microscope (Olympus, BX 51) with 4 years of use were compared with iLED microscope. All of these microscopes were still in good condition at the time of the study (recorded from the latest quarterly supervision report).

All routine smears at the participating sites were read by TB technicians who have never previously worked with FM microscope but had a median of 28.5 years (range

8-29 years) experience with ZN microscopy. Smears were read/rechecked by one of the three full-time PNT laboratory supervisors based on the requirements of each phase, who have a median experience of 5 years with FM (range 1 – 5 years) and 9 years (range 5 – 29 years) with ZN microscopy.

A workshop was organized for all participants involved in the study, including both supervision and demonstration sites. The training focused on knowledge and skills to perform fluorescence-based smear microscopy using the Primo Star iLED. After the training, a proficiency test was performed for all participants. The proficiency reading results showed 100% sensitivity and specificity for all staff.

At the end of the validation phase, a proficiency slide panel of 10 ZN and 10 auramine slides were provided to all participating sites. If there was (1) >95% accordance between results of the demonstration site and supervisory site for fluorescence readings and (2) Quality of auramine stains was acceptable in 100% and (3) ≤ 2 false results in the proficiency testing panel, the participating site was ready to enter the implementation phase. Otherwise, further training was provided until the above targets were met.

At the end of implementation and continuation phases, each technician from the demonstration sites had to read a slide panel including 10 ZN slides and 10 auramine slides by using iLED microscope (both smear positive and negative slides) to determine the reading skill development over time. The sensitivity, specificity, false positive and false negative rates, corresponding results and reading time were recorded for comparison between phases.

All staining reagents and solutions were prepared monthly by PNT laboratory for use at PNT and all study sites. For each preparation batch, quality testing was done by preparation of control slides to ensure all working solutions/reagents met standard requirements (in terms of AFB colour, decolourization, background staining and contamination).

All staining solutions were stored in a place away from heat, humidity, sunlight and explosive/flammable agents. Fuchsine and auramine were filtered twice a week before use. All redundant staining solutions were discarded after a month.

During the study, site visits from supervision site to participation sites were organized according to protocol guidelines for quality assurance; at least one supervisory visit was conducted for each site monthly.

An appraisal form was also completed by staff at each site by the end of each phase to determine end-user assessment at the sites.

4.3.4 Microbiology methods

- Panel slide preparation (phase 1 and fading assessment phase): see **chapter 2, section 2.6.2.2.1.c**
- Sample collection at District Tuberculosis Units (phase 2): see **chapter 2, section 2.4**
- Smear preparation, staining, reading and recording: see **chapter 2, section 2.6.2.2**

4.3.5 Statistical analysis

- The statistical analysis is described in **chapter 2, section 2.8**

4.4 RESULTS

4.4.1 Phase 1: Pilot study

Determination of the effectiveness of Primostar iLED in comparison with conventional light and fluorescence microscopy using evaluation slide panels.

Four panel slide sets including 110 slides each were examined under light microscope (100X), conventional fluorescent microscope (40X), iLED microscope (40X) and iLED microscope (20X), by a single technician with time to result recorded. The sensitivity and specificity of iLED 40X microscope against Phase 1 panel slide reference results were 94.67% and 100%, respectively; and 94.67% and 97.14%, respectively for iLED 20X microscope. There was no significant difference among the methods in terms of sensitivity, specificity, PPV, NPV, identical rate, corresponding rate, false positive and false negative rates (**table 4.2**).

Table 4.2 Effectiveness of light microscope (ZN), conventional fluorescent microscope (FM 40X) and iLED microscope (40X and 20X) in *M.tuberculosis* detection by panel slides

| | ZN (N=110) | FM 40X (N=110) | iLED 40X (N=110) | iLED 20X (N=110) |
|---------------------------|-----------------------|---------------------------|-----------------------------|-----------------------------|
| Sensitivity | P1=0.74 | P2=0.63 | P3=0.87 | P4=0.87 |
| % | 97.3 | 94.7 | 94.7 | 94.7 |
| (95%CI) | 92.8 – 99.9 | 86.9 – 98.5 | 86.9 – 98.5 | 86.9 – 98.5 |
| Specificity | | | | |
| % | 100 | 100 | 100 | 97.1 |
| (95%CI) | 90.0 – 100.0 | 90.0 – 100.0 | 89.9 – 100.0 | 85.0 – 99.0 |
| PPV | | | | |
| % | 100 | 100 | 100 | 98.6 |
| (95%CI) | 95.1 – 100 | 94.9 – 100 | 94.9 – 100 | 95.2 – 99.9 |
| NPV | P1=0.80 | P2=1.00 | P3=0.80 | P4=0.99 |
| % | 94.6 | 89.7 | 89.7 | 89.5 |
| (95%CI) | 85.5 – 99.9 | 75.8 – 97.1 | 75.8 – 97.1 | 75.2 – 97.0 |
| False positive | | | | P4=1.00 |
| % | 0.0 | 0.0 | 0.0 | 1.39 |
| (95%CI) | | | | |
| False negative | P1=0.36 | P2=1.00 | P3=0.22 | P4=1.00 |
| % | 2.8 | 10.3 | 10.3 | 10.5 |
| (95%CI) | | | | |
| Identical rate | P1=0.74 | P2=0.63 | P3=0.87 | P4=0.87 |
| % | 88.2 | 82.7 | 90.9 | 88.2 |
| (95%CI) | 80.6 – 88.2 | 74.3 – 89.3 | 83.9 – 95.6 | 80.6 – 93.6 |
| Corresponding rate | P1=0.88 | P2=1.00 | P3=0.88 | P4=0.96 |
| % | 99.9 | 96.4 | 96.4 | 95.5 |
| (95%CI) | 95.0 – 99.9 | 90.9 – 99.0 | 90.9 – 99.0 | 89.7 – 98.5 |

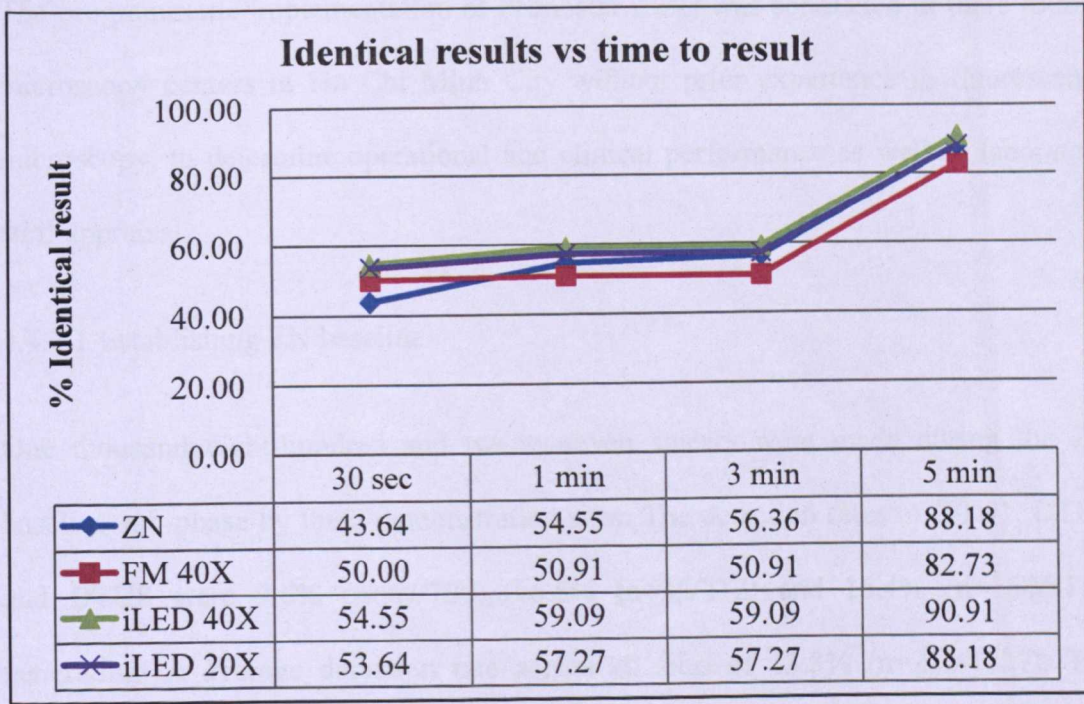
110 panel slides were read by one technician with high experience in fluorescence microscopy. All measurements were compared against Phase 1 panel slide reference results. Pairwise comparisons between groups were performed by Fisher's exact test. P1: ZN vs FM 40X, P2: FM 40X vs iLED 40X, P3: ZN vs iLED 40X and P4: iLED 40X vs iLED 20X. No significant difference was found between the groups.

The time to identical result and time to corresponding result was also recorded for each slide to compare the reading time between methods. Our data shows that 54.6% (n=60/110) and 53.7% (n=59/110) of slides examined by iLED 40X and iLED 20X, respectively had results available which were identical to the reference results, within 30 seconds of examination. These rates increased to 90.9% and 88.2% respectively for iLED 40X and iLED 20X after 5 minutes. This trend was also observed for the light microscope and conventional fluorescent microscope. Similar results were also found when analyzing the correlation between corresponding rate and time to result (figure 4.2).

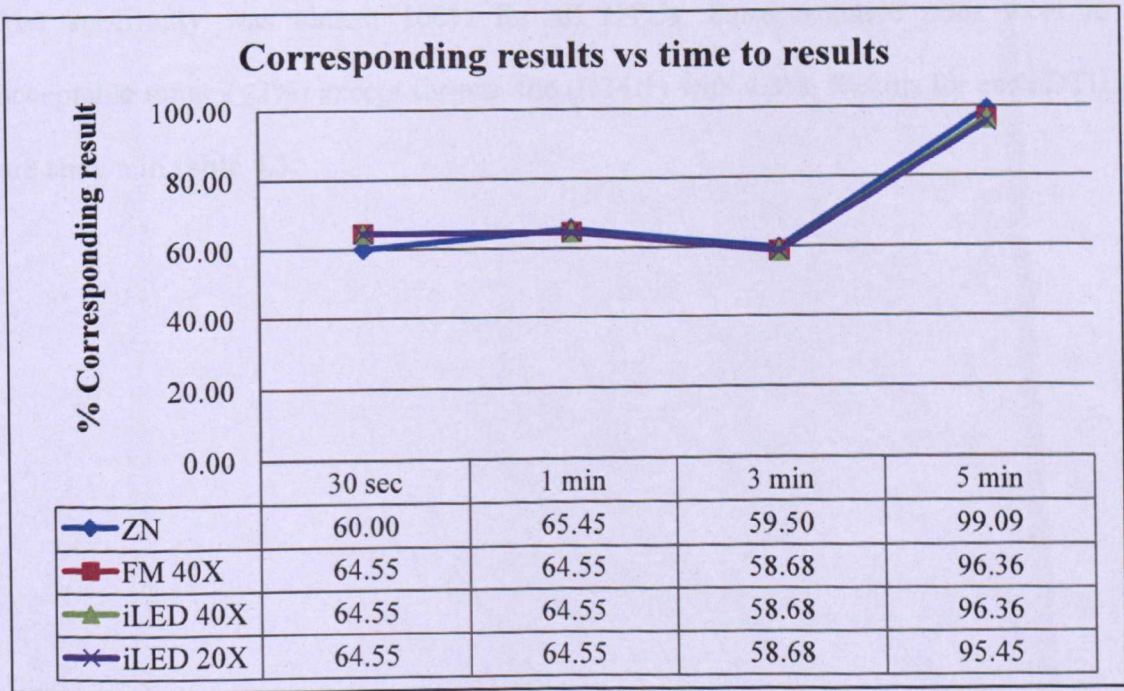
In general, there was no advantage in terms of efficacy or speed when using iLED for a technician highly experienced with both ZN and FM in comparison with conventional light microscope and conventional fluorescence microscope.

Figure 4.2 Identical (a) and corresponding (b) results in association with time to result in panel slide reading.

a.



b.



Accumulated percentage of slides with available results by time was recorded for each reading method. No significance in time to positive was found between microscopy methods. Time to result available was not different between the methods.

4.4.2 Phase 2: Operational study – Programmatic implementation of iLED

microscope.

The programmatic implementation of Primostar iLED was conducted in three routine microscopy centers in Ho Chi Minh City without prior experience in fluorescence microscopy, to determine operational and clinical performance as well as laboratory staff appraisal.

4.4.2.1 Establishing ZN baseline

One thousand eight-hundred and twenty-seven smears were made during the ZN baseline sub-phase by the 3 demonstration sites. The detection rates of DTU1, DTU5 and DTU8 were 9.6% (n=67/700), 16.5% (n=35/212) and 16.4% (n=150/915); generating an average detection rate across all sites of 13.8% (n=252/1827). The sensitivity was varying between peripheral laboratories, ranging from 71.3% to 100%. The specificity was almost 100% for all DTUs. False negative rates were in acceptable range ($\leq 2\%$) except for one site (DTU1) with 4.3%. Results for each DTU are shown in **table 4.3**.

Table 4.3 Rechecking result in ZN baseline

| | DTU1 N=700 | DTU 5 N=212 | DTU 8 N=915 | All sites N=1827 |
|---|-----------------------|------------------------|------------------------|-----------------------------|
| Sensitivity % (95%CI) | 71.3 61.0 – 80.1 | 100.0 89.9 - 100 | 96.1 91.7 – 94.9 | 88.4 84.1 – 91.9 |
| Specificity % (95%CI) | 100.0 99.3 - 100 | 100.0 97.9 - 100 | 99.9 99.2 – 99.9 | 99.9 99.6 – 99.9 |
| False positive % (95%CI) | 0.0 0.0 – 5.3 | 0.0 0.0 – 10.0 | 0.7 0.0 – 3.6 | 0.4 0.01 – 2.2 |
| False negative % (95%CI) | 4.3 2.8 – 6.1 | 0.0 0 – 2.0 | 0.8 0.2 – 1.7 | 2.1 1.4 – 2.9 |
| Identical rate % (95%CI) | 93.1 91.0 – 94.9 | 93.4 89.1 – 96.3 | 92.4 90.4 – 93.9 | 92.8 91.5 – 93.9 |
| Corresponding rate % (95%CI) | 96.1 94.4 – 97.4 | 100.0 98.3 - 100 | 99.2 98.4 – 99.6 | 98.1 97.4 – 98.7 |
| Detection rate % (95%CI) | 9.6 7.4 – 11.9 | 16.5 11.8 - 22.2 | 16.4 14.0 – 18.9 | 13.8 12.2 – 15.4 |

All measurements were calculated against PNT laboratory as the reference results.

