DEVELOPMENT OF ONE-TUBE MULTIPLEX SEMI-NESTED PCR FOR IDENTIFICATION OF RIFAMPICIN-RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Rifampicin resistance is well accepted as a surrogate marker of MDR-TB. Therefore, rapid detection of rifampicin-resistant *M. tuberculosis* will help in diagnosis, treatment and control of TB transmission. This study aims to develop the one-tube multiplex semi-nested PCR for identification of rifampicin-resistant *M. tuberculosis.*

Five primer pairs were designed for amplification of *rpoB* gene in a single tube. One pair was specifically designed for the 81-bp hot spot region of *rpoB* and generated 196-bp product. The remaining four forward primers were allele-specific primers that their 3'-ends located specifically at codons 531, 526, 516 and 511. Mutations of these codons are mostly found to confer rifampicin resistance in *M. tuberculosis*. This method was done in a blind experiment with 216 clinical isolates of *M. tuberculosis* with known susceptibility results obtained from the standard proportion method. For interpretation, the presence of 5 amplification products will be accounted as wild type or a sensitive strain. The absence of band at corresponding codon will be reported as a rifampicin-resistant strain.

The developed PCR method could accurately identify 97 from 103 rifampicin-resistant and 113 of 113 rifampicin-sensitive strains. Six discordant strains were sequenced and shown to have mutations outside the designed region. This reveals sensitivity, specificity, positive predictive value and negative predictive value of 94.2%, 100%, 100% and 94.9%, respectively. In addition, the developed PCR also provided concordantly results of 49 in 50 samples (98%) from the positive automated BACTEC MGIT 960 cultures, comparing with the proportion method. The detection limit of the PCR was 10 pg. The developed PCR required at least 90% of the resistant strain in the mixed population to clearly identify the resistant subpopulation.

The established method showed good results and was more rapid (1 day) than both conventional and automated culture systems (7-28 days). It can be used as a rapid screening method for detection of rifampicin-resistant *M. tuberculosis*, a marker of MDR-TB, and provide information of the mutations in the hot spot region of *rpoB*.

KEY WORDS: MULTIPLEX PCR / RIFAMPICIN RESISTANCE / RPOB
TUBERCULOSIS / IDENTIFICATION

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การพัฒนาวิธี ONE-TUBE MULTIPLEX SEMI-NESTED PCR เพื่อตรวจหาเชื้อวัณโรคที่ดื้อยา rifampicin (DEVELOPMENT OF ONE-TUBE MULTIPLEX SEMI-NESTED PCR FOR IDENTIFICATION OF RIFAMPICIN-RESISTANT *MYCOBACTERIUM TUBERCULOSIS*)

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บทคัดย่อ

การดื้อยา rifampicin เป็นตัวแทนที่ได้รับการยอมรับว่าแสดงถึงการติดเชื้อวัณโรคดื้อยาหลายขนาน ดังนั้นการตรวจหาเชื้อวัณโรคดื้อยา rifampicin ให้ได้รวดเร็ว จะช่วยในการวินิจฉัย การรักษาและการควบคุมการแพร่เชื้อวัณโรค การศึกษานี้มีจุดมุ่งหมายเพื่อพัฒนาวิธี one-tube multiplex semi-nested PCR สำหรับตรวจหาเชื้อวัณโรคดื้อยา rifampicin

primer ทั้ง 5 คู่ถูกออกแบบสำหรับการเพิ่มจำนวนยีน *rpoB* ในหลอดเดียว โดยมีหนึ่งคู่ถูกออกแบบให้จำเพาะกับบริเวณ 81-bp hot spot ภายในยีน *rpoB* โดยให้ดีเอ็นเอที่มีขนาด 196-bp ส่วน forward primer อีก 4 ข้างเป็น primer ที่จำเพาะกับ allele ที่ศึกษาซึ่งให้ด้านปลาย 3' จำเพาะอยู่ที่ codon 531, 526, 516 และ 511 การผ่าเหล่าของตำแหน่งเหล่านี้พบว่าส่วนใหญ่มีผลทำให้เชื้อวัณโรคดื้อยา rifampicin วิธีนี้ถูกทดสอบโดยใช้เชื้อวัณโรคจำนวน 216 ตัวอย่างที่แยกได้จากผู้ป่วยที่ทราบผลการทดสอบความไวของยาด้วยวิธีมาตรฐาน proportion method และได้จัดเรียงเชื้อใหม่เพื่อไม่ให้มีความลำเอียงในการแปลผล วิธีอ่านผล ถ้าพบดีเอ็นเอครบทั้ง 5 ขนาดแสดงว่าเป็นสายพันธุ์ที่ไวต่อยา ถ้ามีดีเอ็นเอไม่ครบทั้ง 5 ขนาดแสดงว่าเป็นสายพันธุ์ที่ดื้อต่อยา rifampicin

วิธี PCR ที่พัฒนาขึ้นมานี้สามารถตรวจพบเชื้อวัณโรคที่ดื้อยา rifampicin ได้ 97 จาก 103 ตัวอย่าง และเชื้อวัณโรคที่ไวต่อยา rifampicin ได้ 113 จาก 113 ตัวอย่าง ส่วนอีก 6 ตัวอย่างที่ได้ผลไม่ตรงกันถูกนำไปอ่านลำดับเบสของดีเอ็นเอ และพบการผ่าเหล่าอยู่นอกเหนือจากบริเวณที่ออกแบบ primer จากผลนี้แสดงถึงค่าความไว, ความจำเพาะ, ค่าทำนายผลบวก และค่าทำนายผลลบ เท่ากับร้อยละ 94.2, 100, 100 และ 94.9 ตามลำดับ นอกจากนี้ได้ทดสอบวิธี PCR กับตัวอย่างเชื้อจากหลอดที่ให้ผลบวกจากเครื่องเพาะเชื้ออัตโนมัติ BACTEC MGIT 960 จำนวน 50 ตัวอย่าง พบว่าได้ผลตรงกับวิธีมาตรฐาน 49 ใน 50 ตัวอย่าง (98%) ปริมาณดีเอ็นเอต่ำสุดที่สามารถใช้วิธี PCR นี้ตรวจหาเชื้อวัณโรคดื้อยาได้เท่ากับ 10 พิโคกรัม โดยวิธีนี้ต้องการประชากรดื้อยาอย่างน้อยร้อยละ 90 ของประชากรผสม จึงสามารถตรวจหาเชื้อดื้อยาได้

วิธีที่พัฒนานี้แสดงผลที่ดีในการตรวจหาเชื้อวัณโรคดื้อยา rifampicin จากการเพาะเชื้อ และใช้เวลาสั้นกว่า (1 วัน) ทั้งวิธีมาตรฐานเดิมและวิธีเพาะเชื้อแบบอัตโนมัติ (7-28 วัน) วิธีนี้จึงสามารถใช้เป็น rapid screening method สำหรับตรวจหาเชื้อวัณโรคดื้อยา rifampicin ซึ่งเป็นตัวแทนแสดงถึงการติดเชื้อวัณโรคดื้อยาหลายขนานและช่วยให้ข้อมูลการผ่าเหล่าในบริเวณ hot spot ของ *rpoB*

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CHAPTER I
INTRODUCTION

Since 1980, eighty-one million tuberculosis (TB) patients have been reported through World Health Organization (WHO)'s surveillance system. From these, seventeen million were notified by the internationally recommended strategy of directly observed therapy short course (DOTS) for TB control programmes since 1995. The financial monitoring system has accounted for US$ 4.3 billion spent on TB control in the 22 high-burden countries of which there are account for approximately 80% of all new TB cases arising each year since its inception in 2002. By the way, once a strain of *Mycobacterium tuberculosis* develops resistance to isoniazid (INH) and rifampicin (RIF), it is defined as multidrug-resistant tuberculosis (MDR-TB) (1). The prevalence of MDR-TB ranges from 0 to 14.1% (2). In addition, MDR-TB patients were identified approximately 250,000 people per year worldwide. Owing to the lack of routine antimycobacterial susceptibility testings, MDR-TB patients were not identified until they had failed one or more courses of treatment over a period of months or years, resulting in the continued transmission of the multi-drug resistant strains (3). In Thailand, tuberculosis is still the major cause of death due to infectious diseases and is the major cause of death among AIDS patients. The incidence of TB in the Thai population is 135/100,000 and new cases of MDR-TB constitute approximately 2.1% (4). Thailand has had nationwide DOTS coverage since 2002 and reached the global target for case detection in 2003. Recent data suggest that the incidence of TB is declining slowly in Thailand. Considerable efforts are being made to extend TB control services to marginalized and deprived population groups, and this has boosted the case detection rate. However, treatment success is still well below the DOTS target and too many patients die, fail to complete their treatment or are lost to follow-up (5). One of the most alarming trends concerning TB is the emergence of drug-resistant *M. tuberculosis* strains, which has become a worldwide health care problem (6). Instead of being eradicated, drug-resistant strains have evolved and have

been documented in every country surveyed (7). INH and RIF are the backbone of the standard regimen for treating tuberculosis, and resistance to these drugs indicates a necessity for alteration of the standard regimen. In order to treat patients properly and to reduce further spread of drug-resistant TB, it is clinically important to provide drug susceptibility results to health care providers as soon as possible (8). A delay in the diagnosis of MDR-TB results in patients presenting with chronic disease, higher bacillary loads, and continuing transmission (9). With the slow growth rate of *M. tuberculosis* complex, the average turnaround time of drug susceptibility results obtained with the standard phenotypic proportion method is 2-4 weeks or more from specimen receipt. A more rapid alternative to the current phenotypic drug susceptibility testing is greatly needed.

Laboratory diagnosis of tuberculosis relies on culture and direct examination of smears by the Ziehl-Neelsen (ZN) stain. Culture is sensitive, but time consuming, because of the slow growth rate of *M. tuberculosis* complex. Microscopic detection of acid-fast bacilli in stained ZN smears is rapid, but not very sensitive (at least 10^4 bacterial cells are needed), and is not specific for pathogenic mycobacteria (10). The proportion method was carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) on Middlebrook 7H10-11 agars which are the most commonly used methods to determine drug susceptibility of *M. tuberculosis* (11). The proportion method is a gold standard for susceptibility testing but it also still needs more time. The use of an automated or semi-automated liquid culture system can detect growth much earlier than the naked eye. The first such system to be used in the laboratory was the BACTEC 460TB instrument. Using a broth with radiolabelled ^{14}C-palmitate as its sole carbon source, the metabolising organism converts this to radiolabelled $^{14}CO_2$, which can then be detected by the BACTEC instrument. The amount of this radioactive metabolite can be quantified and used to detect mycobacterial growth within 1-2 weeks (12). Drug susceptibility testings were performed by inoculation of the test isolate in drug-containing medium and inoculation of one-hundred fold dilution (1%) of the isolate in drug-free medium (13). The time required to obtain results with the BACTEC method ranged from 5 to 21 days (average 11.5 days) following the arrival of the specimens (12). New systems that rely on non-radiometric detection of growth have been developed, such as the

MB/BacT (Organon Teknika), BACTEC 9000 (Becton Dickinson), and the mycobacterial growth indicator tube (MGIT; Becton Dickinson). The BACTEC 460 system remains the fastest and most sensitive, followed by the continuous automated non-radiometric liquid culture systems, with the solid media systems being the slowest. It is important to note that although a system may appear to be faster because its detection method is more sensitive, the biomass present in the culture bottle may be substantially less than that of the other systems (14). These methods are technically advanced, but expensive, require radioactivity or sophisticated equipment and laboratory expertise. Consequently these methods are not universally available and were not suitable for low income countries. Moreover the other phenotypic methods were developed for susceptibility testing, but still time consuming due to culture were mostly required.

Rifampicin is a key component of the WHO-directly observed therapy (DOT) short course regimen because in addition to a significant early bactericidal effect on metabolically active *M. tuberculosis*, RIF also exhibits excellent late sterilizing action on semidormant organisms undergoing short bursts of metabolic activity (15). INH (isonicotinic acid hydrazide) was first synthesized in the early twenty century, and was introduced as an antituberculosis medication in the 1950s. Afterwards RIF was introduced for concomitantly use in antituberculous therapy in the early 1970s (16). Thus RIF-monoresistance is extremely rare and development of INH-resistance usually precedes that to RIF, resistance to the latter is therefore considered to be the MDR marker. It has been shown in many studies that RIF-resistance in up to more than 95% of resistant strains is caused by mutations in the *rpoB* gene encoding the RNA polymerase ß-subunit. These mutations are generally described in the short 81 bp region in *rpoB* gene cluster I which is RIF resistance-determining region encoding amino acids 507 to 533 (17-26). The codon numbers are designated on the basis of *Escherichia coli rpoB* used by Telenti *et al*. (27). In addition, RIF-resistance may be caused by mutations in other parts of *rpoB* outside the hot spot region, such as codon 176 in the N-terminal part (17, 19). Recently, there are several techniques used to detect *rpoB* mutations associated with RIF-resistance for examples; DNA sequencing, real-time PCR (28-29), molecular beacons (20), biprobe analysis (30), PCR-SSCP (27), heteroduplex analysis (31), Line probe assay or INNO-LiPA test (21, 32), PCR-

enzyme-linked immunosorbent assay (PCR-ELISA) (33), in-house Mycobacteriophage-based assay (34) and multiplex detection (19, 23). These techniques have both different advantages and disadvantages. A technique which is rapid, precise, accurate, easy to manipulate and inexpensive is developed for using in low income countries with high MDR-TB burden. The one-tube multiplex semi-nested PCR for identification of RIF-resistant *M. tuberculosis* is proposed to fulfill these objectives.

The principle of the one-tube multiplex semi-nested PCR for identification of RIF-resistant *M. tuberculosis* obtains from the studies of many countries which show that the most common mutations in the short 81-bp hot spot region of *rpoB* gene which determined RIF-resistance is at the codon 531 (range 31-56% of resistant strains). The second most common mutation is at the codon 526 (range 11-43% of resistant strains). The third is at the codon 516 (range 5-16% of resistant strains) (17-18, 21-22, 30, 33, 35-36) and the last one is at the codon 511 which range between 2-7% of resistant strains (17-18, 22, 30, 33, 35-36). From these informations, the base position of each studied codon which are frequently altered is often in the middle of each codon. So the objective of determination of *rpoB* gene mutation that confer RIF-resistance are focused on these four positions namely codon 531, 526, 516 and 511. Ability to detect the mutations of these four codons, will cover about 90-95% of RIF-resistant *M. tuberculosis* strains. At the present study, the principle of the one-tube multiplex semi-nested PCR using two outer primers that flank a region under study and invariably anneal on the conserved DNA targets, plus a wild type-allele specific inner primers that stops in its 3'-end at the targeted codon and amplifies a wild type-allele specific fragment is developed. An alteration of the base that corresponds to the 3'-end of the specific primers at codons 531, 526, 516 and 511 causes the primer template mismatch that prevents polymerase to extend the primer and results in nonamplification of the indicative fragment.

Objectives:

1. To develop the one-tube multiplex semi-nested PCR for a rapid detection of RIF-resistant *M. tuberculosis* from pure cultures.
2. To evaluate the developed PCR for detection of RIF-resistant *M. tuberculosis* clinical isolates in terms of its sensitivity, specificity and accuracy compared with the standard proportion method of antimycobacterial susceptibility testing.

CHAPTER II
LITERATURE REVIEW

1. Diagnostic tools for detection of drug resistant *M. tuberculosis*

TB is one of the leading infectious disease in the world and is responsible for more than 2 million deaths and 8 million new cases annually (5). More than 100 years after its discovery, microscopic examination of sputum is still the only widely available diagnostic tool for tuberculosis in disease-endemic countries. The advantages of acid-fast bacilli (AFB) microscopy are well known that it is inexpensive to perform, is very specific for mycobacteria in high prevalence settings, and detects the most infectious subset of patients. For these reasons, microscopy rightly retains its primary role in case detection. However, technical shortcomings of AFB microscopy seriously limit both the extent and quality of its application, and ultimately, its impact on TB control. It requires equipment that is relatively difficult to maintain in field settings, yields results that depend upon the studious attention of a trained and motivated technician, and is insensitive, especially in programme conditions (37) , (38). Subsequently culture is sensitive, but requires a minimum of four weeks to detect growth of *M. tuberculosis*, and the presence of a quality assured laboratory to undertake such tests. New tools are also needed for rapid drug-sensitivity testing, highlights the need for simple and inexpensive methods to screen for drug resistance, aimed to decrease morbidity, selection of drug-resistant mycobacteria and transmission of MDR-TB strains.

Nowadays, there are many alternative techniques rather than the proportion method as radiometric BACTEC 460 TB method. This system detects the presence of mycobacteria based on their metabolism rather than visible growth. When the ^{14}C labelled substrate present in the medium is metabolized, $^{14}CO_2$ is produced and measured by the BACTEC system instrument and reported in terms of growth index (GI) value. The BACTEC system is also useful in the identification of *M. tuberculosis* using a specific inhibitor, para-nitro-α-acetyl-amino-β-hydroxypropiophenone. Using

the same system, drug susceptibility tests can also be performed for all the anti-tuberculosis drugs when sufficient GI is observed (39). Another technique is MGIT 960 (Mycobacteria growth indicator tube). This is an automated system for the growth and detection of mycobacteria with the capacity to incubate and continuously monitor mycobacteria growth indicator tubes every 60 minutes for increase in fluorescence. Growth detection is based on the AFB metabolic O_2 utilization and subsequent intensification of an O_2 quenched fluorescent dye contained in a tube of modified MGIT. A series of algorithms are to determine presumptive positivity and alert the operator to the presence and location of positive tubes. But these alternative techniques are still time consuming as the proportion method. Moreover the BACTEC 460 system requires isotopes and heavy equipment, consequently is not feasible in most resource-poor setting. The commercial MGIT system, the non-radiometric method, is reliable but still expensive to implement in low-resource countries (40). So there has been enormous research interest in the development of novel approaches to identify MDR-TB. Many genotypic methods which detect specific mutations associated with resistance are developed to replace the phenotypic methods. Their potential advantage is that there is no need for growth of the organisms, and drug susceptibility results can be determined in days rather than weeks. In most cases, the detection of rifampicin resistance has been used as a surrogate for MDR-TB and studies suggest that they are highly reliable for this. All genotypic methods demand DNA extraction, gene amplification, and detection of mutations (*eg*., in the *rpoB* gene for rifampicin resistance). Several studies have demonstrated the value of these assays in middle-income countries such as Russia, China, and Philippines, where there are high rates of MDR-TB both for diagnosis and for characterizing the range of mutations seen (41).

2. The major historical landmarks of tuberculosis therapy

The history of the TB treatment was initiated by the discovery of effective medications of streptomycin (STR) and para-aminosalicylic acid (PAS) in 1944, which were eventuated by Selman Waksman's research in New Jersey and Jorgen Lehman in Sweden respectively. Rapidly pressed into use, these two agents had clearly identifiable activity against clinical TB. Due to a shortage STR, the British

Medical Research Council (BMRC) performed the first randomized clinical trials comparing PAS or STR alone with the combination of both agents (42). The results, which were published in 1950, demonstrated that the combination was more effective at both achieving cures and preventing acquired drug resistance. These insights substantially shaped future treatment trials as shown in table1.

Gerhard Domagk's research was eventuated in the discovery of the anti-TB activity isonicotinic acid hydrazide (INH) in 1952. Adding INH to PAS and STR (triple therapy) resulted in predictable cures for 90-95% of patients. Unfortunately, it required up to 24 months of continuous treatment to achieve this objective (43). This was related to the persistence of viable bacilli in tissues long after sputum cultures had become negative. Perhaps the most fundamentally important principle derived from triple therapy was that such treatment, reliably given, effectively precluded acquired drug resistance.

The replacement of PAS by ethambutol (EMB) in the 1960s had two benefits. EMB was much better tolerated than PAS and it allowed reduction in the duration of treatment to 18 months (44).

The next major advance in therapy was the introduction of rifampicin (RIF). Derived from *Streptomyces mediterranei*, RIF was studied in early trials by the BMRC in East Africa (45) and Hong Kong (46), demonstrating that practical combinations of INH, STR, EMB and RIF resulted in predictable cures in more than 95% of cases in just 8-9 months. The particular activity of RIF that facilitated this compression of treatment was its pronounced capacity of RIF to kill mycobacteria undergoing sporadic metabolism, the so-called "sterilizing effect".

The next step forward was the recognition that the inclusion of pyrazinamide (PZA) allowed a reduction in the duration required to achieve predictable cures. PZA was found to accelerate the time required to achieve culture negativity and to yield more than 95% cure rates in 6 months when combined with INH and RIF (46). It has been speculated that the singular role of PZA is activity against tubercle bacilli in the acidic debris in pulmonary cavity walls (47). This is consistent with the observation that PZA exerts all of its beneficial effects in the first 2 months of the therapy.

The treatment that most organizations recommend for patients who have not been treated previously is RIF, INH, PZA and EMB for two months, followed by RIF and

INH for a period of four months, administered daily or three times per week (48), (49). Some organizations, including the World Health Oraganization (WHO), also recommend twice-weekly administration. The six months short course chemotherapy has been established as the standard regimen for the initial treatment of tuberculosis patients all over the world (50). This regimen is conveniently abbreviated to "2HRZE/4 HR".

Table 1 Landmarks in tuberculosis therapy

Date	Landmark
1944	STR and PAS
1952	Triple therapy, INH/STR/PAS, 24 months
1960s	EMB replaces PAS, 18 months
1970s	RIF added to INH/EMB/STR, 9 months
1980s	PZA added to INH/RIF, 6 months

3. Characteristic basis of the first-line TB drugs

The general descriptions and applications of the five first-line TB drugs namely RIF, INH, EMB, PZA and STR are described here in brief, including genetic basis of the drugs. A detail review of the genetic basis of resistance to the five first-line TB drugs has been published (18).

3.1 Rifampicin (RIF)

Rifampicin (RIF) is a semi-synthetic derivative (Figure 1) of rifamycin which is an antibiotic produced by *Streptomyces mediterranei* that is active against gram-positive bacteria (including *M. tuberculosis*) and some gram-negative bacteria by inhibition of transcription initiation. RIF binds to the β-subunit of ribonucleic acid (RNA) polymerase, encoded by the *rpoB* gene, and apparently blocks the entry of the first nucleotide which is necessary to activate the RNA polymerase, thereby blocking mRNA synthesis. It has bactericidal effect against both intracellular and extracellular *M. tuberculosis*. RIF is lipid-soluble. Following oral administration, it is rapidly

absorbed and distributed throughout the cellular tissues and body fluids; if the meninges are inflamed, significant amounts penetrates the cerebrospinal fluid. This drug acts on the rapid-growth populations of bacilli as well as on slow-multiplying populations. For this reason, its sterilization capacity is considerable (51). The potent sterilizing effect was against tubercle bacilli in both intracellular and extracellular locations. It is also a generally well-tolerated drug although it produces reddish colouring in the urine and other fluids, and it can cause hepatoxicity. Owing to its extensively recycled in the enterohepatic circulation and metabolites formed by deacetylation in the liver are eventually excreted in the feces.

RIF is well tolerated by most patients at currently recommended doses, although gastrointestinal tolerance can be unacceptably severe. Other adverse effects such as skin rashes, fever, influenza-like syndrome and thrombocytopenia are more likely to occur with intermittent administration. Moderate rises in serum concentrations of bilirubin and transaminases, which are common at the outset of treatment, are often transient and without clinical significances. However, dose-related hepatitis can occur which is potentially fatal.

For the genetic basis, it was known for many years that missense mutations and short deletions in the central region of the RNA polymerase beta subunit gene (*rpoB*) result in RIF-resistant *E. coli* (52). This insight led to characterization of the *M. tuberculosis rpoB* gene and to identification of a wide variety of mutations conferring RIF resistance (16), (27). Compilation of data available from many studies indicated that 315 of 329 (96%) of epidemiologically unrelated RIF-resistant patient isolates of *M. tuberculosis* had 35 distinct point mutations, or short insertions and deletions located in an 81-bp core region (rifampicin resistance determining region of *rpoB* codons 507 to 533 encoding 27 amino acids, see Figure 2) (16), (27), (53). Importantly, 43% of the strains had missense mutations in codon 531 (Ser) and 36% of organisms had codon 526 (His) alterations that would result in amino acid replacements. The most common amino acid substitutions were Ser531Leu (42%) and His526Tyr (23%) (16). The mechanism of resistance was not identified in 4% of RIF-resistant clinical isolates because no mutations were detected in the 81-bp core region of *rpoB* nor elsewhere in the *rpoB* gene. Virtually about 95% of RIF-resistant isolates have point mutation in an 81-bp region of the *rpoB* gene, and these mutations are

absent in susceptible isolates, making it an ideal target for development of molecular drug susceptibility testing methods.

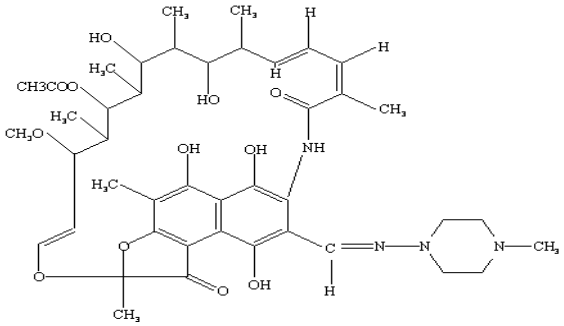

Figure 1 A chemical structure of rifampicin (RIF). (54)

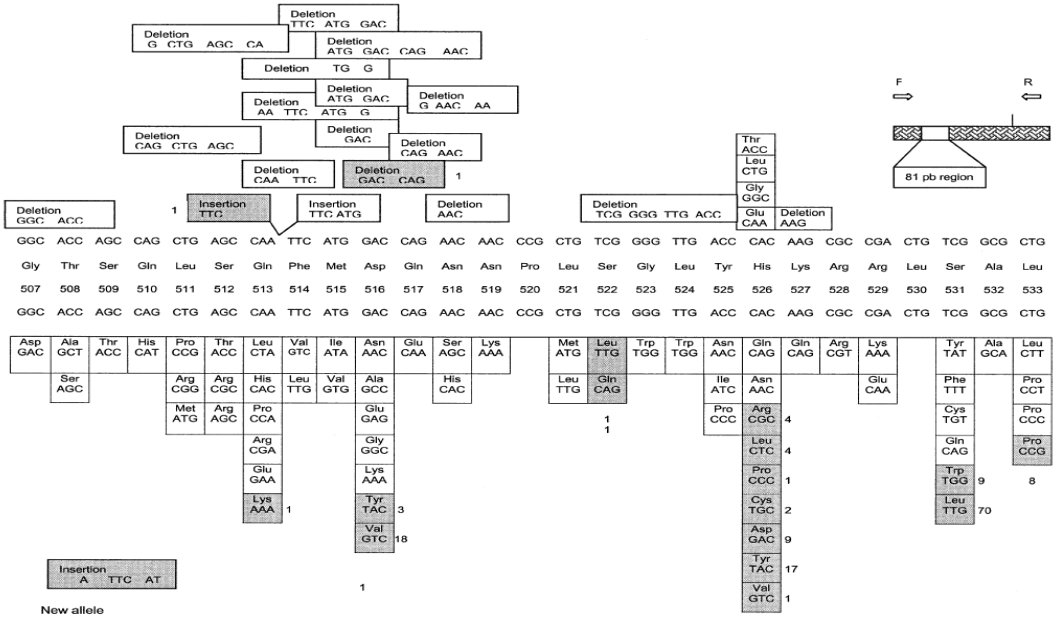

Figure 2 Mutations located at codons 507 through 533 of the *M. tuberculosis rpoB* gene (81-bp region). This included a revision of the mutation data previously found in several countries. The codon numbers are designated on the basis of *Escherichia coli rpoB* (26).

3.2 Isoniazid (INH)

Isoniazid or isonicotinic acid hydrazide (55), is a synthetic bactericidal agent (Figure 3) against replicating tubercle bacilli, including *M. bovis* and *M. kansasii*. It is a derivative of nicotinic acid. Once activated, INH inhibits the synthesis of mycolic acids which is an essential component of the bacterial cell wall. It is readily absorbed following oral administration. It is rapidly absorbed and diffuses readily into all fluids and tissues. INH enters mycobacterial cells via passive diffusion across the bacterial envelope (56). It is a prodrug that requires cellular activation by KatG producing a reactive species with antimicrobial action (Figure 4). Specifically activation is associated with reduction of the mycobacterial ferric KatG catalase-peroxidase by hydrazine and reaction with oxygen to form an oxyferrous enzyme complex (57). At therapeutic levels INH is bactericidal against actively growing intracellular and extracellular *M. tuberculosis*. Specifically INH inhibits InhA which is the enoyl reductase from *M. tuberculosis*, by forming a covalent adduct with the NAD cofactor. It is the INH-NAD adduct that acts as a slow, tight-binding competitive inhibitor of InhA. INH is activated by a mycobacterial peroxidase enzyme and destroys several targets in the cell. It is a powerful bactericide, acting on rapidly multiplied TB (58), and is usually well tolerated.

Decades ago, study of patient isolates of *M. tuberculosis* noted a correlation between INH resistance and loss of catalase-peroxidase activity (59), (60). This observation eventually led to the cloning and sequencing of the structural gene (*katG*) for the enzyme (61). Molecular genetic studies confirmed that *katG* participated in mediating susceptibility to INH (62). Moreover, *katG* was deleted from the chromosome of two strains with high level INH-resistant isolates (63). However many subsequent studies have shown that the vast majority of INH-resistant strains contain the *katG* gene, a result indicating that deletion of this gene is very rare among patient isolates (64), (65), (66), (67).

Although a diverse array of distinct KatG changes are uniquely represented among INH-resistant organisms, amino acid substitutions located at position 315Ser are the most abundant (Figure 5). For example, Musser *et al*. (68) studied 85 INH-resistant strains from global sources and identified changes in amino acid 315 (Ser) in 49 (58%) epidemiologically unassociated isolates. Most organisms had a Ser315Thr

amino acid replacement. Similarly, Haas *et al*. (69) reported that 68% of INH-resistant strains from Africa had codon 315 missense changes (usually Ser315Thr substitutions) and Dobner *et al*. (70) found that 26 of 27 INH-resistant isolates from Germany and Sierra Leone had codon 315 mutations. Marttila *et al*. (67) reported that 22 of 24 INH-resistant isolates from the St. Petersburg area in Russia had Ser315Thr substitutions. Statistically the most common amino acid substitution is AGC (Ser) to ACC (Thr), but ACA (Thr), ATC (Ile), AGA (Arg), CGC (Arg), AAC (Asp) and GGC (Gly) changes also have been identified (16), (65), (68), (69), (66).

A plausible explanation for the abundance of KatG315 amino acid changes in most INH-resistant organisms can be inferred from data published. Wengenack *et al*. (71) studied the enzymatic properties of purified wild type and the Ser315Thr mutant KatG proteins made in *E. coli*. Comparison with the activities for wild type KatG, the catalase activity of the Ser315Thr mutant was reduced 6-fold, whereas the peroxidase activity was only decreased less than 2-fold. The mutant and wild type proteins each had one heme group per subunit, a result indicating that the difference in enzymatic activity of the Ser315Thr mutant protein was not due to incomplete heme cofactor incorporation in the heterogenous *E. coli* host. The wild type KatG was more efficient than the mutant enzyme in ability to convert INH (prodrug) to isonicotinic acid (activated INH). Taken together, the results are consistent with the idea that the Ser315Thr mutant is a competent catalase-peroxidase that has reduced ability to metabolize INH. Hence amino acid substitutions at position 315 appear to strike a balance between the need to maintain active catalase-peroxidase activity to detoxify host antibacterial radicals and reduce conversion of prodrug to active INH, a process that would normally kill the bacterium.

The most commonly occurring polymorphism found in the *katG* gene is a CGG to CTG change resulting in Arg to Leu variation at amino acid position 463 (16). Although an initial analysis suggested that the KatG Arg463Leu substitution was causally involved in INH resistance. Moreover strains with Arg463Leu substitutions have essentially wild type levels of catalase-peroxidase activity (72). Site-directed mutagenesis demonstrated that the presence of Leu at codon 463 (rather than Arg) did not alter catalase-peroxidase activity (73). The Arg463Leu substitution is present in

many INH-susceptible strains, especially organisms recovered in China, the former Soviet Union and certain other areas of Asia (74).

Furthermore the identification of a Ser94Ala substitution in InhA protein which was participated in mycolic acid biosynthesis conferring resistance to INH in the laboratory led to the hypothesis that missense mutations in *inhA*-encoded enoyl reductase would occur in INH-resistant *M. tuberculosis* strains recovered from patients. DNA sequence analysis of the *inhA* locus in 115 INH-resistant clinical isolates identified an Ile16Thr substitution in InhA (68). Fifteen isolates had mutations in the upstream putative regulatory region of the *inhA* locus.

On the basis of identification of mutations in the upstream region of *inhA* locus, it was hypothesized that these changes result in increased InhA protein expression, thereby elevating the drug target levels and producing INH resistance via a titration mechanism. To test this hypothesis, a multicopy plasmid containing the *inhA* gene was transformed into *M. smegmatis* and *M. tuberculosis* strains. High-level INH resistance was conferred only to *M. smegmatis*. The promoter region containing mutations at positions thought to be located at –8 and –15 upstream from translational start site of *mabA* were cloned into an integrating, single copy vector and then transformed into *M. smegmatis* and *M. tuberculosis* (75). The results indicated that the mutations had no measurable effect on INH resistance in *M. smegmatis* but had a modest effect on *M. tuberculosis.* Comparison of the lipid biosynthetic responses to INH treatment revealed that the lipid profiles were different between *M. smegmatis* and *M. tuberculosis.* The *M. smegmatis* profiles were consistent with an inhibition of short-chain fatty acid biosynthesis by INH, presumably due to inhibition of InhA. In contrast, the *M. tuberculosis* lipid profiles suggested that INH directly inhibits desaturation of tetra or hexacosanoic acids. On the basis of these results, it was suggested that InhA is not the major target for activated INH in *M. tuberculosis.* However, studies with *M. smegmatis* and results obtained with *Mycobacterium aurum* support the idea that InhA is a target for KatG activated INH (76). Due to INH activated by catalase-peroxidase binds exclusively to enoyl-reductase-NADH complex in the presence of manganese and oxygen (77).