4.4.2.2 Validation phase

In this phase (one month duration), slides were read at peripheral sites by iLED 40X but were rechecked at PNT laboratory by conventional FM 40X. The overall detection rate, sensitivity, specificity, false positive and false negative rates were 16.2% (n=304/1879, 95%CI: [14.5%, 17.9%]), 93.1% (n=296/318, 95%CI: [89.7, 95.6]), 99.5% (n=1553/1561, 95%CI: [98.9, 99.8]), 2.6% (n=8/304, 95%CI: [1.1, 5.1]) and 1.4% (n=22/1575, 95%CI: [0.9, 2.1]), respectively. No significant difference was observed in comparison with the ZN baseline data, except for the false positive rate. Reading by iLED 40X generated more false positive results than ZN reading under

the study condition, which was 2.6% vs 0.4%, respectively, $P=0.046$. Further analysis was undertaken to determine if any individual site had yielded a high false positive rate that inflated the overall false positive rate. DTU 5 yielded the highest false positive rate (6.1%, $n=3/49$) in comparison with DTU 1 (0%, $n=0/105$, $P=0.01$) and DTU 8 (3.3%, $n=5/150$, $P=0.41$). Importantly, all false positive results were misreading from negative to scanty or 1+ (low positive).

We found that in the first month working with iLED 40X microscope, more false positive results were generated in comparison with ZN reading under the study condition in the technician group with no prior experience on FM microscopy. The remaining measurements were comparable to those from the ZN baseline.

4.4.2.3 The implementation phase

This phase lasted for 3 months. In this phase, both peripheral and supervision sites used iLED 40X for smear reading and rechecking. Since low positive smears resulted in all false positive results in the previous phase (validation phase), all low positive smears ($n=321$) and 20% of all negative smears from each site were rechecked by the supervisory site in the first two months of this phase. The false positive rate was 2.8% ($n=9/321$). In the last month of this phase, slides were rechecked according to LQAS guidelines – the routine QA system of Vietnamese TB programme recommended by WHO (254). The overall detection, sensitivity, specificity, false positive and false negative rates at peripheral sites of the implementation phase were 14.0% [$n=767/5458$, 95%CI: 13.1, 15.0], 97.25% [$n=318/327$, 95%CI: 94.8, 98.7], 98.5% [$n=572/581$, 95%CI: 97.0, 99.3], 2.7% [$n=9/327$, 95%CI: 1.3, 5.2] and 1.6% [$n=9/581$, 95%CI: 0.7, 2.9], respectively. No significant difference was observed in comparison with results obtained from the validation phase ($P=0.053$ for detection

rate, $P=0.69$ for sensitivity, $P=0.87$ for specificity, $P=1.0$ for false positive and $P=0.83$ for false negative). When compared with the ZN baseline phase, all parameters except the false positive rate were similar between the two phases. The implementation phase generated a higher false positive rate than the ZN baseline phase (2.7% vs 0.4%, respectively) ($P=0.049$). To understand if reading by iLED 40X affected the detection rate and yielded higher false positive/negative errors in comparison with routine ZN reading, the respective rates of this phase were compared with those from the same quarter last year (table 4.4). Our data showed that the overall detection rate, false positive and false negative rates of all sites during this phase (3 months) were not different from respective measurements recorded under routine LQAS system during the same period of time last year ($P=0.4$, $P=1.00$ and $P=0.6$, respectively). Importantly, only the detection rate of DTU 8 was improved under the study conditions when iLED 40X was used (16.3% vs. 12.9%, $P=0.002$) compared to ZN reading under LQAS system of the same quarter last year.

In brief, the false positive rate in this phase was significantly higher than that of the ZN baseline phase but comparable to the validation phase. All of the measurements from the implementation phase, including false positive rate, were comparable to those from the same quarter last year when LQAS system was routinely conducted, except the detection rate was improved in one microscopy center.

Table 4.4 Effectiveness of iLED40X in the study condition compared with ZN in routine condition.

| Study site | Routine condition ZN reading – rechecking Feb-April 2008 | | | Study condition iLED40X reading – rechecking Feb-April 2009 | | | P values | | |
|----------------------|--|---------------|---------------|--|----------------|----------------|--------------|------|------|
| | % Detection | % False(+) | % False(-) | % Detection | % False(+) | % False(-) | P1 | P2 | P3 |
| DTU1 (n=x/y) | 11.8 (210/1774) | 0.0 | 0.0 | 9.9 (233/2333) | 4.3 (4/94) | 1.6 (4/258) | 0.08 | 0.57 | 1.00 |
| DTU 5 (n=x/y) | 19.7 (116/608) | 0.0 | 0.0 | 19.8 (146/739) | 0.0 (0/33) | 0.0 (0/67) | 0.79 | NA | NA |
| DTU 8 (n=x/y) | 12.9 (370/2874) | 0.0 | 0.0 | 16.3 (388/2386) | 2.5 (5/200) | 1.9 (5/256) | 0.002 | 1.00 | 1.00 |
| All sites (n=x/y) | 13.2 (696/5256) | 0.0 | 0.0 | 13.9 (767/5458) | 2.7 (9/327) | 1.5 (9/581) | 0.40 | 1.00 | 0.60 |

Data of reading and rechecking from the study was compared with data from the same quarter last year.

P values were calculated for comparison between two time points by Fisher's exact test.

P1 for comparison of detection rate.

P2 for false positive rates.

P3 for false negative rates.

Only detection rate of DTU8 was significantly increased (P1=0.002) when performing iLED40X in comparison with ZN.

4.4.2.4 The Continuation phase

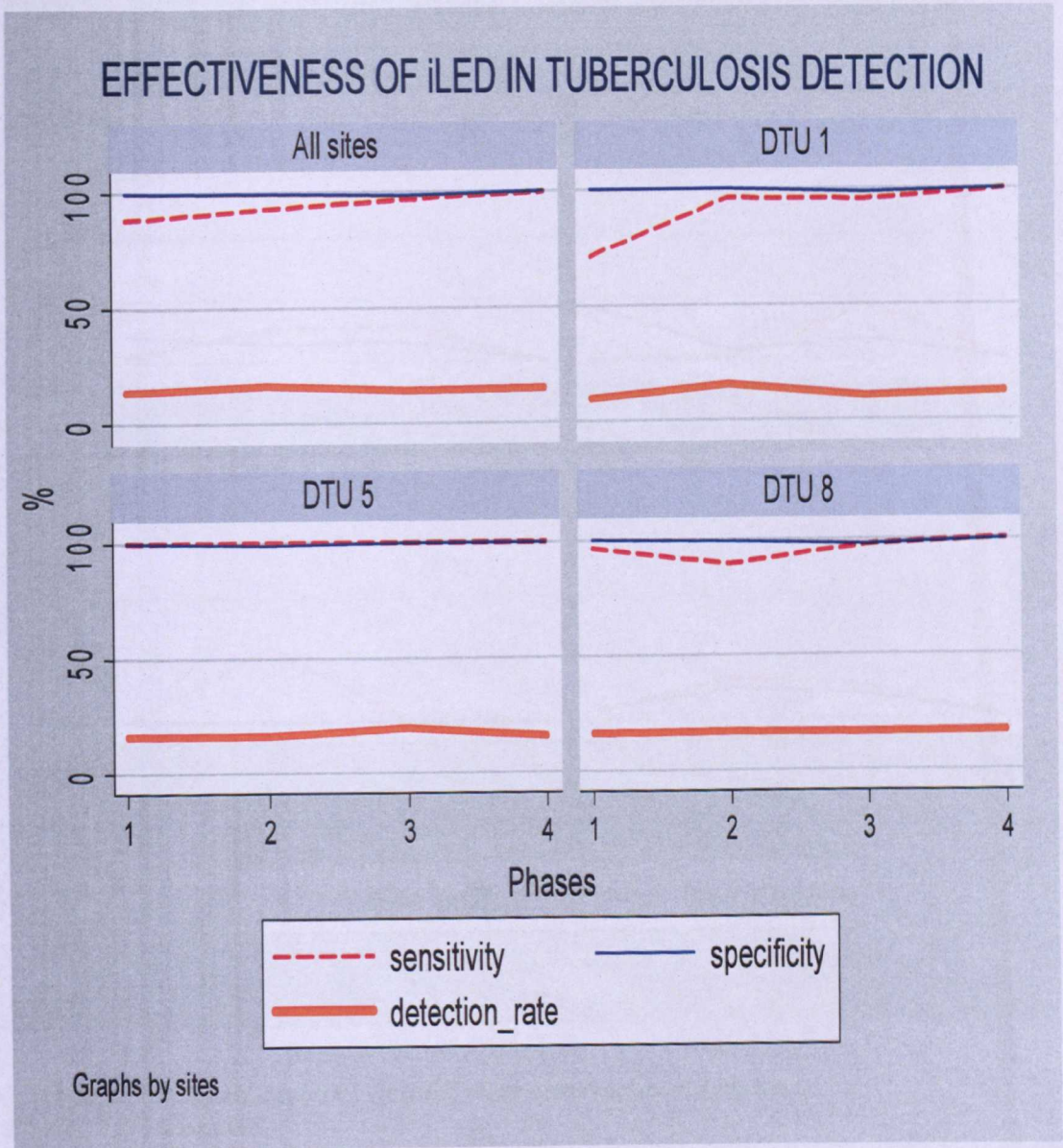
This phase lasted for 6 months (from May 1st to October 30th 2008). During this time, both the peripheral and supervision sites used iLED 40X for reading and rechecking. The rechecking followed the LQAS system. The overall detection, sensitivity, specificity, false positive and false negative rates at peripheral sites were 14.9% [n=1595/10707, 95%CI: 14.2, 15.6], 100% [n=19/19, 95%CI: 82.4, 100],

100% [n=161/161, 95%CI: 97.7, 100], 0.0% [n=0/19] and 0.0% [n=0/161], respectively. By the end of this phase, all measurements were not significantly different from the ZN baseline measurements, including the false positive rate.

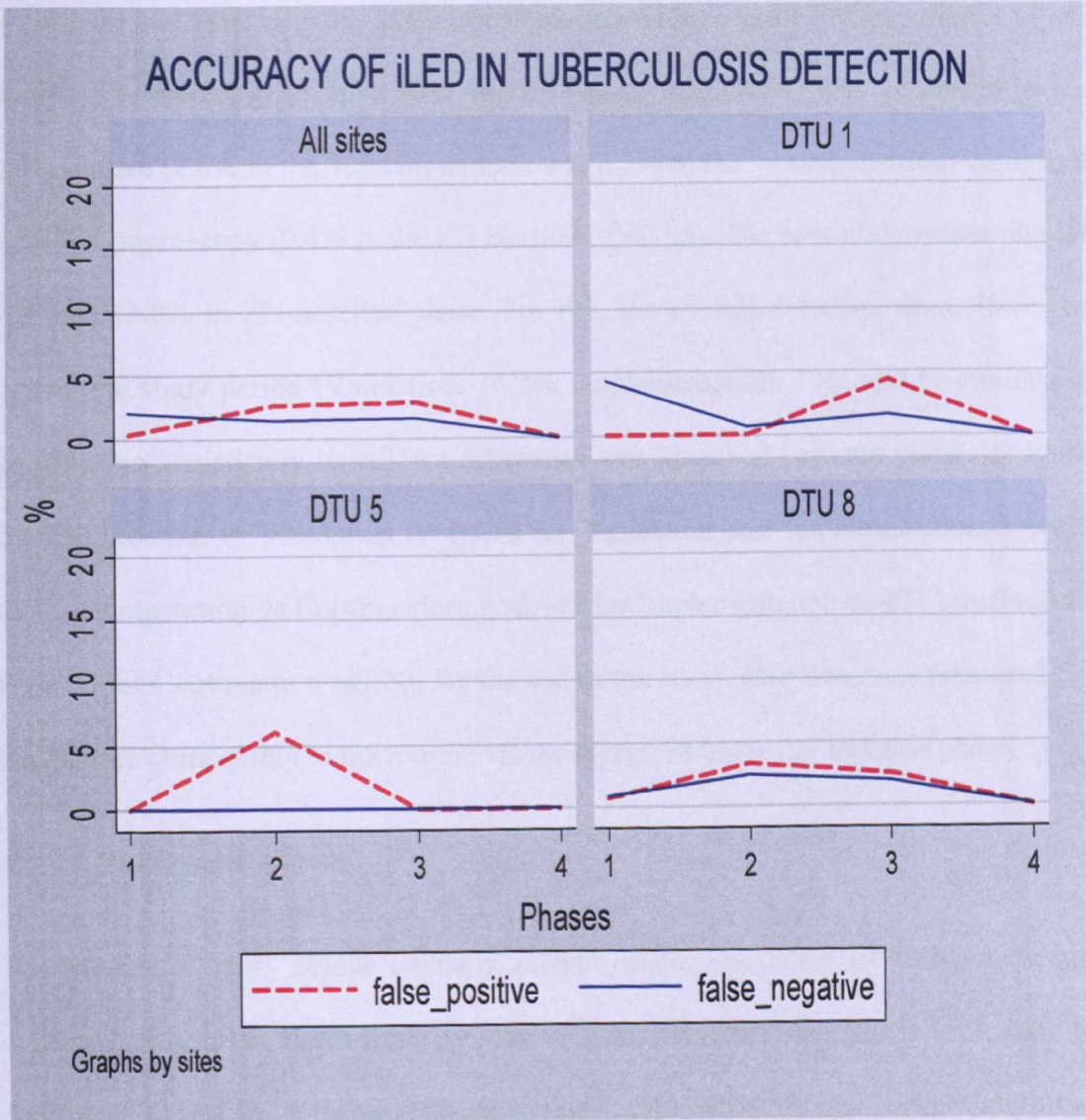
The effectiveness and accuracy of iLED 40X during the study period, by sites, is described in **figure 4.3**.

Figure 4.3 Effectiveness and accuracy of iLED in the 4 phases of the study.

a. Effectiveness of iLED fluorescence microscope



b. Accuracy of iLED fluorescence microscope



The effectiveness and accuracy of iLED were observed over 4 phases:

Phase 1: ZN baseline.

Phase 2: Evaluation or Validation.

Phase 3: Implementation.

Phase 4: Continuation.

The overall detection, sensitivity, specificity and false negative rates were not statistically different between phases when compared between each individual site and in combination of all three demonstration sites, by Fisher's exact test.