Six low-level INH-resistant *M. tuberculosis* isolates were identified that had missense mutations in the *inhA* structural gene, resulting in Ile16Thr, Ile21Thr,

Ile21Val, Ile47Thr, Val78Ala, and Ile95Pro replacements (78). INH-susceptible organisms lacked these variant amino acids. Strains containing the first five amino acid substitutions did not have *katG* mutations that would confer INH resistance. Several papers have described the detailed *in vitro* chemical interactions between INH and mycobacterial proteins. Mutations in *katG* or *inhA* do not account for all INH-resistant strains since 15% to 25% of INH-resistant clinical isolates have both wild type *katG* and *inhA* genes.

Moreover the observation that mutations in *katG* and the *inhA* loci do not account for INH resistance in all patient isolates in part has stimulated additional investigations designed to identify targets of this drug. Automated DNA sequence analysis discovered that four amino acid-altering mutations were present in *kasA*-encoded a presumed β-ketoacyl-ACP synthase in INH-resistant patient isolates (Figure 7). Susceptible strains lacked mutations in this gene. Importantly two of the resistant isolates lacked mutations in *katG* and the *inhA* locus. Therefore KasA is a new INH target useful for the development of new therapeutics and the identification of drug-resistant strains.

In addition, *oxyR* gene controls expression of *katG* and several other genes including *ahpC*, encoding the small subunit of alkylhydroperoxide reductase (79). The susceptibility of *M. tuberculosis* to INH is in part due to loss of OxyR function is that mutations that result in enhanced expression of other genes whose products are involved in the INH pathway might compensate for the loss of *oxyR* gene. Consistent with this idea, mutations in the *ahpC* promoter sequences were identified in a small number of INH-resistant organisms deficient in KatG activity, but were not present in INH-susceptible bacteria (80). The increased AhpC activity could compensate for the loss of KatG activity in the detoxification of organic peroxides. Hence the weight of the genetic and biochemical evidence indicated that the *ahpC* mutations represented compensatory alterations occurring as a consequence of loss of catalase-peroxidase activity. These observations raised the possibility that upregulation of AhpC expression may participate in INH resistance in *M. tuberculosis* strains lacking mutations in *katG* or *inhA* locus.

In summary, mutations in a relatively large number of genes have been associated with INH resistance. Evidence implicates mutations in *katG*, *inhA* and *kasA* as

causally involved in mediating resistance to this critical drug. The *oxyR* in *M. tuberculosis* is a pseudogene. Mutations in the promoter region of *ahpC* occur in many or most catalase-negative, INH-resistant isolates. These mutations appear to be compensatory and are presumably selected after reduction in catalase-peroxidase activity attributable to *katG* changes arising with INH therapy. The AhpC itself does not appear to directly mediate INH resistance.

In contrast to RIF, the genetic basis of resistance to other TB drugs including to INH are more complex. Alterations in a relatively large number of genes have been associated with INH resistance, but most mutations in two genes, *katG* (Figure 5) and *inhA* (Figure 6), are found in 75-85% of INH-resistant *M. tuberculosis* isolates. Moreover mutations conferring INH-resistance were found at *kasA* gene (Figure 7), *oxyR* gene and *ahpC* gene (Figure 8).

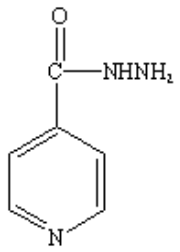

Figure 3 A chemical structure of isoniazid (INH). (54)

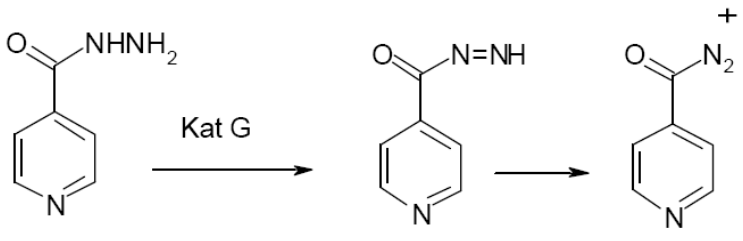

Figure 4 A potential metabolic activation mechanism for INH. KatG mediates 2 electron transfers to produce an activated INH intermediate(s). It is this reactive intermediate that is capable of intracellular acylation of nucleophiles in *M. tuberculosis*, thereby facilitating toxic effects. (55)

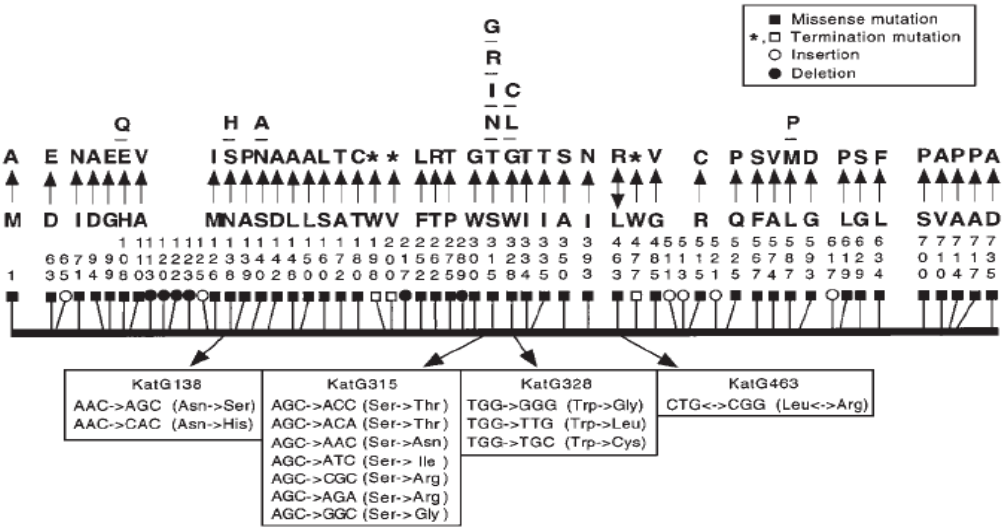

Figure 5 Nucleotide and amino acid changes within *katG* gene, which were associated with INH-resistance. (18)

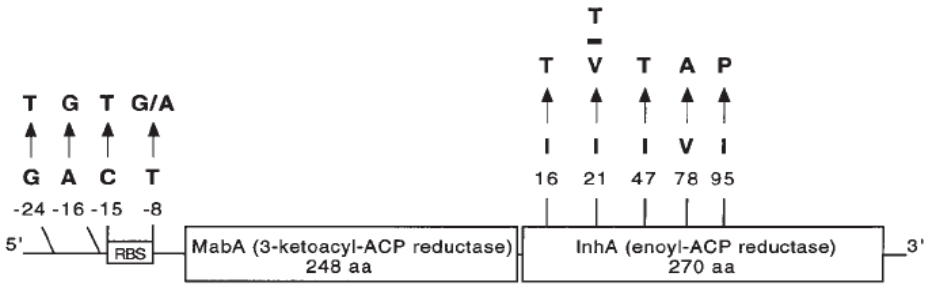

Figure 6 Schematic representation of mutations identified in the *inhA* locus in INH^r isolates of *M. tuberculosis*. The *inhA* locus is composed of two contiguous open reading frames designated mabA (encoding 3-ketoacyl carrier protein reductase) and inhA (encoding enoyl-acyl carrier protein reductase). (18)

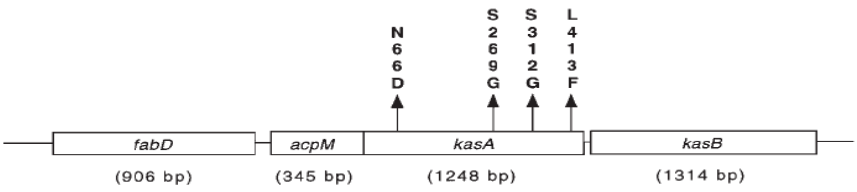

Figure 7 Kas genomic locus from *M. tuberculosis*, most mutation confer INH-resistance were at *kasA* gene which encodes a presumed β-ketoacyl-ACP synthase.

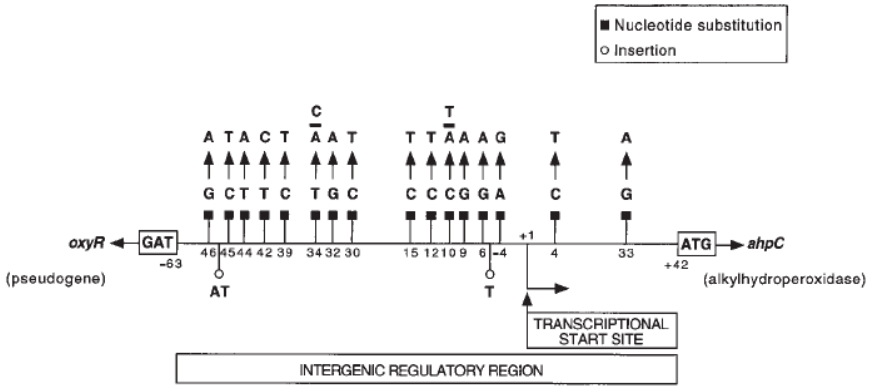

Figure 8 Schematic presentation of the *M. tuberculosis oxyR-ahpC* intergenic region showing nucleotide changes identified in INH-resistance isolates. (18)

3.3 Streptomycin (STR)

Streptomycin (STR) was the first drug used successfully to treat tuberculosis. This is a protein synthesis inhibitor. STR is an aminoglycoside antibiotic that inhibits protein synthesis. STR (Figure 9) was derived from *Streptomyces griseus*. Aminoglycosides work by binding to the bacterial 30S ribosomal subunit, causing misreading of tRNA, leaving the bacterium unable to synthesize proteins vital to its growth. Specifically STR binds to four nucleotides of 16S rRNA and a single amino acid of protein S12 which is involved in the initiation of protein synthesis. This interferes with decoding site in the vicinity of nucleotide 1400 in 16S rRNA of 30S subunit of the bacterial ribosome. This region interacts with the wobble base in the anticodon of tRNA. This leads to interference with the initiation complex, misreading of mRNA so incorrect amino acid are inserted into the polypepide leading to nonfunctional or toxic peptides and the breakup of polysomes into nonfunctional monosomes. The drug had side effect especially on the inner ear (81).

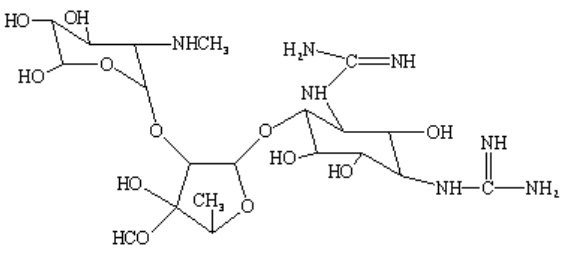

Figure 9 A chemical structure of streptomycin (STR). (54)

Mutations associated with STR resistance in *M. tuberculosis* have been identified in the 16S rRNA gene (*rrs*) and *rpsL* gene encoding ribosomal protein S12 (82). In contrast to other bacteria which have multiple copies of rRNA genes, *M. tuberculosis* complex members have only one copy (83). Hence single nucleotide changes can potentially produce antibiotic resistance. Point mutations in *rrs* are clustered in two regions around nucleotides 530 and 915. The 530 loop of 16S rRNA is highly conserved (Figure 10) and is located adjacent to the 915 region in secondary structure models. Several nucleotides in both regions interact with the ribosomal S12 protein (84). In the 530 loop, C to T transitions are observed at positions 491, 512 and 516 and transversions (A to C or T) have been reported at position 513. In the 915 region, C to A or G alterations at position 903 and A to G changes at position 904 are common. These observations are consistent with footprinting experiments in *E. coli* that show STR interacts directly with the nucleotide sites in the 530 loop and 915 regions of 16S rRNA and that mutations at these sites result in STR-resistant *E. coli* (85).

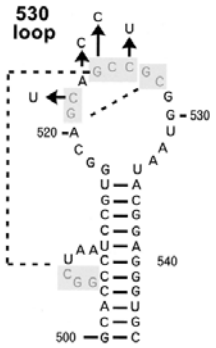

Figure 10 Secondary structure of the 530 loop region (86). This figure demonstrated *rrs* mutations associated with streptomycin (STR) resistance are indicated by an arrow. Nucleotides involved in tertiary structure interaction resulting in pseudoknots are shown in gray and are connected by dotted lines.

The majority of point mutations producing STR resistance occur in *rpsL*. The most common mutation is an AAG to AGG change in codon 43 resulting in a Lys to Arg substitution; less frequently, an AAG to ACG (Lys to Thr) substitution is observed (16), (82) . Mutations also occur in codon 88 and these result in Lys to Arg (AAG to AGG) or Lys to Gln (AAG to CAG) amino acid replacements. The

analogous mutations have been recorded in STR-resistant strains in several bacterial species, presumably because the S12 protein is highly conserved.

In summary, about 65-75% of STR-resistant isolates have resistance-associated changes in RpsL or *rrs*. Failure to identify resistance-associated variation in these genes in 25-35% of organisms indicates that other molecular mechanisms of STR resistance exist.

3.4 Etambutol (EMB)

Ethambutol (EMB) is a first-line antimycobacterial drug that inhibits cell wall synthesis. EMB is a synthetic congener (Figure 11) of 1,2-ethanediamine that is active against *M. tuberculosis, M. bovis* and some non-specific mycobacteria. It is an inhibitor of arabinosyl transferases that is involved in arabinogalactan biosynthesis of mycobacterial cell wall (87). It is an oral chemotherapeutic bactericidal agent which is specifically effective against growing microorganisms of the genus *Mycobacterium*, including *M. tuberculosis*. About 75% to 80% of an orally administered dose of EMB is absorbed from the gastrointestinal tract. It is used in combination with other anti-TB drugs to prevent or delay the emergence of resistant strains and readily absorbed from the gastrointestinal tract and excreted in the urine both unchanged and as inactive hepatic metabolites. It is generally well-tolerated and has few toxic effects. The most commonly recognized toxic effect of EMB is optic neuropathy which generally is considered uncommon and reversible in medical literature. Other side effects that have been observed are pruritus, joint pain, gastrointestinal upset, abdominal pain, malaise, headache, dizziness, mental confusion, disorientation and possible hallucinations. It is therefore not recommended for children who are unable to receive frequent ophthalmological check-ups.

To gain insight into the molecular genetics of arabinan biosynthesis, Belanger *et al*. (88) identified a two gene locus (*embAB*) in *Mycobacterium avium* that encodes arabinosyl transferases mediating polymerization of arabinose into arabinogalactan. Working in parallel, Telenti *et al*. (89) cloned and sequenced genes encoding the putative EMB target in *M. smegmatis*. Three genes organized as an operon were identified and designated as *embCAB* gene. The corresponding *M. tuberculosis embCAB* genes were then identified from an ordered cosmid library. Sequence

analysis and genetic studies revealed that the three genes were organized as a 10 kbp operon. A collection of 72 strains of *M. tuberculosis* including 28 EMB-resistant isolates were examined for mutations in the *embCAB* operon by automated DNA sequencing and SSCP analysis. Among epidemiologically unrelated EMB-resistant isolates, 13 of 28 (47%) had mutations in codon 306 of *embB* that would produce Met to Ile or Met to Val substitutions (Figure 12). These mutations were not present in 44 EMB-susceptible strains. In addition, analysis of *M. tuberculosis* isolates cultured from a single patient before and after development of EMB resistance identified a Met306Val substitution in the resistant strain, but not in the susceptible organism. Mutations located at a site corresponding to codon 306 were also found in EMB-resistant *M. smegmatis* strains. Furthermore gene transfer experiments in *M. smegmatis* showed that mutations in *embB* conferred EMB resistance. Secondary structure analysis suggested that EmbCAB are integral membrane proteins with 12 transmembrane domains. It was postulated that the EMB-resistance determining region (ERDR) in EmbB is located in a cytoplasmic loop that is well conserved among different mycobacterial Emb proteins.

To summarize, *embB* mutations are associated with EMB resistance in roughly 70% of EMB-resistance isolates of *M. tuberculosis* (89). It is likely that the molecular basis of EMB resistance involves altered interaction of the drug with one or more products of the EmbCAB operon. The cause of EMB resistance in many organisms lacking mutations in the EMB-resistance determining region (ERDR) of EmbB is unknown. It is reasonable to anticipate that the identification of additional altered EmbB variants will contribute to a fuller understanding of structure-function relationships in this putative arabinosyltransferase.

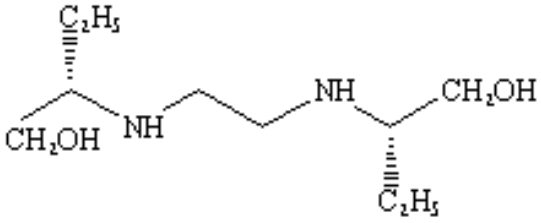

Figure 11 A chemical structure of ethambutol (EMB). (54)

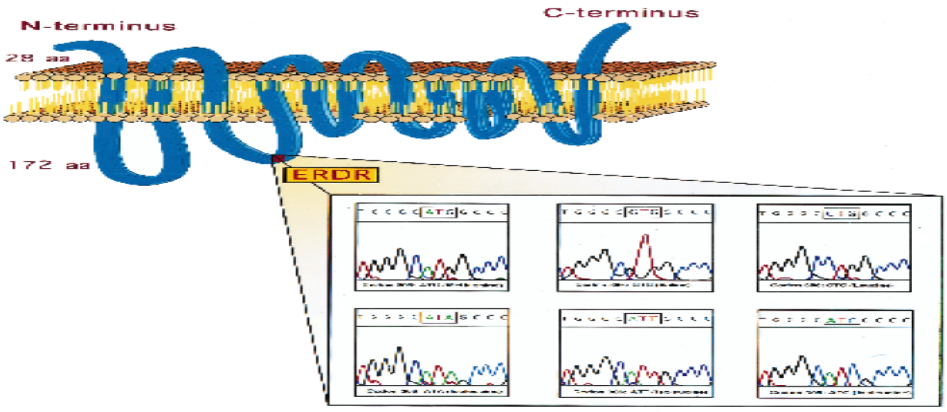

Figure 12 Schematic representation of polymorphism in *embB* codon 306 in EMB-resistant *M. tuberculosis* (18). The DNA sequencing chromatograms show the wild-type and five mutants of *embB* codon 306. The wild type codon in EMB-susceptible isolates is ATG (Met), and the five mutant codons found in ETB-resistant strains are GTG, (Val), CTG (Leu), ATA (Ile), ATT (Ile) and ATC (Ile). EmbB is presumed to be a transmembrane protein on the basis of computer modeling. The exact location of amino acid 306 relative to the membrane is unknown.

3.5 Pyrazinamide (PZA)

Pyrazinamide (PZA) is another first-line antimycobacterial drug that inhibits mycobacterial metabolism. PZA is a synthetic analogue of nicotinamide (90) (Figure 13) that is only weakly bactericidal against *M. tuberculosis*, but has potent sterilizing activity, particularly in the relatively acidic intracellular environment of macrophages and in areas of acute inflammation. PZA is a prodrug that stops the growth of *M. tuberculosis* which produces the enzyme pyrazinamidase which is only active at acidic pH. Functioning exclusively in the acid environment (pH 5.0-5.5) at the site of bacterial infection (91). Pyrazinamidase converts PZA to the active form as pyrazinoic acid (92). Pyrazinoic acid inhibits the enzyme fatty acid synthetase I, which is required by the bacterium to synthesize fatty acids (93). PZA can kill semi-dormant (94) and probably intracellular *M. tuberculosis* (95). This characteristic of PZA makes it possible to reduce total duration of treatment from 12-18 months to 6 months (96), (97). It is well absorbed orally. It crosses inflamed meninges and is an essential part of the treatment of tuberculous meningitis. It is metabolized by the liver and the

metabolic products are excreted by the kidneys. The action of PZA is fundamental in ensuring that duration of treatment is not prolonged. It is highly effective during the first two months of treatment while acute inflammatory changes persist and its use has enabled treatment regimens to be shorten and the risk of relapse to be reduced. It appears to act on slow-multiplication and intercellular bacilli (98). PZA has no other medical uses; in particular, it is not used to treat other mycobacteria such as *M. bovis* and *M. leprae* which are innately resistant to PZA. It is readily absorbed from the gastrointestinal tract and is rapidly distributed throughout all tissues and fluids. The most common (approximately 1%) side effect of PZA is joint pains (arthralgia), but this is not usually so severe that patients need to stop taking the PZA (99), (100). The arthralgia can be distressing to patients, but is never harmful. The most dangerous side effect of PZA is hepatitis, which is dose related and interferes with the metabolism of uric acid by increasing its level. It can produce attacks of gout in individuals who are predisposed to this condition. In the standard four-drug regimen (INH, RIF, PZA and EMB), PZA is the most common cause of drug-induced hepatitis (101). PZA is usually well tolerated. Hypersensitivity reactions are rare, but some patients complain of slight flushing of the skin.

To define the molecular mechanism of PZA resistance, Scorpio *et al*. (102) cloned and characterized the *M. tuberculosis* gene (*pncA*) encoding pyrazinamidase. Sequence analysis reveals that *pncA* encodes a protein with 186 amino acids that is 35.5% identical to *E. coli* nicotinamidase (pyrazinamidase). DNA sequencing of four PZA-resistant clinical isolates were identified missense mutations in codons 63, 138 and 141 in three of the organisms and deletion of nucleotide 162 in the fourth isolate, a change was resulted in the production of a truncated polypeptide. All four strains were lacked pyrazinamidase activity, in contrast, susceptible strains had the identical wild type sequence. Transformation of the wild type *pncA* gene into a PZA-resistant mutant derived from H37Rv restored pyrazinamidase activity and PZA susceptibility. The results were provided strong molecular genetic and biochemical evidence that *pncA* mutations conferred PZA resistance.

To gain additional insight into the role of *pncA* mutations in PZA resistance, Sreevatsan *et al*. (103) sequenced this gene in 67 PZA-resistant and 51 PZA-susceptible *M. tuberculosis* clinical isolates from diverse geographical localtities and

with a wide diversity of IS*6110* subtype. PZA susceptibility testing was done by BACTEC or the proportion method. All PZA-susceptible isolates had the identical *pncA* allele, whereas 72% of PZA-resistant had *pncA* mutations. A total of 17 previously undescribed mutations, including missense changes, upstream presumed regulatory mutations, nucleotide insertions and deletions and termination mutations were found (Figure 14). If these changes occurred in alpha-helical regions, protein structure might be altered, thereby detrimentally affecting pyrazinamidase activity. Lack of *pncA* mutations in 19 of 67 (28%) PZA-resistant isolates suggested the existence of at least one additional gene participating in PZA resistance.

Hence, a remarkably wide array of *pncA* mutations resulting in structural changes in PncA protein have been identified in greater than 70% of drug resistant clinical isolates. It is presumed that these structural changes detrimentally change enzyme function, thereby altering conversion of PZA to its bioactive form. One hypothesis to account for the extreme diversity in resistance-associated mutations is that because the enzyme consists of only 186 amino acids, any amino acid substitution may be likely to detrimentally alter function. Susceptible organisms lack *pncA* changes. The details of the molecular mechanism of PZA-pyrazinamidase interaction and pyrazinoic acid formation are still unknown.

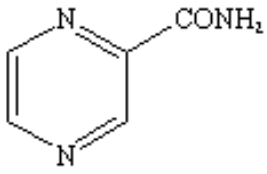

Figure 13 A chemical structure of pyrazinamide (PZA). (54)

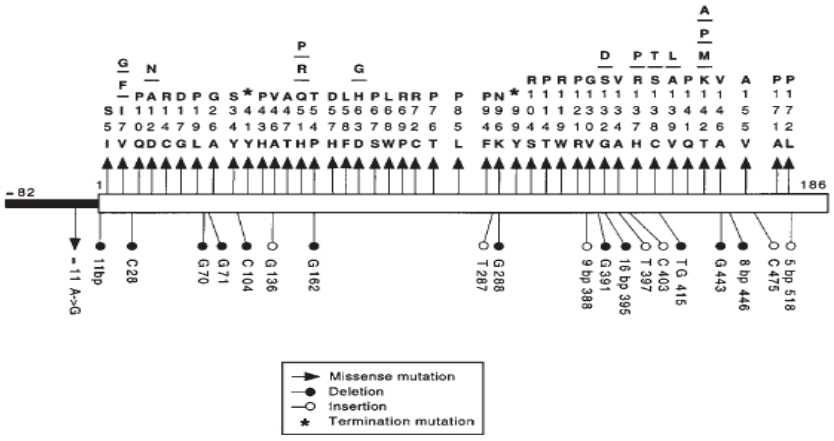

Figure 14 Schematic representation of polymorphism in *pncA* gene in pyrazinamide (PZA)-resistant *M. tuberculosis*. Mutations reported previously are included. Variant amino acids are numbered vertically. (18)

Drug-resistant *M. tuberculosis* is a serious threat to TB control because only a few effective drugs namely INH, RIF, EMB, PZA, STR are available for treatment of this disease. *M. tuberculosis* acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. No plasmids or transposable elements are involved in this process. Individual nucleotide changes (point mutation) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to MDR-TB. Drug-resistant strains emerge when chemotherapy is intermittent or otherwise inadequate (104). The epidemiology of drug resistance has been analyzed in two recent international surveys sponsored by the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD). These surveys documented wide-ranging rates and patterns of drug resistance around the globe (6), (4). Resistance was seen most frequently to INH and STR, the two agents used most extensively over the years (105).

After the primary culture results are available, conventional drug susceptibility testing performed on solid medium takes about 2-4 weeks. When the radiometric BACTEC drug susceptibility testing system is used, five days to twenty-one days are needed to obtain results (12). Advances in molecular biology have made it possible to investigate the genetic mechanisms of drug resistance in *M. tuberculosis* and to develop methods for rapid detection of mutations associated with resistance. The

currently available methods have mostly been developed for detection of RIF-resistance because the genetic basis of resistance to other first-line TB drugs are much more complex. Moreover, resistance to RIF can often be used as a marker of MDR-TB. Therefore this issue was only focused on RIF background.

4. Rifampicin history

RIF was introduced for use in antituberculous therapy in the early 1970s and is a very important component of current treatment regimens. On the basis of detailed structure-function studies conducted with *Escherichia coli* (Figure 15), the molecular mechanism of RIF activity involves inhibition of DNA-dependent RNA polymerase (Figure 16). In *E. coli*, this enzyme is a complex oligomer composed of four different subunits (α, β, β' and σ encoded by *rpoA, rpoB, rpoC* and *rpoD* respectively), which can occur either as a core enzyme ($\alpha_2\beta\beta'$) or a holoenzyme ($\alpha_2\beta\beta'$ plus σ). RIF binds to the β-subunit of *E. coli* RNA polymerase (Figure 17) and results in transcription inhibition, which is indicated as the mechanism of action in strains found to be RIF-resistance by *in vitro* testing (16).

Because resistance to RIF in many *E. coli* strains were known to arise as a result of missense and other mutations occurring in a discrete region of the *rpoB* gene, Honore *et al*. cloned and sequenced the cognate region of the *M. tuberculosis* gene by using sequence information available from the *M. leprae rpoB* gene (106).

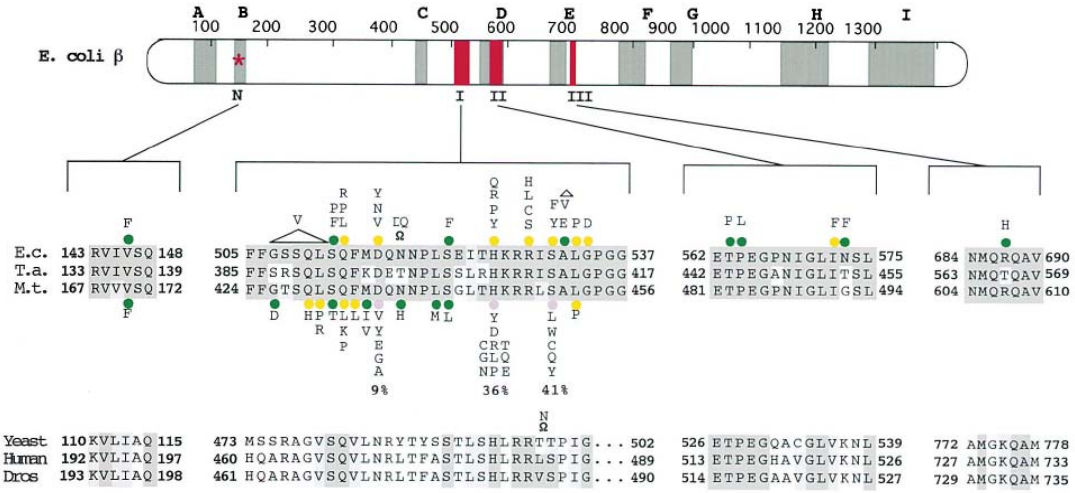

Figure 15 The RIF-resistant regions of the RNA polymerase β-subunit (RpoB) (107). The bar on top schematically represents the *E. coli* β-subunit primary sequence with amino acid numbering shown directly above. Gray boxes indicate evolutionarily conserved regions among all prokaryotic, chloroplast, archaebacterial, and eukaryotic sequences (labeled A–I at the top; (108); (109)). Red markings indicate the four clusters where RIF-resistant mutations have been identified in *E. coli* (110), (111), (112), (52), (113), (114) denoted as the N-terminal cluster (N), and clusters I, II, and III (I, II, III). Directly below is a sequence alignment spanning these regions of the *E. coli* (E.c.), *Thermus aquaticus* (T.a.), and *M. tuberculosis* (M.t.) RpoB. Amino acids that are identical to *E. coli* are shaded dark gray, and those that are homologous (ST, RK, DE, NQ, FYWIV) are shaded light gray. Mutations that confer RIF-resistance in *E. coli* and *M. tuberculosis* are indicated directly above (for *E. coli*) or below (for *M. tuberculosis*) as follows: D for deletions, V for insertions, and colored dots for amino acid substitutions (substitutions at each position are indicated in single amino acid code in columns above or below the positions). Color coding for the amino acid substitutions (for reference to subsequent figures) is as follows: yellow, residues that interact directly with the bound RIF; green, residues that are too far away from the RIF for direct interaction; purple, three positions that are substituted with high frequency (noted as a % immediately below the substitutions) in clinical isolates of RIF-resistant *M. tuberculosis* (18). Below the three prokaryotic sequences is a sequence alignment of three eukaryotic sequences with shading as above. The dots indicate a gap in the alignment.

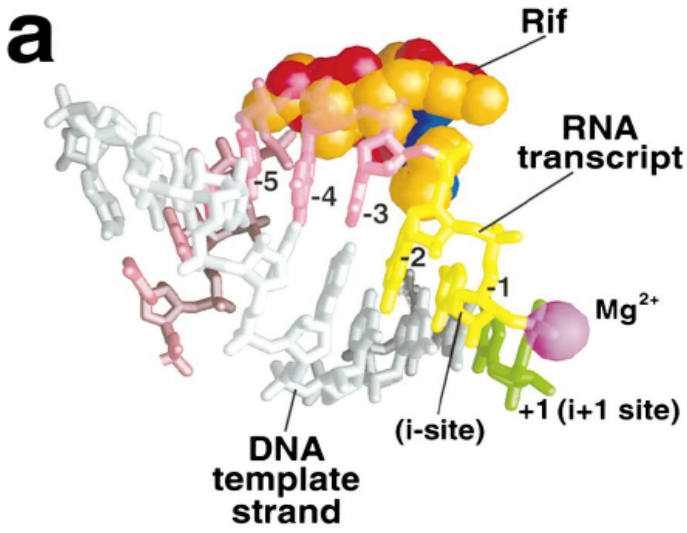

Figure 16 Mechanism of RNA polymerase inhibition by RIF (107). The RNA polymerase active site Mg^{2+} (magenta sphere) and the 9 bp RNA/DNA hybrid (from +1 to -8) from a model of the ternary elongation complex (115) are shown. The RNA polymerase itself and the rest of the nucleic acids are omitted for clarity. The incoming nucleotide substrate at the +1 position is colored green, the -1 and -2 positions, which can be accommodated in the presence of RIF, are colored yellow. The RNA further upstream (-3 to -8), which cannot be accommodated in the presence of RIF, is colored pink. The template strand of the DNA is colored gray. Also shown is a CPK atoms (carbon, orange; oxygen, red; nitrogen, blue) representation of RIF as it would be positioned in its binding site on the β-subunit. The RIF is partially transparent, illustrating the RNA nucleotides at -3 to -5 that sterically clash.

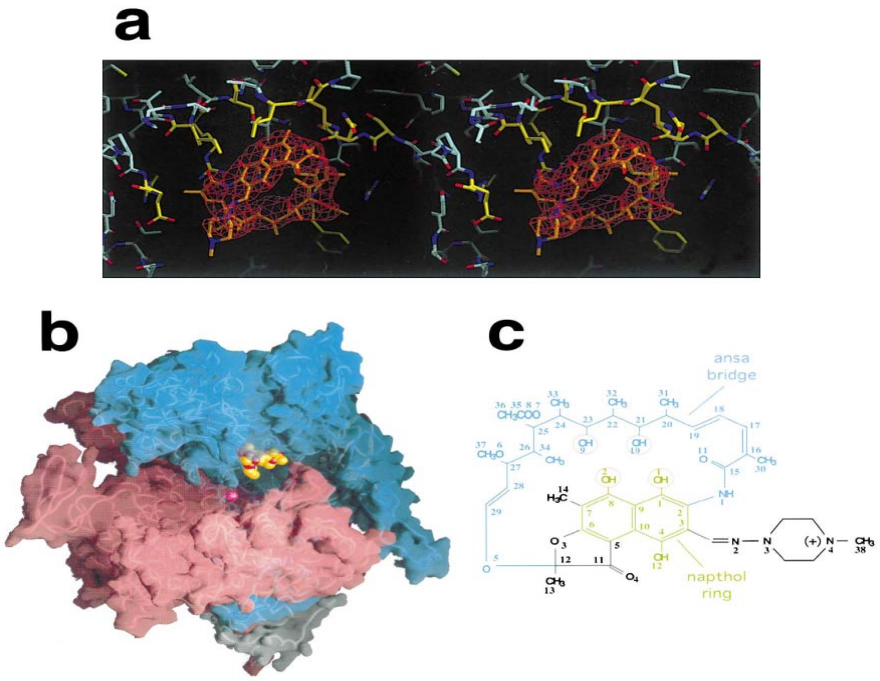

Figure 17 RIF-RNA polymerase cocrystal structure (107).

(a) Stereo view of the RIF binding pocket of *T. aquaticus* core RNAP, generated using O (116). Carbon atoms of the RNA polymerase β-subunit are cyan or yellow, while carbon atoms of the inhibitor are orange. Oxygen atoms are red, nitrogen atoms are blue, and sulfur atoms are green.

(b) Three-dimensional structure of *T. aquaticus* core RNA polymerase in complex with RIF. The backbone of the RNA polymerase structure is shown as tubes, along with the color coded transparent molecular surface (β, cyan; β', pink; ω, white; the α subunits are behind the RNA polymerase and are not visible). The Mg^{2+} ion chelated at the active site is shown as a magenta sphere. The RIF is shown as CPK atoms (carbon, orange; oxygen, red; nitrogen, blue).

(c) Structural formula of RIF. Features of the structure discussed in the text are color coded (ansa bridge, blue; napthol ring, green). The four oxygen atoms critical for RIF activity (117) are shaded with red circles.

5. Techniques for detection of drug resistant *M. tuberculosis* especially RIF

The World Health Organization has estimated that MDR-TB accounted for 3.5% of all new cases of TB in year 2000 (118). Methods that generate rapid and correct drug susceptibility test results are increasingly important tools in the clinical

management of tuberculosis. The results guide the treatment and help minimizing the spread of drug-resistant strains in the community. There are two different principles for development of new methods using in detection of drug-resistant *M. tuberculosis* namely by phenotypes or genotypes.

5.1 Phenotypic methods

The proportion method of antimycobacterial susceptibility testing is currently a standardized method for estimating drug resistance and is also applied in the rapid testing methods such as BACTEC 460 and MGIT 960. The BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 (Becton Dickinson) is a non-radiometric fully automated culture system that was recently FDA-cleared for drug susceptibility testing bypasses some of the disadvantages related to the BACTEC 460 system (119). It is based on continuous monitoring of bacterial growth measured as increasing fluorescence. The BACTEC system was not well standardized due to it used only a single critical concentration of drug and requirement for technical expertise in performing and interpreting the results (120). These methods are still heavy equipment, expensive to implement in low-income countries. Recently developed phenotypic methods are microplate tetrazolium reduction assay and micro-well alamar blue assay, mycolic acid index susceptibility assay. Microscopic observation of broth cultures for drug susceptibility assay, PhaB assay, luciferase reporter phage assay and others are also being developed (121).

5.1.1 The proportion method

The agar proportion method for susceptibility testing of slowly growing mycobacteria was developed in the early 1960s and is the method most commonly used in mycobacteriology laboratories in the United States. The proportion method, as presently used in the United States has undergone various modifications. A proposed standard method was published by the NCCLS and this procedure will be updated in an expanded form in 1990s. The preferred medium for the proportion test is Middlebrook 7H10 agar and drugs used in the proportion method can be prepared from reference powders or added as drug-impregnated disks. For this method, the bacilli are cultured on two media, one with and one without the drug and the ratio of

the number of colonies obtained on the drug-containing medium to the number of colonies obtained on the drug-free medium indicates the proportion of resistant bacilli (122).

According to World Health Organization (WHO) protocol (123), antimycobacterial drugs are adjusted in the LJ medium to final concentrations of 40 μg/ml for RIF. One hundred microliter of prepared bacterial inoculum is inoculated on LJ medium, containing or not containing drug for test or as a control, followed by incubation at 37°C for 21-28 days. Resistance is defined as growth on drug containing tubes equal to or greater than 1% of the growth on drug free control medium for RIF.

The National Committee for Clinical Laboratory Standards (NCCLS), proportion method (11) is performed by dispensed the disk aseptically into individual quadrants of sterile Petridishes. Complete OADC-enriched Middlebrook 7H10 agar medium is prepared and dispensed 5.0 ml of this medium into each quadrant, overlaying the disk and keeping it approximately centered. Delivery of exactly 5.0 ml of medium to each quadrant is essential to assure correct concentration of drug, allowed the agar to solidify at room temperature. And next bearing include interpretation as same as above proportion method.

The proportion methods require 3-4 weeks of incubation, so these methods are technically complicated to prepare plates with appropriate concentration and time consuming.

5.1.2 Radiometric BACTEC 460 TB method

The radiometric BACTEC 460 TB method is specific for mycobacterial growth, wherein ^{14}C labelled palmitic acid in M7H12 medium is used. This system detects the presence of mycobacteria based on their metabolism rather than visible growth. When the ^{14}C labelled substrate present in the medium is metabolized, $^{14}CO_2$ is produced and measured by the BACTEC system instrument and reported in terms of growth index (GI) value. The BACTEC system is also useful in the identification of *M. tuberculosis* using a specific inhibitor, para-nitro-α-acetyl-amino-β-hydroxypropiophenone. Using the same system, drug susceptibility tests can also be performed for all the anti-tuberculosis drugs when sufficient GI is observed. As for drug susceptibility testing, its use was performed by inoculation of the test isolate in drug-containing medium and

inoculation of one-hundred fold dilution (1%) of the isolate in drug-free medium (13). The critical concentration of RIF used for the BACTEC assays was 2.0 μg/ml. Mycobacteria in clinical samples can be detected in half the time compared to conventional culture methods (39). The BACTEC 460 system is a variant of the reference proportion method and provides drug susceptibility testing results within 4 to 12 days (124). The system brought considerable improvement in culturing of mycobacteria but suffers from certain disadvantages primarily the need for radioactive isotopes and use of needles. Due to the BACTEC 460 system requires isotopes, require expertise to set up and interpret and expensive, consequently is not feasible in most resource-poor setting.

5.1.3 Mycobacteria growth indicator tube (MGIT) 960

The MGIT 960 is an automated system for the growth and detection of mycobacteria with the capacity to incubate and continuously monitor mycobacteria growth indicator tubes every 60 minutes for increase in fluorescence. The MGIT method uses a fluorescence quenching-based oxygen sensor embedded in the base of a tube containing a modified Middlebrook 7H9 broth. The fluorescence that indicates the presence of mycobacterial growth can be detected by transillumination with a 365 nm UV light such as a simple Wood's lamp. Growth detection is based on the acid fast bacilli metabolic O_2 utilization and subsequent intensification of an O_2 quenched fluorescent dye contained in a tube of modified MGIT. A series of algorithms are to determine presumptive positivity and alert the operator to the presence and location of positive tubes. The system's software algorithms evaluate the relative growth in the drug-containing tubes and compare it to the drug-free control tube.

Susceptibility data generated by the new, the fully automated BACTEC MGIT 960 system are well comparable to those generated by the radiometric BACTEC 460TB system. Concerning time to result there is no statistically significant difference between the two systems (6.8 days for BACTEC 460TB and 7.7 days for BACTEC MGIT 960) (125). In being as efficient as the BACTEC 460TB System, the new nonradiometric BACTEC MGIT 960 system is a promising candidate to replace radiometric antimicrobial susceptibility testing of *M. tuberculosis* complex and thus overcomes the limitations of radiometry in particular, safety and regulatory principles.

The commercial MGIT system, the non-radiometric method, is reliable but still expensive to implement in low-resource countries (40).

5.1.4 Microplate tetrazolium reduction assay and microwell alamar blue assay

Oxidation-reduction dyes such as tetrazoliums, have been used to obtain drug susceptibility measurements of mycobacteria (126). Yajko *et al.* (126) reported as a result of tests with clinical isolates a good correlation between the proportion technique and a broth method with alamar blue that is a novel proprietary, resazurin-based oxidation-reduction indicator which delivered colorimetric MICs for *M. tuberculosis* isolates in 14 days. Franzblau *et al*. (127) reported that 100% of results of 34 *M. tuberculosis* isolates including both pansensitive and multidrug-resistant strains and H37Rv were available within 8 days by using bacterial suspensions prepared directly from solid media. A microplate version of the alamar blue assay with modified medium composition, reaction time and temperature, and inoculum preparation was evaluated as a high-throughput screen by comparing the MICs of 30 antimicrobial agents for *M. tuberculosis* H37Ra and H37Rv obtained by microplate version of the alamar blue assay to the MIC obtained with the BACTEC 460 system (128).

The microplate tetrazolium reduction assay and microwell alamar blue assay are colorimetric based on the oxidation-reduction of the dye dimethylthiazol-diphenyl tetrazolium bromide (MTT) or alamar blue. Drug resistance is detected by the reduction of the dye from yellow to pink for MTT or blue to pink for alamar blue due to the oxidation-reduction metabolism of viable organisms (127). MTT in a microplate assay would give results similar to those obtained by alamar blue. The microplate assay that either use alamar blue or tetrazolium-type compounds is simple, rapid, low-cost, appropriate technology which does not require expensive instrumentation and which makes use of a nontoxic, temperature-stable reagent. But this method may give the ambiguous results for observing breakpoints.

5.1.5 Mycolic acid index (MAI) susceptibility testing

The mycolic acid analysis using high-performance liquid chromatography (HPLC) and *p*-bromophenacyl bromide derivative reagent for UV detection is a well-established method for identification of mycobacterial strains isolated from clinical specimens (129). A modification of this method that uses a coumarin as the fluorescent derivative agent of mycolic acids allowed the detection of mycobacteria directly from clinical specimens (130), since the use of the fluorescence increases the sensitivity of detection to a level at least 200-fold better than with UV detection. In addition, Garza-Gonza *et al.* (131) described a linear relationship between the total area under the mycolic acid (TAMA) chromatographic peaks of a culture of *M. tuberculosis* and log CFU per milliliter, suggesting the possibility of using TAMA as a good estimator of mycobacterial growth and also as a means of susceptibility testing of *M. tuberculosis*. The development of the new derivative method of mycolic acids allowed the possibility of improving the use of TAMA for susceptibility testing of *M. tuberculosis*, since the chromatographic signal of the mycolic acids was increased, thus increasing the difference between cultivated strains in the presence or absence of an anti-TB drug and reducing the volume of the liquid medium needed (132).

The results of the MAI susceptibility method were accessible clearly within 5 days after the clinical isolates were incubated in the presence or absence of an anti-TB drug (133). The MAI susceptibility method is based on the ability of *M. tuberculosis* to synthesize mycolic acids during growth. In nonviable mycobacteria or mycobacteria susceptible to anti-TB drugs, synthesis of new mycolic acids are reduced due to the absence or decreased metabolic activity of the organisms. Besides the rapidity and objectivity of the MAI susceptibility method, a major advantage of this method is its ability to confirm the identification of *M. tuberculosis* and the diagnosis of TB. However, there is an important shortcoming in the use MAI for susceptibility testing of *M. tuberculosis*. Although the test is rapid, accurate, and reproducible, many clinical laboratories do not have the facilities to perform the procedure. Nevertheless, the test could be used by public health laboratories or large reference laboratories with a biosafety level 3 containment. It is recommended that after an injection into the HPLC system, the syringe was cleaned at least five times with HPLC-grade methylene chloride and the injector loop was cleaned one time with 1 ml of the same solvent; it is

also recommended that a blank injection be used between samples when the prior mycolic acid signal is high. Furthermore, the reagents and supplies for HPLC are cheap compared with those needed for other rapid-susceptibility methods such as the BACTEC radiometric method (BACTEC TB system; Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.). The BACTEC and MAI methods should be able to obtain results after the same length of time because they use almost the same culture medium; however the BACTEC method does not allow estimation of the percentage of resistant organisms and is vulnerable to false susceptibility or resistance results due to the possibility of mixed populations of mycobacterial species (134); these deficiencies are not a problem in their MAI susceptibility method. Since mycolic acid analysis can be done at present directly with young cultures (130), the MAI susceptibility method could be carried out in a direct form instead of the indirect form.

In conclusion, the MAI susceptibility method can be used to perform susceptibility testing of *M. tuberculosis* clinical isolates and shows great promise as a rapid, effective, accurate, and reliable susceptibility method for a mycobacteriology laboratory. But this method is complicated, difficult to perform, high cost system and need biosafety cabinet level 3 which contain only in public health laboratories or large reference laboratories as well.

5.1.6 Microscopic observation of broth cultures-drug susceptibility assay (MODS)

M. tuberculosis grows more rapid in liquid medium, where it grows as strings and tangles, than on solid medium (135). Based on this, Caviedes *et al.* (136) developed a new, simple, efficient, reliable, and inexpensive method that permits simultaneous *M. tuberculosis* detection and determination of drug susceptibility in less than 2 weeks. The MODS uses microscopy to detect early growth of *M. tuberculosis* as strings and tangles of bacterial cells in Middlebrook 7H9 broth medium with or without antimicrobial agents of interest (Figure 18). A concentration of 1.0 μg/ml was the lowest concentration of RIF used when testing drug susceptibility with MODS, to improve concordance with microwell alamar blue assay.

The MODS was developed as a simple adaptation of the microagar M7H11 test, which uses microscopic observation to detect *M. tuberculosis* on agar. In MODS,

liquid medium is used to replace agar. Their results confirm the advantage of using liquid medium: both liquid culture methods used, MODS and MGIT, were at least 14% more sensitive and 4 to 5 days faster than either the microagar 7H11 or LJ culture (136).

Sputum samples positive for tuberculosis (136) were firstly digested to reduce viscosity and to kill other bacteria and then concentrated by centrifugation. They are diluted into M7H9 broth and distributed to wells of a 24-well plastic culture tray with and without antimicrobial agents. Susceptibility results using MODS were available between 5 and 20 days (median, 9.5 days) after the inoculation. Subsequently *M. tuberculosis* growth is observed as the formation of characteristic strings and tangles by simple light microscopy. The MODS unlike other rapid detection systems, does not require either radioactive isotopes or fluorescent indicators.

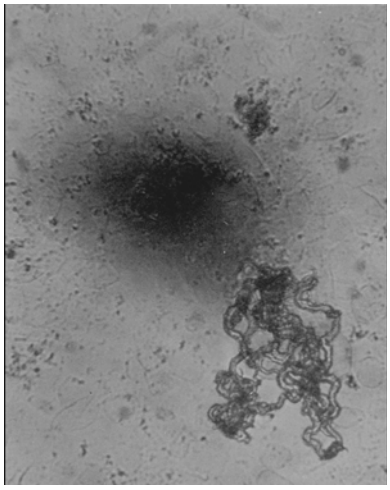

Figure 18 Appearance of *M. tuberculosis* at 100X magnification after 25 days of culture in broth. Note the serpentine, ropy aspect of the microcolonies (136).

5.1.7 Phage amplified biologically (PhaB) assay

Phage-based assays utilize bacteriophages to infect and detect the presence of *M. tuberculosis* in clinical specimens and culture isolates (137). Two main approaches are used to detect *M. tuberculosis* namely (i) amplification of phages or PhaB assay (Figure 19) after their infection of *M. tuberculosis*, followed by detection of progeny phages using sensor cells as plaque formation (138) and (ii) detection of light

produced by luciferase reporter phages (LRP) (Figure 20) after their infection of live *M. tuberculosis* (139) that is being explained for next topic below.

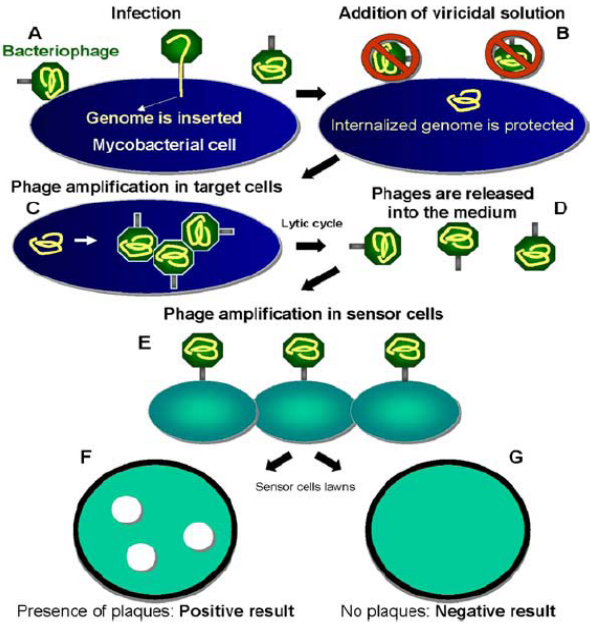

Figure 19 An overview of the phage amplification assay (PhaB assay) (140).

The PhaB assay is based on the ability of viable *M. tuberculosis* to support the replication of an infecting mycobacteriophages and non-infecting exogenous phages are inactivated by chemical treatment. The number of endogenous phages, which is an indication of the original number of viable *M. tuberculosis*, is determined after cycles of infection, replication and release in rapidly growing mycobacteria. In the case of drug-resistant *M. tuberculosis*, bacilli will remain viable and protect the mycobacteriophage. Any mycobacteriophage protected within viable bacilli replicate and ultimately lyse their host. For rapid detection, the released mycobacteriophages are mixed with rapidly growing *M. smegmatis* host in which they undergo rapid cycle of infection, replication and lysis. The lysis is easily seen as clear areas or plaques in a lawn culture of *M. smegmatis*. The number of plaques generated from a given sample is directly proportional to the number of protected mycobacteriophages, which is dependent on the number of tubercle bacilli that remain viable after drug treatment.

Phage-based assays are available as commercial kits (e.g. FASTPlaque-TBw and PhageTek-MBw, a variant of the FASTPlaque-TB, Biotec Laboratories Ltd, U.K.) and

as in-house (laboratory-developed) assays (137). In-house assays use either amplification technology (e.g. PhaB) or LRPs. Some of the phage-based assays are specifically designed to rapidly detect RIF resistance (e.g. FASTPlaque-TBMDRiw, earlier called FASTPlaque-TB-RIFw) in culture isolates. Newer kits are being developed for the rapid detection of drug resistance directly from clinical specimens such as sputum (e.g. FASTPlaque-TB-Responsew).

Phage-based assays for the detection of RIF resistance suggests that phage assays are associated with fairly high sensitivity and specificity, when applied to culture isolates. Once an isolate was drawn from a growing culture, the average turnaround time for phage-based assays was 48 to 72 hrs, whereas with conventional drug susceptibility testing, the turnaround time might vary from 1 to 2 weeks for BACTEC and MGIT and 3 to 4 weeks for solid media-based methods. In addition to rapidity, phage-based assays have the advantage of being less expensive and technologically simpler than liquid-media based tests such as MGIT, BACTEC. This offers some advantages in resource-limited settings.

In contrast, some studied revealed that phage-based assays do not show such high accuracy for the direct detection of *M. tuberculosis* in clinical specimens. Phage-based assays have performance characteristics that are fairly similar to that of sputum microscopy in high specificity but modest and variable sensitivity.

Albert and colleagues (141) evaluated the new FASTPlaque-TB-Response test directly on sputum specimens and demonstrated a sensitivity and specificity of 100% and 99%, respectively. However, Butt and co-workers (142) using the FASTPlaque-TB-RIF test showed a modest sensitivity of 86% and specificity of 73%. This difference in accuracy could be partly due to the variation in definitions used for RIF resistance and cut-points used for valid results. In the study by Albert and colleagues, considered valid if ≥100 plaques were observed on the plate without RIF, and a strain was determined to be resistant if ≥50 plaques were seen on the plate with RIF (141). In the study by Butt and colleagues, results were considered valid if ≥20 plaques were seen on the plate without RIF, and a strain was determined to be resistant when ≥20 plaques were seen on plate with RIF (142). This variability in cut-points emphasizes the need for better standardization of phage assays.

The PhaB assay has many disadvantages include unexplaining low sensitivity and specificity in some studies, and potential for contamination and uninterpretable results. Some studies reported low specificity estimates in the range of 80% (143). In settings with a relatively lower prevalence of MDR-TB, false positive rates in the range of 10% to 20% can result in significant false over-reporting of MDR-TB. Because bacteriophages can replicate in non-tuberculous mycobacteria (NTM) as well as *M. tuberculosis*, there is always a potential for false positive results when phage assays are directly applied to sputum specimens. Some NTM such as *M. smegmatis* are naturally resistant to RIF. In populations with high background prevalence of NTM, there is a risk of over-diagnosing MDR-TB. To minimize false positive results, a second confirmatory test such as conventional drug susceptibility testing for first line drugs may be necessary to confirm and validate all positive phage results. This method may provide uninterpreted results and must be careful for the contamination which is leading to false positive result.

5.1.8 Luciferase reporter phage assay (LRP assay)

A method which utilizing bacteriophage to introduce the firefly luciferase gene (*fflux*) into any viable isolate of *M. tuberculosis*, thus yielding visible light in the presence of cellular ATP and the exogenous substrate luciferin (Figure 20). Easily detectable signals are seen a few minutes after the infection of *M. tuberculosis* with reporter phages. When drug-susceptible *M. tuberculosis* strains are incubated with specific anti-tuberculosis drugs, they fail to produce light after infection with luciferase reporter phages. Effective antimycobacterial drugs will abrogate light production in this system either by decreasing ATP stores, by impairing luciferase protein production, or by interfering with productive phage infection. Antimicrobial susceptibility testing can be performed within 2 to 3 days (144). In contrast, drug-resistant strains are unaffected by the drugs and produce light at levels equivalent to those documented for untreated controls after infection with reporter phages (145). These tests have generally good sensitivity and reproducibility but are yet to be used in routine clinical laboratories.

The greatest cost issue with this method is the requirement for a luminometer to quantify the light output. The photographic detection of light from recombinant lytic

phage-infected *M. tuberculosis* was used a custom-made light-tight box which accommodates a 96-well plate and a polaroid film cassette. A photographic detection apparatus, designated the Bronx Box, which greatly simplifies the detection protocol and removes the requirement for the most costly component in the protocol such a luminometer was subsequently developed. The semiquantitative output which results is simple to interpret and accurate compared to that from the luminometer. Furthermore, this method can be applied directly to primary isolates of *M. tuberculosis* grown on solid agar, available in 3 to 4 weeks from the time of sample acquisition and still the most common means of cultivating *M. tuberculosis* worldwide (6).

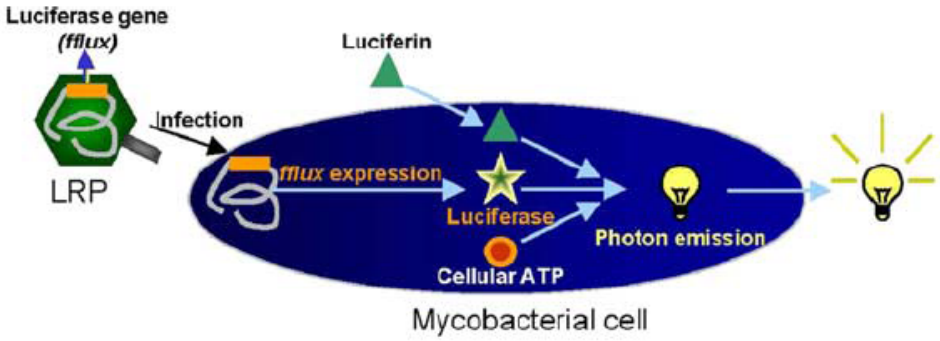

Figure 20 An overview of the luciferase reporter phage assay (140).

5.2 Genotypic methods

Due to the phenotypic methods required 2 to 6 weeks for turnaround time til the susceptibility testing result were obtained. Therefore the genotypic methods were developed for rapid susceptibility testing.

Genetic and molecular analysis of drug resistance in MDR-TB suggest that the bacilli usually acquire resistance either by alteration of the drug target by mutation or by titration of the drug through over-production of the target. In contrast to other bacteria, drug resistance in *M. tuberculosis* is not plasmid mediated. Most of the molecular events relating to the chromosomal basis of drug resistance have been elucidated. The MDR-TB usually result from accumulation mutations of individual target genes.

Delineation of the molecular mechanisms of antimicrobial agent resistance has resulted in the development and application of several PCR-based strategies designed

to rapidly detect mutations associated with resistance. A variety of diagnostics have capitalized on the recent elucidation of molecular mechanisms of drug resistance, especially to RIF, which is served as a surrogate marker for the detection of MDR-TB and which is almost always caused by a limited number of mutations in the single gene *rpoB* (18). In addition, more than 95% RIF-resistant isolates are also INH resistance.

RIF is one of the potent anti-TB agents, most effective in killing metabolically active *M. tuberculosis*. Ninety six percent of RIF-resistant strains of *M. tuberculosis* possess genetic mutations resulting in amino acid change within an 81-bp RIF resistance determining region of *rpoB* gene (81-bp hot spot region), corresponding to codons 507 to 533 [*E. coli* codon numbering system] (146), (27). Mutations outside the RIF resistance determining region of *rpoB* have also been reported, with a frequency of less than 2% (17). This is of importance in the design of rapid diagnostic methodology for detection of MDR-TB.

The different techniques that are applied for the genotypic analysis of resistance in *M. tuberculosis* can be grouped into three categories namely DNA sequencing, electrophoresis and hybridization-based assays. The sequencing and hybridization-based assays directly detect mutations and can define the precise mutations involved, whereas electrophoretic assays provide an indirect method of detecting the existence of mutations without having the ability to define the exact nucleotidic substitution involved. These methods include DNA sequencing of PCR products, real-time PCR, single strand conformation polymorphism (SSCP) analysis, heteroduplex analysis, RNA/RNA duplex which is base pair-mismatch assay, a reverse hybridization-based line probe assay and other strategies. These methods are designed for identify mutations that found in resistant strains and are absent in susceptible organisms.

5.2.1 DNA sequencing assay

Among the molecular techniques available to detect *M. tuberculosis* drug resistance, DNA sequencing of PCR amplified products are most widely used and is becoming the gold standard for this purpose. It has been performed by both manual and automated procedures. The DNA sequencing is used for characterization of the mutation responsible for drug resistance (147). The automated DNA sequencing is the

most commonly applied technique to characterize mutations in the *rpoB* gene for genotypic prediction of RIF resistance (148). The detection of RIF resistance by DNA sequencing is rapid and advantageous that provide a confirmatory and reproducible results. DNA sequencing technique is a rapid, conclusive and more advantageous technique than the conventional susceptibility testing for detection of RIF resistance in terms of the risk involved and time consumption. But the automated DNA sequencing assay is still costly technique, require expensive instrument which is not appropriate for developing countries to use for MDR-TB identification.

For example, the automated DNA sequencing was carried out using ABI 310 Genetic Analyser (Applied Biosystems Inc., Foster City, CA, USA) using both the forward and reverse primers to confirm the results. The sequence data was assembled and analyzed by ClustalW (http://www.clustalw.genome.ad.jp) (148).

5.2.2 Real-time PCR

The use of fluorescence labels and the inclusion of optical devices or CCD cameras in thermocyclers have recently made it possible to observe an amplification reaction directly while it is occurring. The so called 'real-time PCR' involves real-time monitoring of a DNA amplification reaction by measuring the fluorescence signal accumulated by amplification products. Different real-time PCR instruments are available, some based on conventional peltier-based thermocyclers and others on air-heating and air-cooling thermocylers which, in combination with capillary tubes, also allow a rapid-cycle PCR. Different reaction formats have been developed to monitor a PCR reaction in real time by the use of fluorescent dyes. If the purpose is to detect specific mutations in the amplified region, then the fluorescent dye must be bound to DNA probes designed to be complementary to the template DNA under analysis. Different formats of labeled probes can be adapted to the detection of mutations. Among the most frequently used are TaqMan, molecular beacons, and fluorescence-resonance-energy transfer (FRET) probes. These probes are included in the PCR reaction together with the other reagents and are designed to be homologous with the DNA region in which a mutation is to be searched for. Although these methods are rapid, high specificity, high sensitivity but still require expensive instrument and technically interpretation.

TaqMan probes

The TaqMan probes are fluorescence-labeled at the 5'-end and include a fluorescence quencher at the 3'-end. The probe anneals to the amplified target in each of the annealing steps of the PCR. While it is bound to its homologous sequence, the real-time PCR instrument does not measure fluorescence, because the quencher absorbs the energy of the dye. In the elongation step of the PCR, the 5'-3'exonuclease activity of the *Taq* polymerase releases the fluorescent dye which enhances the emission of fluorescence owing to the separation of the quencher from the dye. This fluorescence is measured by the real-time PCR instrument. Of note is the fact that fluorescence is only emitted if the probe is correctly anchored to its homologous site to allow the *Taq* polymerase to release the dye. This is the basis for the detection of mutations using TaqMan probes; if a mismatch (mutation) impairs the binding of the probe, the *Taq* polymerase would not meet the probe and the fluorescense would remain quenched. Therefore, the absence of fluorescence indicates the presence of a mutation in the genetic region covered by the probe. One study has used this kind of probe to detect resistance mutations for RIF and INH in *M. tuberculosis* in cultured strains and clinical samples (149) obtaining a 100% specificity and a sensitivity of 97% and 40%, for cultured strains and clinical samples, respectively. A requirement of real-time PCR assays using TaqMan probes is the addition to the PCR reaction of internal amplification controls, with their respective complementary probes, to guarantee that the absence of fluorescence is due to the existence of a mutation and not to deficiencies in the PCR.

Molecular beacons

Current methods of analyzing DNA sequences have been restricted largely to research and specialized clinical laboratories due to their technological complexity and cost. An approach to DNA sequence analysis is simple to perform, robust and accurate. This method is based on the sequence-dependent hybridization of fluorogenic reporter molecules called molecular beacons in real-time PCR. A set of molecular beacons, each specific for a different target sequence within an amplicon and each overlapping a neighboring target sequence, is used to probe the sequence of that amplicon. Molecular beacons are single-stranded nucleic acid molecules that

posses a stem-and-loop structure. The loop portion of the molecules serves as a probe sequence that is complementary to a target nucleic acid. The stem is formed by the annealing of two complementary arm sequences that are on either side of the probe sequence. A fluorescent moiety is attached to the end of one arm and a nonfluorescent quenching moiety is attached to the end of the other arm. The stem hybrid keeps the fluorophore and the quencher so close to each other that fluorescence does not occur. When the molecular beacon encounters a target molecule, it forms a probe-target hybrid that is stronger and more stable than the stem hybrid. The probe then undergoes a spontaneous conformational reorganization that forces the arm sequences apart, separating the fluorophore from the quencher and permitting the fluorophore to fluoresce. The power of molecular beacons lies in their ability to hybridize only to target sequences that are perfectly complementary to the probe sequence, to contain different colored fluorophores and to detect PCR reaction products in hermetically sealed tubes (150). Internal amplification controls must be added to the PCR to determine whether the reduction in fluorescence is really due to mutations, or alternatively, to amplification inhibitions.

Beacons were first adapted for the detection of RIF-resistant *M. tuberculosis* (150) by including independent probes in different reaction tubes. Recently, a version of these beacons, called wavelength-shifting molecular beacons, allows each probe to be labeled with different dyes which permit the simultaneous analysis of the whole *rpoB* region in a single reaction tube by following the reaction through independent channels in the real-time instrument (20).

Varma-Basil *et al.* (151) reported the single-well molecular beacon assay used five molecular beacons, each hybridizing to a different target segment within the *rpoB* core region and each labeled with a differently colored fluorophore. Each molecular beacon was designed to be so specific that it could not bind to its target if the target sequence differed from the wild-type *M. tuberculosis rpoB* sequence by even a single nucleotide substitution. Because molecular beacons fluoresce only when they are bound to their targets, the absence of fluorescence from any fluorophore in the assay indicates the presence of a mutation and thus predicts RIF resistance. The molecular beacon assay appears to have sensitivity and specificity of 89% and 99% respectively.

The molecular beacon assays have significant advantages over other techniques in such a way that it can detect unknown mutations in the *rpoB* region, the assay is simple, rapid, specific, and highly sensitive. Amplification, molecular beacon hybridization, and analysis are all performed simultaneously in sealed wells. The entire assay, including analysis, can be completed in less than 3 hrs (152). However the molecular beacon assay requires expensive equipment that is not yet widely available. The main limitation of the assay is that it is unable to detect RIF resistance caused by mutations outside of the *rpoB* core region. In addition, a potential problem of the assay is that it would be expected to detect silent *rpoB* mutations when they were present and would falsely identify such mutants as RIF resistant. Silent mutations are exceedingly rare in *M. tuberculosis* (153), thus, this problem does not have an important effect on specificity.

Fluorescence-resonance-energy transfer (FRET) probes

The FRET probes are paired probes which are designed to anneal to the DNA template in a head-to-tail orientation with each probe close to the next. One probe (anchor) is fluorescence-labeled at its 3'end and the adjacent probe (sensor) is labeled at 5' with a dye which can be excited by the fluorescence of the anchor. The sensor probe is designed to overlap with the mutation which is to be searched for. When the FRET probes are correctly bound to their homologous target at the end of the annealing step of the PCR, a process of energy transfer enables emission of the fluorescein to excite the dye in the adjacent probe. The fluorescence emission from the sensor probe is measured by the real-time instrument. This energy transfer only occurs if the distance between the probes is 1–5 nucleotides, which is only the case if the two probes are specifically bound to their homologous target.