False positive increased in the early phases. By the end of the study, false positive rate was 0% and comparable to the ZN baseline phase

In general, iLED 40X did not show any advantage against conventional light microscope and conventional fluorescence microscope in terms of sensitivity, specificity and false negative rates in technicians with no prior FM-experience in our study. A relatively high false positive rate (using iLED 40X) was generated in the early phases (2.6% in the validation phase and 2.7% in the implementation) compared with ZN microscopy (0.4% in the ZN baseline, $P < 0.05$). The overall detection rate for ZN was 13.8% in ZN-baseline phase. For FM, the overall detection rates fluctuated during the study period (Validation: 16.2%, Implementation: 14% and Continuation: 14.9%). No statistically significant difference was observed between phases ($P = 0.08$ for ZN baseline vs Validation, $P = 0.053$ for Validation and Implementation, $P = 0.21$ for Implementation vs Continuation, $P = 0.809$ for Implementation vs ZN baseline and $P = 0.28$ for Continuation vs ZN). By the end of the study, all parameters measured for iLED were comparable to the routine values described in the ZN baseline phase.

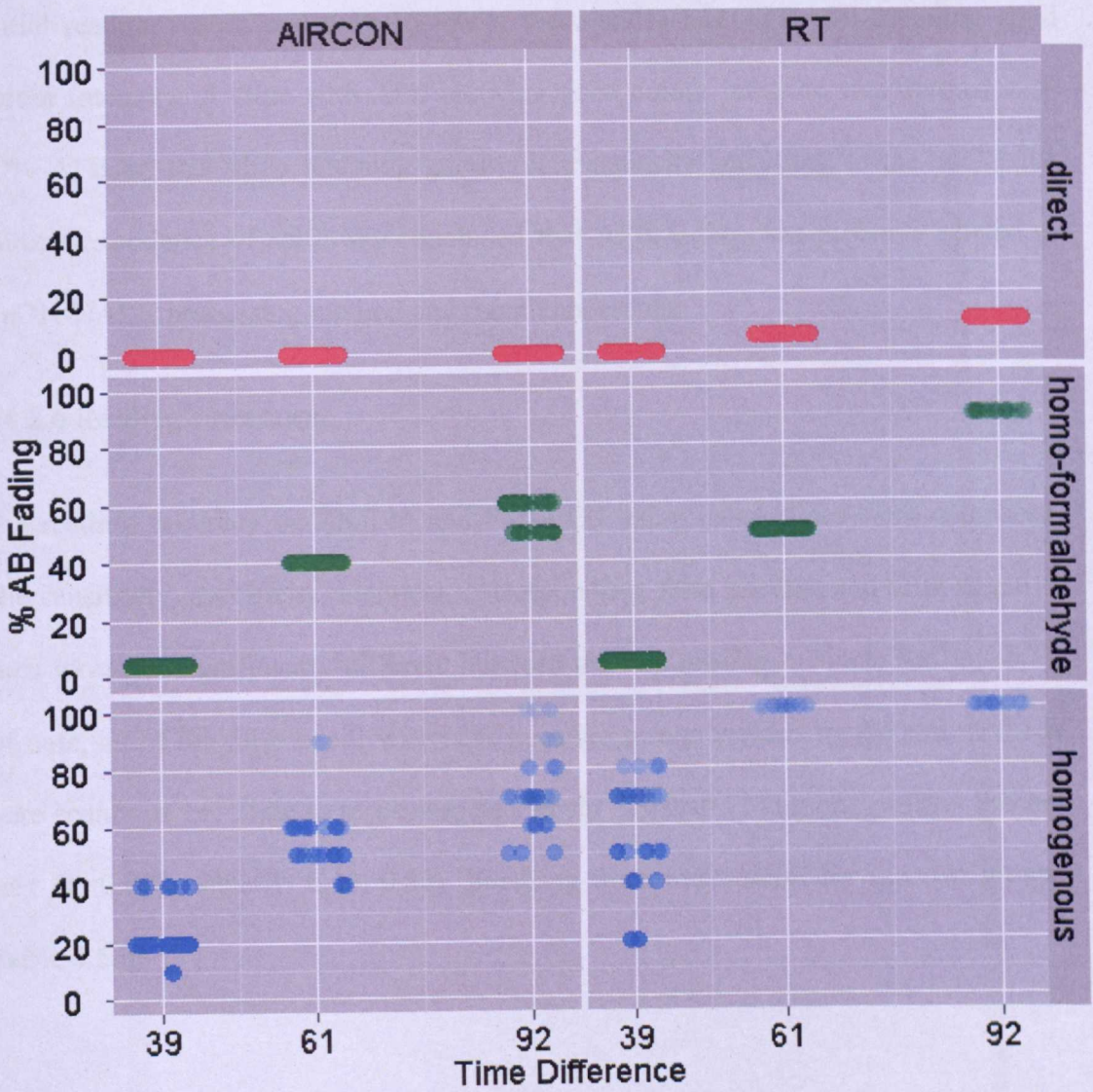
4.4.2.5 Fading assessment

To evaluate if AFB colour intensity affects reading accuracy of technicians, we collected 90 routine slides (priority was for slides selected for LQAS from June to August 2009 and the remaining slides were selected randomly and sequentially from LQAS collection so that the final set contained 45 positive smears and 45 negative smears) and stored the slides under routine conditions at Pham Ngoc Thach laboratory. These slides were then rechecked and AFB subjective colour intensity and quantitative results were recorded. Our data showed that with the median storage time of 113 days (83 days – 140 days), a median of 60% AFB on each of 45 positive slides was still in good color. After such a storage time, 28.9% ($n = 13/45$) positive smears had a two level-difference in quantitative result between initial reading result and the

final rechecking result, of which only 15.4% (n=2/13) smears had AFB still in good colour. A two level-difference refers to 2+ and scanty (n=8/13), 3+ and 1+ (n=2/13), and scanty and negative (n=3/13). The colour of AFB affected quantitative reading results, P=0.002 [95%CI: -0.07, -0.01].

An attempt was made to investigate possible factors that would affect AFB colour. Sample processing method and storage condition were evaluated in this study. The colour of AFB was likely to be faded if the sample was processed by concentrated method (NaOH-NALC) and the slide was stored at room temperature (28°C – 32°C, 70% - 85% humidity). Under this condition, almost 70% AFB on each slide were faded after 39 days and fading reached 100% by 2 months (**figure 4.4**).

Figure 4.4 Effect of storage conditions and sample processing methods on AFB colour observed under fluorescence iLED microscope.



*This graph shows % of AFB in faded condition on each slide by processing method and storage conditions. Color of AFB was likely to be faded if the sample was processed by concentrated method (homogenous smear) and the slide was stored at room temperature. Time difference was measured in days
 Homo: refers to concentrated method (Homogenous smear)
 Homo+formal: refers to NaOH + NALC + formaldehyde for preparation of panel slides
 Direct: refers to no processing method used (unprocessed samples)*

Overall, under the routine storage conditions at microscopy centers and Pham Ngoc Thach laboratory, with a median storage time of 113 days, 28.9% positive smears were incorrectly read as a two level reduction in quantitative result compared with the initial reading result; and only 15.4% of these slides had AFB still retaining good colour intensity. A slide with AFB showing good colour intensity was defined as $\geq 70\%$ AFB on that slide retaining good colour intensity according to the subjective routine experience of staff. We concluded that AFB fading was probably related to NaOH-NALC processing method and room temperature.

4.4.2.6 Reading assessment

The reading accuracy on ZN-LM and FM-iLED using panel slides were compared. The sensitivity, specificity, identical, corresponding, false positive and false negative rates were not significantly different between the two reading methods (**table 4.5.a**). Of note, all of the false positive or false negative results yielded by the two methods were scanty or 1+. Time to result was shorter for ZN than FM among positive smears only ($P=0.007$, 95%CI: 0.40, 0.86). Focusing time was similar for the two groups (**table 4.5.b**)

Table 4.5 Reading assessment (Combined data of both sites peripheral site and supervisory site).

a. Efficacy of ZN compared to FM in iLED microscope

| | iLED 40X, N=150 | | ZN-LM, N=150 | | P value |
|-----------------------|-----------------|------------|----------------|------------|---------|
| | % (n) | 95% CI | % (n) | 95% CI | |
| False (+) | 5.6 (3/54) | 1.2, 15.4 | 0.0 (0/55) | N/A | 0.24 |
| False (-) | 6.3 (6/96) | 2.3, 13.1 | 5.3 (5/95) | 1.7, 11.8 | 1 |
| Sensitivity | 89.5 (51/57) | 78.5, 96.0 | 91.7 (55/60) | 81.6, 97.2 | 0.9 |
| Specificity | 96.8 (90/93) | 90.8, 99.3 | 100 (90/90) | 95.9, 100 | 0.9 |
| Identical results | 84.0 (126/150) | 71.1, 89.5 | 84.0 (126/150) | 71.1, 89.5 | 1 |
| Corresponding results | 94.0(141/150) | 88.9, 97.2 | 96.7 (145/150) | 92.4, 98.9 | 0.9 |

Efficacy of ZN-LM microscopy and FM-iLED microscopy were similar, by Fisher's exact test. All of false positive and false negative results were from smears scanty or 1+.

b. Time to result and focusing time

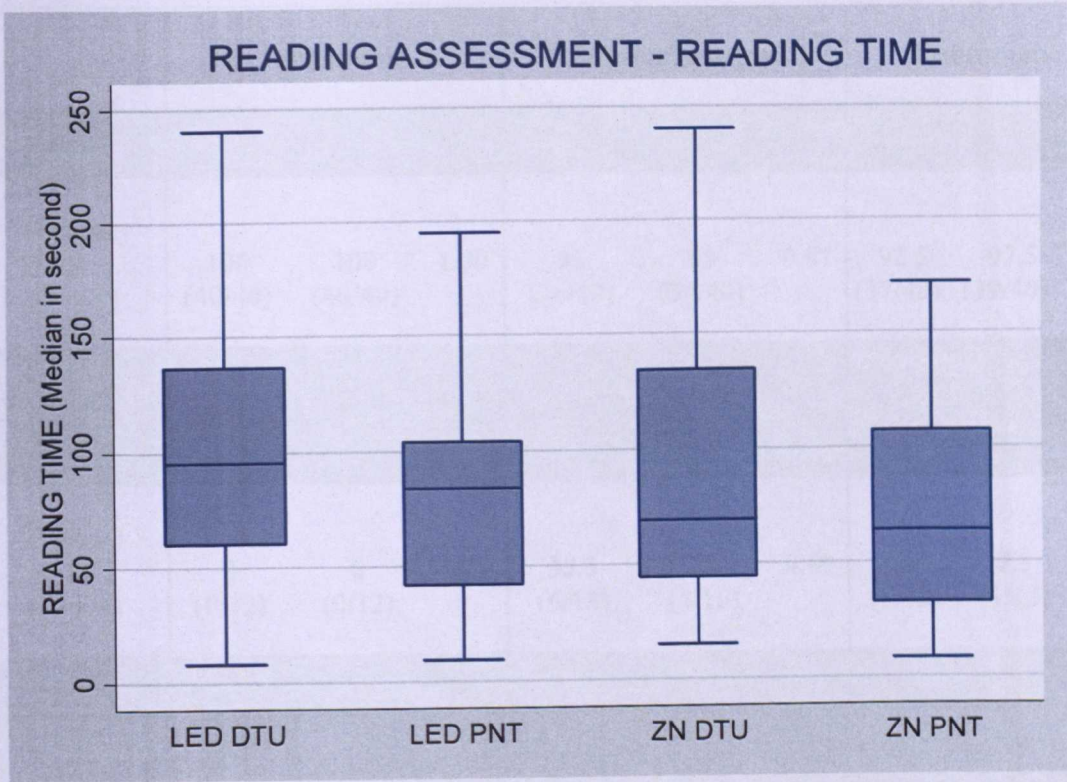
| | iLED 40X, N=150 | | ZN-LM, N=150 | |
|------------------------------------|-----------------|---------------|--------------|--------------|
| | Median | IQR | Median | IQR |
| Time to results (in second) | | | | |
| Negative | 101 | 80 - 141 | 100 | 64 - 149.25 |
| Scanty | 113.5 | 82.5 - 128.75 | 91 | 51.5 - 113.5 |
| 1+ | 60 | 39.5 - 80 | 36 | 29 - 58.25 |
| 2+ | 40 | 32 - 45 | 30 | 18 - 35 |
| 3+ | 22 | 14.5 - 31.5 | 20 | 14.25 - 27 |
| All slides | 92 | 57 - 124.5 | 68 | 40.5 - 120 |
| Focusing time (in seconds) | 8 | 6 - 10 | 8 | 6 - 12 |

ZN-LM reading was faster than FM-iLED reading, especially for positive smears (P=0.007, 95%CI: 0.40, 0.86), by Wilcoxon Rank-sum test.

When data was stratified by peripheral site and supervisory site, no significant difference between ZN-LM and FM-iLED 40X was observed between the two groups in terms of sensitivity, specificity, identical, corresponding, false positive and false negative rates. The reading time of ZN-LM and FM-iLED40X was comparable for both supervisor group ($p=0.25$) and peripheral group ($p=0.1$) (**figure 4.5**).

In general, the accuracy of ZN-LM compared to FM-iLED was comparable for both supervision site and microscopy centers. The reading time was shorter for ZN-LM than FM-iLED 40X in positive smears only when observed at both sites in combination, but it was comparable when data was stratified by sites or groups.

Figure 4.5 Time to result of reading assessment (data stratified by sites)



LED: Reading FM40X-iLED microscope.

ZN: reading ZN-light microscope (ZN-LM).

DTU: peripheral group.

PNT: supervisor group.

Reading time of ZN-LM and iLED40X were comparable for peripheral group ($p=0.1$) and supervisor group ($p=0.25$), by Wilcoxon Rank-sum test.

4.4.2.7 Proficiency testing for peripheral site

Proficiency testing was done at microscopy centers at the end of each sub-phase (validation, implementation and continuation phase). Each technician read 10 ZN slides and 10 FM slides by iLED microscope. By the end of the continuation phase (or completion of the study), reading accuracy and false positive/negative rates were comparable for the two reading methods. The reading time of FM- iLED40X was faster than ZN-iLED, 38.5 second vs 51.5 second, respectively, ($p=0.01$). The testing results are summarized in **table 4.6**.