In this format, the mutation analysis is different than that explained for TaqMan or beacon probes, and this analysis is performed in a melting step at the end of the PCR. This melting step involves a slow increase in temperature (0.05–0.2°C/ s) to release the sensor probe from its target and thus monitor the melting of the probes by measuring the progressive reduction in fluorescence emission. The analysis software represents the melting of the probe as a melting peak with a melting temperature value (Tm). This melting temperature is constant for a pair of probes when bound to the wild

type sequence. If a mutation maps in the region covered by the probe, the mismatch between the probes and the template leads to a decrease in the Tm of the probes. Therefore, deviations in the Tm of the probes with respect to the reference wild type value correspond to the presence of a mutation in the region covered by the probe.

The studied of Garcia de Viedma *et al.* (29) offered a simplified model for detecting, in a single reaction tube, all the mutations related to RIF-resistance and the most prevalent for INH-resistance with only three pairs of FRET probes and two independent measurement channels. This was achieved by a design of FRET probes with both paired probes acting as mutation sensors, different from the conventional anchor-sensor design. This design succeeded in detecting 14 different mutations affecting nine codons in the same reaction. This could serve as a reference for adapting real-time PCR to the detection of multiple mutations using simple and less expensive designs which are suitable for different geographic settings. A blind test was performed with a panel of 15 different susceptible and resistant strains from throughout Spain, and the results were also in 100% agreement with the sequencing data.

Unlike TaqMan or beacons, FRET probes do not require amplification controls in the reaction, because the detection of mutations is not based on the presence or absence of a fluorescent signal but rather, on deviations in the Tm values of a positive amplification signal.

Biprobes

Biprobes are single probes which are fluorescently labeled at the 5'end. In real-time PCR, biprobes are included in the reaction mix together with Sybr-Green which is a dye that emits fluorescence when it binds to double-strand DNA. Thus, at the end of each annealing step of the PCR, the Sybr-Green excites the probe fluorophore by energy transfer, emitting fluorescence which can be measured by the real-time PCR instrument. The analysis of mutations involves monitoring of melting as was explained for FRET probes. Deviations in the Tm of the biprobe indicate the presence of mutations. In one report, this kind of probe correctly assigned all mutations in 46 RIF-resistant strains by using three independent biprobes which involved the performance

of three independent reactions (30). But this method is low specitivity due to the Sybr-Green may bind non-specific DNA when the system is contaminated.

5.2.3 Electrophoresis-based techniques

The different electrophoresis-based approaches search for mutations after analyzing the electrophoretic mobility of DNA fragments which include the genetic regions involved in resistance are developed. Mobility shifts in these DNA fragments, when compared with the mobility of wild type and reference sequences, indicate the presence of mutations. In order to facilitate the detection of mobility shifts, these methods analyse DNA structural variants (single-strand, heteroduplexes) and select special electrophoretic conditions (pH gradients, denaturing gels).

PCR-single strand conformation polymorphism assay (PCR-SSCP)

The PCR-SSCP assay is based on the conformational distortion that a nucleotide substitution can cause in a single-strand DNA fragment. It is based on the property of single-stranded DNA to fold into a tertiary structure whose shape depends on its sequence. Single strands of DNA differing by only one or a few bases will fold into different conformations with different mobilities on a gel, producing what is called a single strand conformation polymorphism (SSCP). The conformational change leads to an electrophoretic mobility different to that of the wild type single-strand fragment. The procedure involves amplification by PCR of DNA fragments including the region of interest, denaturation of these fragments, and running it in a polyacrylamide gel together with the denatured wild type reference sample. Mobility shifts in the clinical sample would indicate the presence of a mutation in the region analyzed.

The advantages of this method are that it is inexpensive, easy and quick. The ability to perform SSCP in a nonradioactive, automated fashion will use to screen a large number of isolates in 24 hrs. It has frequently been used to search for resistance mutations against RIF and INH (154), with higher sensitivity for RIF-resistant than INH-resistant mutations. Trials to adapt this technique to other drugs with more genetically complex mechanisms of resistance such as PZA or EMB have failed (155), (156).

Some authors obtained positive results by using SSCP directly with clinical samples, especially to rule out the presence of mutations in susceptible cases (157). However not all nucleotide changes are well detected by SSCP, depending on the substitution and the relative position of the affected codon within the amplified fragment. In addition, interpretation is difficult and ambiguous and only under extreme electrophoretic conditions do some mutant strains.

Telenti *et al*. (158) reported some potential limits to PCR-SSCP in terms of sensitivity. It was apparent from their data that a significant load of *M. tuberculosis* should be present in the sample to unequivocally establish the RIF genotype. In addition, if there was a mixed population of resistant and susceptible organisms, the minority strain should probably represent more than 15% of all organisms in the sample for SSCP to identify the presence of a mixed pattern.

Furthermore Bobadilla del Valle M. *et al.* (154) revealed that the practical implications of their study were that the PCR-SSCP method might not be a reliable tool for the detection of resistance to RIF in *M. tuberculosis*. Due to the sensitivity of the assay was 31.4%, although its specificity was 100%.

PCR-heteroduplex fragment assay (PCR-HDF)

Another method of detecting mutations based on electrophoretic band-shifts is the detection of heteroduplexes in polyacrylamide gels. The technique depends on the PCR amplification of the region of interest in samples. This amplicon from the samples is denatured and then mixed with an equivalent amount of a denatured amplicon from a reference wild type strain or, in other designs, from a universal heteroduplex generator (159). The mixture of amplicons is allowed to cool rapidly to reconstitute double strand DNA-renatured molecules. If the clinical strain has no mutations in the region analyzed, the homology between the single strands of the samples and reference amplicons would be complete, with the result that the renatured duplexes would be identical to the clinical and reference original DNA homoduplexes. On the contrary, if a mutation exists in the clinical amplicon, heteroduplexes could be obtained by the renaturation of one strand from the sample mutant amplicon and another from the reference strain. These heteroduplexes have an electrophoretic mobility different than the original amplicons.

Williams *et al.* (159) showed that the PCR-HDF was used to detect all RIF resistant strains having mutation within the 305-bp region of the *rpoB* gene. The sensitivity and specificity for the assay were 83% and 98.2% respectively. This assay could be used cost-effectively, a short turnaround time of 72 hours (160). But this method is still difficult to interpret and sometimes provide ambiguous result.

Some authors improved the sensitivity of the detection of heteroduplexes by performing special electrophoresis techniques such as double-gradient denaturing gradient gel electrophoresis (DG-DGGE) which used a double gradient (temperature and polyacrylamide) (161). A modification of the heteroduplex assay is RNA/RNA mismatch assay, in which the detection of heteroduplex is performed on RNA hybrid detected after treatment with RNAse, which cleaves the heteroduplex where there is a mismatch. The RNA/RNA mismatch assay can return a result within 24 hrs. The specificity and sensitivity of the mismatch assay in detecting RIF resistance were 100% and 96%, respectively when tested against 46 RIF-resistant and RIF-susceptible strains of *M. tuberculosis* (162). However, the mismatch assay utilizes a transcription step and subsequent manipulation of RNA, although with proper precautions to avoid RNase contamination, this should cause a problem.

5.2.4 Hybridization-based techniques

Hybridization-based techniques comprise a heterogeneous group. Some of them are performed on PCR assays which include specific DNA probes, and others are conventional hybridization assays in solid or liquid formats. All of them are based on the hybridization of two strands of DNA, one from the clinical strain and the other from the consensus probe or oligonucleotides. The hybridization of the two strands is highly stable when it involves fully complementary sequences, if a mismatch occurs in some nucleotide position, owing to genetic differences between the clinical strain and the consensus sequence, the binding of the two strands is thermodynamically impaired. In order to appreciate more clearly the peculiarities of these techniques, all PCR-based assays using hybridization probes have been grouped under the heading ‘real-time PCR’ as described above, and in this section only the immobilized hybridization formats are reviewed.

Hybridization on strips

One adapted hybridization design for the detection of resistance to RIF involves the use of commercial membrane strips in which a set of ten oligonucleotide probes are immobilized (INNOLiPA; Innogenetics, Gent Belgium). The LiPA test is line probe assay. Five of these probes are homologous with five subregions which together constitute the *rpoB* core region, four other probes are designed to be homologous with frequent RIF-resistant mutations, and another probe is an amplification control for the *M. tuberculosis* complex. The method follows a reverse-hybridization design in which the labeled probe is constituted by the amplicon of the *rpoB* core region obtained from the clinical sample by PCR. The label (biotin) is incorporated in the amplicon during the PCR. The labeled amplicon is hybridized with the immobilized probes under stringent conditions. The hybridized complexes are detected by incubation with peroxidase-steptavidine and subsequent addition of a chromogen. If any of the five probes which are homologous with wild type sequences fail to hybridize, a mutation is suspected for the region covered by that probe. Furthermore, if any of the probes that are homologous with a mutation give a positive hybridization signal, it is possible to specify the mutation responsible for the resistance.

The technique is simple and needs no expertise in molecular biology, but it does require a pre-PCR step to amplify and label the clinical amplicon, and a subsequent hybridization step, which requires a specific device. Its application to clinical isolates shows a good correlation with DNA sequencing (only 2-4% of discrepancies with DNA sequencing and phenotypic assays) (163), as well as a good performance directly on clinical samples (164). With regard to the ability to assign the specific resistance mutation, a number of cases have been reported in which certain mutants, frequently insertions, could not be detected (163).

In contrast, Morgan *et al*. (165) revealed the application of LiPA to diagnose RIF-resistant TB within 24 to 48 hours of sample collection, but the test had relatively lower sensitivity when used directly on clinical specimens. The LiPA is a highly sensitive and specific test for the detection of RIF resistance in culture isolates. Twelve of 14 studies that applied LiPA to isolates had sensitivity greater than 95%, and specificity of 100%. The four studies that applied LiPA directly to clinical specimens had 100% specificity, and sensitivities that ranged between 80% and 100%.

The LiPA has shown a high degree of accuracy when used on culture isolates, but this requires 2 to 6 weeks for primary isolation. Moreover the cost of the commercial LiPA kit is US $45 per sample tested. When additional costs for import and transport are taken into account, the actual cost per sample is as high as US $116. This may be prohibitively expensive for routine use in the regions of the world with the highest prevalence and incidence of TB and MDR-TB.

Hybridization in microplates

A PCR-enzyme-linked immunosorbent assay (PCR-ELISA) has also been developed for the detection of RIF-resistant strains (33). In this assay, five independent oligonucleotides, homologous to the corresponding wild type sequences, which cover the core *rpoB* gene, were designed as capture probes, and immobilized to the wells of a microtiter plate. The *rpoB* region from the clinical isolate is amplified and labeled with digoxygenin and hybridized to the immobilized probes. After developing, positive signals correspond to amplicons lacking a mutation in the region covered by the probe and negative signals indicate the presence of a mutation in that region. This format was tested successfully with a collection of clinical strains and also directly on clinical samples.

The PCR-ELISA was able to detect as little as 100 fg of purified *M. tuberculosis* DNA per amplification reaction and the equivalent of 10^3 CFU/ml in spiked culture-negative sputum. The PCR-ELISA is therefore sufficiently sensitive not only for detection of RIF resistance in cultured organisms but also for direct detection in clinical specimens. The PCR-ELISA is specific for *M. tuberculosis* complex, thereby avoiding misinterpretation of results due to amplification of nontuberculous mycobacteria present in the sample.

The PCR-ELISA utilizes commonly available reagents and equipment and is simple to perform, requiring only a basic understanding of molecular techniques. The entire procedure, including DNA extraction, PCR amplification and ELISA detection, can be performed in a single working day. However the PCR-ELISA cannot identify the specific mutation causing RIF resistance but does indicate the region in which the mutation is located. This is an important disadvantage of the method, in case of silent mutation that not determining to RIF resistance.

5.2.5 Allele-specific multiplex PCR

Some authors have developed PCR assays designed to allow the direct discrimination of certain nucleotidic substitutions at specific codons. These designs are multiplex PCR which include outer primers in the reaction to generate the amplicon for analysis, and inner primers, which are designed to alternatively bind one or the other, depending on the substitution found at a certain codon. The codon in the analysis is placed asymmetrically in the amplicon, causing the length of the PCR fragments to vary, according to the direction of the inner primer which binds to the codon in analysis.

Mokrousov *et al*. (19) reported the allele-specific multiplex PCR (MAS-PCR), which has been successfully assayed to assign resistance to RIF associated with the 81-bp hot spot region of *rpoB*. The MAS-PCR method in their design used two outer primers that flank a region under study and invariably anneal on the conserved DNA targets, plus a wild type-allele specific inner primer that stops in its 3'-end at the targeted codon and amplifies a wild type-allele specific fragment. An alteration of the base that corresponds to the 3'-end of the specific primer causes the primer template mismatch that prevents polymerase to extend the primer and results in nonamplification of the indicative fragment. Notably, *rpoB* mutations in three codons namely 516, 526 and 531 were accounted for the majority of RIF-resistant strains that covered for 70% to 95%. These three respective codons were experimented by individually three reaction tubes. The MAS-PCR method was used for analysis of purified DNA preparations from 287 RIF-resistant *M. tuberculosis* isolates that recovered from unlinked patients, collected from 1996 to 2002. The results revealed the MAS-PCR could identify 247 of 287 RIF-resistant isolates (86.1%) which demonstrated mutations in one of the analyzed *rpoB* codons. In this study they reported a simple, rapid, and inexpensive assay based on the allele-specific PCR methodology targeting *rpoB* gene mutations to detect RIF-resistant *M. tuberculosis*. However this MAS-PCR method required three reaction tubes for identification of one sample that was experimentally complicated.

Many new possibilities have emerged for the detection of drug resistance in *M. tuberculosis* and for performing drug susceptibility tests. These novel methods rely on new information concerning molecular mechanisms of drug resistance or in new

approaches in detecting mycobacterial growth. The genotypic methods have the advantage of being rapid and specific. However, not all of the molecular mechanisms of drug resistance are known; hence the current molecular tools cannot detect all resistant strains. The phenotypic methods are more diverse; some of them though simple in their procedure, still require expensive equipments, not always available in laboratories of TB endemic countries. Some of them are very useful, being low-tech and simple but take a long time that can be routinely used in developing countries, while others need further evaluation and validation to obtain acceptable levels of sensitivity, specificity and reproducibility before they replace the current drug susceptibility test procedures. It is also to be noted that most of the new techniques described involve prohibitive expenditure in terms of instrumentation, expertise and reagents, putting them out of reach of many laboratories in developing countries.

CHAPTER III
MATERIALS AND METHODS

MATERIALS

1. *Mycobacterium tuberculosis* isolates and mycobacterial reference strains

Two hundred and sixteen clinical isolates of *M. tuberculosis*, which were obtained from the Molecular Mycology and Mycobacteriology Laboratory, Drug Resistant Tuberculosis Research Fund, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, were used in the study. They comprised of 113 RIF-susceptible strains and 103 RIF-resistant strains determined by the standard proportion method (see Appendix). *M. tuberculosis* H37Rv was used as the reference susceptible strain. The susceptibility patterns and source of the RIF-resistant isolates were shown in Table 2. In addition, some RIF-resistant clinical isolates were selected and used as the reference mutant strains of each codon for optimizing the developed PCR method as listed in Table 3. All RIF-resistant strains had sequencing data of the *rpoB* gene hot spot region (see Appendix) except strains S1, S4, S7, S9, and RIF 163.

Thirty-seven mycobacterial *rpoB* sequences were used for *in silico* primer design by multiple alignments of ClustalW program as listed in Table 4. Twenty-five mycobacterial reference strains were used for determining the specificity of the developed PCR method as listed in Table 5.

Table 2 The susceptibility patterns of RIF-resistant *M. tuberculosis* isolates

Source of clinical isolates	Susceptibility pattern						Total
	Sensitive	Resistant					
		R	HR	HRS	HRE	HRSE	
Sputum	108	5	40	14	7	28	202
Lymph node	3	_	2	1	_	3	9
Pus	2	_	_	_	_	_	2
Pleural fluid	_	_	1	_	_	_	1
Abscess	_	_	_	1	_	_	1
Unspecified	_	_	_	_	1	_	1
Total	113	5	43	16	8	31	216

H, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol

Table 3 RIF-resistant *M. tuberculosis* clinical isolates used for optimization of each codon

Strain	Use for optimization of respective codons			
	codon 511	codon 516	codon 526	codon 531
S1			+	
S4		+		
S7				+
S9			+	
RIF 049				+
RIF 167	+	+		
RIF 172		+		
RIF 182			+	

Table 4 List of 37 mycobacterial *rpoB* sequences used for *in silico* primer design

No.	Mycobacteria	Accession number
1	*M. abscessus*	AY147164
2	*M. africanum*	AY271330
3	*M. asiaticum*	AF057455
4	*M. aurum*	AF057456
5	*M. avium*	AF060366
6	*M. bovis* BCG	AF057453
7	*M. celatum*	AY271337
8	*M. chelonae*	AY271898
9	*M. flavescens*	AF057463
10	*M. fortuitum*	AY147173
11	*M. gastri*	AF057466
12	*M. genavense*	AY271338
13	*M. gordonae*	AY271341
14	*M. haemophilum*	AY271331
15	*M. interjectum*	AF057470
16	*M. intracellulare*	AY271328
17	*M. kansasii*	AY271333
18	*M. leprae*	AF057474
19	*M. malmoense*	AF057475
20	*M. marinum*	AF057476
21	*M. microti*	AY271315
22	*M. mucogenicum*	AY147171
23	*M. neoaurum*	AF057477
24	*M. nonchromogenicum*	AF057478
25	*M. peregrinum*	AF057481
26	*M. phlei*	AF057480
27	*M. scrofulaceum*	AF057482
28	*M. senegalense*	AF057483
29	*M. shimoidei*	AF057486
30	*M. simiae*	AY271339

Table 4 List of 37 mycobacterial *rpoB* sequences used for *in silico* primer design (continued)

No.	Mycobacteria	Accession number
31	*M. smegmatis*	AY271345
32	*M. szulgai*	AF057487
33	*M. terrae*	AF057488
34	*M. thermoresistibile*	AF057489
35	*M. triviale*	AF057490
36	*M. ulcerans*	AY271899
37	*M. xenopi*	AY271332

Table 5 Mycobacterial reference strains used for assessment of specificity

No.	*Mycobacterium* spp.	Strain
1	*M. austroafricanum*	ATCC 3005
2	*M. avium*	ATCC 700898
3	*M. avium*	212
4	*M. bovis* BCG	ATCC 35735
5	*M. bovis* BCG	KK Tokyo 12-02
6	*M. chelonae*	ATCC 23016
7	*M. duvalii*	MNC 442
8	*M. flavescens*	ATCC 23035
9	*M. fortuitum*	ATCC 23048
10	*M. gordonae*	330
11	*M. gordonae*	ATCC 11470
12	*M. intracellulare*	ATCC 13950
13	*M. kansasii*	ATCC 12478
14	*M. kansasii*	302
15	*M. marinum*	329
16	*M. marinum*	ATCC 927
17	*M. nonchromogenicum*	ATCC 19530
18	*M. scrofulaceum*	ATCC 19981
19	*M. simiae*	ATCC 25275
20	*M. smegmatis*	ATCC 16941
21	*M. szulgai*	JATA 3201
22	*M. szulgai*	352
23	*M. terrae*	ATCC 15755
24	*M. tuberculosis*	H37Ra
25	*M. tuberculosis*	H37Rv

ATCC; American Type Culture Collection, Rocksville, Maryland

JATA; Japan Anti-Tuberculosis Association, Kiyose-Shi, Tokyo

KK; Mycobacteria Collection, Research Institute of Tuberculosis, Tokyo, Japan

MNC; Mycobacteria Nocardia Collection, Copenhagen, Denmark

2. Purified *Mycobacterium tuberculosis* DNA

Four *M. tuberculosis* strains, which were susceptible to four first-line antituberculous drugs (INH, RIF, EMB and STR), were used in this study (Table 6). DNAs from these strains were isolated by using the enzymatic method (166) and kindly provided by Ms. Jutaporn Yorsangsukkamol.

Table 6 Purified DNAs of susceptible strains used in the study

***M. tuberculosis* strains**	**Source of the strains**
43-06042	Cerebrospinal fluid
43-19186	Cerebrospinal fluid
45-08716	Cerebrospinal fluid
T2-SCMI-0019	Sputum

3. Molecular-size markers

3.1 25-bp DNA ladder — Invitrogen, U.S.A

3.2 Allele-specific marker — This study

4. Instruments

4.1 Autoclave — Huxley Speedy HL-340, Taiwan

4.2 Automatic pipette — Gilson, France

4.3 Biological safety cabinet class II, Gelman BH2000 series — Gelman Sciences, Australia

4.4 Freezer -20°C — Sanyo, Thailand

4.5 Gel electrophoresis apparatus — Mupid II, Japan

4.6 GeneSnap UV transilluminator — Syngene, Canada

4.7 PTC-200 DNA Engine thermal cycler — MJ Research, Massachusetts, U.S.A

4.8 Touchgene Gradient thermal cycler — Techne, Cambridge, U.S.A

4.9 Vortex mixer, Genie-2^{TM} — Scientific Industries, U.S.A

5. Microbiological media

5.1 Löwenstein-Jensen medium — BBL, U.S.A

6. Chemical

6.1 Acetic acid — Merck, Germany

6.2 Bromophenol blue — GE Healthcare Bio-science, U.S.A

6.3 EDTA	Merck, Germany
6.4 Glycerol	Ajax Finechem, Australia
6.5 Hydrochloric acid (HCl)	Merck, Germany
6.6 Rifampicin	BBL, U.S.A
6.7 Sodium hydroxide (NaOH)	Merck, Germany
6.8 Tris	Amresco, Ohio, U.S.A
6.9 Reagents for PCR amplification	
6.9.1 Deoxynucleoside triphosphates (dNTPs)	Fermentas, U.S.A
6.9.2 KCl buffer	Fermentas, U.S.A
6.9.3 $MgCl_2$	Fermentas, U.S.A
6.9.4 Primers	Bio Basic Inc., Canada
6.9.5 *Taq* DNA polymerase	Fermentas, U.S.A
6.10. Reagents for gel electrophoresis	
6.10.1 Ethidium bromide	Bio Basic Inc., Canada
6.10.2 Nusieve 3:1 agarose	Cambrex Bio Science, Inc., U.S.A
6.10.3 Agarose	Research organics, Inc., U.S.A

7. Miscellaneous

7.1 Filtered tip	Axygen scientific Inc., U.S.A
7.2 Microcentrifuge tube 0.2 ml	Axygen scientific Inc., U.S.A
7.3 Microcentrifuge tube 1.5 ml	Axygen scientific Inc., U.S.A
7.4 Pipette tips	Axygen scientific Inc., U.S.A

METHODS

1. Isolation of mycobacterial DNA from cultures

This study required both RIF-resistant and RIF-susceptible *M. tuberculosis* isolates for optimizing the one-tube multiplex semi-nested PCR. Hence to assure that the isolated DNAs were obtained from resistant strains, the RIF-resistant *M. tuberculosis* strains were cultured on Löwenstein-Jensen media (LJ) containing RIF with the final concentration of 40 μg/ml (123). The RIF-susceptible strains were cultured on Löwenstein-Jensen media without the drug. The cultures were incubated at 37°C for 3-4 weeks or until the colonies appeared.

Mycobacterial DNA was isolated by using the boiling method modified from previously studies (10), (167). One loopful of *M. tuberculosis* colonies (3-4 weeks) grown on plain Löwenstein-Jensen medium (LJ) or LJ containing 40 μg/ml RIF was suspended into 200 μl of TE buffer in the 1.5 ml microcentrifuge tube and mixed well by vortexing. The suspension was boiled for 20 minutes to kill viable mycobacteria and to induce cell lysis, and then centrifuged at 13,000 rpm for 2 minutes. The supernatant was transferred to a new sterile tube and stored at -20°C until used for PCR.

2. Optimization of the one-tube multiplex semi-nested PCR

2.1 Primers design and assessment of their specificity

The *rpoB* gene sequence of *M. tuberculosis* covering the 81-bp hot spot region, which mostly contains mutations confering RIF-resistance, was selected and used as target for the developed PCR method. Two primers, namely RPOF/RPOR, based on the sequence flanking the 81-bp hot spot region was initially designed as outer primers and generate the 149-bp amplification products. Subsequently, the reverse primer namely RPOR-1 was designed to improve A531F/RPOR amplification. The primers RPOF/RPOR-1 should generate the 196-bp amplification products. In addition, these outer primers were designed and used for function as the internal control for amplification.

DNA was synthesized from 5’ to 3’ direction by reading DNA template in 3’ to 5’ direction and adding complementary nucleotides. Each wild type-allele specific inner primer was, therefore, designed by that the 3’-end of each allele-specific primer was positioned to pair with the second base of the targeted codon. Thus, in the absence of mutation at the corresponding codon, the wild-type allele-specific fragment would be amplified whereas none of allele-specific PCR products would be generated, if there is a mutation at the targeted codon(s).

In this study, the primers specific to the wild type codons 531, 526, 516 and 511 within the 81-bp hot spot region of the *M. tuberculosis rpoB* gene were selected and used as the allele-specific inner forward primers, namely A531F, A526F, A516F and

A511F, respectively (Figure 21). These inner forward primers were initially paired with the RPOR reverse primer for each amplification and provided 68-, 82-, 115- and 129-bp PCR products, respectively (Table 7, Figure 21). On the other hand, the inner forward primers were subsequently paired with the RPOR-1 reverse primer for each amplification and provided 115-, 129-, 162- and 176-bp PCR products, respectively (Table 7, Figure 21). The RPOF and A526F primer was originally described by Mokrousov *et al*. (19). The A531F and A516F primers were slightly modified from Mokrousov *et al.* (19) by deleted the first base at the 5'-end of the primers to adjust the melting temperature (Tm). The remaining primers were designed in this study. All designed inner forward primers should have similar Tm and the primer sequences were finally examined *in silico* with BLAST program (www.ncbi.nlm.nih.gov) for determining their specificity. The primers were also tested *in vitro* with DNAs from twenty-five strains of reference mycobacteria as list in the Table 5. By the way, primers TR9 and TR2b that were described by Telenti *et al* (27), were used for sequencing covered hot spot region of *rpoB* gene to identification of reference RIF-resistant strains which were known individually all four mutations namely codons 531, 526, 516 and 511 (Table 7).

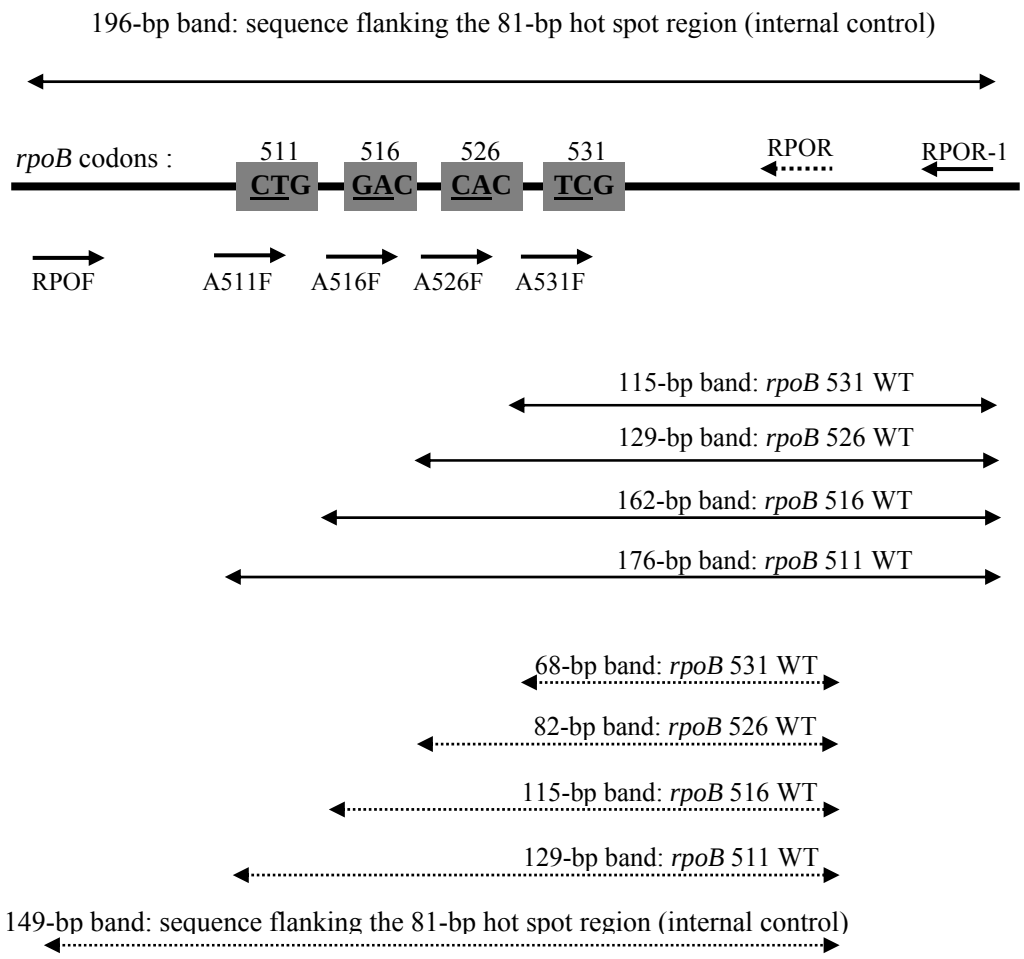

Figure 21 Schematic view of the *rpoB* gene fragment amplified by different pairs of primers. Short arrows depict the primers, long double-headed arrows represent PCR fragments; lengths not to scale. The targeted *rpoB* codons were in shaded boxes; the mutated bases were underlined.

Table 7 Oligonucleotide primers used for amplification

Primer		Tm (°C)	Type	Reference
RPOF	(5'-TCGCCGCGATCAAGGAGT 3')	58	forward primer	(27)
A531F	(5'-CAAGCGCCGACTGTC 3')	50	forward primer	Modified from (19)
A526F	(5'-GTCGGGGTTGACCCA 3')	50	forward primer	(19)
A516F	(5'-CTGAGCCAATTCATGGA 3')	50	forward primer	Modified from (19)
A511F	(5'-CGGCACCAGCCAGCT 3')	52	forward primer	This study
RPOR	(5'-GCGGACCTCCAGCCCGG 3')	60	reverse primer	This study
RPOR-1	(5'-GGGGTTTCGATCGGGCA 3')	56	reverse primer	This study
TR9	(5'- TCGCCGCGATCAAGGAGT 3')	58	forward primer	(27)
TR2b	(5'-TACGGCGTTTCGATGAACC 3')	58	reverse primer	(27)

2.2 *rpoB* sequencing for identification of reference RIF-resistant strains

The RIF-resistant *M. tuberculosis* strains DNAs were amplified to yield a 277 bp fragment of *rpoB* gene. The amplification reactions were performed in a final volume of 50 µl containing 2.5 mM $MgCl_2$, 50 mM KCl, 1 U of *Taq* DNA polymerase, 200 µM deoxynucleoside triphosphates (dNTPs), 10 pmol TR9 (5'-TCGCCGCGATCAAGGAGT-3'), 10 pmol TR2b (5'-ATGCCGCAAAGCTACTTGG-3') and 5 µl DNA template. The PCR reactions were performed in a PTC-200 DNA Engine thermal cycler under the following conditions: initial denaturation at 94°C for 5 minutes; 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and final elongation at 72°C for 10 minutes. The presence of the 277 bp PCR product was analyzed by mixing 5 µl of the amplified product with 3 µl of loading dye solution in a 2.5% agarose gel. The gel was run at 100 volt for 25 minutes, stained with ethidium bromide and photographed. The 277-bp PCR products were subjected to sequencing by previously performimg with Gel Extraction Kit (NucleoSpin® Extract II, Macherey-Nagel, USA) by the protocol recommended by the manufacturer.

2.3 Optimization of the one-tube multiplex semi-nested PCR

At first all five primer pairs were optimized separately and multiplex PCR was subsequently performed. The PCR condition and profile were adjusted until the best condition was reached. If the PCR products were scarce, the conditions were adjusted by increasing the amount of primers and/or decrease annealing temperature. If non-specific products appeared, the condition was changed by increasing annealing temperature and/or decreasing amount of template and enzyme. If all adjustments failed, some additive such as glycerol was used. If all failed, fresh dNTPs were used. And the last option was reselection of primer sequences. The outline of optimization process was shown in Figure 22.

The optimization was initiated by performing simplex PCR of each primer pair using the same condition. Firstly, the simplex PCR using each primer pair was performed with 100 ng purified DNA of *M. tuberculosis* H37Rv. Annealing temperatures were varied from 41°C to 70°C by using the program of Touchgene Gradient DNA thermal cycler. A negative control (blank), which was the PCR mixture without DNA template, was required in all experiments. Ten picomoles of each primer were constantly used throughout these experiments. The simplex PCR was then tested with 15 RIF-resistant clinical isolates with known mutations at codons 531, 526, 516 or 511, namely RIF 77, RIF 116, RIF 152, RIF 153, RIF 160, RIF 166, RIF 167, RIF 171, RIF 172, RIF 174, RIF 182, RIF 185, RIF 196, RIF 202 and RIF 203 (for details see Appendix).

Furthermore, if the optimized PCR still did not generate specific products, Mg^{2+} concentrations (1.0, 1.5, 2.0, 2.5, 3.0 mM) and then primer concentrations (5, 10, 20 pmol) were subsequently adjusted. If all failed, the last option was reselection of primer sequences. After the simplex PCR was successfully optimized, a multiplex PCR with all five primer pairs was performed with the optimized condition used for the simplex PCR.

A set of clinical isolates contained eight strains with known mutations at each codon was used as reference resistant strains in the multiplex PCR for differentiation RIF-resistant from RIF-susceptible strains (see Table 3). Ten picomoles of primers A526F, A516F and A511F, were used for initial optimization whereas the remained primers A531F, RPOF and RPOR-1, were initially used with 20, 2.5 and 40 pmol,

respectively. The optimized multiplex PCR was initiated by using the PCR profile as same as that of the simplex PCR. If the used PCR profile was failed to generate a specific product, the following step was performed by changing PCR profile from the one PCR cycling profile (consisted of 35 cycles of 94°C 1 minute, 69°C, 1 minute and 72°C 1 minute) to two PCR cycling profile (consisted of 15 cycles of 94°C 1 minute, 69°C, 1 minute and 72°C 1 minute followed by 35 cycles of 94°C 1 minute, 57°C, 1 minute and 72°C 1 minute). The new PCR profiles was optimized by that the first PCR cycle round suitable for outer primers amplification and the second for inner primers amplification. Annealing temperature of both PCR cycle rounds were adjusted by initiating from melting temperature of outer primers and inner primers for first and second amplification, respectively. The annealing temperatures were varied from 41°C to 70°C by using the program of Touchgene Gradient DNA thermal cycler. In addition, annealing time, extension time and number of cycles were considered for the PCR profile optimizations.

Beside concomitantly annealing temperatures and PCR profile optimization, the PCR was optimized by varying primer concentrations (0.02-60 pmol), dNTPs concentrations (200, 400 μM) and *Taq* DNA polymerase concentrations (1, 2 U). Another way to improve PCR performance was the use of fresh PCR components. If all adjustments failed, some additive such as glycerol was used. The optimizations were performed by adding glycerol (10% final concentration) to the PCR mixture and compared a result with and without glycerol in each PCR reactions at various temperatures from 50°C to 70°C. But if all failed, the last option was reselection of primer sequences.

Moreover, type of buffers used with *Taq* DNA polymerase and type of DNA thermal cycler were evaluated in the study. The KCl buffer (Fermentas, U.S.A) and the $(NH_4)_2SO_4$ buffer (Fermentas, U.S.A) were compared by performing the same PCR reaction different only the used buffer. The buffer generated strongest PCR products after gel electrophoresis were chosen for using in further study. The type of DNA thermal cyclers by using the same PCR mixture and condition for amplification were also compared. The thermal cycler giving stronger amplification was selected and used throughout the study.

2.4 Detection of amplification products

The allele-specific marker was established in this study by combining all amplification products from the simplex PCR using five primer pairs. Each PCR product was determined by 4% agarose gel electrophoresis before mixing together as the allele-specific markers and stored at 4°C. The amounts per well were adjusted by using 5 µl per well. The largest amplified product was 196-bp and contained the 81-bp hot spot region of *rpoB* gene. The four remaining PCR products were specifically positioned for codons 511, 516, 526 and 531 which were 176-, 162-, 129- and 115-bp, respectively. The allele-specific markers were necessarily required for each gel as the internal marker of the respective codons.

The obtained amplified products were detected by 4% nusieve 3:1 agarose gel electrophoresis with Mupid II. The 300 ng of 25 bp DNA ladder and the 5 µl of the allele-specific markers were electrophoresed in every gel. Seven microliters of the amplified products were mixed with 3 µl of gel loading dye and electrophoresed in 17 wells-nusieve agarose gel at 100 V for 38 minutes or until the loading dye reached the lower edge of the gel. The gel was then stained with 1 µg/ml of ethidium bromide solution for 30 seconds. After destaining with distilled water each for 15 minutes, the amplification product was visualized and photographed by GeneSnap UV transilluminator using resolution of 900 ms. The size of amplified products were estimated by comparing with that of the markers.

For interpretation, the presence of all five specific bands were refered to wild type *M. tuberculosis* strain. In contrast, if the results reveal less than 5 bands, it refers to a mutant strain with a mutation located at the disappeared band. The 196-bp product was used as internal control band which should appear in both wild type and mutant strain in every amplification. The pre- and post-PCR amplification areas were separated to avoid the carry-over contamination. There was a separated set of supplies and pipetting devices dedicated for the specific handling of PCR products.

2.5 Determination of the detection limit

Detection limit of the multiplex PCR was determined with the 10-fold dilution of purified *M. tuberculosis* DNA from 100 ng to 10 fg. The purified DNA was obtained from *M. tuberculosis* wild type strain, which was susceptible to INH, RIF, EMB and

STR and was 10-fold diluted from originally 1 µg/µl. The multiplex PCR was performed using the optimized condition and was repeated at least three different occasions for reproducibility testing. The detection limit of the optimized PCR was the least DNA concentration that clearly generated all specific PCR products.

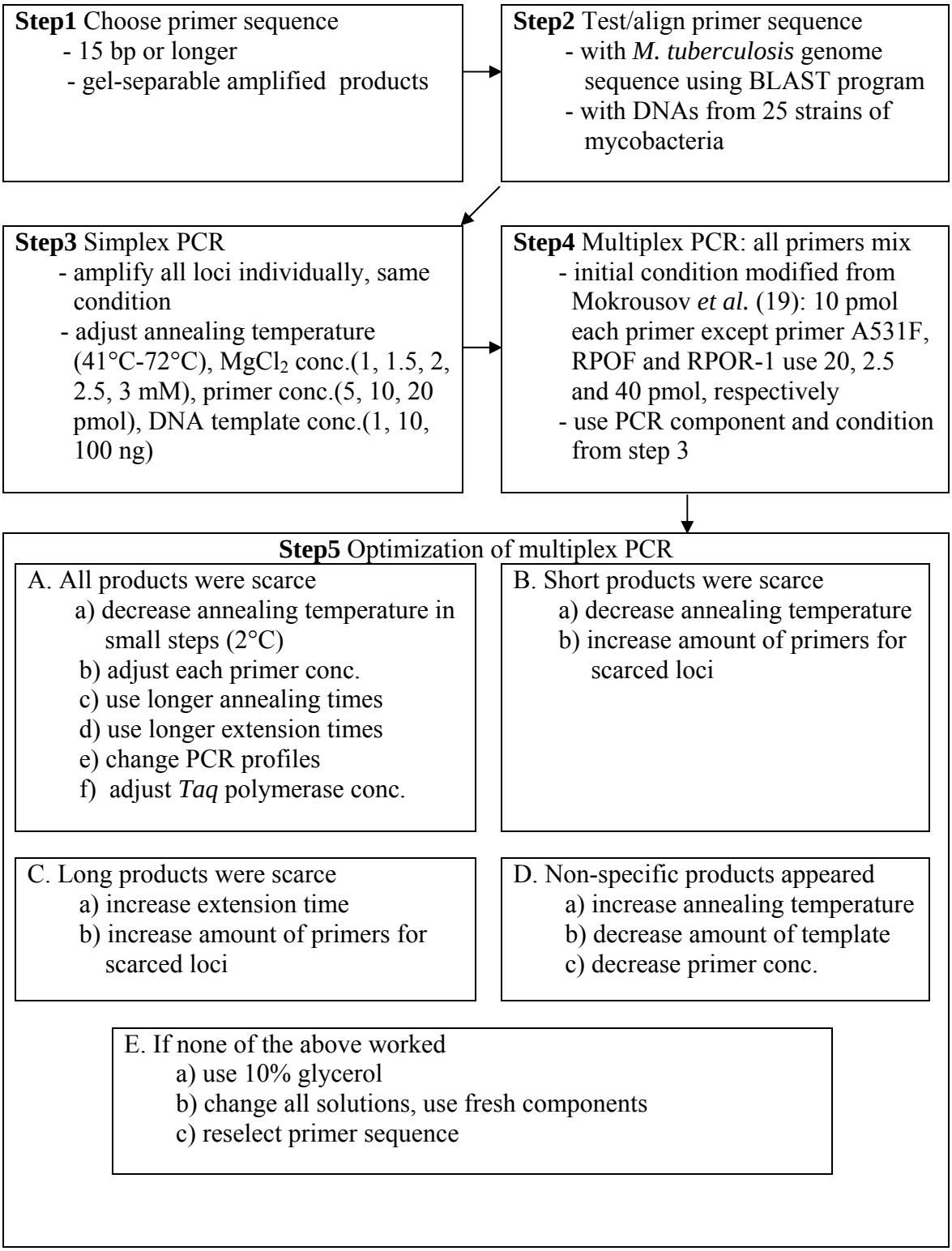

Figure 22 The outline of optimization processes for the one-tube multiplex semi-nested PCR.

3. Evaluation of the developed PCR method for detection of RIF-resistant *M. tuberculosis* clinical isolates

The optimized one-tube multiplex semi-nested PCR was tested with blind DNA samples obtained from 216 *M. tuberculosis* clinical isolates with known patterns of antimycobacterial susceptibility and type of mutations as shown in the Appendix. Of 216 *M. tuberculosis* isolates, there were 103 RIF-resistant and 113 RIF-susceptible *M. tuberculosis* isolates. Results of PCR and susceptibility testing were compared and the sensitivity, specificity, PPV and NPV were subsequently evaluated as follows.

%Sensitivity = [TP / (TP+FN)] x 100

%Specificity = [TN / (FP+TN)] x 100

% PPV = [TP/(TP+FP)] x 100

% NPV = [TN/(FN+TN)] x 100

TP, True positive; TN, True negative; FP, False positive; FN, False negative

4. Simulated mixed population of *M. tuberculosis*

The interpretation of susceptibility result according to the standard proportion method demonstrates resistance when an equal or more than 1% of the population shows the resistant phenotype to the drug tested (123). In general, isolation of *M. tuberculosis* from clinical samples always used the media without any drugs, resulted in a presence of mixed *M. tuberculosis* subpopulations. Therefore, this study was planned to evaluate the possible use of the developed PCR method for detection of the RIF-resistant *M. tuberculosis* subpopulation by determining the least percentage of the RIF-resistant *M. tuberculosis* subpopulation that could be detected by the developed method.

Two *M. tuberculosis* strains, susceptible *M. tuberculosis* H37Rv and RIF-resistant *M. tuberculosis* DS 10238 (mutation at codon 531), were used for this experiment. Cultivation of *M. tuberculosis* H37Rv was done on LJ medium without RIF whereas the resistant strain was cultured on LJ medium containing RIF. In addition, *M. tuberculosis* H37Rv was also cultured on LJ medium containing RIF as a control for drug quality and used microorganism. The cultures were incubated at 37°C for 3-4 weeks. The McFarland No.1 of *M. tuberculosis* H37Rv and RIF-resistant strains were prepared and mixed together in a total volume of 1 ml with the following

ratios, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 5:95, 2:98 and 1:99. All mixed *M. tuberculosis* strains were centrifuged at 12,000 rpm for 10 minutes. After the supernatant was removed by pipetting out, 50 µl of TE buffer was subsequently added and followed by vortexing for 30 seconds. The DNA was isolated by using the boiling method as previously mentioned. The experiment was independently repeated four times.

5. Evaluation of the developed PCR method for direct detection from automated MGIT cultures

The optimized one-tube multiplex semi-nested PCR was tested with 50 blind automated MGIT cultures. Of 50 samples, *M. tuberculosis* was isolated as 5 RIF-resistant and 45 RIF-susceptible isolates (Table 11 in Appendix). Results of PCR and susceptibility testing were compared and the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were subsequently evaluated as mentioned above.

CHAPTER IV
RESULTS

1. Primers design and assessment of their specificity

Sequences of the designed primers were initially *in silico* tested their specificity using the ClustalW program (http://www.ebi.ac.uk/clustalw). The *rpoB* DNA sequence covered the hot spot region of *M. tuberculosis* H37Rv (accession no. U12205) was aligned with the *rpoB* DNA sequences of 37 mycobacteria and *M. tuberculosis* complex DNA sequences. Locations of the seven primers, namely RPOF, A511F, A516F, A526F, A531F, RPOR and RPOR-1, within the *rpoB* gene were shown in Figure 23. All primer sequences were finally examined *in silico* with BLAST program (http://www.ncbi.nlm.nih.gov) for checking their specificity.

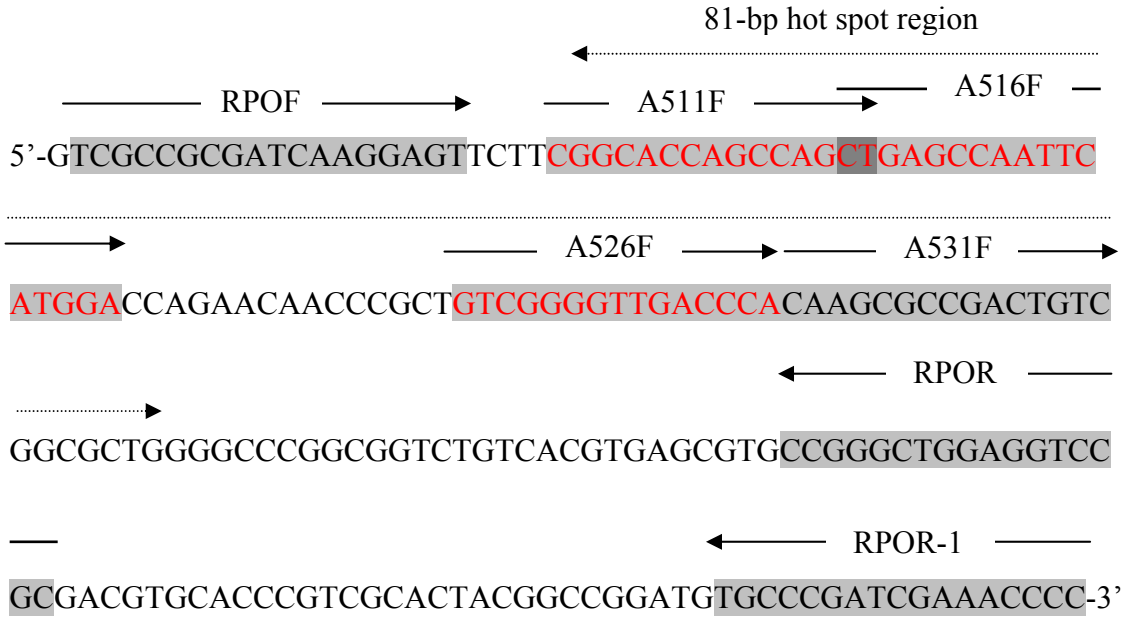

Figure 23 Seven primers used in the study namely RPOF, A511F, A516F, A526F, A531F, RPOR and RPOR-1 were positioned within the *rpoB* gene that covered the 81-bp hot spot region.

Results showed that beside *M. tuberculosis* complex, the outer primers RPOF and RPOR-1 could bind to many nontuberculous mycobacteria (NTM) and some bacteria as summarized in Table 7. But only *M. shimoidei* and *M. xenopi*, in theory, could generate the specific products. However, these two species were rarely found in clinical samples in Thailand.

The forward inner primer A531F was specific for *M. tuberculosis* complex and crossed hybridize with *M. leprae*. In addition, alignment of A531F primer sequence with the whole genome of *M. tuberculosis* strain H37Rv revealed that this primer could bind to many positions within the *M. tuberculosis* genome as shown in Figure 24. Therefore, non-specific products were expected to occur when PCR was performed. The forward inner primer A526F was crossed hybridize with some bacteria. Whereas the primer A516F was specific only for *M. tuberculosis* complex. The primer A511F was crossed hybridize with some nontuberculous mycobacteria, other bacteria and some virus (Table 7).

The statistic score (bits) is calculated from multiple alignments, if it shows high score, refers to high probability of match appearing by chance. The Expect value (E) is a parameter that describes the number of hits; one can expect to see just by chance when searching a database of a particular size. It decreases exponentially with the score that is assigned to a match between two sequences. Essentially, the E value describes the random background noise that exists for matches between sequences. The lower the E-value, or the closer it is to zero, the more significant matching is.

Table 8 Specificity of each primer used in the study

Primer	Sequences producing significant	Score (bits)	E Value	% Query coverage
RPOF	*M. tuberculosis*	36.2	0.50	100
	M. africanum	36.2	0.50	100
	M. arupense	36.2	0.50	100
	M. asiaticum	36.2	7.9	88
	M. botniense	36.2	0.50	100
	M. bovis	36.2	0.50	100
	M. bovis BCG	36.2	0.50	100
	M. caprae	36.2	0.50	100
	M. chimaera	36.2	0.50	100
	M. conspicuum	36.2	0.50	100
	M. cookii	36.2	0.50	100
	M. flavescens	32.2	7.9	88
	M. gordonae	32.2	7.9	88
	M. haemophilum	32.2	7.9	88
	M. heidelbergense	32.2	7.9	88
	M. hiberniae	36.2	0.50	100
	M. holsaticum	32.2	7.9	88
	M. interjectum	36.2	0.50	100
	M. intermedium	36.2	0.50	100
	M. intracellulare	36.2	0.50	100
	M. kansasii	36.2	0.50	100
	M. kumamotonense	36.2	0.50	100
	M. lacus	36.2	0.50	100
	M. lentiflavum	36.2	0.50	100
	M. madagascariense	32.2	7.9	88
	M. malmoense	36.2	0.50	100
	M. microti	36.2	0.50	100
	M. montefiorense	36.2	0.50	100
	M. nebraskense	36.2	0.50	100
	M. palustre	36.2	0.50	100

Table 8 Specificity of each primer used in the study (continued)

Primer	Sequences producing significant	Score (bits)	E Value	% Query coverage
RPOF (continued)	*M. parascrofulaceum*	32.2	7.9	88
	M. pseudoshottsii	32.2	7.9	88
	M. saskatchewanense	36.2	0.50	100
	M. scrofulaceum	36.2	0.50	100
	M. shimoidei	32.2	7.9	88
	M. smegmatis	32.2	7.9	88
	M. szulgai	36.2	0.50	100
	M. terrae	36.2	0.50	100
	M. triplex	32.2	7.9	88
	M. triviale	36.2	0.50	100
	M. ulcerans	32.2	7.9	88
	M. xenopi	32.2	7.9	88
	Agreia bicolorata	36.2	0.50	100
	Agrococcus jenensis	36.2	0.50	100
	Agromyces cerinus	36.2	0.50	100
	Aspergillus clavatus	34.2	2.0	94
	Burkholderia cenocepacia	32.2	7.9	88
	Candida glabrata	32.2	7.9	88
	Clavibacter michiganensis	36.2	0.50	100
	Cryobacterium psychrophilum	36.2	0.50	100
	Curtobacterium flaccumfaciens	36.2	0.50	100
	Frigoribacterium faeni	36.2	0.50	100
	Leifsonia poae	36.2	0.50	100
	Neurospora crassa	32.2	7.9	88
	Nocardia asteroides	36.2	0.50	100
	Nocardia farcinica	32.2	7.9	88
	Okibacterium fritillariae	36.2	0.50	100
	Pseudomonas aeruginosa	32.2	7.9	88
	Pseudomonas citronellolis	34.2	2.0	94
	Pseudomonas multiresinivorans	32.2	7.9	88

Table 8 Specificity of each primer used in the study (continued)

Primer	Sequences producing significant	Score (bits)	E Value	% Query coverage
RPOF (continued)	*Pseudomonas nitroreducens*	32.2	7.9	88
	Pseudomonas putida	32.2	7.9	88
	Pseudomonas resinovorans	32.2	7.9	88
	Rhodococcus equi	36.2	0.50	100
	Streptomyces avermitilis	32.2	7.9	88
	Streptomyces coelicolor	32.2	7.9	88
	Subtercola boreus	36.2	0.50	100
	Subtercola frigoramas	36.2	0.50	100
RPOR-1	*M. tuberculosis*	34.2	2.0	100
	M. africanum	34.2	2.0	100
	M. bovis	34.2	2.0	100
	M. bovis BCG	34.2	2.0	100
	M. caprae	34.2	2.0	100
	M. microti	34.2	2.0	100
	M. shimoidei	34.2	2.0	100
	M. xenopi	34.2	2.0	100
	Corynebacterium matruchotii	34.2	2.0	100
	Geobacter bremensis	34.2	2.0	100
	Pelobacter masselenicus	34.2	2.0	100
	Pseudomonas aeruginosa	34.2	2.0	100
	Pseudomonas balearica	34.2	2.0	100
	Pseudomonas citronellolis	34.2	2.0	100
	Pseudomonas cremoricolorata	34.2	2.0	100
	Pseudomonas fulva	34.2	2.0	100
	Pseudomonas mendocina	34.2	2.0	100
	Pseudomonas multiresinivorans	34.2	2.0	100
	Pseudomonas nitroreducens	34.2	2.0	100
	Pseudomonas resinovorans	34.2	2.0	100
	Pseudomonas stutzeri	34.2	2.0	100
	Rhodospirillum rubrum	34.2	2.0	100

Table 8 Specificity of each primer used in the study (continued)

Primer	Sequences producing significant	Score (bits)	E Value	% Query coverage
RPOR-1 (continued)	*Rytigynia bagshawei*	34.2	2.0	100
	Trichlorobacter thiogenes	34.2	2.0	100
A531F	*M. tuberculosis*	30	7.7	100
	M. africanum	30	7.7	100
	M. bovis	30	7.7	100
	M. bovis BCG	30	7.7	100
	M. leprae	30	7.7	100
A526F	*M. tuberculosis*	30	7.7	100
	Corsican bluetonque virus	30	7.7	100
	Neurospora crassa	30	7.7	100
	Ralstonia solanacearum	30	7.7	100
A516F	*M. tuberculosis*	34.2	2.0	100
	M. bovis	34.2	2.0	100
A511F	*M. tuberculosis*	30	7.7	100
	M. fortuitum	30	7.7	100
	M. mageritense	30	7.7	100
	M. peregrinum	30	7.7	100
	M. septicum	30	7.7	100
	Anopheles gambiae	30	7.7	100
	Brucella suis	30	7.7	100
	Brucella melitensis	30	7.7	100
	Chromobacterium violaceum	30	7.7	100
	Human adenovirus type 17	30	7.7	100
	Treponema pallidum	30	7.7	100

Blast Server Results
URL: http://www.sanger.ac.uk/cgi-bin/blast/getblast?id=204E86cG90T7582098080

Query = primer A531F (15 letters)

Database: TB.seq
1 sequences; 4,411,532 total letters.

Sequences producing High-scoring Segment Pairs:	High Score	Smallest Sum Probability P(N)	N
Mycobacterium tuberculosis H37Rv	75	0.98	1

>*Mycobacterium tuberculosis* H37Rv [Full Sequence]

Length = 4,411,532

Plus Strand HSPs:

Score = 75 (17.3 bits), Expect = 4.0, P = 0.98
Identities = 15/15 (100%), Positives = 15/15 (100%), Strand = Plus / Plus
[HSP Sequence]

```
Query:      1 CAAGCGCCGACTGTC 15
              |||||||||||||||
Sbjct: 761141 CAAGCGCCGACTGTC 761155
```

Score = 60 (15.1 bits), Expect = 4.0, P = 0.98
Identities = 12/12 (100%), Positives = 12/12 (100%), Strand = Plus / Plus
[HSP Sequence]

```
Query:       4 GCGCCGACTGTC 15
              ||||||||||||
Sbjct: 1923264 GCGCCGACTGTC 1923275
```

Figure 24 Alignments of allele-specific inner primer A531F with the whole genome of *M. tuberculosis* strain H37Rv.

Score = 60 (15.1 bits), Expect = 4.0, P = 0.98
Identities = 12/12 (100%), Positives = 12/12 (100%), Strand = Plus / Plus
[HSP Sequence]

```
Query:      4 GCGCCGACTGTC 15
              ||||||||||||
Sbjct: 346816 GCGCCGACTGTC 346827
```

Minus Strand HSPs:

Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
Identities = 11/11 (100%), Positives = 11/11 (100%), Strand = Minus / Plus
[HSP Sequence]

```
Query:     15 GACAGTCGGCG 5
              ||||||||||||
Sbjct: 787076 GACAGTCGGCG 787086
```

Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
Identities = 11/11 (100%), Positives = 11/11 (100%), Strand = Minus / Plus
[HSP Sequence]

```
Query:    15 GACAGTCGGCG 5
             ||||||||||||
Sbjct: 28416 GACAGTCGGCG 28426
```

Figure 24 Alignments of allele-specific inner primer A531F with the whole genome of *M. tuberculosis* strain H37Rv. (continued)

Unexpectedly, after performing PCR with DNAs from twenty-five strains of reference mycobacteria, it was found that besides *M. tuberculosis* complex, seventeen mycobacterial species were also amplified by the outer primer pair, except *M. duvalii, M. flavescens*, *M. marinum* and *M. nonchromogenicum* as shown in Figures 25 and 26.

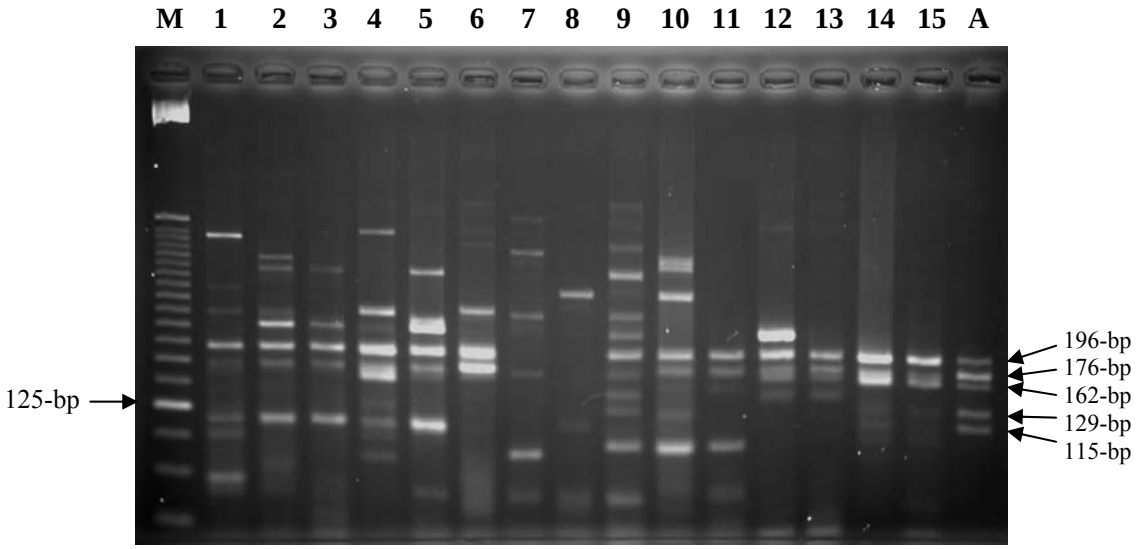

Figure 25 The 4% Nusieve 3:1 agarose gel electrophoresis showed the amplified products of mycobacterial reference strains.

Lane M : 25-bp DNA ladder
Lane 1 : *M. austroafricanum* strain ATCC 3005
Lane 2 : *M. avium* strain ATCC 700898
Lane 3 : *M. avium* strain 212
Lane 4 : *M. bovis* BCG strain 35770
Lane 5 : *M. bovis* BCG strain Tokyo KK 12-02
Lane 6 : *M. chelonae* strain ATCC 23016
Lane 7 : *M. duvalii* strain MNC 442
Lane 8 : *M. flavescens* strain ATCC 23035
Lane 9 : *M. fortuitum* strain ATCC 23048
Lane 10 : *M. gordonae* strain 330
Lane 11 : *M. gordonae* strain ATCC 11470
Lane 12 : *M. intracellulare* strain ATCC 13950
Lane 13 : *M. kansasii* strain ATCC 12478
Lane 14 : *M. tuberculosis* strain H37Rv
Lane 15 : 1 ng purified *M. tuberculosis* H37Rv DNA
Lane A : Allele-specific markers

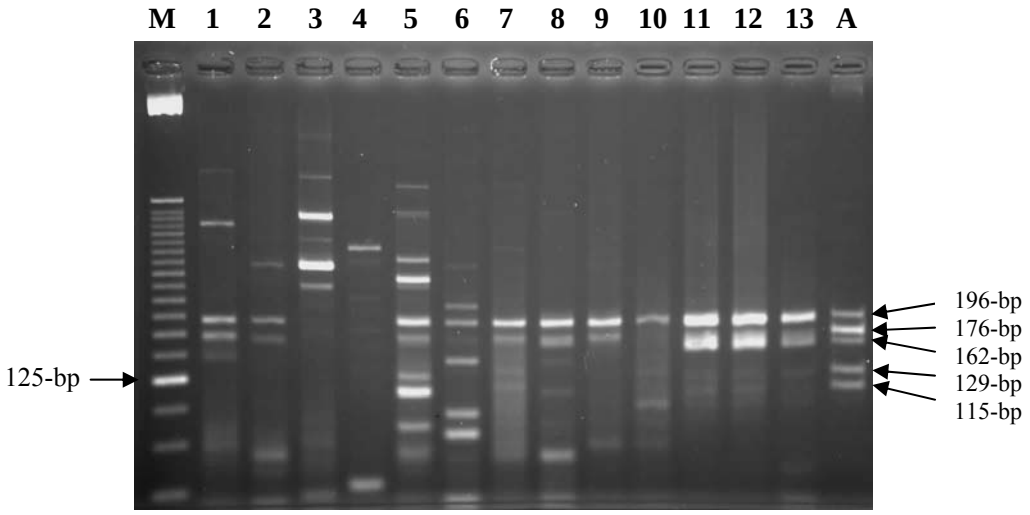

Figure 26 The 4% Nusieve 3:1 agarose gel electrophoresis showed the amplified products of mycobacterial reference strains.

Lane M : 25-bp DNA ladder
Lane 1 : *M. kansasii* strain 302
Lane 2 : *M. marinum* strain 329
Lane 3 : *M. marinum* strain ATCC 927
Lane 4 : *M. nonchromogenicum* strain ATCC 19530
Lane 5 : *M. scrofulaceum* strain ATCC 19981
Lane 6 : *M. simiae* strain ATCC 25275
Lane 7 : *M. smegmatis* strain 16941
Lane 8 : *M. szulgai* strain JATA 3201
Lane 9 : *M. szulgai* strain 352
Lane 10 : *M. terrae* strain ATCC 15755
Lane 11 : *M. tuberculosis* strain H37Ra
Lane 12 : *M. tuberculosis* strain H37Rv
Lane 13 : 1 ng purified *M. tuberculosis* H37Rv DNA
Lane A : Allele-specific markers

2. *rpoB* sequencing for identification of reference RIF-resistant strains

Polymerase chain reaction was used to amplify a region of *rpoB* gene associated with RIF-resistant phenotype in *M. tuberculosis*. The 277-bp amplified fragments of *rpoB* gene were produced by using primers TR9 and TR2b during amplification reaction and DNA sequences were then analyzed. Finally results revealed eight known mutation strains namely S1, S4, S7, S9, RIF 049, RIF 167, RIF 172 and RIF 182 (Table 3), had individually all four mutations covered respective codons namely codons 531, 526, 516 and 511 for using as reference RIF-resistant strains.

3. Optimization of the one-tube multiplex semi-nested PCR condition

3.1 Optimization of simplex PCR

The optimization was initiated by performing the simplex PCR of each primer pair with the same condition. Ten picomoles of each primer were constantly used with 100 ng of *M. tuberculosis* purified DNA as templates. Individual simplex PCRs were performed using annealing temperatures at 41°C, 46°C, 51°C, 56°C, 61°C and 65°C. Results revealed that using the annealing temperature at 65°C, strong specific bands were clearly amplified from all allele-specific primers such as RPOF/RPOR, A526F/RPOR, A516F/RPOR and A511F/RPOR (Figure 27, 29-31) except A531F/RPOR (Figure 28), which provided a weak specific band, strong primer dimers and non-specific bands.

Attempts to optimize PCR condition of primer A531F/RPOR by adjusting Mg^{2+} concentrations (1.0, 1.5, 2.0, 2.5, 3.0 mM), primer concentrations (5, 10, 20 pmol), annealing temperatures (60°C-72°C) and DNA template concentrations (1, 10, 100 ng) were not successful. Alternatively, performing PCR by adding only one primer in the PCR mixture, followed by adding another primer simultaneously with DNA template, could still not solve the problem.

The accomplished resolve was ultimately manipulated by that a new reverse primer, namely RPOR-1, was designed. Specificity of the RPOR-1 primer was determined by BLAST program and the result was shown in Table 8. The simplex PCR was performed by using the same PCR component and profile to test individual primer pairs (Figure 32). Results revealed that specific amplified products for all primer pairs were generated, especially that the amplification products of

A531F/RPOR-1 showed a stronger 115-bp specific band, even if non-specific bands were generated as shown in Figure 34. PCR of the remaining four forward primers with the new reverse primer were clearly amplified as shown in Figures 33, 35-37.

To improve the specificity of A531F/RPOR-1, annealing temperature was then optimized by varying from 64°C to 69°C to increase the specificity. The results showed that the maximum annealing temperature at 69°C provided an allele-specific band (codon 531) whereas non-specific bands were disappeared. Finally, the appropriate PCR condition of simplex PCR of A531F/RPOR-1 used the annealing temperature at 69°C as summarized in Figure 38. However, at this annealing temperature, primers A516F/RPOR-1 could not be amplified. Therefore this condition could not be used for all simplex PCR. The results of individual simplex PCR by this condition were shown in Figures 39 and 40.