Table 4.6 Proficiency testing in the 4 study phases.

| | Validation | | | Implementation | | | Continuation | | |
|---|-----------------|----------------|-------------|---------------------|---------------------|-------------|-----------------|-------------------|----------------|
| | ZN-iLED | iLED 40X | P | ZN-iLED | iLED 40X | P | ZN-iLED | iLED 40X | P ⁹ |
| Accuracy % (n=x/y) | 100 (40/40) | 100 (40/40) | 1.00 | 90 (36/40) | 85 (34/40) | 0.87 | 92.5 (37/40) | 97.5 (39/40) | 0.87 |
| False(+) % (n=x/y) | 0 (0/28) | 0 (0/28) | - | 0 (0/22) | 0 (0/24) | | 0 (0/25) | 0 (0/27) | - |
| False(-) % (n=x/y) | 0 (0/12) | 0 (0/12) | - | 33.3 (6/18) | 25 (4/16) | 0.69 | 20 (3/15) | 7.7 (1/13) | 0.61 |
| Reading time Median(sec) (IQR) | 39.5 (19-64) | 35 (22-48) | 0.18 | 60.5 (30.5-99.5) | 40.5 (19.5-65.5) | 0.14 | 51.5 (33-86) | 38.5 (20-49.5) | 0.01 |

The accuracy of reading results and reference results (positive or negative results), false positive and false negative rates and median of reading time were evaluated during the proficiency testing of each Phase.

By the end of the study (end of continuation Phase), FM-iLED40X reading was faster than ZN-iLED reading (p=0.01). The percentages of accuracy, false positive and false negative were comparable for both reading methods.

⁹ P values were calculated by Fisher's exact test for comparison accuracy, false positive and false negative; and by Wilcoxon Rank-sum test for comparison reading time.

4.4.2.8 Laboratory staff appraisal

The laboratory staff appraisal was evaluated based on a questionnaire designed by FIND (table 4.7). In brief, laboratory staffs were satisfied with the design of the iLED microscope and found that it was easy to use. Satisfaction with the contrast, colour intensity and background of the iLED microscope also increased over time, from 43% at the validation phase to 100% at the end of continuation phase, without modification of the microscope or the staining method. The homogeneity of reading field was comparable to the standard fluorescent microscope. Interestingly, in the continuation phase (the last phase of the operational study), all technicians agreed that they spent less time on smear reading with FM-iLED microscope than standard light microscope; and the advantage of fluorescence microscopes over light microscopes in terms of reading time was recorded in the proficiency testing of this phase (38.5s vs 51.5s, respectively) but FM-iLED and light-iLED microscope (rather than standard light microscope) was used for evaluation. The light-iLED microscopes have lower clarity lenses than the standard light microscopes (subjective assessed by technicians) and therefore the comparison may be biased in favour of FM-iLED. Study participants found that a dark room was not required for iLED use. The participants preferred to use iLED-FM 40X rather than iLED-FM 20X for reading. In addition, AFBs were less well distinguished by light-iLED microscope than standard light microscope. Therefore, the technicians would prefer to use iLED-FM 40X rather than iLED-FM 20X or light-iLED microscopes for TB detection if possible. During the study period, the manufacturer improved the battery-life of the Primostar iLED microscope, approximately 22 hours vs 1.5 hours. Last but not least, participants suggested that 5 to 7 days are required for iLED training for staff with smear

experience but for staff naïve to this technique, they suggested a full training course of 25 – 30 days. Details of the appraisal form results are shown in table 4.7.

Table 4.7 Results of staff appraisal questionnaire

| Questions | Validation phase (N=7) % | Implementation phase (N=6) % | Continuation phase (N=4) % |
|--|--------------------------------|------------------------------------|----------------------------------|
| Part I: Installation and first use | | | |
| 1. Was the installation/first use of Primostar iLED by a microscopist: | | | |
| Self-explanatory, can be done without reading the user manual | | | |
| Easy, but a user manual with instructions is required | 100 | 100 | 100 |
| Rather difficult; some problems were faced during installation/first use | | | |
| Very difficult; cannot be expected of a microscopist | | | |
| 2. Was the installation/first use of the battery pack by a microscopist: | | | |
| Self-explanatory, can be done without reading the user manual | | 33 | |
| Easy, but a user manual with instructions is required | 86 | 67 | 100 |
| Rather difficult; some problems were faced during installation/first use | 14 | | |
| Very difficult; cannot be expected of a microscopist | | | |
| 3. How satisfied were you with the Primostar iLED user manual: | | | |
| Easy to read and understand; covers all questions I had during installation/use | 100 | 100 | 100 |
| Most sections easy to read and understand, with some weaknesses in sections: Missing topics: | | | |
| Rather cumbersome to read (information required is not found easily; not enough pictures that allow understanding at first glance), weaknesses especially in the following sections: Missing topics: | | | |
| Comments: | | | |
| Part II: Training | | | |
| 1a. For a microscopist trained in ZN microscopy, how intensive should the training for Primostar iLED be?days | | | |

| | | | |
|---|-----|-----|-----|
| 5 days | 57 | 50 | |
| 7 days | 43 | 50 | 100 |
| 1b. For someone without prior training in smear microscopy, how intensive should the training for Primostar iLED be?..... days | | | |
| 10 days | | 17 | |
| 22 days | | 33 | |
| 24 days | | 17 | |
| 25 days | | 0 | 25 |
| 30 days | 100 | 33 | 75 |
| 2. How satisfied were you with the Primostar iLED training manual: | | | |
| Can be used by NTPs for implementation of LED microscopy without major changes | 100 | 100 | 100 |
| Can be used by NTPs for implementation of LED microscopy but requires some major changes | | | |
| Requires complete revision | | | |
| Suggestions for changes: | | | |
| Part III: Optics and Handling | | | |
| 1. How satisfied are you with contrast, color intensity and signal-to-noise (background) ratio of Primostar iLED? | | | |
| Very satisfied (better than for the available light microscope and where applicable fluorescence microscope) | 43 | 67 | 100 |
| Satisfied (comparable to available light microscope and where applicable fluorescence microscope) | 57 | 33 | |
| Not satisfied (not as good compared to those of the available light microscope and where applicable fluorescence microscope) | | | |
| Comments: | | | |
| 2. How satisfied are you with the color impression for ZN stain of the Primostar iLED (white LED) in comparison to a standard light microscope (halogen bulb)? | | | |
| AFBs can be better distinguished | 14 | 50 | |
| Same | | | |
| AFBs can be less well distinguished | 86 | 50 | 100 |
| 3. How satisfied are you with the resolution and depth of focus of Primostar iLED? | | | |

| | | | |
|---|----|-----|-----|
| Very satisfied (better than for the available light microscope and where applicable fluorescence microscope) | 43 | 67 | |
| Satisfied (comparable to available light microscope and where applicable fluorescence microscope) | 57 | 33 | 100 |
| Not satisfied (not as good compared to those of the available light microscope and where applicable fluorescence microscope) | | | |
| Comments: | | | |
| 4. Was there a difference between the homogeneity of fluorescence illumination in the field of view compared to your standard microscope? | | | |
| Field of view of Primostar iLED is more homogeneously illuminated | 57 | 67 | |
| Same | 43 | 33 | 100 |
| Field of view of Primostar iLED is less homogeneously illuminated | | | |
| 5. How satisfied are you with the overall handling features of the microscope (on/off switch, intensity regulation of bright light and fluorescence light, variable viewing height, focus mechanism (coarse and fine focus)? | | | |
| Very satisfied (better than for the available light microscope and where applicable fluorescence microscope) | 57 | 67 | |
| Satisfied (comparable to available light microscope and where applicable fluorescence microscope) | 43 | 33 | 100 |
| Not satisfied (not as good compared to those of the available light microscope and where applicable fluorescence microscope) | | | |
| Comments: | | | |
| 6. Is the procedure for switching between bright field and fluorescence light convenient and do you easily understand the symbols used for white light and fluorescence light? | | | |
| Very convenient | 43 | 67 | |
| Convenient | 57 | 33 | 100 |
| Not convenient | | | |
| Comments: | | | |
| 7. Is focusing with the fluorescence unit (due to black background): | | | |
| Very difficult | | | |
| Difficult, but only a matter of training | 29 | | |
| Easy, I quickly got used to it | 71 | 100 | 100 |

| | | | |
|--|-------------------|-----|-----|
| 8. Do you use the option of opening the slider on the white light source to focus with the fluorescence unit (dark background gets structured which makes focusing easier) | | | |
| Yes, I always use this to facilitate focusing | | 17 | |
| Sometimes | | 17 | |
| Never | 100 | 64 | 100 |
| 9. Are the blue LEDs on both sides of the microscope that indicate the intensity level of the bright field illumination convenient or rather disturbing/dazzling? | | | |
| Convenient | 100 | 100 | 100 |
| Disturbing/Dazzling | | | |
| 10. Are the 4pcs objectives with magnifications: 10x, 20x 40x and 100x the best choice for the applications Auramine O fluorescence and Ziehl Neelsen bright field detection of pulmonary tuberculosis? | | | |
| Yes | 100 | 100 | 100 |
| No; I would prefer to have a magnification | | | |
| Comments: | | | |
| 11. Which magnification do you prefer for fluorescence detection of AFBs: 20 times or 40 times? | | | |
| 20x | 29 | | |
| 40x | 71 | 100 | 100 |
| 12. In your opinion, can Primostar iLED be used without a darkroom? | | | |
| No darkroom is needed | 100 | | 100 |
| Darkroom is needed | | 100 | |
| 13. Do you use the dazzling protection for the eyepieces? | | | |
| Yes, they are useful | 57 | 33 | 25 |
| No, I do not need them (no dazzling) | 43 | 67 | 75 |
| No, I would need them, but they are not comfortable/convenient | | | |
| 14. Did you have any technical problems with your microscope until now (repair, replacement)? | | | |
| Yes, describe: | 43 ⁽¹⁾ | | 25 |
| No | 57 | 100 | 75 |
| Part IV: Application questions | | | |

| | | | |
|---|-------------------|-------------------|--------------------|
| <p>1. In your daily work, do you plan to switch between bright field and fluorescence contrast using just the Primostar iLED microscope or would you rather use the iLED for fluorescence detection only and a second microscope for bright field detection (Ziehl-Neelsen)?</p> <p>I would use the Primostar iLED for both fluorescence and bright field and would switch between the two modes at least once per day</p> | 14 | 17 | |
| <p>I would use the Primostar iLED for fluorescence only and will use a second microscope for bright light microscopy</p> <p>I do not think a bright field microscope will be needed in the future anymore for TB detection, i.e. I will only use it for fluorescence</p> | 86 ⁽²⁾ | 83 ⁽²⁾ | 100 ⁽²⁾ |
| <p>2. For which applications would you use the Primostar iLED?</p> <p>for TB detection only</p> | 100 | 100 | 100 |
| <p>for Malaria or HAT detection only</p> | | | |
| <p>for various applications (such as TB, Malaria, Blood Cell Counts, urine analysis, Trypanosomiasis)</p> | | | |
| <p>3. Do you see a significant gain in speed when reading slides with Primostar iLED (40 fields) compared to ZN (100 fields)?</p> <p>Yes</p> | 86 | 67 | 100 |
| <p>Yes, for negative and low positive slides only</p> | 14 | 33 | |
| <p>No</p> | | | |
| <p>4. If you had to decide whether to change a majority of microscopy centers in your country from light microscopy to LED based fluorescence microscopy, would you recommend to the Head of the National Health Program to switch to LED?</p> <p>Yes. Reasons:</p> | 57 ⁽³⁾ | 67 ⁽³⁾ | 100 ⁽³⁾ |
| <p>In principle, yes. But I would prefer using another microscope and not the Primostar iLED. Reasons:</p> <p>Only for low volume microscopy centers. Reasons:</p> | | | |
| <p>Only for high volume microscopy centers. Reasons:</p> | | | |
| <p>Only in specific settings. Define setting: ... Reasons:</p> | | | |
| <p>No. Reasons:</p> <p>No. But I would switch centers that currently have and use a conventional fluorescence microscope to LED fluorescence. Reasons:.....</p> | 43 ⁽⁴⁾ | 33 ⁽⁴⁾ | |

(1) Mechanical stage movement

(2) Due to low clarity lenses of light-iLED microscopes compared with standard light microscopes.

(3) Because iLED is faster

(4) Because iLED is cheaper and more convenient

4.5 SUMMARY OF FINDINGS

Sensitivity, specificity, and false negative rates of the iLED 40X were comparable to those of ZN reading (ZN-light microscope) under the study condition (ZN baseline) and routine LQAS system (previous quarter data).

Overall detection rates of iLED 40X were comparable over the phases in comparison with ZN reading (ZN baseline). Although the raw data showed slight variation from FM results using iLED 40X (ZN baseline: 13.8%, Validation: 16.2%, Implementation: 14% and Continuation: 14.9%), the difference was borderline significant in the early phases ($P=0.08$ for ZN baseline vs Validation and $P=0.053$ for Validation and Implementation) and non-significant for the late phases ($P=0.21$ for Implementation vs Continuation, $P=0.809$ for Implementation vs ZN baseline and $P=0.28$ for Continuation vs ZN). The detection rate of FM-iLED reading at one peripheral site (DTU8) was increased by 3.4% in comparison with routine ZN (light microscope, data from previous quarter) (16.3% vs 12.9%, $p=0.002$).

False positive rates using iLED increased in the early stages (Validation and Implementation phases) but then were comparable to the ZN-baseline value and the routine value (continuation phase).

Colour of AFB affected quantitative smear reading results ($P=0.002$). Sample processing method (NaOH-NALC) and slide storage condition at room temperature ($28^{\circ}\text{C} - 32^{\circ}\text{C}$, 70%-85% humidity) were probably responsible for 70% of AFB fading on each positive slide after 39 days; and the fading reached 100% by 2 months.

Reading time of FM-iLED 40X was shorter than light-iLED in proficiency testing but reading by standard light microscope was faster than FM-iLED 40X in reading

assessment. By the end of the study, the reading time of FM-iLED 40X was improved compared with at the initiation (38s recorded in the proficiency testing of the continuation phase compared with 92s in the reading assessment phase); and this FM-iLED 40X reading time (38 seconds) was faster than the one recorded for reading standard light microscope in the reading assessment phase (68 seconds). All staff agreed that FM reading saved more time than ZN reading when completing the questionnaire.