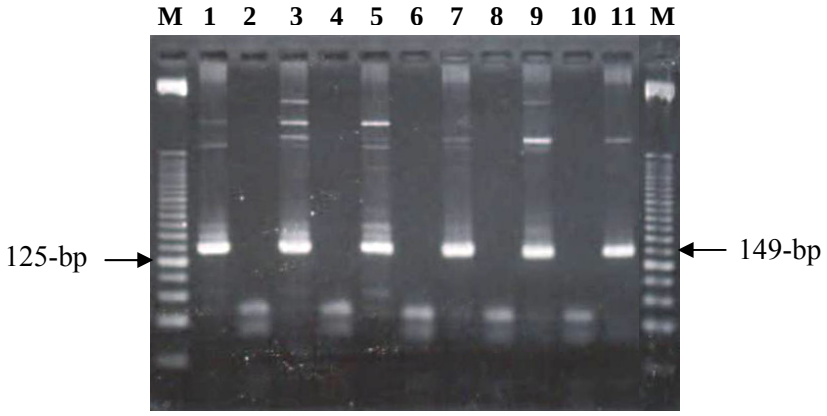

Figure 27 The 4% agarose gel electrophoresis showed the amplified products using 100 ng *M. tuberculosis* DNA as positive control and the primers RPOF/RPOR in simplex PCR by varying temperature from 41°C to 65°C. The expected specific product was 149-bp.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : Positive and blank control set at 41°C
Lanes 3 and 4 : Positive and blank control set at 46°C
Lanes 5 and 6 : Positive and blank control set at 51°C
Lanes 7 and 8 : Positive and blank control set at 56°C
Lanes 9 and 10 : Positive and blank control set at 61°C
Lane 11 : Positive control set at 65°C

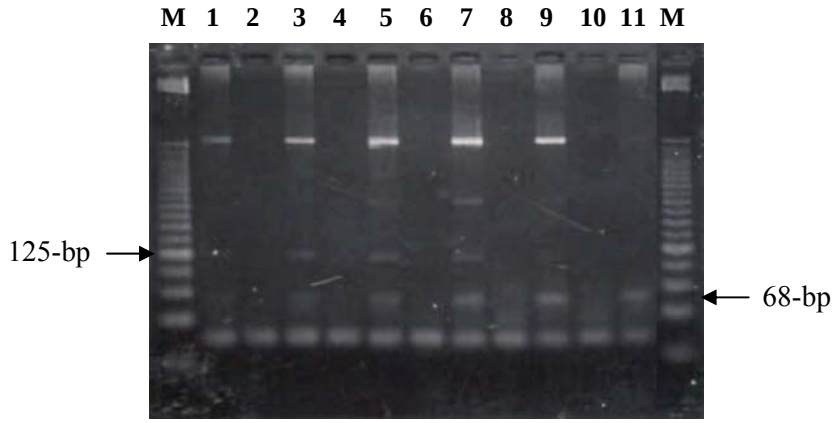

Figure 28 The 4% agarose gel electrophoresis showed the amplified products using 100 ng *M. tuberculosis* DNA as positive control and the primers A531F/RPOR in simplex PCR by varying temperature from 41°C to 65°C. The expected specific product was 68-bp.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : Positive and blank control set at 41°C
Lanes 3 and 4 : Positive and blank control set at 46°C
Lanes 5 and 6 : Positive and blank control set at 51°C
Lanes 7 and 8 : Positive and blank control set at 56°C
Lanes 9 and 10 : Positive and blank control set at 61°C
Lane 11 : Positive control set at 65°C

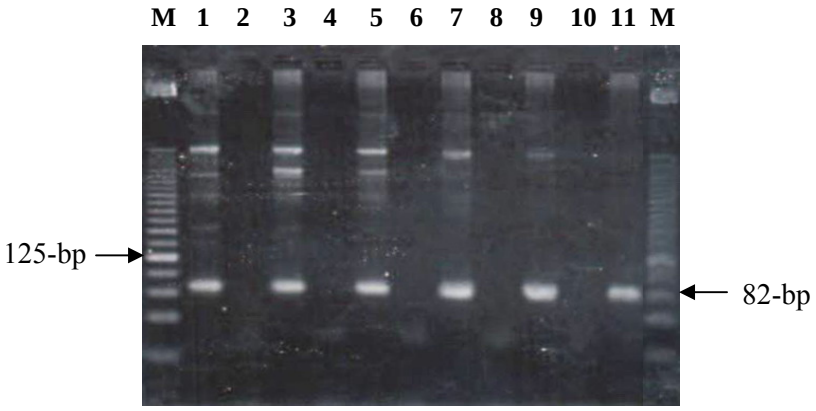

Figure 29 The 4% agarose gel electrophoresis showed the amplified products using 100 ng *M. tuberculosis* DNA as positive control and the primers A526F/RPOR in simplex PCR by varying temperature from 41°C to 65°C. The expected specific product was 82-bp.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : Positive and blank control set at 41°C
Lanes 3 and 4 : Positive and blank control set at 46°C
Lanes 5 and 6 : Positive and blank control set at 51°C
Lanes 7 and 8 : Positive and blank control set at 56°C
Lanes 9 and 10 : Positive and blank control set at 61°C
Lane 11 : Positive control set at 65°C

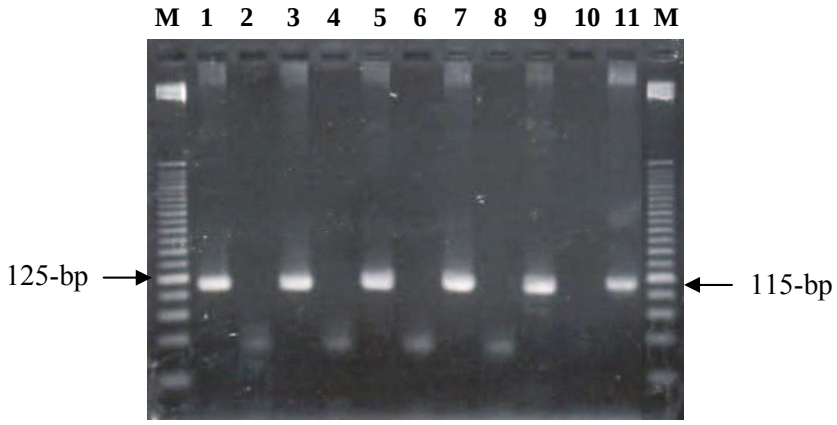

Figure 30 The 4% agarose gel electrophoresis showed the amplified products using 100 ng *M. tuberculosis* DNA as positive control and the primers A516F/RPOR in simplex PCR by varying temperature from 41°C to 65°C. The expected specific product was 115-bp.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : Positive and blank control set at 41°C
Lanes 3 and 4 : Positive and blank control set at 46°C
Lanes 5 and 6 : Positive and blank control set at 51°C
Lanes 7 and 8 : Positive and blank control set at 56°C
Lanes 9 and 10 : Positive and blank control set at 61°C
Lane 11 : Positive control set at 65°C

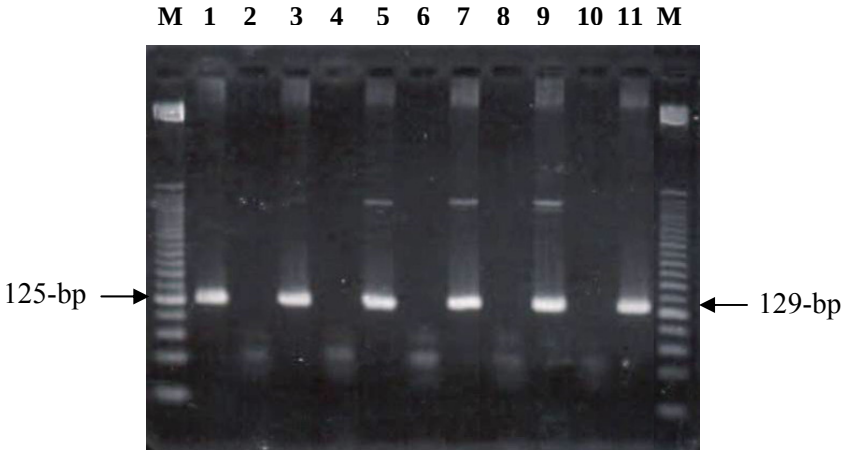

Figure 31 The 4% agarose gel electrophoresis showed the amplified products using 100 ng *M. tuberculosis* DNA as positive control and the primers A511F/RPOR in simplex PCR by varying temperature from 41°C to 65°C. The expected specific product was 129-bp.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : Positive and blank control set at 41°C
Lanes 3 and 4 : Positive and blank control set at 46°C
Lanes 5 and 6 : Positive and blank control set at 51°C
Lanes 7 and 8 : Positive and blank control set at 56°C
Lanes 9 and 10 : Positive and blank control set at 61°C
Lane 11 : Positive control set at 65°C

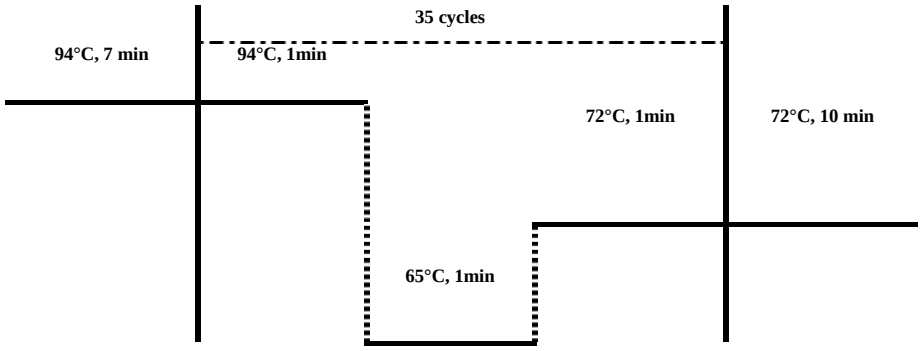

Figure 32 PCR conditions of the simplex PCR for amplification of all primer pairs

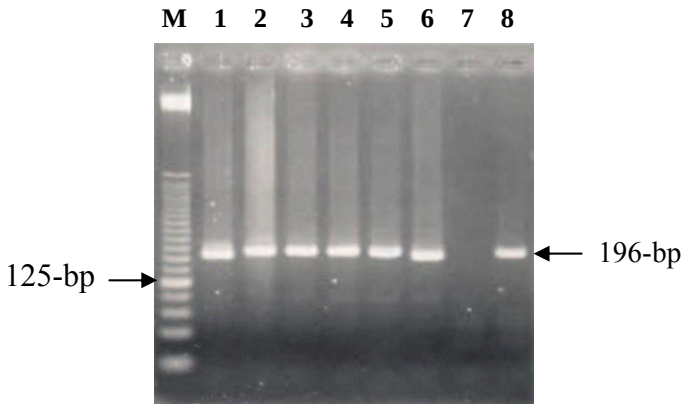

Figure 33 The 4% agarose gel electrophoresis showed the amplified products of the RPOF/RPOR-1 simplex PCR with crude DNAs from clinical isolates. The expected product was 196-bp.

Lane M : 25-bp DNA ladder
Lane 1 : RIF-resistant clinical isolate RIF 172: mutation at codon 526
Lane 2 : RIF-resistant clinical isolate RIF 166: mutation at codon 526
Lane 3 : RIF-resistant clinical isolate RIF 182: mutation at codon 526
Lane 4 : RIF-susceptible clinical isolate RIF 202: mutation at codon 522
Lane 5 : RIF-susceptible clinical isolate RIF 167: double mutations at codon 511 and 516
Lane 6 : RIF-susceptible clinical isolate RIF 068: deletion codon 513-516
Lane 7 : Blank control
Lane 8 : 10 pg purified *M. tuberculosis* H37Rv DNA

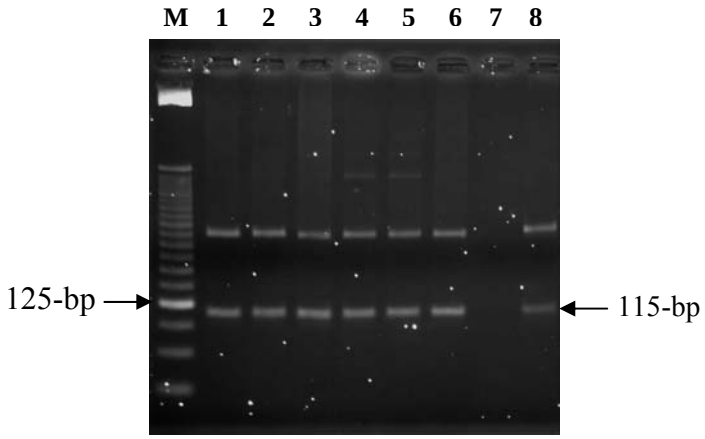

Figure 34 The 4% agarose gel electrophoresis showed the amplified products of the A531F/RPOR-1 simplex PCR with crude DNAs from clinical isolates. The expected product was 115-bp.

Lane M : 25-bp DNA ladder
Lane 1 : RIF-resistant clinical isolate RIF 172: mutation at codon 526
Lane 2 : RIF-resistant clinical isolate RIF 166: mutation at codon 526
Lane 3 : RIF-resistant clinical isolate RIF 182: mutation at codon 526
Lane 4 : RIF-susceptible clinical isolate RIF 202: mutation at codon 522
Lane 5 : RIF-susceptible clinical isolate RIF 167: double mutations at codon 511 and 516
Lane 6 : RIF-susceptible clinical isolate RIF 068: deletion codon 513-516
Lane 7 : Blank control
Lane 8 : 10 pg purified *M. tuberculosis* H37Rv DNA

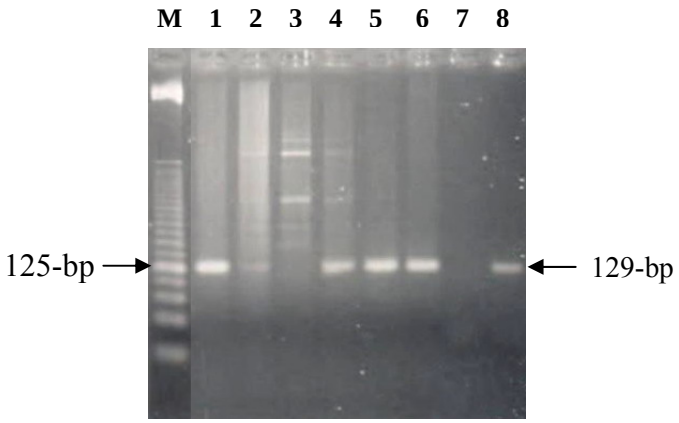

Figure 35 The 4% agarose gel electrophoresis showed the amplified products of the A526F/RPOR-1 simplex PCR with crude DNAs from RIF-susceptible and resistant clinical isolates. The expected product was 129-bp and should be absent when performing with isolates containing mutations at codon 526.

Lane M : 25-bp DNA ladder
Lane 1 : RIF-resistant clinical isolate RIF 172: mutation at codon 526
Lane 2 : RIF-resistant clinical isolate RIF 166: mutation at codon 526
Lane 3 : RIF-resistant clinical isolate RIF 182: mutation at codon 526
Lane 4 : RIF-susceptible clinical isolate RIF 202: mutation at codon 522
Lane 5 : RIF-susceptible clinical isolate RIF 167: double mutations at codon 511 and 516
Lane 6 : RIF-susceptible clinical isolate RIF 068: deletion codon 513-516
Lane 7 : Blank control
Lane 8 : 10 pg purified *M. tuberculosis* H37Rv DNA

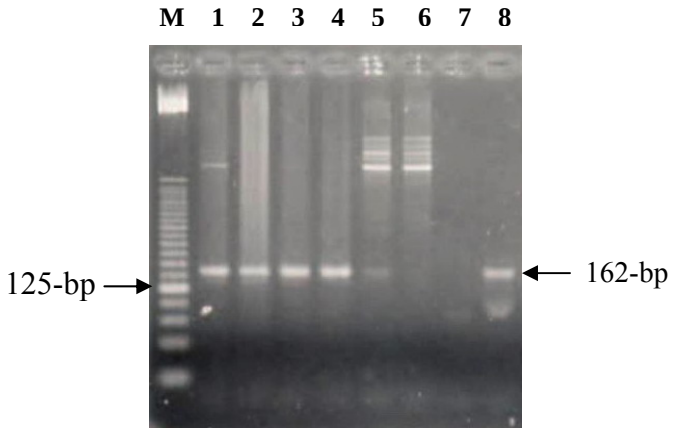

Figure 36 The 4% agarose gel electrophoresis showed the amplified products of the A516F/RPOR-1 simplex PCR with crude DNAs from RIF-susceptible and resistant clinical isolates. The expected product was 162-bp and should be absent when performing with isolates containing mutations at codon 516.

Lane M : 25-bp DNA ladder
Lane 1 : RIF-resistant clinical isolate RIF 172: mutation at codon 526
Lane 2 : RIF-resistant clinical isolate RIF 166: mutation at codon 526
Lane 3 : RIF-resistant clinical isolate RIF 182: mutation at codon 526
Lane 4 : RIF-susceptible clinical isolate RIF 202: mutation at codon 522
Lane 5 : RIF-susceptible clinical isolate RIF 167: double mutations at codon 511 and 516
Lane 6 : RIF-susceptible clinical isolate RIF 068: deletion codon 513-516
Lane 7 : Blank control
Lane 8 : 10 pg purified *M. tuberculosis* H37Rv DNA

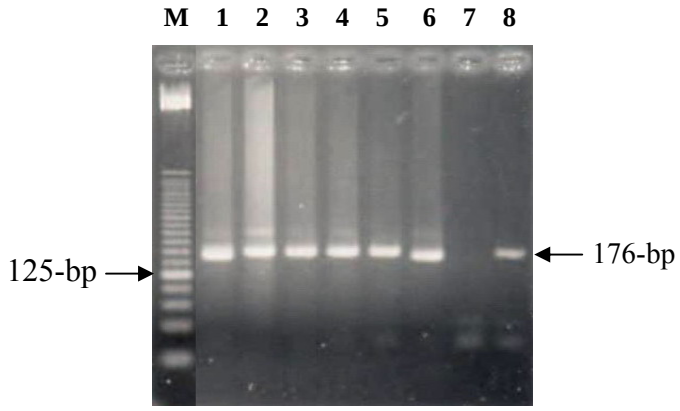

Figure 37 The 4% agarose gel electrophoresis showed the amplified products of the A511F/RPOR-1 simplex PCR with crude DNAs from RIF-susceptible and resistant clinical isolates. The expected product was 176-bp and should be absent when performing with isolates containing mutations at codon 511.

Lane M : 25-bp DNA ladder
Lane 1 : RIF-resistant clinical isolate RIF 172: mutation at codon 526
Lane 2 : RIF-resistant clinical isolate RIF 166: mutation at codon 526
Lane 3 : RIF-resistant clinical isolate RIF 182: mutation at codon 526
Lane 4 : RIF-susceptible clinical isolate RIF 202: mutation at codon 522
Lane 5 : RIF-susceptible clinical isolate RIF 167: double mutations at codon 511 and 516
Lane 6 : RIF-susceptible clinical isolate RIF 068: deletion codon 513-516
Lane 7 : Blank control
Lane 8 : 10 pg purified *M. tuberculosis* H37Rv DNA

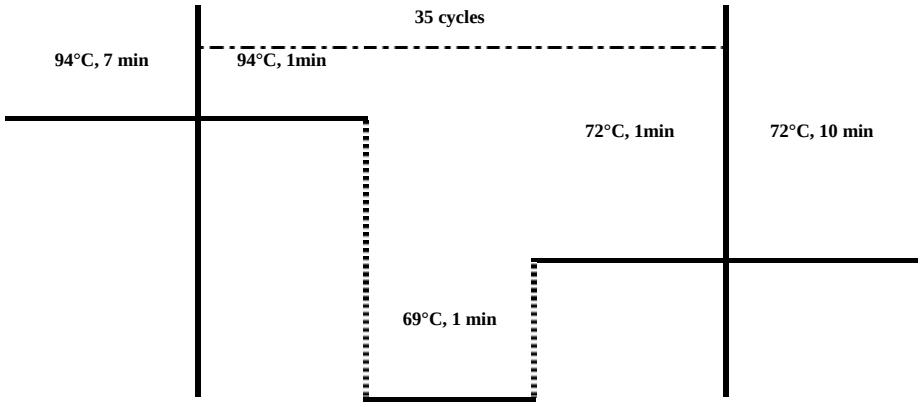

Figure 38 The optimized PCR conditions of the simplex PCR for amplification of primers A531F/RPOR-1

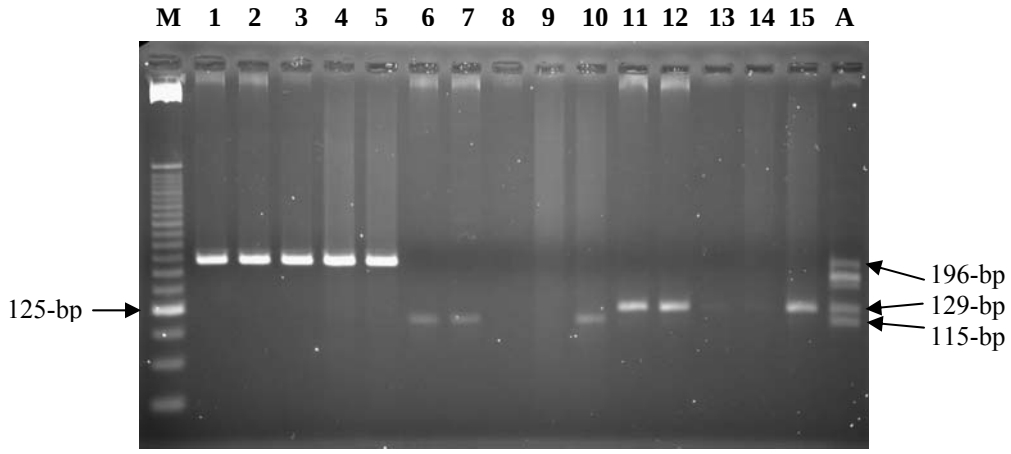

Figure 39 The 4% agarose gel electrophoresis showed the amplified products of the simplex PCR using the annealing temperature at 69ºC of primers RPOF/RPOR-1 (Lanes 1-5), A531F/RPOR-1 (Lanes 6-10) and A526F/RPOR-1 (Lanes 11-15). The expected products were 196-bp, 115-bp and 129-bp, respectively.

Lane M : 25-bp DNA ladder
Lane 1 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 2 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 3 : RIF-resistant clinical isolate RIF 003: mutation at codon 531
Lane 4 : RIF-resistant clinical isolate RIF 049: mutation at codon 531
Lane 5 : RIF-susceptible clinical isolate RIF 014
Lane 6 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 7 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 8 : RIF-resistant clinical isolate RIF 003: mutation at codon 531
Lane 9 : RIF-resistant clinical isolate RIF 049: mutation at codon 531
Lane 10 : RIF-susceptible clinical isolate RIF 014
Lane 11 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 12 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 13 : RIF-resistant clinical isolate RIF 019: mutation at codon 526
Lane 14 : RIF-resistant clinical isolate RIF 062: mutation at codon 526
Lane 15 : RIF-susceptible clinical isolate RIF 014
Lane A : Allele-specific markers

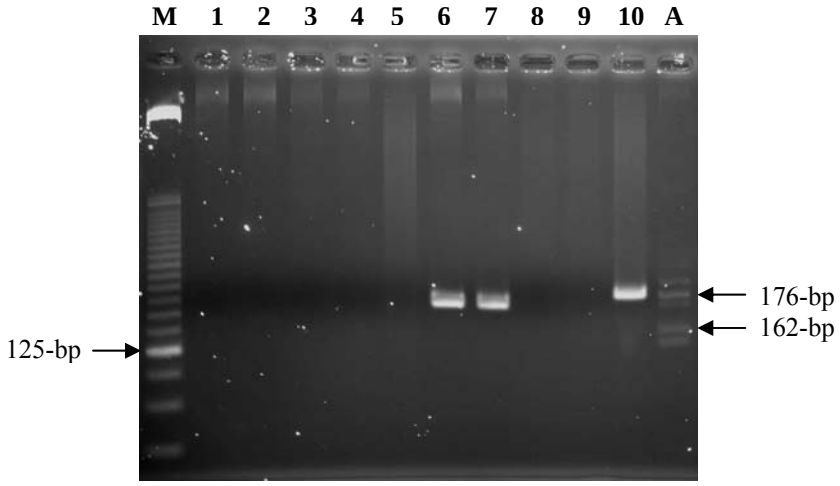

Figure 40 The 4% agarose gel electrophoresis showed the amplification products of the simplex PCR using the annealing temperature at 69ºC of primers A516F/RPOR-1 (Lane 1-5) and A511F/RPOR-1 (Lane 6-10). The expected products were 162-bp and 176-bp, respectively.

Lane M : 25-bp DNA ladder
Lane 1 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 2 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 3 : RIF-resistant clinical isolate RIF 026: mutation at codon 516
Lane 4 : RIF-resistant clinical isolate RIF 103: mutation at codon 516
Lane 5 : RIF-susceptible clinical isolate RIF 014
Lane 6 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 7 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 8 : RIF-resistant clinical isolate RIF 167: mutation at codon 511
Lane 9 : RIF-resistant clinical isolate RIF 167: mutation at codon 511
Lane 10 : RIF-susceptible clinical isolate RIF 014
Lane A : Allele-specific markers

3.2 Optimization of multiplex PCR

For initial optimization, the multiplex PCR was performed with DNAs from eight RIF-resistant strains with known mutations. Ten picomoles of primers A526F, A516F, and A511F were used and the remaining primers, RPOF, A531F and RPOR-1, were initially used at 2.5, 20 and 40 pmol, respectively. The multiplex PCR was initiated by using the PCR profile that optimize for most primers as shown in Figure 32.

Results revealed that the optimization of multiplex PCR was unsuccessful either using crude DNAs from six clinical isolates (Figure 41) or using purified DNA at concentrations of 10 ng, 1 ng and 100 pg as templates (Figure 42). Only the 196-bp amplification product of the outer primers (RPOF/RPOR-1) was detected, whereas other four allele-specific bands were not seen by this condition.

Further adjustment was performed by changing PCR profile from initial denaturing at 94°C 7 minutes, 35 cycles of denaturing at 94°C 1 minute, annealing at 69°C 1 minute, extension at 72°C 1 minute (one cycling profile) to 94°C 7 minutes, 15 cycles of denaturing at 94°C 1 minute, annealing at 69°C 1 minute, extension at 72°C 1 minute followed by 35 cycles of denaturing at 94°C 1 minute, annealing at 61°C 1 minute, extension at 72°C 1 minute and final extension at 72°C, 10 minutes (two cycling profiles) giving an opportunity that the outer primer pair was firstly amplified, and followed by amplification of the inner primers. Therefore, the used annealing temperature of the first PCR cycling profile was 69°C as same as the condition used for the simplex PCR. Considering the melting temperatures (Tm) of all used primers, it was shown that a set of outer primers had higher Tm of 8°C than the allele-specific inner primers. Hence, the annealing temperature of the second PCR cycling profile was selected at 61°C. The result of the adjusted PCR profile condition demonstrated the 196-, 176- and 129-bp amplification products as shown in Figure 43.

The multiplex PCR was then performed with a set of clinical isolates containing eight strains with known mutations at each codon by varying the annealing temperatures from 65°C to 72°C for the first PCR cycling profile and 45°C to 68°C for the second PCR cycling profile of PCR condition. In addition, annealing time (60 and 90 seconds), extension time (60 and 90 seconds) and number of cycles (15 and 25, 10 and 30, 5 and 35, 10 and 35, 15 and 35, and 20 and 35 for the first and second PCR cycling profiles, respectively) were considered for the optimization. After that, the

optimization was done by varying primer concentrations (0.2-60 pmol) namely, RPOF (0.2, 0.6, 1.0, 1.25 and 2.5 pmol), A531F (10, 20, 30, 40, 50 and 60 pmol), A526F (2.5, 5, 10, 20, 30 and 40 pmol), A516F (0.2, 0.5, 0.8, 1.0, 1.25, 1.5, 2.0, 2.5, 5, 7 and 10 pmol), A511F (0.2, 0.4, 0.5, 0.75, 1.0, 1.25, 2, 2.5, 5 and 10 pmol) and RPOR-1 (20, 30 and 40 pmol). Finally the used primer concentrations of RPOF, A531F, A526F, A516F, A511F and RPOR-1 were 0.2, 40, 20, 1, 0.5 and 30 pmol, respectively. The PCR was performed with annealing temperature of 70ºC and 58ºC for 1 minute and the cycle numbers of 15 and 35 cycles for the first and second PCR cycling profiles, respectively. The result with the eight reference resistant strains was shown in Figure 44, revealed the 129-bp amplified product for mutation at codon 526 was not clearly quite disappear.

In order to increase efficiency of amplification, the optimizations were then performed by adding glycerol (at 10% final concentration) and results were compared between PCR with and without glycerol at various annealing temperatures from 58°C to 68°C. The results revealed that amplified products from PCR reaction with glycerol (Figure 45) were weaker than those without glycerol (Figure 46), indicating that supplement of 10% glycerol did not support PCR amplification.

Unexpectedly, amplification of primers A511F and RPOR-1 was not differentiated between wild type and mutant strains at codon 511. The amplification product was generated from both wild type and mutant strains. Optimization was repeated by changing all solutions of PCR mixtures and using fresh dNTPs. The PCR was adjusted by varying primer concentrations (0.2-60 pmol) and annealing temperatures (54°C-69°C). An important parameter of the optimization, which is critical for the multiplex PCR, was primer concentrations. Due to the binding of primer A511F to its target was challenged with primer A516F (Figure 23) which begins from the last two bases of the 3'-end of primer A511F. From principle, if point mutation at codon 511 was presented, an allele-specific band for codon 511 should not be generated. However, the results demonstrated that the allele-specific band could be detected, even if the codon 511 mutant strain was used as template. Considering the Tm of primer A511F was higher than that of primer A516F about 2°C. Therefore, the opportunity to bind the target and continue extension of primer A511F was higher than that of primer A516F when using the high annealing temperature. From this

hypothesis, primer concentration of A511F was reduced to less than that of A516F about 15 times, in order to obtain an amplification of primers A511F and RPOR-1 and, therefore, revealed difference between wild type and codon 511 mutant strain. The amplified product was generated only from wild type, and not from mutant strain.

Furthermore, types of buffer [KCl and $(NH_4)_2SO_4$] used and DNA thermal cycler were considered whether they had some influence on amplification. PCR using different buffer was performed and results showed that the KCl buffer gave a better result than the $(NH_4)_2SO_4$ buffer in this study. No significant difference between two DNA thermal cyclers, Touchgene Gradient Thermal Cycler (Techne, Cambridge, U.S.A) and PTC-200 DNA Engine Thermal Cycler (MJ Research, Massachusetts, U.S.A), was found in this study (data not show).

Finally, the optimized multiplex PCR was performed by adding 5 µl DNA template to the PCR mixture (final volume 50 µl) containing 2.5 mM $MgCl_2$, 50 mM KCl, 2 U of *Taq* DNA polymerase, 200 µM deoxynucleoside triphosphates (dNTPs), 0.2 pmol RPOF, 30 pmol RPOR-1, 40 pmol A531F, 2.5 pmol A526F, 7.5 pmol A516F and 0.5 pmol A511F. The PCR reactions were performed in a PTC-200 DNA Engine thermal cycler under the following conditions: initial denaturation at 94°C for 7 minutes; 15 cycles of 94°C for 1 minute, 70°C for 1 minute, 72°C for 1 minute 30 seconds; 35 cycles of 94°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute 30 seconds and final elongation at 72°C for 10 minutes as summarized in Figure 47. By the way, the extension time of both PCR cycling profiles were used as 1 minute 30 seconds due to increase opportunity for all primers extension especially A531F which could bind with other non-specific sites within *rpoB* gene.

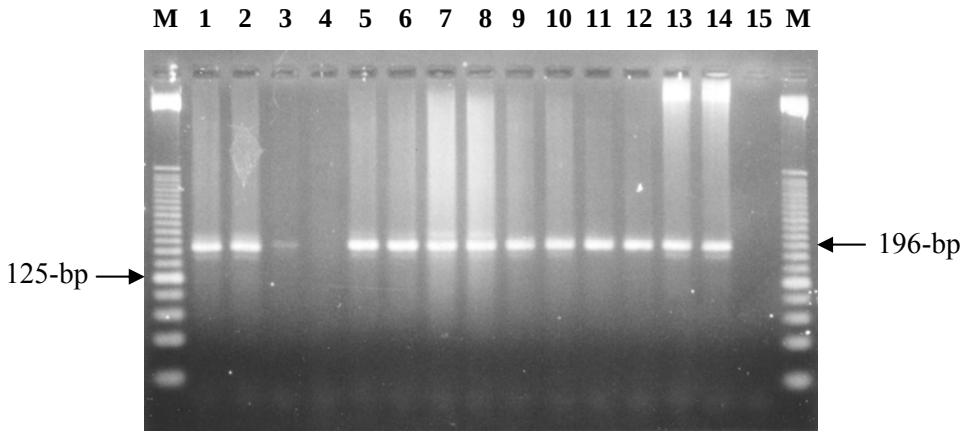

Figure 41 The 4% Nusieve 3:1 agarose gel electrophoresis of the initially optimized multiplex PCR that used PCR profile of the simplex PCR. The used DNA templates were crude DNA from six clinical isolates. The result demonstrated only the 196-bp amplification products of outer primers.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : RIF-resistant clinical isolate RIF 172: mutation at codon 526
Lanes 3 and 4 : RIF-susceptible clinical isolate RIF 077: mutation at codon 526
Lanes 5 and 6 : RIF-susceptible clinical isolate RIF 174: mutation at codon 516
Lanes 7 and 8 : RIF-resistant clinical isolate RIF 166: mutation at codon 526
Lanes 9 and 10 : RIF-resistant clinical isolate RIF 182: mutation at codon 526
Lanes 11 and 12 : RIF-susceptible clinical isolate RIF 167: double mutations at codon 511 and 516
Lanes 13 and 14 : 100 ng purified *M. tuberculosis* H37Rv DNA
Lane 15 : Blank control

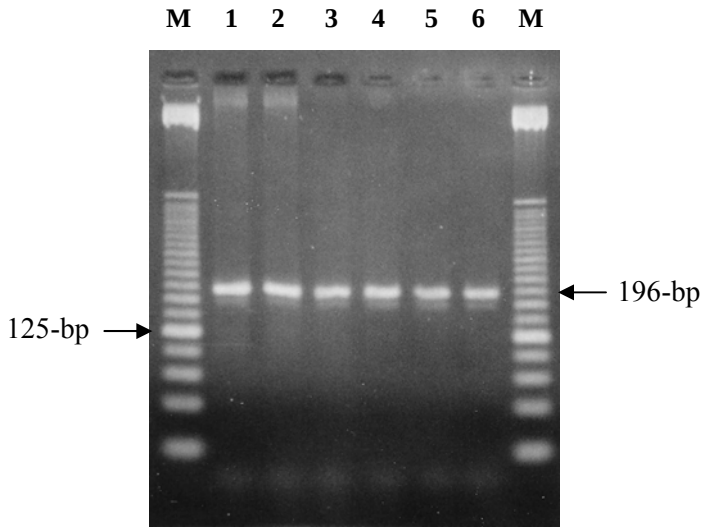

Figure 42 The 4% Nusieve 3:1 agarose gel electrophoresis showed the results of multiplex PCR that used PCR profile from the simplex PCR. The used DNA templates were purified DNA concentrations of 10 ng, 1 ng and 100 pg. The results demonstrated only the 196-bp amplification products of outer primers.

Lane M	:	25-bp DNA ladder
Lanes 1 and 2	:	10 ng purified *M. tuberculosis* H37Rv DNA
Lanes 3 and 4	:	1 ng purified *M. tuberculosis* H37Rv DNA
Lanes 5 and 6	:	100 pg purified *M. tuberculosis* H37Rv DNA

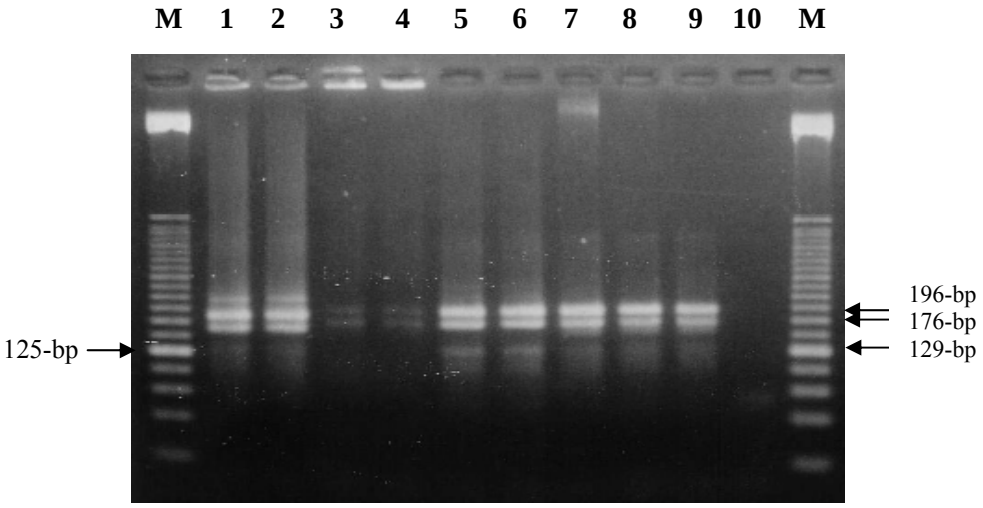

Figure 43 The 4% Nusieve 3:1 agarose gel electrophoresis showed the results of multiplex PCR optimization that used new adjusted PCR profile which was two cycling profiles: the annealing temperature of the first and second PCR cycling profiles were 69°C and 61°C, respectively. The result demonstrated the 196-, 176- and 129-bp amplification products.

Lane M	:	25-bp DNA ladder
Lanes 1 and 2	:	RIF-resistant clinical isolate RIF 172
Lanes 3 and 4	:	RIF-resistant clinical isolate RIF 077
Lanes 5 and 6	:	RIF-resistant clinical isolate RIF 167
Lane 7	:	10 ng purified *M. tuberculosis* H37Rv DNA
Lane 8	:	1 ng purified *M. tuberculosis* H37Rv DNA
Lane 9	:	100 pg purified *M. tuberculosis* H37Rv DNA
Lane 10	:	Blank control

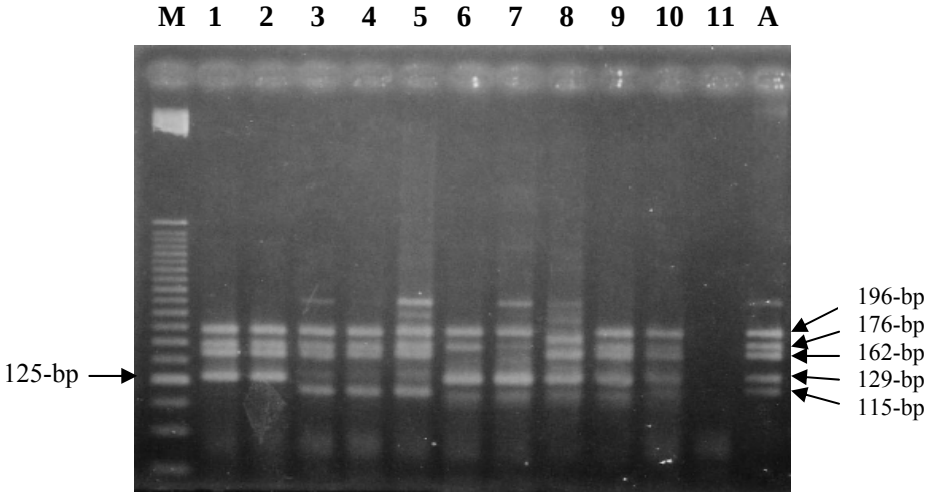

Figure 44 The 4% Nusieve 3:1 agarose gel electrophoresis showed the result of multiplex PCR optimization with known resistant *M. tuberculosis* strains. The used primer concentrations of RPOF, A531F, A526F, A516F, A511F and RPOR-1 were 0.2, 40, 20, 1, 0.5 and 30 pmol, respectively. The PCR was performed with annealing temperature of 70ºC and 58ºC for 1 minute and the cycle numbers of 15 and 35 cycles for the first and second PCR cycling profiles, respectively.

Lane M : 25-bp DNA ladder
Lane 1 : S7 containing mutation at codon 531
Lane 2 : S8 containing mutation at codon 531
Lane 3 : S1 containing mutation at codon 526
Lane 4 : S9 containing mutation at codon 526
Lane 5 : RIF 182 containing mutation at codon 526
Lane 6 : S4 containing mutation at codon 516
Lane 7 : RIF 167 containing double mutations at codons 516 and 511
Lane 8 : RIF 068 containing deletion between codons 513 to 516
Lane 9 : 1 ng purified *M. tuberculosis* H37Rv DNA
Lane 10 : 100 pg purified *M. tuberculosis* H37Rv DNA
Lane 11 : Blank control
Lane A : Allele-specific markers

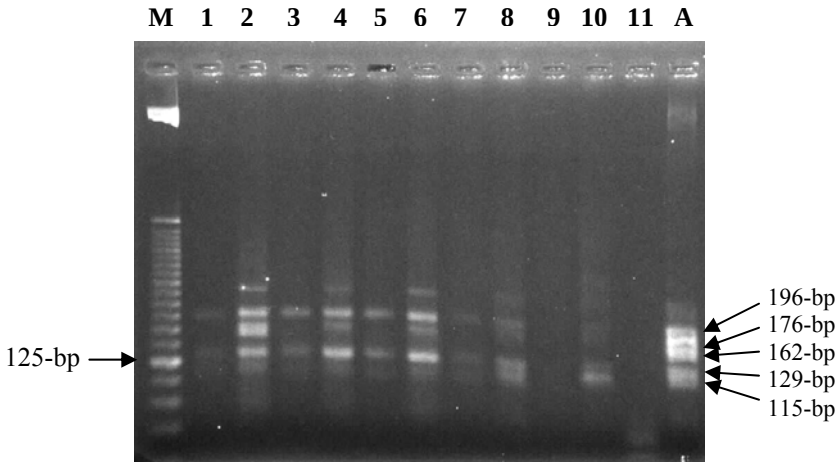

Figure 45 The 4% Nusieve 3:1 agarose gel electrophoresis showed the results of PCR reactions with 10% glycerol. PCR was performed by varying the annealing temperature from 58°C to 68°C. DNA from the mutant strain RIF 167 and wild type *M. tuberculosis* strain H37Rv was used as template.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : Mutant and wild type *M. tuberculosis* strains set at 58°C
Lanes 3 and 4 : Mutant and wild type *M. tuberculosis* strains set at 61°C
Lanes 5 and 6 : Mutant and wild type *M. tuberculosis* strains set at 64°C
Lanes 7 and 8 : Mutant and wild type *M. tuberculosis* strains set at 66°C
Lanes 9 and 10 : Mutant and wild type *M. tuberculosis* strains set at 68°C
Lane 11 : Blank control
Lane A : Allele-specific markers

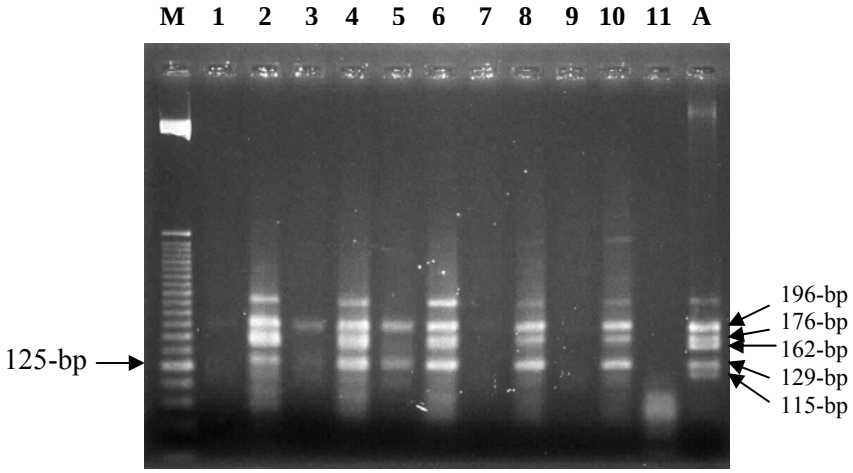

Figure 46 The 4% Nusieve 3:1 agarose gel electrophoresis showed the results of PCR reactions without 10% glycerol. PCR was performed by varying the annealing temperature from 58°C to 68°C. DNA from the mutant strain RIF 167 and wild type *M. tuberculosis* strain H37Rv was used as template.

Lane M : 25-bp DNA ladder
Lanes 1 and 2 : Mutant and wild type *M. tuberculosis* strains set at 58°C
Lanes 3 and 4 : Mutant and wild type *M. tuberculosis* strains set at 61°C
Lanes 5 and 6 : Mutant and wild type *M. tuberculosis* strains set at 64°C
Lanes 7 and 8 : Mutant and wild type *M. tuberculosis* strains set at 66°C
Lanes 9 and 10 : Mutant and wild type *M. tuberculosis* strains set at 68°C
Lane 11 : Blank control
Lane A : Allele-specific markers

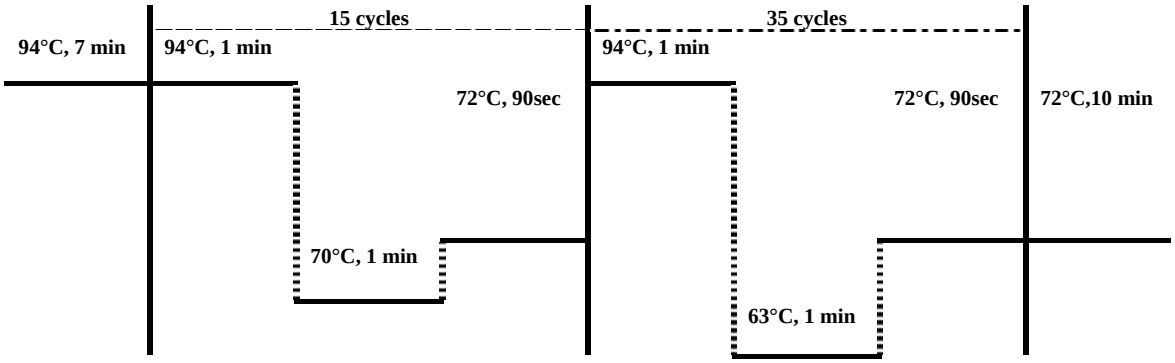

Figure 47 The optimized PCR condition of the one-tube multiplex semi-nested PCR

4. Determination of the detection limit of the multiplex PCR

The detection limit of the optimized PCR was elucidated in order to determine the least DNA concentration that could be detected by the established PCR method. The purified DNA templates were ten-fold diluted from 100 ng to 10 fg. Amplification was performed with each DNA concentration (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg and 10 fg) and performed independently at least 3 times. The PCR was performed with the optimized condition as shown in Figure 47. Results revealed that at concentration of 10 pg DNA template was the least detection limit of the developed PCR as shown in Figure 48.

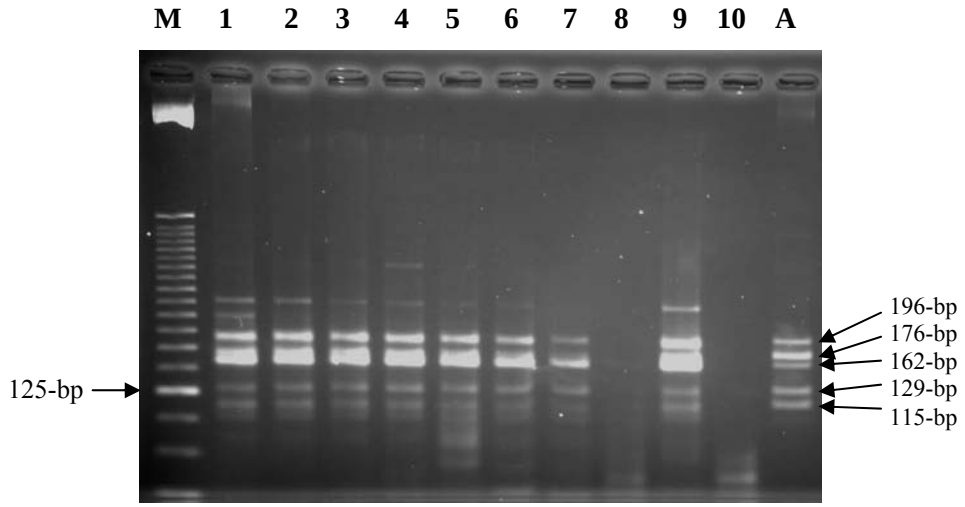

Figure 48 The 4% Nusieve 3:1 agarose gel electrophoresis showed the results of DNA detection limit of the one-tube multiplex PCR. The ten-fold dilution of purified DNA of *M. tuberculosis* H37Rv from 100 ng to 10 fg were tested.

Lane M : 25-bp DNA ladder

Lanes 1-8 : purified DNA of *M. tuberculosis* (H37Rv) 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg and 10 fg, respectively

Lane 9 : 20 ng purified *M. tuberculosis* H37Rv DNA (positive control)

Lane 10 : Blank (negative control)

Lane A : Allele-specific markers

5. Evaluation of the multiplex PCR for detection of RIF-resistant *M. tuberculosis* clinical isolates

The developed one-tube multiplex semi-nested PCR was used to blindly test with DNAs obtained from 216 *M. tuberculosis* clinical isolates (103 RIF-resistant and 113 RIF-susceptible isolates) with known patterns of antimycobacterial susceptibility testing as shown in Table 11 (Appendix). Results showed that the developed PCR could concordantly identify 113 of 113 RIF-susceptible and 97 of 103 RIF-resistant *M. tuberculosis* clinical isolates when compared with the proportion method (Table 8). With advantage of this method, PCR could identify mutations at codons 531, 526, and 516 with the frequency of 52.6% (51/97), 37.1% (36/97), 8.2% (8/97), respectively. For the remaining two resistant isolates, the multiplex PCR identified one isolate containing double mutations at codon 511 and 516 whereas the other contained the deletion between codons 511 and 516. PCR results of some isolates were shown in Figure 49 and 50. Comparing with the DNA sequencing results, all identified isolates showed the identical results; 95 isolates had a single point mutation either at codon 531, 526, or 516, one isolate had a double mutation at codon 511 and 516, and one contained a deletion between codons 513 and 516 as listed in Table 11 (Appendix).

Table 9 The results of the developed PCR blindly tested with 216 *M. tuberculosis* strains compared with the proportion method of susceptibility testing

		Proportion method		Total
		Susceptible	Resistant	
PCR	Susceptible	113	6	119
	Resistant	0	97	97
	Total	113	103	216

For the other 6 discordant isolates, which were identified as RIF-susceptible isolates (negative result) by the PCR method, 5 isolates except RIF 163, had DNA sequencing results that revealed the mutations outside the designed region, such as codon 176 and codon 533 (Table 9). Comparing with the proportion method, the

overall sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of this multiplex PCR was 94.2%, 100%, 100% and 94.9%, respectively.

Table 10 The six RIF-resistant isolates that showed negative PCR results

No.	**Samples**	**Results**		
		Proportion method	**PCR method**	***rpoB* sequencing**
1.	RIF 058	resistant	susceptible	Mutation at codon 533 (Leu to Pro)
2.	RIF 100	resistant	susceptible	Mutation at codon 176 (Gln to Lys)
3.	RIF 101	resistant	susceptible	Mutation at codon 533 (Leu to Pro)
4.	RIF 144	resistant	susceptible	Mutation at codon 533 (Leu to Pro)
5.	RIF 163	resistant	susceptible	Not available
6.	RIF 184	resistant	susceptible	Mutation at codon 176 (Gln to Lys)

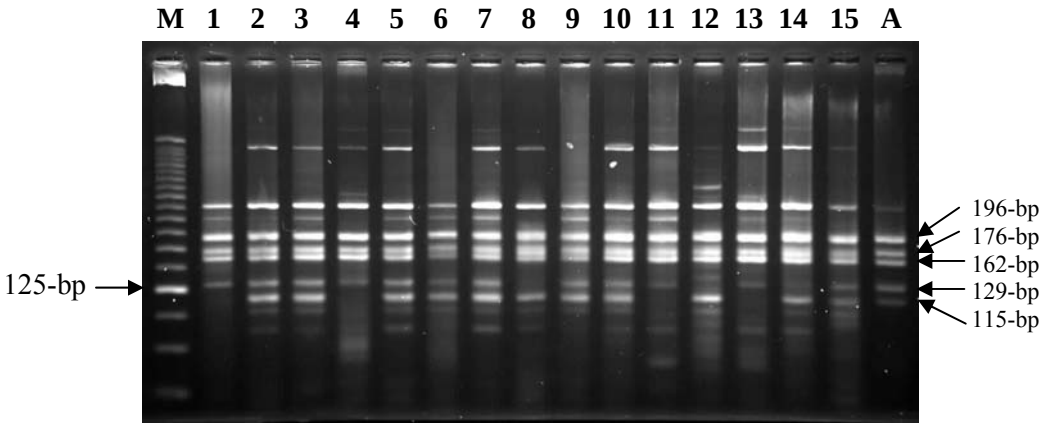

Figure 49 The 4% Nusieve 3:1 agarose gel electrophoresis showed some results of one-tube semi-nested multiplex PCR for identification of RIF-resistant *M. tuberculosis* strains.

Lane M : 25-bp DNA ladder
Lane 1 : RIF 012 containing mutation at codon 531
Lane 2 : RIF 013 containing wild type pattern
Lane 3 : RIF 014 containing wild type pattern
Lane 4 : RIF 015 containing mutation at codon 531
Lane 5 : RIF 016 containing wild type pattern
Lane 6 : RIF 017 containing wild type pattern
Lane 7 : RIF 018 containing wild type pattern
Lane 8 : RIF 019 containing mutation at codon 526
Lane 9 : RIF 020 containing wild type pattern
Lane 10 : RIF 021 containing wild type pattern
Lane 11 : RIF 022 containing mutation at codon 531
Lane 12 : RIF 023 containing mutation at codon 526
Lane 13 : RIF 024 containing mutation at codon 531
Lane 14 : RIF 025 containing mutation at codon 526
Lane 15 : 10 ng purified DNA of *M. tuberculosis* H37Rv
Lane A : Allele-specific markers

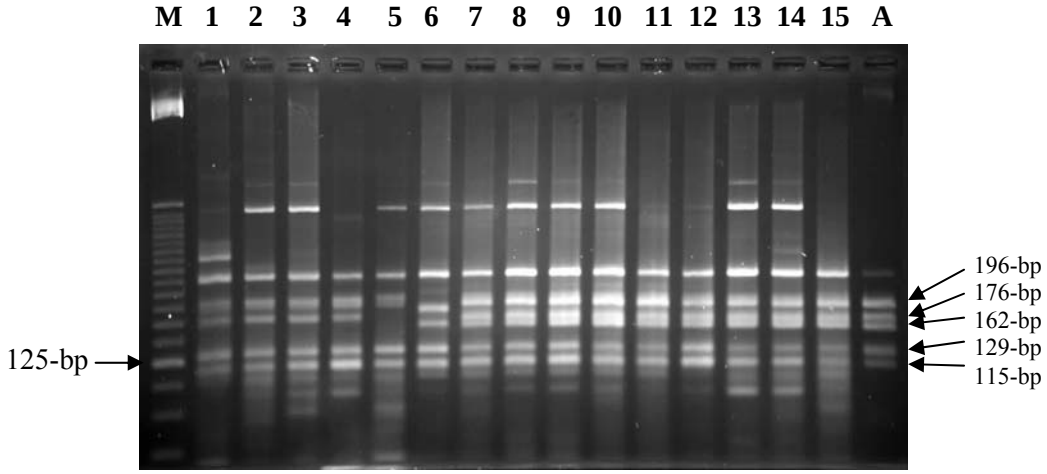

Figure 50 The 4% Nusieve 3:1 agarose gel electrophoresis showed some results of one-tube semi-nested multiplex PCR for identification of RIF-resistant *M. tuberculosis* strains.

Lane M : 25-bp DNA ladder
Lane 1 : RIF 075 containing mutation at codon 516
Lane 2 : RIF 103 containing mutation at codon 516
Lane 3 : RIF 174 containing mutation at codon 516
Lane 4 : RIF 221 containing mutation at codon 516
Lane 5 : RIF 167 containing double mutation at codons 516 and 511
Lane 6 : RIF 068 containing deletion between codon 513 to 516
Lane 7 : RIF 001 containing wild type pattern
Lane 8 : RIF 002 containing wild type pattern
Lane 9 : RIF 013 containing wild type pattern
Lane 10 : RIF 021 containing wild type pattern
Lane 11 : RIF 040 containing wild type pattern
Lane 12 : RIF 058 containing wild type pattern
Lane 13 : RIF 033 containing wild type pattern
Lane 14 : RIF 078 containing wild type pattern
Lane 15 : 10 ng purified DNA of *M. tuberculosis* H37Rv
Lane A : Allele-specific markers

6. Simulated mix population of resistant and susceptible *M. tuberculosis* strains

The different ratios of populations of *M. tuberculosis* susceptible strain, and the *M. tuberculosis* RIF-resistant strain were prepared and tested by the developed multiplex PCR. Results showed that the developed PCR required at least 90% of the resistant strain in the mixed population to clearly identify the resistant subpopulation as shown in Figure 51.

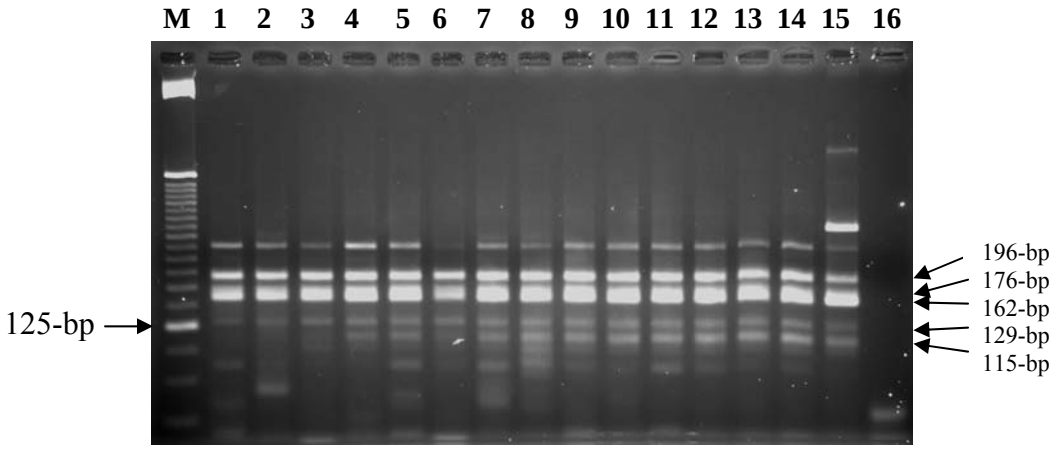

Figure 51 The 4% Nusieve 3:1 agarose gel electrophoresis showed the results of different combinations of *M. tuberculosis* susceptible strain (*M. tuberculosis* H37Rv), and the *M. tuberculosis* RIF-resistant strain (*M. tuberculosis* DS10238) which was spontaneously mutated at codon 531, were mixed with the following ratio; 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 5:95, 2:98, 1:99 and 0: 100 in lane 1-14 respectively.

Lane M : 25-bp DNA ladder

Lanes 1-14 : Different ratios of resistant : sensitive strains *M. tuberculosis* from 100:0 to 0:100

Lane 15 : 10 ng purified *M. tuberculosis* H37Rv DNA

Lane 16 : Blank control

7. Evaluation of the developed PCR method for direct detection of RIF-resistant strains from positive MGIT tubes

The optimized one-tube multiplex semi-nested PCR was tested with 50 blind DNA samples, prepared from the positive automated BACTEC MGIT 960 cultures. *M. tuberculosis* were isolated from all samples and susceptibility testing was performed by the proportion method on LJ. The isolated RIF-resistant strains were identified in 5 of 50 and the remaining were RIF-susceptible isolates. Comparison with the proportion method, PCR concordantly identify 44 of 45 positive RIF-susceptible and 5 of 5 RIF-resistant *M. tuberculosis* clinical isolates as summarized in Table 10 and 12. Comparing with the proportion method, the sensitivity, specificity, PPV and NPV of this multiplex PCR was 100%, 97.8%, 83.3% and 100%, respectively.

Table 11 The result of the developed PCR tested with 50 blind *M. tuberculosis* automated MGIT samples compared with the proportion method

The developed PCR	Proportion method		Total
	Resistant	Susceptible	
Resistant	5	1	6
Susceptible	0	44	44
Total	5	45	50

CHAPTER V
DISCUSSION

Early detection of drug resistance TB allows starting of an appropriate treatment, which has an impact in the better control of the disease. MDR-TB constitutes a major threat to TB control. Several new approaches have been proposed to rapidly detect MDR-TB including both genotypic and phenotypic methods. In many cases, the genotypic methods especially have been directed to detect RIF resistance since it was shown to be a good surrogate marker for MDR-TB. Genotypic methods have the advantage of a shorter turnaround time, no need for cultivation of the organism, possibility for direct application in clinical samples, less biohazard risks, and feasibility for automation; not all molecular mechanisms of drug resistance, however, are known (168). Genotypic methods determine genetic determinants of resistance rather than the resistance phenotype and involve two basic steps, namely a molecular nucleic acid amplification such as PCR to amplify sections of the *M. tuberculosis* genome known to be altered in resistant strains and a second step of assessing the amplified products for specific mutations correlating with resistance.

For example, DNA sequencing has been the most widely used method; it is accurate and reliable and it has become the gold standard for mutation detection, but making the cost high. Two solid-phase hybridization techniques have been currently commercialized, namely the Line Probe Assay (INNO-LiPA Rif TB Assay; Innogenetics, Ghent, Belgium) for the detection of RIF resistance and the GenoType MTBDR assay (Hain Lifesciences, Nehren, Germany) for the simultaneous detection of INH and RIF resistance. The LiPA assay was introduced several years ago and is based on the hybridization of amplified DNA from cultured strains or clinical samples to 10 probes covering the RIF resistance determining region of the *rpoB* gene of *M. tuberculosis*, immobilized on a nitrocellulose strip. The GenoType MTBDR, on the other hand, detects resistance to INH and RIF from cultivated samples or pulmonary smear-positive clinical samples (169), (170) based on the detection of the most

common mutations in the *katG* and *rpoB* genes, respectively. Both solid hybridization methods have shown to be relatively simple to perform although basic expertise in molecular biology and PCR techniques are required. As with other genotypic methods the sensitivity of the test depends on the amount of DNA present in the sample and also the presence of inhibitors could cause false-negative results. Due to the high cost involved, the use of these methods for detecting drug resistance TB is still beyond the reach of most clinical mycobacteriology laboratories. Real-time PCR techniques have also been introduced for rapid detection of drug resistance. Different probes have been used like the TaqMan probe, fluorescence resonance energy transfer (FRET) probes, molecular beacons and biprobes. By the way, advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination. The main disadvantages would be the requirement for expensive equipment and reagents, and the need for skilled technical personnel.

Previously, a multiplex allele-specific PCR (MAS-PCR) assay based on standard PCR and agarose gel electrophoresis to detect mutations in *katG315* (171) and *embB306* (172) in *M. tuberculosis* strains have been described and a similar assay to detect *rpsL43*, *rpoB531*, and *katG315* mutations has very recently been published by Victor *et al.* (173). The MAS-PCR method had a drawback that it was required three reaction tubes for identification of only one sample, leading to quite experimentally cumbersome.

Therefore, a technique which is easy to perform, rapid, accurate and inexpensive, is ideally required for detection of RIF-resistant *M. tuberculosis* as a surrogate marker for detection of MDR-TB. The one-tube multiplex PCR is becoming a rapid and convenient assay for use in both clinical and research laboratories. In the present study, the one-tube multiplex semi-nested PCR was developed for identification RIF-resistant *M. tuberculosis* strains, which was applied the same principle and concept as that of the previously reported allele-specific PCR method (19). Another advantage of this method beside required a shorter turnaround time only 1 to 2 days from positive culture was low-cost about 90 baht/test/4 mutations whereas the other method like InnoLipa test was up to 2800 baht/test/4 mutations. Several studies revealed that RIF resistance in up to 95% to 98% of resistant strains is caused by mutations in the 81-bp hot spot region of *rpoB* gene within codons 507 to 533 (27). In addition, RIF

resistance may be resulted from mutations in other parts outside the hot spot region, such as codon 176 in the N-terminal part (27), (17). Moreover, some other mutation(s) occurred outside the sequenced part of the *rpoB* gene or in a gene affecting the permeability of RIF influenced the level of resistance in these mutants (174). Nucleotide sequencing of the entire *rpoB* gene may be necessary to answer this question.

Notably, *rpoB* mutations in three codons namely 531, 526, or 511 were accounted for the majority of RIF-resistant strains (70% to 95%), especially in the areas with high incidence of MDR-TB (18). Previous studies also demonstrated that a mutation at codon 511 within the hot spot region was frequently concerned (17), (18), (33), (35). Therefore the detection of this codon was included in the study, resulted in high potential for more covering RIF-resistant *M. tuberculosis* detection.

In this study, the multiplex PCR was developed to target simultaneously 4 loci, at which RIF resistance-associated mutations are most frequently observed, in a single tube and was assessed for the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) in comparison with the culture based phenotypic method using 216 *M. tuberculosis* clinical isolates. The developed PCR was blindly tested with DNAs obtained from 103 RIF-resistant and 113 RIF-susceptible *M. tuberculosis* clinical isolates with known patterns of antimycobacterial susceptibility testing. Results showed that the developed PCR could concordantly identify 113 of 113 RIF-susceptible and 97 of 103 RIF-resistant *M. tuberculosis* clinical isolates when compared with the proportion method. For the six discordant isolates, which were identified as RIF-susceptible isolates (negative result) by the PCR method, were submitted for DNA sequencing and found the mutations outside the designed region, such as codons 176 and 533 leading to misidentification. Comparing with the proportion method, the sensitivity, specificity, PPV and NPV of the multiplex PCR were 94.2%, 100%, 100% and 94.9%, respectively. Therefore, susceptible strains identified by this PCR method in case of highly clinical suspected for RIF-resistant or MDR-TB strain should be further confirmed by the standard culture-based or sequencing methods. On the other hands, due to the detection limit of the developed PCR was 10 pg, therefore the PCR was not appropriate for direct detection of RIF-resistant *M. tuberculosis*.

In addition, the developed PCR were also tested with 50 blind DNA samples isolated from AFB culture-positive MGIT tubes of the automated BACTEC MGIT 960 culture system. For every experiment, if the sample gave ambiguous result, the sample should be retested until the result was confirmed. Result revealed that the PCR could concordantly identify 44 of 45 RIF-susceptible and 5 of 5 RIF-resistant *M. tuberculosis* clinical isolates when compared with the proportion method of antimycobacterial susceptibility testing in automated MGIT 960. The sensitivity, specificity, PPV and NPV of this multiplex PCR for use with MGIT samples were 100%, 97.8%, 83.3% and 100%, respectively. Therefore the developed multiplex PCR may be applied for using as rapid RIF-resistance screening test of samples directly obtained from positive MGIT tubes.

In the present study, mutations in four codons, namely 531, 526, 516 and 511, were found in 90.3% (93/103) of RIF-resistant strains, concordantly with previous studies. Other mutations within the hot spot region beyond the four respective codons were codons 533, 522, and 513. Mutation at codon 533 was found in 3 strains (2.9%) whereas mutation at codons 522 and 513 was found in one strain (0.9%). The multiplex PCR could not identify the codon 533 mutant strains, leading to misidentify as RIF-susceptible strain. Unexpectedly, codon 513 mutant (RIF 027) and codon 522 mutant (RIF 202) were identified as resistant strain with allele-specific A516F and A526F primer, respectively. These might be resulted from the inability of those primers to bind with the mutated sequence, even if located in the 5'-part of the primers when using the high-stringency PCR condition.

In addition, the developed multiplex PCR could identify the deletion-mutant strain like strain RIF 068, which contained the deletion between codon 513 to 516. The developed PCR could detect deletion due to 162-bp amplified product (codon 516) disappear, 176-bp (codon 511) and 196-bp (internal control) amplified products shift down whereas 129-bp (codon 526) and 115-bp (codon 531) still appear as actually wild type pattern. These revealed that some part of DNA downstream of codon 511 and codon 516 were deleted. However, the developed PCR could not identify some resistant strains containing mutations outside the designed region, as in this study the codon 176 mutation was found in two strains (1.9%). Therefore, as

present in this study, all RIF-resistant strains had mutations in the *rpoB* gene and the developed PCR could overall identify the resistant strains up to 94.2%.