4.6 DISCUSSION

The first part of our study showed that the efficacy of Primostar iLED fluorescent microscope was similar to that of conventional fluorescent microscope and light microscope in the hands of microscopists experienced working with a fluorescent microscope. The sensitivity and specificity of iLED 40X against phase 1 panel slide reference results were more than 94% and 97% respectively. The same was observed for iLED 20X. Interestingly, no significant differences were observed in terms of identical, corresponding rates, false positive and false negative rates for the iLED 40X in comparison with conventional FM microscope and conventional light microscope. In terms of reading time, over 95% of slides had correct results available in 5 minutes for all methods. The technician performing this evaluation had 5 five years experience with both FM and ZN, and reads approximately 100 ZN slides and 30 FM slides per day using conventional light and fluorescent microscopes in daily work, which is much higher than the minimum number of slides recommended to be read daily by WHO. According to WHO recommendations, at least 2-3 smears should be read by a microscopist daily, with a maximum of 20 to maintain and improve their reading skill (254). Therefore, continuously working with smear reading under high workload

pressure probably improved his reading skill in both ZN and FM smears. It is likely that the pre-existing high proficiency of this technician on both ZN and FM microscopy, contributed to the results showing no difference in terms of efficacy and reading time. Another probable reason to explain the comparable efficacy between ZN and FM is closely following the ZN and FM procedures recommended by WHO. Proficiency testing results from 167 laboratories in the United States and Switzerland showed that the sensitivity of ZN staining method and fluorochrome staining methods can be similar if slides are prepared and examined according to the standard recommendations in terms of stain concentration, decolorization time, counterstaining time and number of field examined (174). Therefore, a high level of experience and close monitoring of smear performance may result in equal performance between ZN and FM reading on conventional LM, conventional FM and FM-iLED microscopes.

In technicians without FM experience, iLED 40X still did not show advantages against conventional LM and conventional FM in terms of sensitivity, specificity and false negative at any stage of the study. Although the detection rate fluctuated slightly during the study period this was not statistically significant, furthermore the false positive rate increased in the early stages of the study but all parameter measurements were comparable to the routine values at the end of the study period. World TB day (24th March) occurred during the study period and during that time a media campaign was conducted to raise awareness of TB symptoms and increased presentation of patients to TB clinics. More patients were detected than usual during the period of this campaign (routine NTP data). Furthermore, lack of familiarity with the FM microscope may influence the reading capacity of technicians, reducing detection rates and yielding more false positives. Finally, it is possible that working with the FM microscope for one year (the study period), the experience of technicians with FM

accumulated during the study was insufficient to gain advantages over ZN microscopy

Our findings were interestingly opposite to previous studies. One study from Uganda suggested that fluorescence microscope was 11% more sensitive than conventional light microscope ($P < 0.001$) (30). However, in this study, fluorescence smears were prepared from processed samples while ZN smears were from unprocessed (direct) samples. Studies in the literature have shown that sputum processing methods increase the detection rate by approximately 9% in comparison with direct smear (181). Therefore, the processing method is likely to have affected the sensitivity of FM observed in the Ugandan study and FM itself may not have been responsible for the improvement over conventional LM. The Ugandan study also found that more false positives were observed using FM than LM with culture as the gold standard. We also recognized that high false positive (by using iLED 40X) was generated in the early phases (Validation and Implementation) compared with ZN reading under the study conditions (ZN baseline); and the gold standard in our study was the rechecking process from the supervision site (PNT laboratory). Of note, like the study from Uganda, all of the false positives in our study were smear scanty or 1+, such errors usually result from lack of experience. Our peripheral technicians did not have any experience with FM before participating in the 5-day study training course; which may have been too brief. In addition, different gold standards may yield different levels of false results. The best gold standard for rechecking is not clear, LQAS system and culture have been used by different studies. In the National TB program, smear microscopy is the main microbiology test for TB diagnosis and therefore LQAS system is widely applied. First, the same smear preparation and reading system is followed by both peripheral and supervision sites. Second, no further equipment is

needed for supervisory site. Last, it is relatively cheap and not labour intensive. In a research or reference laboratory, culture yields higher sensitivity than microscopy and therefore it is a preferred gold standard to establish if positive results by a new microscopy methodology are true positives. However, not all TB laboratories are equipped for culture performance. In addition, time to culture positive, training, human resource, reagent/media supply and cost are important draw backs to the wide implementation of culture for QA. Moreover, it is unreasonable to use culture as the gold standard for smear microscopy because culture detects viable TB bacilli while smear microscopy detects both viable and dead TB bacilli. Patients on treatment may expectorate dead bacilli and furthermore, during the sample processing procedure, approximately 30% to 60% of viable TB bacteria will be killed depending on the decontamination method (214). These bacteria cannot be detected by culture but they can be observed by microscopy. Therefore, some smear positive cases will be incorrectly classified as false positive using culture as a gold standard, although a positive smear result is clinically misleading if detecting bacilli which are not viable. In order to minimize bias we applied the LQAS system at the supervisory site as the gold standard and only staff experienced with smear microscopy at supervisory site (PNT hospital) were involved in the study, although microbiological methods are widely used to identify the presence of bacteria in samples, neither smear or culture are 100% reliable for TB diagnosis. Patient management is not only based on laboratory results but also on clinical symptoms and radiology. However, clinical symptoms are subjective and radiology only shows possible pathological sign of the disease. Therefore, no perfect gold standard for TB diagnosis is available.

Interestingly, a recent study from Uganda reported that FM-LED was more sensitive than standard light microscope (5.6% vs 9.4%) and FM-LED was also more sensitive

than conventional/routine FM (69.8% vs 54.7%) against culture as the gold standard. In addition, examination time for FM-LED was 2 and 4 times less than for standard light microscope (2). However, the readers performing conventional/routine FM were from Mulago hospital and were not under the quality assurance management of the study because they were out of the study team. Two readers performing ZN-LM and FM-LED smears were from FIND tuberculosis research laboratory and under quality assurance system. Therefore, there was an inequivalent comparison of conventional FM with ZN-LM and FM-LED due to differences in staff proficiency. Furthermore, ZN reading skill of the two readers from FIND laboratory may not have been optimal because major errors were detected through intra and inter rechecking, leading to the reading result favouring FM-LED. The study also showed that the difference in sensitivity and specificity among these reading methods was not significant. If all readers had similar skill in ZN reading or FM reading and quality assurance was tightly managed, the sensitivity of detection of AFB among these methods would not vary.

Peripheral technicians in our study only had a one year period to learn, familiarize and work with FM-iLED microscope, which may be a short time in comparison with the experience gained working with ZN-LM reading, the advantage of FM reading against ZN reading may not have been maximized. FM-iLED should be performed as a routine test to replace for ZN-LM reading over at least one or two years for further evaluation. Therefore, the replacement of conventional light microscope with iLED40X at the moment should be carefully considered in high TB burden countries where most of the laboratory technicians are familiar with and have high experience using the ZN method. A full cost:benefit analysis is needed examining costs including

training, equipment replacement and maintenance and reagent requirements in operational settings.

A sub-study to understand if routine storage conditions (temperature, humidity) results in fading, which reduces the reading performance, was also conducted. We found that if direct FM smears were stored for one month at room temperature (DTU conditions, $28^{\circ}\text{C} - 32^{\circ}\text{C}$) with a relative humidity of 80% - 85%, followed by storage in an air conditioned room ($22^{\circ}\text{C} - 25^{\circ}\text{C}$, 60% humidity) during working hours after approximately 4 months, 28.9% of positive smears had a 2 level difference in quantitative results compared to previous reading results and only 15% of these smears still retained AFB in good colour intensity after a median storing time of 113 days. Storage conditions in terms of temperature, duration and humidity were proven to be a risk for slide fading. A study from Bangladesh showed that the fading process occurred on ZN stained smears in 2-5 months if smears were stored at a high degree of relative humidity (at least 80%), combined with temperature of 30°C (209). Therefore, our routine storage conditions favored the fading process, especially after 3 months for FM smears. Interestingly, if samples were processed with NaOH-NALC before smear preparation and smears were stored at room temperature for at least one month, the slides faded. It is likely that NaOH-NALC, room temperature and humidity affected the process of fluorescence decay in our study.

However, under the study conditions (not in fading assessment section) and routine LQAS system, direct routine slides from 3 study sites were stored at room temperature but they were stored at PNT condition for one month only, which is too short to cause AFB fading and cause reading errors. We therefore excluded fading as the reason of the high false positive rate at some DTUs during the study time.

The reading time assessment was conducted to compare time to results of ZN reading and FM-iLED reading. Two types of assessment were conducted during the study period: reading assessment (at the first month of the implementation phase for comparison between ZN-LM and FM-iLED) and proficiency testing (at the end of validation phase, implementation phase and continuation phase for comparison between ZN-iLED and FM-iLED). We showed that in the reading assessment, ZN-LM reading was faster than FM-iLED 40X reading, especially in positive smears ($p=0.007$) when data was combined from both peripheral site and supervisory site. However, when data was stratified by site, ZN-LM reading time and FM-iLED40X reading time were comparable for peripheral sites (69 second vs 95.5 second, respectively, $p=0.1$) and supervisory site (64 second and 84 second, respectively, $p=0.25$). Some observations may explain this finding. **First**, all of the participants at peripheral sites had a median of 28.5 years working with a light microscope; and read approximately 20 - 50 ZN slides per day. Therefore, adapting to operating the FM microscope was simple. The important issue was to recognize fluorescent acid-fast bacilli and to distinguish between AFB and artifacts with similar shape and colour present on the slide. **Second**, none of peripheral technicians had experience with a fluorescence microscope prior to the five days of iLED training, and at the time of the reading assessment, all participants from microscopy centers had used a fluorescence microscope for only one month, which may be too short to achieve a reading speed as expected. **Third**, concern about incorrectly recognizing AFB by FM- iLED microscope may have slowed down the reading speed on FM-iLED microscope and resulted in similar reading times on ZN-iLED and iLED40X. **Last but not least**, psychological factors during the reading assessment such as trying to avoid false

reading and the feeling of “being closely observed” by supervisors may have influenced the observed reading time.

When conducting proficiency testing at the end of each study phase, we found that FM-iLED40X reading was faster than ZN-iLED reading in staff with no prior FM experience. By the end of continuation phase, the reading time of FM-iLED and ZN-iLED was 38.5 second vs 51.5 second, respectively, $P=0.01$). This may suggests that time to result was improved when FM reading experience increased during the study period (9 months) in non-FM-experienced staff. However, there is an important point about the 100X objective of iLED microscope that might slow down the reading time of ZN-iLED. The objective 100X of iLED microscope was not as clear as that of the conventional light microscope. Therefore, it took more time to confirm both positive and negative results on ZN-iLED reading. Our finding was similar to the report from a systematic review in 2006, which showed that FM reading was faster than ZN reading, and 1 minute is required to record a result for FM microscopy compared to 4 minutes for conventional light microscopy (180). Interestingly, although our reading time was half that described in the review, we generated a high false positive rate in the early phase. Further evaluation should be conducted to clarify if faster reading time is related to high false positive rate. More studies should address the reading time recommended for FM reading to limit the false reading rate.

Unfortunately, we did not conduct proficiency testing for the supervisory site at the end of the study so we are unable to determine if there was an improvement of reading time for this group. However, we conducted an experiment on panel slides for this group to compare the percentage of slides with correct results available after 30 second, 1 minute, 3 minutes and 5 minutes. This evaluation showed no difference

between FM-iLED 40X, conventional light microscope and conventional fluorescence microscope 40X. It is possible that if we had conducted proficiency testing for this group at the end of the study, we would not have observed a reading improvement because this group was already familiar with FM reading prior to the study.

We also did not conduct an assessment to compare reading time between FM-iLED 40X and conventional LM in routine samples for non FM-experienced staff to determine if replacing conventional LM with FM-iLED 40X in the NTP system would practically result in time savings. Data showed that reading time reduced with experience (92s for FM-iLED in reading assessment – early phase vs 38.5s for FM-iLED in proficiency testing – late phase) and by the end of the study, the reading time of FM-iLED (38.5s) was shorter than that of standard light microscope recorded in the reading assessment phase (68s). Large scale studies should be conducted to evaluate time saving of FM-iLED in comparison with standard light microscope.

The design of the iLED microscope met the requirements of the microscopists such as good contrast, no requirement for a dark room, power saving and good battery-life. Another advantage of the iLED microscope is that the cost is almost 10 times lower than the conventional FM, which is promising for wide application. However, the 100X objective lens had rather poor clarity compared with the conventional light microscopes currently in use (AFB colour is less well distinguished by white LED (iLED 100X) in comparison with conventional light microscope, recorded from staff appraisal forms and discussion with Catharina Boehme during her visits. Therefore, currently, iLED microscope only shows high performance using fluorescence. Assessment and improvements should be made to the 100x objective lens, although this may increase costs of the microscope.

In general, although the Primostar iLED microscope presented many advantages against conventional fluorescence microscopes in terms of design and construction, the proposal to replace the conventional light microscope which is currently used in high TB burden countries should be carefully considered. **First**, the sensitivity, specificity and detection rates of the Primostar iLED microscope did not show improvements over the conventional light microscope (data during the study period was compared to those from the same period of previous year). A higher false positive rate was generated by the FM-Primostar iLED microscope in comparison with ZN-LM (ZN baseline phase), especially; most of the false positive results were incorrectly read as scanty and 1+ smears. **Second**, although by the end of the study, reading time of FM-iLED 40X was shorter than that of ZN-iLED (proficiency testing), we cannot conclude that FM reading will save more time than ZN reading because the 100X objective of iLED was not as clear as expected and this may prolong the reading time of ZN-iLED. In addition, we did not conduct an assessment to compare the efficacy of FM-iLED40X with conventional light microscope in non FM-experienced group, although data showed that reading time reduced with experience by the end of the study. Therefore, further evaluation should be conducted before replacing conventional LM with FM-iLED 40X. However, the comparison was made in FM-experienced group and the results were comparable for conventional light microscope, conventional fluorescence microscope, FM-iLED 40X and FM-iLED 20X.

Conversely, in countries where fluorescence microscope is routinely implemented at some clinics and well-trained, FM-experienced staff is available, FM-iLED should replace damaged/out of order conventional FM because it has advantages over the conventional FM, as discussed above. In 2009, World Health Organization has endorsed the replacement of conventional fluorescent microscope by LED microscope

in laboratories where conventional fluorescent microscopes are available. In settings where ZN staining is the main method for use in TB diagnosis, the implementation of FM-LED has also been encouraged for stepwise replacement of light microscopes by WHO (238). However, prior to wide application of FM-LED, each setting should conduct studies to optimize training material preparation and training time as these will vary between settings due to different background experience of technicians, evaluation and monitoring of detection rate and outcome of cases diagnosed by FM-LED to determine the accuracy of FM-LED, establishment of quality control and quality assurance procedure for technical control and management, waste management (because auramine is considered possibly carcinogenic to humans although there is inadequate evidence for carcinogenic effect (41, 255), and microscope maintenance.

4.7 CONCLUSION

The option of replacing light microscopes with FM microscopes for wide application in national TB programs should be carefully considered in settings where ZN microscopy performance is high. Further studies should be conducted in routine conditions over a longer period of time for better evaluation of iLED performance. The relative costs of implementing programs to improve and maintain ZN microscopy performance vs. implementation of FM microscopy should be carefully evaluated in our setting.

Chapter 5

OVERVIEW DISCUSSION – CONCLUSION – FUTURE DIRECTIONS

The aim of this thesis was to address two questions:

1. What is the efficacy and feasibility of MODS in diagnosis of paucibacillary TB and multidrug resistant TB?
2. What are the accuracy and performance characteristics of the iLED fluorescence microscope in comparison to light microscope and traditional fluorescence microscopes in diagnosis of TB?

The purpose of this thesis was to evaluate if MODS and fluorescence iLED microscope are accurate as routine TB diagnostic tests in high burden settings with limited resources and to contribute to the development of a roadmap for the implementation of these methods in these countries. The following discussion summarises the principal conclusions of this thesis, the outstanding questions and presents an overview of the potential impact on the TB epidemic of improved diagnosis and the challenges in assessing and implementing novel TB diagnostics in national TB programmes of resource poor settings.

5.1 SUMMARY OF FINDINGS

The MODS study showed that with a turnaround time of 7 – 8 days and a pooled sensitivity, across all sub-studies, of 13% higher than homogenous smear microscopy but comparable to MGIT culture, MODS is an appropriate test for early diagnosis of paucibacillary TB in resource limited settings.

In terms of diagnosis of MDR-TB, MODS had low sensitivity (77.8%) and low PPV (87.5%) but high specificity (99.7%) and high NPV (99.3%). The low sensitivity and PPV of DST-MODS in this study was probably due to low bacterial load samples and a high critical INH concentration (0.4µg/ml rather than the now recommended 0.1µg/ml) and the low MDR-TB rate in this community (3.8%), respectively. MODS is also a reliable method for MDR-TB detection if a critical concentration of 0.1µg/ml is used for INH.

The expected advantages of fluorescence iLED 40X microscope against light microscope and conventional fluorescence microscope were not clearly observed in this study in terms of accuracy. The reading time of FM-iLED microscope (38s, recorded in the last phase of the study) showed an improvement compared with that of the standard light microscope (68s recorded in the early phase of the study). Further studies should be conducted to determine if a real time-saving occurs with FM-iLED compared with standard light microscope in routine use. AFB fading of auramine stained smears was shown to be related to homogenous smear preparation and storage conditions at room temperature.

5.2 OUT STANDING QUESTIONS

5.2.1 What needs to be done for scale- up of MODS in resource constrained settings?

MODS has been proven to be an effective, accurate and reliable test for diagnosis of TB in Viet Nam, where it was evaluated at the reference laboratory for this thesis. An operational study in Peru demonstrated increased sensitivity of the MODS assay against MBBacT and LJ culture (132). However, for rolling out MODS in large scale implementation, more evidence is needed to assure the validity of MODS in national TB programmes, including the reproducibility of MODS, percentage of TB patients diagnosed and receiving benefits from early detection by MODS, treatment outcome of MDR-TB patients detected by MODS and the cost effectiveness of MODS in NTPs. An evaluation of decentralization of MODS in Peru showed that MODS was reliable and effective for use at regional TB laboratories within the NTP (119). Recently, accreditation of MODS was performed for 2 urban regional TB reference laboratories in Peru and Universidad Peruana Cayetano Heredia (UPCH, where the MODS technique was developed) with GenoType® MTBDR*plus* as the gold standard (47). This study showed that concordance between the two methods for detection of INH resistant isolates and RIF resistant isolates were 94.5% and 97.0%, respectively; and consistency of findings was observed across evaluated laboratories. This accreditation scheme should be duplicated in other settings. The decentralization and accreditation processes contribute to the stepwise implementation and external quality assurance (EQA) of MODS. This data is encouraging and suggests MODS may be successfully scaled-up to routine laboratories.

5.2.2 Can MODS distinguish *M.tuberculosis* and Mycobacterium other than tuberculosis (MOTT) based on cording characteristic?

The presumptive identification of *M.tuberculosis* by cording development has a wide range of sensitivity, from 64% to 99% (8, 93). In the MODS evaluation (**chapter 3**), of 29 positive MODS cultures presenting with atypical cording (very short cording, slow cording or yellow colonies), only one sample was confirmed as MOTT by INNO-LiPA. In addition, of 22 positive cultures identified as MOTT by INNO-LiPA, none had atypical cording observed in the MODS assay. The ability to distinguish specific cording of *M.tuberculosis* from atypical cording of MOTT may vary between observers due to the level of experience. Mycobacterium species other than *M.tuberculosis* can produce loose, incomplete “pseudocords” that may be misinterpreted as true cording. In settings with a very low prevalence of MOTT infection, few MOTT will occur in routine samples; therefore less experience differentiating cording is gained, especially in early growth cultures. In settings with a high prevalence *M.tuberculosis*, incubation of 4 days or less may be not enough to determine non-cording (101). In addition, TB treatment affects the cording characteristic of *M.tuberculosis*. In a study carried out by McCarter *et al.* (115), almost 11% of *M.tuberculosis* did not exhibit cording. Of them, 54% were isolated from patients on TB treatment. Interestingly, all isolates previously isolated from these patients had exhibited cording. This study also found that less than 7 days incubation time may not be sufficient for cording formation.

In general, it is challenging to differentiate MOTT from *M.tuberculosis* based on cording formation observed in the MODS assay. However, the current diagnostic test, smear microscopy, also does not differentiate MOTT from *M.tuberculosis*. The addition of rapid speciation tests, such as MPT64 antigen tests, will add cost and complexity to the test and may not be cost-effective in high burden settings. Further studies are required to determine if a simple speciation technique can be applied.

5.2.3 Can samples other than sputum samples be used for MODS assay?

The MODS study described in this thesis has shown that CSF (n=332) and gastric fluid (n=35 samples) are appropriate for diagnosis of TB by MODS. However, more studies should be conducted to confirm this conclusion, particularly for gastric fluid samples because of the small sample size in these studies.

Currently, most accurate TB diagnostic tests have been developed for use on sputum samples rather than other specimens (nasopharyngeal aspirate, bronchial lavage, gastric fluid, urine, stool, blood or other body fluids) and the majority of publications for evaluation of diagnostic tools were conducted in adults. However, specimens other than sputum are often more appropriate for diagnosis of extrapulmonary TB in the most vulnerable patient groups: children, HIV infected individuals and patients with TBM. No serological tests have proven to be accurate (178) and therefore there is an urgent need to determine which microbiological or molecular tests can be applied to samples from these patient groups. MODS appears to be suitable for application to paucibacillary fluids and more extensive evaluation of extrapulmonary specimens is warranted.

5.2.4 What is the appropriate volume of processed sample for inoculation on MODS to achieve a high sensitivity?

The MODS experiments performed with 100µl processed sample yielded a non-significant higher detection rate than with 250µl sample, when the MODS assay was done on 48 well-plates. This observation was described for sputum and CSF samples. The 100µl volume was also recommended in the MODS SOP (90). Practically, not only the volume of processed sample but also the quantity and the quality of clinical sample affect the recovery rate of a test. Therefore, all of the requirements related to sampling, processing and testing should be strictly followed to achieve the highest yield.

5.2.5 Is contamination a major concern when performing the MODS assay?

Although the contamination rate and cross-contamination of MODS were less than 2% in this study and also low in reports from other groups using MODS (33, 131, 144), the technique is considered as a method with a potentially high risk of contamination when attempted for wide application because MODS is a liquid culture based technique; and manipulations related to sample transfer to the MODS plate increase the risk of contamination and cross-contamination. To minimize the contamination rate, technical issues related to handling the plate, opening the lid of the plate, transferring samples to wells and manipulations with pipettes should be performed following aseptic technique and this needs to be included in training. Data from this and previous studies shows that contamination is not an issue in controlled

settings, however, contamination will be more difficult to both control and evaluate in wide-scale implementation.

5.2.6 Can MODS be used for screening or ruling out active TB in HIV-infected individuals?

The potential of MODS for screening for active TB among HIV-infected individuals prior to Isoniazid Preventive Therapy (IPT) is an important consideration. In our study, the sensitivity of MODS in detection of TB among people living with HIV was 70.7% which is lower than that of MGIT (74.9%, $P < 0.03$); and the negative predictive values (NPV) of MODS and MGIT were 22.4% and 25.3%, respectively, $P = 0.711$). However, the sensitivity of MODS was higher than that of smear (56.9%, $P < 0.001$). The NPV values of MODS and MGIT in our study were rather low because we did not have a true non-TB group for comparison, all patients enrolled in our study had clinical symptoms suspected of tuberculosis due to the referral setting. Therefore, from our data, MODS can add sensitivity to smear microscopy in screening for active TB among HIV-infected patients. Although MGIT is more sensitive than MODS, MODS is cheaper and faster than MGIT. Therefore, MODS is more appropriate than MGIT in screening for active TB in the HIV infected population in resource-limited settings. For better screening results, MODS and smear or two MODS cultures can be used in combination. A higher NPV will be obtained in a general population of HIV patients. A study from Peru showed that in screening TB among HIV positive patients, MODS detected more cases (6.2%, $n = 27/435$) than smear (1.6%, $n = 7/435$) and LJ (5.1%, $n = 22/435$). All MODS cultures were positive by day 21; NPV value of

MODS was 100% for 2 sputum samples which is higher than that of 2 smears (95.3%) and 2 LJ cultures (93.8%) (161). This study demonstrated that an algorithm for TB screening combining clinical presentation, chest X-ray and smear microscopy is less effective than two liquid cultures in detection and exclusion of active TB; and MODS may have a potential role in ruling out TB in people living with HIV prior to IPT initiation. People living with HIV are at high risk of TB infection and at high risk of loss to follow-up during diagnosis and treatment. Therefore, if a screening algorithm is insensitive or the screening procedure takes too long, NTP might lose these infectious cases in the TB control and management system. Additionally, MODS has an advantage in terms of lower reagent and consumable costs, therefore, MODS can potentially be widely applied in high burden settings for screening and ruling out active TB in HIV-infected individuals.

5.2.7 Can MODS be used for screening or ruling out MDR-TB in TB suspects?

Although the sensitivity of DST-MODS for detection of INH resistant and RIF resistant isolates was rather low (72.6% and 72.7%, respectively) in our study, data from previous studies showed that DST-MODS is sensitive and specific in detection of INH and RIF resistant isolates as well as MDR-TB isolates (33, 132, 133). In addition, its specificity was very high (97.9% for INH resistance and 99.7% for RIF resistance) and the agreement between DST-MODS and DST-LJ for detection of MDR-TB isolates was very high (99%, kappa: 0.8, $P < 0.001$). MODS can be used for screening patients suspected of infection with MDR-TB or early diagnosis of MDR-TB patients in low income countries where an accurate, cheap and rapid screening

method is currently not available. In this study, the low PPV of MODS in diagnosis of MDR-TB (87.5%) may result in substantial mis-diagnosis which would result in patients receiving inappropriate treatment. To achieve higher accuracy, if resources permit, alternative methods can be combined in an algorithm; such as two MODS DST tests, 1 MODS-DST and confirmation of MDR-TB positives with Genotype@MTBDR*plus* or confirmation of RIF resistance using GeneXpert; and laboratory results should be considered in conjunction with the clinical picture and assessment of risk-factors for MDR-TB.

5.2.8 Does MODS need specific training, quality control and quality assurance?

For wide implementation of the MODS assay, training materials as well as quality control (QC) and quality assurance (QA) for MODS assay should be carefully developed and applied to ensure sustained accuracy. In laboratories where staff have a high level of experience working with microscopy, training on MODS reading and recognition of *M.tuberculosis* cording should take approximately 3 weeks to one month, in our experience. For staff who have no experience with microscopy, a longer training period will be required dependent on individual ability. Training materials should cover all issues related to sample handling, microscopy skills, pipetting and culturing, biosafety and biohazard. Proficiency testing at the completion of training should be standardized. QC-QA should also ideally cover all of these issues. Although QC and QA for the MODS assay have been developed by David Moore (130), countries intending to perform the MODS assay should assess local requirements and

resources in developing and applying modified QA-QC materials to achieve the highest effectiveness.

5.2.9 What is the advantage of the LED microscope?

The LED microscope assessed in this thesis (manufactured by Carl Zeiss, Germany) has some advantages in design (use of LED bulb, no requirement for a dark room during operation, battery powered and electricity saving) and a competitive cost that makes it feasible for replacement of conventional fluorescence microscope (4825US\$ on the market but special pricing of 1250US\$ for high burden countries) (126). The LED microscope weighs 9.5kg which may not be appropriate for portability, especially for use in remote areas. Recently, a compact size (7.5 x 13 x 18cm), minimal-weight (1kg), battery powered (2 AA batteries) and low cost (240US\$) fluorescence microscope has been developed and evaluated (Global Focus microscope) (123). These characteristics of a fluorescence microscope may contribute to the expansion of using fluorescence microscope in low income countries.

5.2.10 Does the fluorescence microscope practically increase case detection?

It was unclear from the study presented in chapter 5 if application of the FM-LED microscope would increase case detection in Vietnam. A multi-country, cross-sectional evaluation in Africa (51) reported that the FM-LED microscope was 10% more sensitive than the standard light microscope in culture positive samples; and FM-LED detected 1% more cases than the standard FM-microscope. However, the number of patients with a correct diagnosis was lower by FM-LED (89%) than using

a standard FM-microscope (91%). The majority of incorrectly diagnosed cases by FM-LED had scanty smear results. The balance between advantages and disadvantages of FM-LED implementation to specific TB control programmes should be evaluated carefully before implementation.