Determination of a mutation site might be useful and helpful for a rapid and accurate diagnosis (175). A mutation at codons 381, 511, 513, 514, 516, 526, 531, or 533 within *rpoB* gene led to resistance to RIF. Among them, a mutation at codons 513, 526 or 531 led to high MICs (MIC ≥ 50 μg/ ml). Especially, a mutation at codons 526 or 531 occurred most frequently. However, even located in the cluster I region of the *rpoB*, a mutation at codons 514, 521, or 533 has been shown to confer low-level of resistance (MIC = 12.5 μg/ ml) or had no effect on resistance phenotype (MIC < 0.39 μg/ ml). These results suggested that mutations in the *rpoB* gene were, mostly, but not necessarily, associated with RIF resistance of *M. tuberculosis*, and the sites of mutations on the *rpoB* gene will affect the level of resistance to RIF.

At the beginning of the study, the outer primer pair of the developed PCR was designed to be specific for *M. tuberculosis* complex. Due to a mistake of BLAST format setting, which was set too low only to 100 for the number of descriptions and the number of alignments. Actually, alignment result showed up to more than 100 DNA isolates which could bind to the designed *M. tuberculosis*-specific primer, including some nontuberculous mycobacteria. Thus, the outer primer pair was then only used for nested amplification and functioned as internal control for determining the presence of PCR inhibitors.

A successful optimization of the multiplex PCR assay was depending on the relative concentrations of the primers at the various loci, the type of the PCR buffer, the cycling temperatures, the magnesium chloride concentration and deoxynucleotide concentration. The fidelity of the *Taq* DNA polymerase is higher at lower dNTP concentrations, using the KCl-based buffer, which requires much less dNTP, can be beneficial when the PCR products need to be further analyzed for mutations (176).

The primers were used with different concentration depending on the Tm value, target binding position, stringency and base composition at 3'-end of each primer. Due to the binding of A511F primer to its target was challenged with A516F primer (Figure 26), which begins from the last two bases of the 3'-end of A511F primer. Considering the Tm of A511F primer was higher than that of A516F primer about 2°C. The opportunity to bind the target and continue extension of A511F primer was

higher than that of A516F primer when using the high annealing temperature. Therefore, A516F primer concentration was used higher than that of primer A511F. Owing to A531F primer could bind with many positions of *M. tuberculosis* DNA sequence, the A531F primer was used with high concentration because at low annealing temperature (low stringency), non-expected amplified products were more generated, in contrast, at high annealing temperature (high stringency), even the expected amplified product was amplified, but it was more difficult to amplify, leading to decreased some non-specific products. Therefore, the high A531F primer concentration was used to challenge opportunity of primer binding and extension under high stringency. However, the amplified product of A531F/RPOR-1 was still subsequently weaker than those of other primer pairs for the multiplex PCR. The A526F and A516F primers had equal Tm value, so almost the same primer concentrations were used. The RPOF forward outer primer concentration was low due to the primer was located far away from challenge between other primers. And the RPOR-1 reverse primer concentration was high due to this primer was function as reverse primer of both one outer primer pair and four allele-specific inner primer pairs. Furthermore owing to binding position of the A531F and A526F primers were adjacent, resulting in primer concentrations of both were differed about 8 folds. In the same way, binding position of the A516F and A511F primers were overlapping, resulting in different primer concentrations of both about 16 folds.

Various authors recommended to use glycerol to improve amplification efficiency (higher amount of product) and specificity (no nonspecific products) of PCR, when used in concentrations varying between 5-10% (v/v) (176). Glycerol is also used to aid as a protein stabilizer. The addition of glycerol is also reported to improve the PCR amplification for GC-rich DNA. In optimization of the multiplex PCR by adding 10% glycerol revealed no improvement of PCR but also generated less products than PCR without glycerol. However, in the multiplex reaction, addition of this adjuvant gave better and more specific PCR products. Therefore, the usefulness of the adjuvant needed to be tested in each case.

For interpretation, the presence of all five bands (at 196-, 176-, 162-, 129- and 115-bp) indicated the wild type genotype of *M. tuberculosis* strains and should be sensitive to RIF. The disappearance of any allele-specific bands indicated that they

had mutation(s) at the corresponding allele-specific codon and resist to RIF. In case of the presence of band for codon 531, it showed the paler band when compared to other bands in the same reaction. In contrast to the absence of this band, it showed clear disappearance, which indicated to be the resistant strain. Particularly, the interpretation always required comparison with the wild type positive control bands for differentiation between wild type and mutant strain (presence or absence).

Results should be carefully interpretated due to all *M. tuberculosis* isolates were isolated from LJ media without RIF. Thus, the cultured strain should consider to the simultaneously mixed population of wild type and resistant subpopulation. The mixed populations of *M. tuberculosis* complex strains, which was prepared from different relative proportions of cells from RIF-susceptible and RIF-resistant strains, were tested with the PCR assay. The developed multiplex PCR assay could detect a resistant population when it contained at least 90% of the RIF-resistant population. Therefore, the presence of resistant strains less than 90% may be interpreted as RIF-susceptible, whereas, resistance interpretation of phenotypic susceptibility testing was defined as growth on drug containing media equal to or greater than 1% of the growth on drug free medium (123). In addition, Telenti *et al*. (158) reported that if there was a mixed population of resistant and susceptible organisms, the minority strain should probably represent more than 15% of all organisms in the sample for single-strand conformational polymorphism (PCR-SSCP) to identify the presence of a mixed pattern. Even though the PCR-SSCP had more sensitivity of resistant detection, but this method was difficult to interpret. Unfortunately, the developed multiplex PCR required up to 90% of resistant population for correct identification of RIF-resistant *M. tuberculosis*, therefore some resistant strains would be mis-identified as sensitive one. However, the obtained RIF-resistance results of conventional proportion method had actually more than 90% of RIF-resistant subpopulation in mixed population. Therefore the opportunity of mis-identification as susceptible strain was too low. In highly suspected cases from clinical data to contain resistant isolate but the developed multiplex PCR could not reveal such resistance, the conventional proportion antimycobacterial susceptibility testing or other molecular testing should be performed.

Additionally, this developed one-tube multiplex semi-nested PCR showed some other limitations that it should use only for known cultures of *M. tuberculosis* because the amplified product of the RPOF/RPOR-1 outer primer pair was not specific only for *M. tuberculosis* complex strain. Besides *M. tuberculosis* complex, the outer and inner primers could amplify several nontuberculous mycobacterial strains; some could be found in clinical specimens, such as *M. flavescens, M. gordonae, M. intracellulare, M. kansasii, M. smegmatis* and *M. fortuitum.* Another limitation of this method, including other molecular method, was that they could detect only known mutations but, however, not all mutations conferring resistance to RIF were known and prevalence of mutations may vary by geographic area; identification of a resistance-associated mutation could be informative, but lack of a mutation in the target sequence must be interpreted with caution.

CHAPTER VI
CONCLUSION

The study aimed to evaluate the developed one-tube multiplex semi-nested PCR for identification of RIF-resistant *M. tuberculosis*. The PCR results were compared to those obtained from the proportion method, which was used as the gold standard. DNAs from 216 clinical isolates of *M. tuberculosis* consisting of 103 isolates of RIF-resistant and 113 isolates of RIF-susceptible strains were blindly tested with the multiplex PCR. Twenty-five mycobacterial reference strains were used for determining the specificity of the developed PCR method.

The one-tube multiplex semi-nested PCR showed concordant results for all 113 RIF-susceptible and 97 of 103 RIF-resistant *M. tuberculosis* clinical isolates when compared with the proportion method. The 6 discordant RIF-resistant strains, which showed negative results by the PCR method, were submitted for *rpoB* gene sequencing and found the mutations outside the designed region such as codons 176 and 533. This revealed the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 94.2%, 100%, 100% and 94.9%, respectively. The detection limit of the developed PCR method was 10 pg DNA. With advantage of this method, PCR could identify mutations at codons 531, 526, and 516 with the frequency of 52.6% (51/97), 37.1% (36/97), 8.2% (8/97), respectively. For the remaining two resistant isolates, the multiplex PCR identified one isolate containing double mutations at codons 511 and 516 whereas another contained the deletion between codons 511 and 516. Therefore, the developed PCR showed some advantages for providing the epidemiological information that determine locations of mutation conferring RIF-resistance (either point mutation or deletions at codons 531, 526, 516, 511). Besides, the method was more rapid while it needed only two working days instead of 2-4 weeks testing with MGIT system or proportion agar or LJ method. It costed about 90 bahts per sample. Unfortunately, the multiplex PCR assay had some drawbacks that it

could detect a RIF-resistant strain when there was at least 90% of resistant subpopulation, comparing with 1% of phenotypic method.

In addition, the developed multiplex PCR was blindly performed with 50 DNA samples obtained from MGIT AFB positive tubes, which consisted of 5 RIF-resistant and 45 RIF-susceptible *M. tuberculosis* isolates. The results showed that the PCR could concordantly identify 44 of 45 RIF-susceptible and 5 of 5 RIF-resistant *M. tuberculosis* clinical isolates compared with the proportion method. For the remained one discordant isolate was identified as RIF-resistant isolate with unexplained reason. Comparing with the proportion method, the sensitivity, specificity, PPV and NPV of the PCR, when using with MGIT positive cultures were 100%, 97.8%, 83.3% and 100%, respectively.

The established PCR method could identify RIF-resistant *M. tuberculosis* from cultures and provide more rapid result (1-2 days) than the standard method using both conventional and automated culture system (7-28 days). Although the developed multiplex PCR, as any other molecular method, could not yet completely replace the culture-based phenotypic susceptibility test, it could be used as a rapid screening method for detection of RIF-resistant *M. tuberculosis*, a surrogate marker for MDR-TB, and provide the epidemiological information of the mutations within the *rpoB* gene, including codons 531, 526, 516 and 511 which made totally of 97.9% coverage of RIF-resistant mutation found in hot spot *rpoB* region. Further optimization and evaluation of this method for direct detection of *M. tuberculosis* drug resistance mutations in clinical specimens of TB patients remain an important focus of a future study.

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APPENDIX

APPENDIX

I. Media

1. Löwenstein-Jensen medium with/without RIF drug

Löwenstein-Jensen medium (BBL, USA)	37.3	g
Distilled water	588	ml
Glycerol	12	ml

Mix and sterilize by autoclaving for 15 minutes at 121°C.

Homogenized whole egg	1000	ml
RIF for LJ with drug (final concentration 40 µg/ml)	3.2	ml (from stock 20 mg/ml RIF preparation by dissolving 100 mg RIF into 5 ml dimethyl formamide and stored in a tube which was wraped with foil at -70°C or -20°C)

Then 1000 ml homogenized whole fresh eggs were mixed throughout with sterile LJ broth. Subsequently 3.2 ml of 20 mg/ml RIF was added into homogenized media with aseptic technique. The mixture was aliquoted for 7 ml per a sterile screw-capped tube and slanted them at 85°C for 3 hours (inspissation). After they cooled down, stored at 4°C until use.

II Reagents

1. TE buffer (10mM Tris-HCl; pH 8.0, 1 mM EDTA)

1 M Tris-HCl, pH 8.0 (see 1.1)	1	ml
0.5 M EDTA, pH 8.0 (see 1.2)	200	µl
Distilled water	98.8	ml

Mix until completely dissolved before sterilize by autoclaving for 15 minutes at 121°C.

1.1. 1M Tris-HCl, pH 8.0

Tris base	12.1	g
Distilled water	95.8	ml
1N HCl	4.2	ml

Dissolve 12.1 g Tris base in 80 ml distilled water, mix until completely dissolved before adjust pH to 8.0 with 1 N HCl ~ 4.2 ml (see 1.3). And then add distilled water to 100 ml and sterilize by autoclaving for 15 minutes at 121°C.

1.2. 0.5 M EDTA (ethylenediaminetetraacetic acid), pH 8.0

$Na_2EDTA•2H_2O$	9.3	g
Distilled water	50	ml
NaOH	1	g

Dissolve 9.3g $Na_2EDTA•2H_2O$ in 40 ml distilled water and stir vigorously on magnetic stirrer, mix until completely dissolved before adjust pH to 8.0 with ~ 1 g NaOH. Then add distilled water to 50 ml and sterilize by autoclaving for 15 minutes at 121°C.

1.3. 1N HCl

Conc. HCl	8.3	ml
Distilled water	91.4	ml

2. 50X Tris-acetate EDTA (TAE) electrophoresis buffer (2M Tris-acetate, 0.1 mM EDTA; pH 8.3)

Tris base	242	g
0.5 M EDTA (from 1.2)	200	ml
Distilled water	742.9	ml
Glacial acetic acid	57.1	ml

Mix until completely dissolved before adjust pH by using glacial acetic acid (~57.1 ml) to pH 8.3

3. Gel loading dye

Glycerol	6	ml
Bromophenol blue	20	mg
Distilled water	4	ml

Mix until completely dissolved before store at 4°C

4. Ethidium bromide staining solution (final concentration 1 µg/ml)

Ethidium bromide from stock 10 mg/ml	60	µl
Distilled water	600	ml

Mix until completely dissolved before store at room temperature in darkness

III Antimycobacterial susceptibility testing by the proportion method

Inoculum preparation

A loopful of freshly grown colonies (3-4 weeks) from LJ medium was transferred to a tube containing 2-3 drops of Middlebrook 7H9 and 6 to 10 sterile glass beads (5mm in diameters). The tube was vigorously agitated on a vortex mixer. Three to five milliliters of Middlebrook 7H9 were then added into the tube. The tube was vortexed and left in a safety cabinet for 30 minutes in order to allow the large clumps of cells to settle. The supernatant was transferred to a new sterile tube and adjusted with Middlebrook 7H9 to the density of 1.0 McFarland. After that, 10-fold dilution was done and selected the dilution of 10^{-2} and 10^{-4} as the inoculum for the proportion method (123).

The proportion method procedure

The proportion method was performed as recommended by World Health Organization (WHO) (123). Antimycobacterial drugs were adjusted in the LJ medium to a final concentration of 40 µg/ml, 0.2 µg/ml, 2.0 µg/ml and 4.0 µg/ml for RIF, INH, EMB and STR, respectively. One hundred microliters of prepared bacterial inoculum was inoculated on LJ medium with or without drug for test or control, respectively, and followed by incubation at 37°C for 21-28 days. Resistance was defined as growth on drug containing tubes equal to or greater than 1% of the growth on drug free medium.

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
001	DS 8216	sputum	S	S	S	S		
002	DS 7898	sputum	S	S	S	S		
003	DS 4130	sputum	R	R	R	R	TCG→TTG	Ser531Leu
004	DS 8466	sputum	S	S	S	S		
005	DS 4129	sputum	R	R	R	R	TCG→TTG	Ser531Leu
006	DS 8471	pus	S	S	S	S		
007	DS 8530	sputum	S	S	S	S		
008	DS 6354	sputum	R	R	R	R	TCG→TTG	Ser531Leu
009	DS 4085	sputum	R	R	R	R	TCG→ TTG	Ser531Leu
010	DS 6278	sputum	R	R	R	S	TCG→TTG	Ser531Leu
011	DS 6265	sputum	R	R	R	R	TCG→TTG	Ser531Leu
012	DS 5985	sputum	S	R	S	S	TCG→TTG	Ser531Leu
013	DS 8387	sputum	S	S	S	S		
014	DS 7937	sputum	S	S	S	S		
015	DS 5537	sputum	R	R	R	S	TCG→TTG	Ser531Leu
016	DS 8368	sputum	S	S	S	S		
017	DS 7749	sputum	S	S	S	S		
018	DS 8350	sputum	S	S	S	S		
019	DS 4340	sputum	R	R	R	S	CAC→CTC	His526Leu
020	DS 8508	sputum	S	S	S	S		
021	DS 7183	sputum	S	S	S	S		
022	DS 6053	sputum	R	R	S	S	TCG→TTG	Ser531Leu
023	DS 6926	sputum	R	R	R	R	CAC→TAC	His526Tyr
024	DS 6000	sputum	R	R	R	R	TCG→TTG	Ser531Leu
025	DS 5899	sputum	R	R	R	R	CAC→CTG	His526Leu

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
026	DS 5839	sputum	R	R	S	R	GAC→GTC	Asp516Val
027	FT 0424	sputum	R	R	R	R	CAA→GAA	Gln513Glu
028	DS 8049	sputum	S	S	S	S		
029	DS 8447	sputum	S	S	S	S		
030	DS 7811	sputum	S	S	S	S		
031	DS 7918	sputum	S	S	S	S		
032	DS 5760	sputum	R	R	R	R	TCG→TTG	Ser531Leu
033	DS 8303	sputum	S	S	S	S		
034	DS 8250	sputum	S	S	S	S		
035	DS 8276	sputum	S	S	S	S		
036	DS 7201	sputum	S	S	S	S		
037	DS 8218	sputum	S	S	S	S		
038	DS 5573	sputum	R	R	R	S	TCG→TTG	Ser531Leu
039	DS 8497	sputum	S	S	S	S		
040	DS 8219	sputum	S	S	S	S		
041	DS 8538	sputum	S	S	S	S		
042	DS 7912	pus	S	S	S	S		
043	DS 6858	sputum	R	R	R	S	TCG→TTG	Ser531Leu
044	FT 0402	sputum	R	R	S	R	TCG→TTG	Ser531Leu
045	DS 8457	sputum	S	S	S	S		
046	DS 8343	sputum	S	S	S	S		
047	DS 6913	lymph node	R	R	S	S	TCG→TTG	Ser531Leu
048	DS 6277	sputum	S	R	S	S	GAC→GTC	Asp516Val
049	DS 6600	sputum	R	R	R	R	TCG→TTG	Ser531Leu
050	DS 7181	sputum	S	S	S	S		

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
051	DS 8098	sputum	S	S	S	S		
052	DS 8485	sputum	S	S	S	S		
053	DS 8481	sputum	S	S	S	S		
054	DS 6088	lymph node	R	R	R	R	TCG→TTG	Ser531Leu
055	DS 8349	sputum	S	S	S	S		
056	DS 7995	sputum	S	S	S	S		
057	DS 6698	sputum	R	R	R	S	TCG→TTG	Ser531Leu
058	DS 5996	sputum	R	R	R	R	CTG→CCG	Leu533Pro
059	DS 8525	sputum	S	S	S	S		
060	DS 6308	sputum	R	R	S	R	CAC→TAC	His526Tyr
061	DS 6156	sputum	R	R	R	S	TCG→TTG	Ser531Leu
062	DS 6646	sputum	R	R	R	R	CAC→CGC	His526Arg
063	DS 8396	sputum	S	S	S	S		
064	DS 8450	sputum	S	S	S	S		
065	DS 8384	sputum	S	S	S	S		
066	DS 8152	sputum	S	S	S	S		
067	DS 8461	sputum	S	S	S	S		
068	DS 5326	sputum	R	R	S	S	deletion (ATTCATGGA)	deletion (codon 513-516)
069	DS 5538	sputum	R	R	S	R	TCG→TTG	Ser531Leu
070	DS 4181	sputum	R	R	R	S	TCG→TTG	Ser531Leu
071	DS 8406	sputum	S	S	S	S		
072	DS 8478	sputum	S	S	S	S		
073	DS 8456	sputum	S	S	S	S		
074	DS 8409	sputum	S	S	S	S		
075	DS 6279	sputum	R	R	R	R	GAC→GTC	Asp516Val

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
076	DS 7552	sputum	S	S	S	S		
077	DS 4224	sinus d/c	R	R	S	S	CAC→TAC	His526Tyr
078	DS 8380	sputum	S	S	S	S		
079	DS 6336	sputum	R	R	S	S	CAC→TAC	His526Tyr
080	DS 8269	sputum	S	S	S	S		
081	DS 8443	sputum	S	S	S	S		
082	DS 6623	sputum	R	R	S	S	CAC→TAC	His526Tyr
083	DS 8094	sputum	S	S	S	S		
084	DS 7209	sputum	S	S	S	S		
085	FT 0476	sputum	R	R	R	S	TCG→TTG	Ser531Leu
086	DS 8182	sputum	S	S	S	S		
087	DS 5539	sputum	R	R	S	R	GAC→GAG TCG→TTG	Asp516Glu Ser522Leu
088	DS 8426	sputum	S	S	S	S		
089	DS 6692	sputum	R	R	R	S	TCG→TTG	Ser531Leu
090	DS 8417	sputum	R	R	R	R	CAC→CGC	His526Arg
091	DS 8438	sputum	S	S	S	S		
092	DS 8386	sputum	R	R	R	R	CAC→CTC	His526Leu
093	DS 8410	sputum	S	S	S	S		
094	DS 8182	sputum	S	S	S	S		
095	DS 8423	sputum	S	S	S	S		
096	DS 8393	sputum	S	S	S	S		
097	DS 8313	sputum	S	S	S	S		
098	DS 7845	sputum	S	S	S	S		
099	DS 8229	sputum	S	S	S	S		
100	DS 5816	sputum	R	R	S	R	GTC→TTC	Gln176Lys

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
101	DS 4135	abscess	R	R	R	S	CTG→CCG	Leu533Pro
102	DS 6549	sputum	R	R	R	R	TCG→TTG	Ser531Leu
103	DS 4352	sputum	R	R	R	S	GAC→GTC	Asp516Val
104	DS 8284	sputum	S	S	S	S		
105	DS 8524	sputum	S	S	S	S		
107	DS 8103	sputum	S	S	S	S		
109	DS 8246	sputum	S	S	S	S		
110	DS 6882	sputum	R	R	S	R	CAC→TAC	His526Tyr
111	FT 0206	sputum	R	R	S	S	TCG→TTG	Ser531Leu
112	FT 0369	sputum	R	R	R	S	CAC→CGC	His526Arg
113	DS 8371	sputum	S	S	S	S		
114	FT 0690	sputum	R	R	R	R	TCG→TTG	Ser531Leu
116	DS 0408	sputum	R	R	S	S	TCG→TTG	Ser531Leu
117	DS 6554	sputum	R	R	R	R	TCG→TTG	Ser531Leu
118	DS 8501	sputum	S	S	S	S		
119	DS 8411	sputum	S	S	S	S		
120	DS 8370	lymph node	S	S	S	S		
121	DS 4160	sputum	R	R	R	S	CAC→TAC	His526Tyr
122	H37Rv	sputum	S	S	S	S		
123	DS 6723	sputum	R	R	S	S	CAC→GAC	His526Asp
124	DS 8401	sputum	S	S	S	S		
125	DS 7975	sputum	S	S	S	S		
126	DS 5695	sputum	R	R	S	S	TCG→TTG	Ser531Leu
127	DS 8317	sputum	S	S	S	S		
128	DS 8468	sputum	S	S	S	S		

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
129	DS 8093	sputum	S	S	S	S		
130	DS 8173	sputum	S	S	S	S		
131	DS 6345	sputum	R	R	R	R	CAC→TAC	His526Tyr
132	DS 6509	sputum	S	R	S	S	CAC→TAC	His526Tyr
133	DS 8344	sputum	S	S	S	S		
134	DS 8351	sputum	S	S	S	S		
135	DS 6277	sputum	S	R	S	S	TCG→TTG	Ser531Leu
137	DS 7890	sputum	S	S	S	S		
138	DS 7982	sputum	S	S	S	S		
139	DS 8385	sputum	S	S	S	S		
140	DS 8044	sputum	S	S	S	S		
141	FT 0483	sputum	R	R	R	R	TCG→TTG	Ser531Leu
142	DS 8131	sputum	S	S	S	S		
143	DS 6567	sputum	R	R	S	S	TCG→TTG	Ser531Leu
144	DS 5983	sputum	R	R	R	R	CTG→CCG	Leu533Pro
145	DS 8377	sputum	S	S	S	S		
146	DS 4152	sputum	R	R	S	S	CAC→TAC	His526Tyr
147	DS 8528	sputum	S	S	S	S		
148	DS 8296	lymph node	S	S	S	S		
149	DS 7062	sputum	R	R	S	S	CAC→CGC	His526Arg
150	DS 7927	sputum	S	S	S	S		
151	DS 8372	lymph node	R	R	R	R	TCG→TTG	Ser531Leu
152	DS 2667	sputum	S	R	S	S	CAC→TAC	His526Tyr
153	DS 4074	sputum	R	R	S	S	TCG→TTG	Ser531Leu
154	DS 8360	sputum	S	S	S	S		

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
157	DS 8261	sputum	S	S	S	S		
158	DS 8347	sputum	S	S	S	S		
159	DS 8455	sputum	S	S	S	S		
160	DS 0209	sputum	R	R	S	S	CAC→CGC	His526Arg
161	FT 0177	sputum	R	R	R	R	CAC→CTC	His526Leu
162	DS 8420	sputum	S	S	S	S		
163	DS 6645	other	R	R	S	R	NA	NA
164	DS 8391	sputum	S	S	S	S		
166	DS 4314	sputum	R	R	S	S	CAC→TAC	His526Tyr
167	DS 3315	sputum	R	R	S	S	CTG→CGG GAC→TAC	Leu511Arg Asp516Tyr
168	DS 6488	sputum	R	R	S	S	CAC→GAC	His526Asp
169	DS 5810	sputum	R	R	R	R	CAC→TAC	His526Tyr
170	DS 8346	sputum	S	S	S	S		
171	DS 4571	sputum	R	R	S	S	TCG→TTG	Ser531Leu
172	DS 4630	sputum	R	R	S	S	CAC→TAC	His526Tyr
173	DS 8486	sputum	S	S	S	S		
174	DS 4230	sputum	R	R	S	S	GAC→GTC	Asp516Val
175	FT 0593	sputum	R	R	R	R	TCG→TTG	Ser531Leu
176	DS 8454	lymph node	S	S	S	S		
177	DS 7843	sputum	S	S	S	S		
178	DS 7892	sputum	S	S	S	S		
179	DS 5738	sputum	R	R	R	R	TCG→TTG	Ser531Leu
180	DS 7300	sputum	S	S	S	S		
181	DS 8121	sputum	S	S	S	S		
182	DS 0502	sputum	R	R	S	S	CAC→CTC	His526Leu

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
183	FT 0578	sputum	R	R	R	R	TCG→TTG	Ser531Leu
184	DS 6481	sputum	R	R	S	S	GTC→TTC	Gln176Lys
185	DS 0211	sputum	R	R	S	S	TCG→TTG	Ser531Leu
186	DS 8395	sputum	S	S	S	S		
187	DS 8206	sputum	S	S	S	S		
188	DS 8441	sputum	S	S	S	S		
189	DS 5295	lymph node	R	R	R	S	TCG→TTG	Ser531Leu
190	DS 8094	sputum	S	S	S	S		
191	DS 6088	lymph node	R	R	R	R	TCG→TTG	Ser531Leu
192	DS 8219	sputum	S	S	S	S		
193	DS 8164	sputum	S	S	S	S		
194	DS 8343	sputum	S	S	S	S		
195	DS 8435	sputum	S	S	S	S		
196	DS 4543	sputum	R	R	S	S	TCG→TTG	Ser531Leu
197	DS 6157	sputum	R	R	R	R	TCG→TTG	Ser531Leu
198	DS 8145	sputum	S	S	S	S		
200	DS 7722	sputum	S	S	S	S		
201	DS 8418	sputum	S	S	S	S		
202	DS 2578	sputum	R	R	S	S	TCG→TTG	Ser522Leu
203	DS 4951	sputum	R	R	S	S	CAC→TAC	His526Tyr
204	DS 5904	sputum	R	R	S	S	CAC→GAC	His526Asp
205	DS 9288	sputum	S	S	S	S		
206	DS 8677	sputum	R	R	S	S	GAC→GTC	Asp516Val
207	DS 9255	sputum	R	R	S	S	TCG→TTG	Ser531Leu
208	DS 9270	sputum	R	R	S	S	TCG→TTG	Ser531Leu

Table 12 Characteristics of all *M. tuberculosis* isolates used in the study (continued)

Blinded strains (RIF No.)	Original strains (Isolate No.)	Clinical source	Susceptibility pattern from the proportion method				*rpoB* gene sequencing results*	
			INH	RIF	STR	EMB	Nucleotide alteration	Amino acid alteration
209	DS 9278	sputum	R	R	S	S	CAC→TAC	His526Tyr
210	DS 9376	sputum	R	R	S	S	CAC→AGC	His526Ser
211	DS 9102	sputum	S	S	S	S		
212	DS 9442	sputum	R	R	S	S	CAC→TAC	His526Tyr
213	DS 9469	sputum	R	R	S	S	TCG→TTG	Ser531Leu
214	DS 9596	sputum	R	R	S	S	CAC→GAC	His526Asp
215	DS 9636	sputum	R	R	S	S	TCG→TTG	Ser531Leu
216	DS 9482	sputum	S	S	S	S		
217	DS 9851	lymph node	R	R	S	S	TCG→TTG	Ser531Leu
218	DS 9854	pleural fluid	R	R	S	S	TCG→TTG	Ser531Leu
219	DS 9915	sputum	R	R	S	S	TCG→TTG	Ser531Leu
220	DS 10197	sputum	R	R	S	S	CAC→TAC	His526Tyr
221	DS 10216	sputum	R	R	S	S	GAC→GTC	Asp516Val
222	DS 9478	sputum	S	S	S	S		
223	DS 10217	sputum	R	R	S	S	CAC→GAC	His526Asp
224	DS 10238	sputum	R	R	S	S	TCG→TTG	Ser531Leu
S1	DS 3561	sputum	R	R	R	R	NA	NA
S4	DS 5574	sputum	R	R	R	R	NA	NA
S7	DS 6157	sputum	R	R	R	R	NA	NA
S9	DS 6765	sputum	R	R	R	R	NA	NA

* Sequencing of the *rpoB* gene was performed as described by Heep *et al.* (17).

NA = Not available

The *rpoB* sequencing for identification of 103 RIF-resistant phenotype *M. tuberculosis* isolates used in assessment of the study

The *rpoB* sequencing results were kindly provided by Mr. Wattana Cheunoy. Nucleotide sequence of a 749-bp fragment covered the *rpoB* hot spot region from 103 RIF-resistant phenotype of *M. tuberculosis* were examined by using of TBB-1 primer (5'-ATCACACCGCAGACGTTG-3') and TBB-2 primer (5'-ACGTAGTGTCACTACATCAGC-3'). The amplification reactions were performed in a final volume of 50 µl containing 1.5 mM $MgCl_2$, 50 mM KCl, 1 U of *Taq* DNA polymerase, 200 µM deoxynucleoside triphosphates (dNTPs), 30 pmol of each primer and 5 µl DNA template. The PCR reactions were performed in a PTC-200 DNA Engine thermal cycler under the following conditions: initial denaturation at 94°C for 5 minutes; 40 cycles of 94°C for 45 seconds, 64°C for 1 minute, and 72°C for 2 minutes and final elongation at 72°C for 7 minutes. The 749-bp PCR products were performimg with Gel Extraction Kit (NucleoSpin® Extract II, Macherey-Nagel, USA) by the protocol recommended by the manufacturer and were subjected to sequencing at Macrogen Unit, South Korea.

Table 13 Characteristics of 50 *M. tuberculosis* clinical samples which cultured from MGIT and used for direct detection by the developed PCR

No.	FT No.	Routine No.	Results				
			PCR method	Susceptibility pattern			
				RIF	INH	STR	EMB
1	1520	8540	S	S	S	R	S
2	1608	11069	S	S	S	R	S
3	1621	11261	S	S	S	S	S
4	1630	11493	S	S	S	S	S
5	1648	12077	S	S	S	S	S
6	1653	12191	S	S	S	S	S
7	1596	10973	S	S	S	S	S
8	1622	11270	S	S	S	R	S
9	1639	11808	S	S	S	R	R
10	1642	11880	S	S	S	R	R
11	1648	12077	S	S	S	S	S
12	1653	12191	S	S	S	S	S
13	1655	12259	S	S	S	S	S
14	1657	12268	S	S	S	R	S
15	1673	12550	deletion516	R	R	R	R
16	1654	12258	S	S	S	S	S
17	1656	12255	S	S	S	S	S
18	1682	12901	S	S	S	S	S
19	1723	13931	S	S	S	S	S
20	1725	13933	S	S	R	R	S
21	1719	13820	S	S	R	S	S
22	1737	14134	S	S	S	S	S
23	1740	14271	S	S	S	S	S
24	1741	14314	531	R	R	R	R
25	1742	14343	S	S	S	S	S

Table 13 Characteristics of 50 *M. tuberculosis* clinical samples which cultured from MGIT and used for direct detection by the developed PCR (continued)

No.	FT No.	Routine No.	Results				
			PCR method	Susceptibility pattern			
				RIF	INH	STR	EMB
26	1755	14878	S	S	S	S	S
27	1758	14888	S	S	S	S	S
28	1809	15866	S	S	S	S	S
29	1821	16228	531	R	R	R	S
30	1702	13539	531	R	R	R	R
31	1799	15743	S	S	S	S	S
32	1805	15816	S	S	S	S	S
33	1827	16457	S	S	S	S	S
34	1845	16791	S	S	S	S	S
35	1847	16865	S	S	S	S	S
36	1849	17019	S	S	S	S	S
37	1859	17210	526	S	R	S	R
38	1862	17333	S	S	S	S	S
39	1872	17479	S	S	S	S	S
40	1886	18181	S	S	S	S	S
41	1890	18272	S	S	S	S	S
42	1891	18273	S	S	S	S	S
43	1916	18992	516	R	R	R	R
44	1920	19132	S	S	S	R	S
45	1923	19236	S	S	R	S	S
46	1924	19237	S	S	S	S	S
47	1928	19419	S	S	S	S	S
48	1980	242	S	S	S	S	S
49	1981	243	S	S	S	S	S
50	2063	2076	S	S	S	S	S

S; susceptible, R; resistant, RIF; Rifampicin, INH; Isoniazid, STR; Streptomycin, EMB; Ethambutol

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