5.2.11 Should fluorescence LED microscopes replace standard light microscopes in National TB programmes?

Fluorochrome acid-fast microscopy is currently assumed to be the most rapid microbiological method for detection of acid-fast bacilli in clinical specimens. Acid-fast microscopy is a useful tool for initiating treatment and monitoring the progress of anti-tuberculous drug therapy and the smear microscopy networks are currently the keystone of TB diagnosis and management in high burden settings. Although data in this LED study showed that the accuracy of the LED microscope was comparable to that of the light microscope and conventional fluorescence microscope, data from other studies strongly suggested advantages of fluorescence microscope over light microscope in terms of sensitivity (31, 171) although a significant difference has not always been shown (2). In addition, data from WHO describes an increase in sensitivity of FM-LED microscopy of 6% compared to ZN microscopy and by 5% compared to conventional fluorescence microscopy but the specificity was comparable between these methods (242). Therefore, fluorescence LED microscope was endorsed by WHO in 2011 to replace ZN microscopy in both high and low volume laboratories (238, 242).

The reading time of both methods observed in the LED study was rapid (<2 minutes), therefore it will be challenging to further increase the speed of detection without introducing reading errors.

In general, little advantage from FM-LED microscope in comparison with the standard light microscope was observed in the LED study of this thesis. Therefore, the replacement of light microscope by FM LED microscope for wide application in national TB programmes should be considered carefully, especially in settings where the light microscope is being used as the main tool for diagnosis of TB and technicians are very experienced in working with standard light microscopes. Further studies should be conducted to evaluate LED microscope in routine conditions for a longer period of time to have a precise conclusion on large scale implementation of this method.

5.2.12 If FM-LED microscopes replace standard light microscopes, what should be considered in terms of QC-QA?

An important consideration in designing QC-QA for auramine stained smears is the fading of colour intensity over time (91). The iLED study found that the sample processing agent (NaOH-NALC) and room temperature (28⁰C – 32⁰C) lead to AFB fading after 39 days. Recently, Minion *et al* also observed the fading of auramine stained mycobacterial smears over time when these slides were exposed to light and temperature. The study showed that among positive slides exposed to light at room temperature (22⁰C), only 24% still had positive results after 4 weeks. When exposed to room temperature (22⁰C), humidified incubator (30⁰C) and refrigerator (4⁰C), the

fading rates per month (with changing result from positive to negative) determined by linear least square regression were 21%, 23% and 26%, respectively (125). Therefore, auramine staining smears should be kept away from high temperature, daylight, sunlight and humidity to minimize fading. This is a huge challenge for settings in the tropical and subtropical regions. This evidence will help to develop external quality assurance guidelines for fluorescence microscopy.

For quality control and quality assurance of FM-LED microscopy, all staining, reading, recording and reporting procedures must comply with WHO guidelines (216). Blind rechecking should be applied to all positive smears and at least 20% of negative smears until quality targets are met (eg. <2% false positive and negative results) to confirm diagnosis at the individual level and investigate errors. Slides with reading errors should be restained with ZN staining method for final confirmation. When technical issues are controlled, blind rechecking by Lot quality assurance system (LQAS) method should be applied to assess overall laboratory performance. This QA-QC strategy has been successfully applied in implementation and monitoring of the ZN-staining method. The AFB fading should be taken into account when developing QA-QC manual for Auramine-staining method.

5.2.13 Is there any concern about waste management in Auramine staining procedure?

Auramine is classified as an industrial chemical related to human cancer (165, 200). The implementation of LED microscope should be carefully considered in terms of waste management because auramine is a carcinogenic agent. Since the LED

microscope is intended for use at all laboratory levels, it is important to have a waste management system under strict control to prevent or minimize environmental contamination with Auramine. The waste management system at peripheral level laboratories in resource constrained settings may lack sufficient regulation.

5.3 OVERVIEW CHALLENGES

Although MODS has advantages in the early diagnosis of TB as demonstrated in this thesis, some challenges remain in implementation of this method which need to be addressed. **First**, since liquid cultures pose an increased risk of infection and transmission, biosafety should be considered when handling MODS assay and a culture laboratory equipped with class I cabinet is required. **Second**, MODS is a relatively laborious technique because of the need for frequent plate reading (alternate days from day 5 to day 15, then twice a week from day 16 to day 30), it is probably not appropriate for use in laboratories with a high workload and personnel shortages. Most peripheral TB laboratories in high burden countries have one to two technicians working full-time, so there may not be sufficient staff for implementation of MODS in these laboratories. **Third**, the availability of a MODS kit may assist in ease of implementation. Recently, a TB MODS kitTM has been developed by Hardy Diagnostics Company and is now under evaluation by PATH (www.PATH.org). This kit may be a suitable solution for scaling up standardized MODS in TB diagnosis worldwide but the cost may be increased. **Fourth**, waste management and biohazard are important issues because all end-products of the MODS culture procedure need to be autoclaved before discard as medical waste. **Last**, for MODS implementation, a

laboratory should be equipped with centrifuge, vortex, incubator, Gilson pipettes, inverted light microscope, autoclave, biosafety cabinet, refrigerator and inverted microscope which are not routine laboratory tools at peripheral laboratories, in high burden countries. Periodic maintenance of this equipment is also a major concern. These are challenges in scaling up MODS in resource constrained settings. An inverted microscope costs approximately \$US 5000 which is more expensive than a normal light microscope. Recently, a group of scientists in Peru attempted to develop a low-cost inverted microscope for use in resource limited settings, which would encourage the use of MODS assay in low income countries (264).

It is challenging to scale up the implementation of FM-LED microscopes in high burden countries. The fading of auramine stained slides may compromise the external quality assurance (EQA) system. If all auramine stained slides are restained for EQA, the increased costs of FM-LED implementation will reduce its cost-effectiveness. In addition, the benefit of FM-LED versus light microscope in terms of case detection and the cost of false positive diagnosis remain unclear. Therefore, further studies of the operational impact of FM-LED microscope are required.

Tuberculosis diagnosis and management is a complex and interactive process, in which novel diagnostic tests are only a single component. Therefore, to achieve global targets for TB elimination, efforts from many fields must be co-ordinated. **National TB programmes are attempting to implement many new interventions across diagnosis, prevention and management and competitive demands for resources must be carefully considered to prioritise strategies with the biggest potential**

impact. Improvements in diagnosis will have little effect if NTPs are unable to cope with the increased case-load and especially, to treat drug-resistant cases appropriately. The effectiveness of novel diagnostic tests therefore depends not only on their efficacy, but on integration of investment across all aspects of TB control programmes.

5.4 POTENTIAL IMPACT OF IMPROVED DIAGNOSTIC TESTS ON THE TB PANDEMIC

New diagnostic tools with increased sensitivity and shortened turnaround time may have positive impacts on TB epidemiology within a community. However, a rapid and widely available diagnostic for tuberculosis with $\geq 85\%$ sensitivity for smear-positive and smear-negative cases, and 97% specificity, which could save approximately 400,000 lives annually, is not yet available (96). Mathematical models exploring the potential impact of novel diagnostics either alone or in combination with other enhanced intervention strategies, can assist policy makers to determine the optimal strategy and allocation of resources. However, there is no standardised consensus model for tuberculosis, where latency complicates the derivation of appropriate models. An age-structure mathematical model of TB applied for the WHO South East Asia region (1) estimated that without novel interventions of vaccination, drug regimens or diagnostics tools, the cumulative incidence for 2015-2050 would be approximately 100 million active TB cases and 18 million TB-related deaths. The intervention of new diagnostic tools could have an important impact on reduction of TB incidence and mortality by 2050. In this model, LED microscope, nucleic acid

amplification tests or a hypothetical dipstick for antigen or antibody methods would reduce the TB incidence by 13%, 42% and 28% respectively. The increase in case detection will shorten the infectious duration, reduce infectious reservoirs and enhance the decrease in mortality if diagnosed patients receive effective therapy. However, even with a combined triple intervention of novel diagnostics, shortened treatment and vaccination, the reductions in incidence were insufficient to reach STOP TB partnership target for TB elimination (<1 case per million/year) by 2050. The introduction of new tools and current diagnostic algorithms do not help people who do not access health services (107). The poorest and most vulnerable groups are known to face barriers when accessing health services. Therefore, advocacy and community action play an important role in targeting this population and maximizing the uptake of novel diagnostics to reduce the infection source in the community. Those diagnosed must also receive immediate and effective treatment for a novel diagnostic test to have an impact on the epidemic.

5.5 ACTION FROM NATIONAL TB PROGRAMMES

Despite the systematic evaluation and endorsement by WHO of many novel TB diagnostic tests over the last decade (229, 235, 238, 248) there is no perfect TB diagnostic test available. While the ideal 'dipstick-style' point of care diagnostic test remains elusive, action must be taken by TB control programmes now using existing strategies to target tuberculosis and so a choice must be made between imperfect solutions.

MODS is simple, sensitive, specific, rapid and low cost but labourious; Fluorescence microscope (LED microscope) is rapid, sensitive and specific but its benefit relative to cost of implementation is unclear in settings where the existing ZN staining method is of high quality; Commercial liquid culture/DST method is sensitive, specific and rapid but the cost remains beyond the reach of poor patients; GeneXpert is simple, sensitive, specific and extremely fast (2 hours) but the cost remains high (17USD/cartridge); and the Line probe assay is sensitive, specific and rapid but is only applicable on smear positive sputum samples and technical skill is required. Commercial serological tests currently on the market have been proven to be inaccurate and in 2010, WHO issued an unprecedented negative policy statement regarding these tests. National TB control programmes and governments should take action to eliminate the use of these tests (250). TB control programmes should develop capacity to adopt the implementation of new validated diagnostic tests which are appropriate for local conditions.

Ethically, every TB patient has a right to receive equal access to high quality health care services (204). Every service in the TB control system must be strengthened to accomplish this mission. Although laboratory services are known as the “backbone” of TB programmes, they have long been neglected and are not sufficiently invested. The weakness of these services is now accentuated in the context of MDR-TB and XDR-TB; and this weakness is a major bottleneck for scaling up management and control of TB in high burden countries which receive limited funds for this activity.

The current condition of TB laboratories can vary dramatically depending on national investment (249). **First**, many TB laboratories in high burden countries are equipped with inadequate and unsafe infrastructure such as small rooms, no or poorly maintained biosafety cabinet, no or unreliable electricity supply. **Second**, Staff usually work with no or limited personal protective equipment. **Third**, country-level strategic decision makers often do not include laboratory services as an important part in the national development plans because annual reports rarely include laboratory efficiency data. **Fourth**, there is a lack of skilled staff. **Fifth**, the current diagnostic tools, smear microscopy and culture, are ineffective in the drug resistant era. **Last but not least**, there is a lack of technical assistance and technology transfer from national and international levels which could strengthen the laboratory “backbone” of TB control programmes. The Global Laboratory Initiative (GLI) is attempting to address this problem (www.stoptb.org/wg/gli).

All of these drawbacks contribute to the inaccurate and unreliable results reported from laboratories and insufficient case detection. An annual report from the Western Pacific region (240) (Cambodia, China, Laos, Mongolia, Papua New Guinea, Philippines and Viet Nam) showed that in 2007, among 7997 laboratories that can perform smear microscopy (generating 0.5 lab/100,000 pop), only 78% (n=6262/7997) of them were in external quality assessment programmes (EQA) and over 80% of laboratory in the EQA system needed to take corrective actions. Furthermore, only 6% (n=463/7997) and 3% (n=224/7997) of microscopy laboratories were equipped for performing culture (generating 1.3 lab/5 million pop.) and DST (generating 1.3 lab/10 million pop.) According to WHO, at least 2000 new

laboratories capable of doing culture and DST and 20,000 newly trained lab technicians worldwide are needed to ease the current global laboratory bottleneck (38).

With the current microbiological diagnostic tests, it will be difficult to achieve the Millennium Goal Development (MGD6) and the Stop TB partnership targets (by 2015, to reduce TB prevalence and death by 50% compared with a baseline of 1990 and by 2050, to eliminate TB as a public health problem) due to the emergence and spread of HIV and MDR-TB in Africa and Eastern Europe after 1999 (201) and the limitations of the current diagnostic tests. To improve diagnostic efficiency globally, in terms of research, it is crucial to develop novel diagnostic tests which can overcome the disadvantages of the current methods, target the population who are responsible for 90% of the TB epidemic worldwide and are appropriate for use in low income countries. If the endorsed novel tests (229, 235, 238, 248) are introduced immediately, they could potentially have an impact on increasing case detection, enrolling more TB cases into treatment promptly, reducing infectious source, and for long term benefit, the TB incidence and prevalence would decrease and the Millennium Goal could be achieved.

One of the important considerations in development of a new test is evaluation of the test using samples collected from the target population. To facilitate this, a bank of reference clinical materials to support the development and evaluation of new diagnostic methods was created in 1999 and led by the Special Programmes for Research and Training in Tropical Diseases (TDR). In this project, samples of 2587

TB suspects have been collected, including sputum, saliva, blood and urine, from 12 countries (South Africa, Uganda, Gambia, Brazil, Canada, Spain, Viet Nam, Bangladesh, Kenya, Columbia, Peru and Zambia). Among 95 requests from the test manufacturers which were approved by TDR, 63% (n=60) were related to immunological methods and only a very small number of requests related to microbiological tests (n=4). Unfortunately, results from these studies did not have any impact on utility for global TB diagnosis (139). It can be inferred that although there is clearly an urgent need for a more sensitive TB diagnostic, no novel test was introduced because, of those developed, none were appropriate for use in samples collected from high burden countries. Aside from accuracy of the test, cost is clearly an important issue in selecting new tests for use in low resource settings. Since TB is a disease of poverty, expensive tests are not feasible for application in resource constrained settings. Recognizing this problem, FIND and WHO have negotiated preferential pricing to high burden countries for novel tests approved by WHO (Genotype®MTBDR-*plus* and GeneXpert). This contributes to the scaling up novel diagnostic methods worldwide.

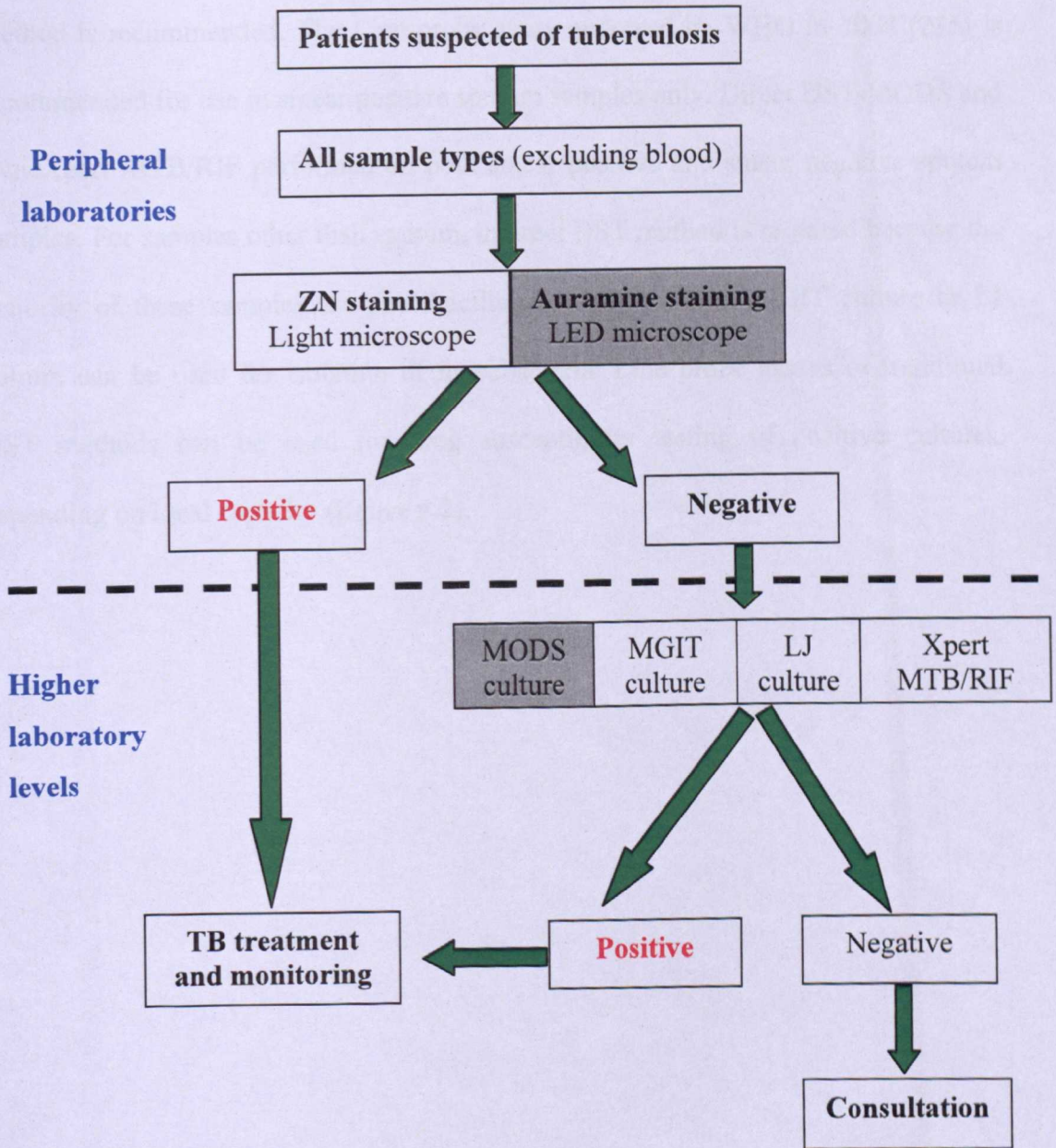
Tests may be introduced into NTPs individually or in combination. Significant achievements have been made since the millennium, but novel diagnostic technologies have yet to reach the majority of people at risk of TB. Sustained commitment from international donors, national governments and expert technical advisors will be required to ensure that implementation of tests proven to be effective is achieved and reaches the high-burden settings.

5.6 PROPOSED ALGORITHM FOR EARLY DIAGNOSIS OF TB AND MDR-TB (applying smear, culture and molecular methods)

The peripheral tuberculosis unit is the first health care centre that the majority of TB suspects contact. Peripheral laboratories within these units use direct ZN -smear microscopy, read using a light microscope. Direct auramine staining smear microscopy (LED microscope) should gradually replace ZN staining to improve efficacy of smear microscopy according to WHO recommendations (238). If the smear result is positive, the patient should be recruited for TB treatment according to WHO/NTP guidelines (220). For smear negative results, culture methods and molecular testing are suggested by WHO for further diagnosis. Diagnosis of smear negative TB is potentially done at intermediate/regional or reference laboratories. At the present time, due to financial shortages, personnel shortages and insufficient infrastructure in high burden countries, it is more feasible to implement MODS culture, MGIT culture, LJ culture or GeneXpert MTB/RIF at regional or reference levels. GeneXpert MTB/RIF is recommended for use at peripheral laboratories because of its simplicity and safety. Although the negotiations between WHO/FIND and Cepheid company have reduced the price of the GeneXpert system to 25% of the market prices for low and middle income countries, the running cost of each test is still high (18US\$) compared with other novel tests (eg. MODS: \leq 2US\$ for in house test or around 6 US\$ for commercial tests, excluding labour costs). The decision of which method should be used is dependent on the internal capacity of the individual NTP programme. In the future, if the “call for action” (147) receives positive feedback from government, policy makers, international organizations, more funds

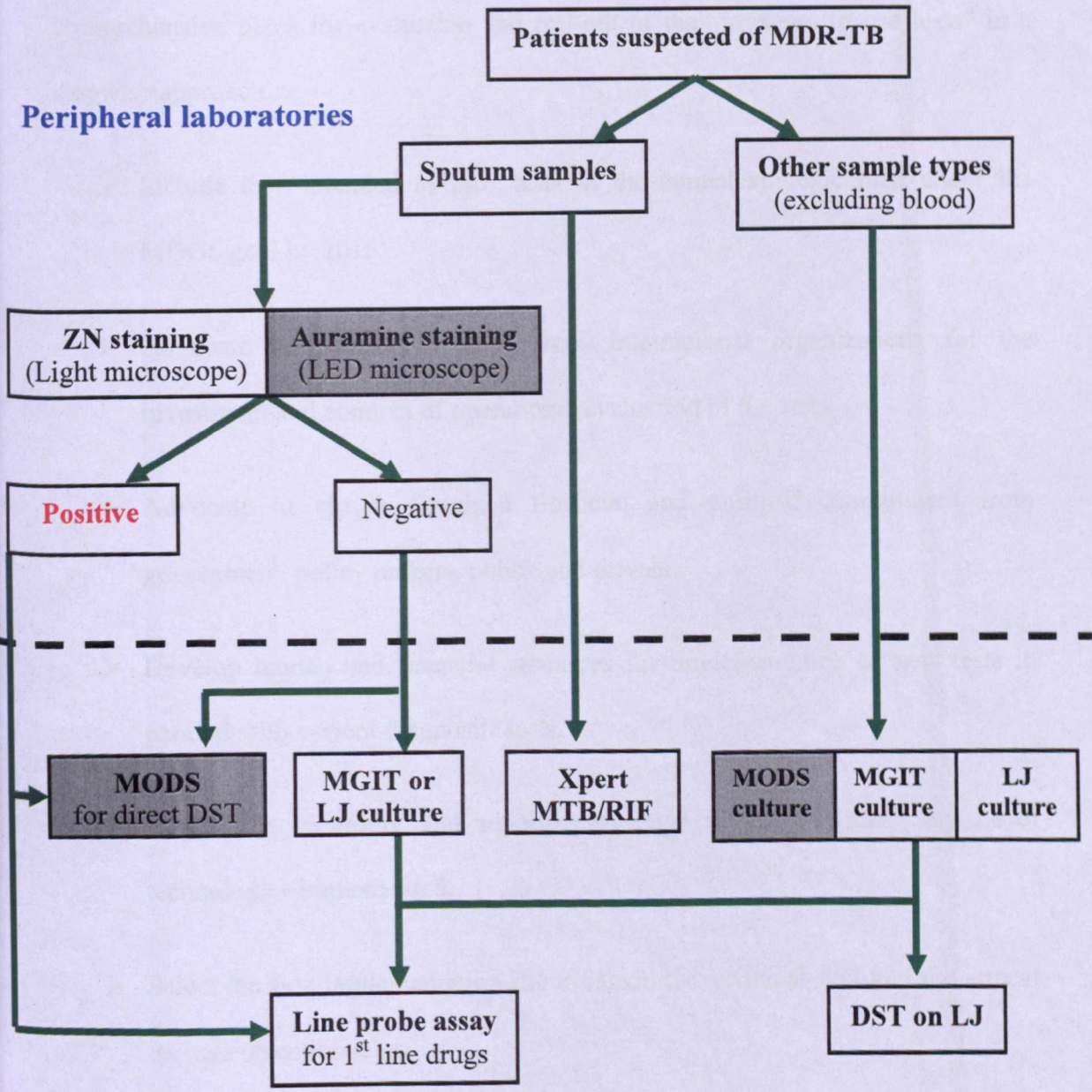
may be available to TB control Programmes which could allow novel diagnostic tests to be applied at all laboratory levels recommended by WHO. **Figure 5.1** depicts a suggested algorithm for initial introduction of microbiological and novel molecular diagnostic tests endorsed by WHO for TB diagnosis.

Figure 5.1 Initial implementation of new diagnostic tests in TB laboratory network for early diagnosis of tuberculosis



As for TB diagnosis, the diagnosis of MDR-TB should be centralised at provincial/regional or reference laboratories. For sputum samples, a direct DST method is recommended. The Line probe assay endorsed by WHO in 2008 (235) is recommended for use in smear positive sputum samples only. Direct DST-MODS and GeneXpert MTB/RIF performed on both smear positive and smear negative sputum samples. For samples other than sputum, indirect DST method is required because the majority of these samples are paucibacillary. MODS culture, MGIT culture or LJ culture can be used for isolation of mycobacteria. Line probe assays or traditional DST methods can be used for drug susceptibility testing of positive cultures, depending on local capacity (**figure 5.2**).

Figure 5.2 Initial implementation of new diagnostic tests in the TB laboratory network for early diagnosis of multidrug resistant tuberculosis



Higher laboratory levels

Although novel tests have been endorsed by WHO for implementation in high burden countries, the impact of these tests may vary among settings due to differential skills and experience of staff. Therefore, individual TB control Programmes should develop comprehensive plans for evaluation and roll-out of the “intended to use tests” in a stepwise approach.

- Include the “intended to use” tests in the annual strategic plan under the MDG6 goal by 2015.
- Advocate and access support from international organizations for the investment and conduct of operational evaluation of the tests.
- Advocate to ensure sustained financial and political commitment from government, policy makers, public and private.
- Develop human and financial resources for implementation of new tests in parallel with current diagnostic tests.
- Revise the recording and reporting systems to accommodate any novel technologies implemented.
- Select the first implementation site to maximize technical skill and the impact on case detection rates.
- Evaluate the first implementation stage, in terms of satisfaction from both patients and staff for accuracy, cost, technical issues and ease of use.

- Introduce further novel tests or multiply the implementation of the first test to further sites, depending on individual NTP capacity.

The most feasible test, in my opinion, is MODS, because it provides sensitive, specific and reliable results in one week. Especially, TB and MDR-TB can be diagnosed by this method with a low cost. The infrastructure needed is the basic equipment for a culture laboratory plus an inverted microscope and tissue culture plates; and this test may be easy to adopt in settings that have a strong resource of experienced microscopists. In 2009, WHO recommended that MODS should be used as an ‘interim solution’ on the way to automated molecular test (GeneXpert) for detection of MDR-TB (238). However, large scale implementation of MODS will require enormous investment including training, supplies of reagents and equipment, quality control assurance, maintenance, biosafety and personnels. The magnitude of investment will not reflect the duration of implementation if MODS is applied only as ‘interim’ solution. In addition, GeneXpert is still out of reach of the poor because of its high cost (despite preferential pricing). Therefore, I believe MODS should be applied as an alternative solution rather than an interim one.

5.7 IMPLEMENTATION IN VIET NAM

The TB laboratory network in Viet Nam is constructed of three levels: National level, provincial level and peripheral level (district level). The provincial laboratory in Ho Chi Minh City based at Pham Ngoc Thach Hospital is functioning as the regional laboratory of Southern provinces.

The microbiological methods for diagnosis of tuberculosis in Viet Nam are smear microscopy (light microscope and conventional fluorescence microscope), LJ culture and MGIT culture. Light microscopes are supplied to all laboratory levels by the NTP. Fluorescence microscopes and MGIT culture must be funded by local tuberculosis programme budgets, rather than centrally by the NTP. For LJ culture, all equipment and reagents (with the exception of some centrifuges) must be purchased with local budgets. At the present time, not all routine 'gold standard' diagnostic tests available in Viet Nam are functioning at provincial laboratories due to budget limitation.

Since the national budget for the TB control and prevention programme is limited, report by WHO as gaps for implementation DOTS and gaps for supplies of first-line anti-TB drugs (244), and there is a chronic shortage of personnel at all laboratory levels, the approved novel diagnostic methods cannot be implemented widely at all laboratory levels. These technologies should be used in appropriate case finding strategies as suggested by WHO (243) at sites with sufficient local infrastructure investment. In my opinion, these methods should be prioritized for implementation at national and regional levels for operational evaluation. The integration of new diagnostic tests can be applied as presented in the **figure 5.1** and **figure 5.2**. If the operational evaluation shows this model works well, the NTP can then multiply this model at other provincial laboratories and then peripheral laboratories following WHO recommendations on the appropriate laboratory level for each technology.

5.8 FUTURE WORK

Novel diagnostic tests which were endorsed by WHO for improving TB diagnosis cannot replace traditional diagnostic methods at least in the near future because no single test satisfies all the characteristics of an ideal test suggested by WHO and studies on large scale implementation, clinical impact and cost effectiveness of these new tools should be locally performed to verify their benefit. At the present time, the role of novel tests, in combination with smear microscopy and culture, is to target patients presenting to health services to increase case detection and reduce the transmission source. The most effective strategy is to detect and manage index cases first present in the community. Currently, all approved new tests are only suitable for implementation from the peripheral laboratory level upwards. Future development efforts will focus on a point of care diagnostic test which can be applied at clinic level. FIND aim to develop such a test by 2013, however this is probably optimistic given the current results from biomarker discovery projects (256). The introduction of diagnostic tools at all levels of the health care system and community will have a major impact on the global epidemiology of tuberculosis providing parallel investment in treatment programmes ensures those diagnosed receive effective treatment (96).

Systematic efforts to evaluate new TB diagnostics in the last decade have clearly demonstrated that more effective tools are available than those currently applied in high burden settings, where smear microscopy remains the only widely applied test. The greatest challenge in the next decade will be to sustain political commitment to

investing in the scale-up of application of these tests to those most at need. Without significant scale-up and investment in TB control, the tuberculosis pandemic will continue to claim 2 million lives a year,

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