

การพัฒนาวิธีการเพิ่มปริมาณยีนดื้อยาในเชื้อวัณโรคด้วยมัลติเพล็กซ์พีซีอาร์
สำหรับการวิเคราะห์ลำดับสารพันธุกรรมสมรรถนะสูง

DEVELOPMENT OF MULTIPLEX PCR AMPLIFICATION METHOD OF
DRUG RESISTANT GENES IN *Mycobacterium tuberculosis*
FOR NEXT-GENERATION SEQUENCING

วิชชุดา ภัทรกุลชัย

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
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สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง

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WITCHUDA PATTARAKUNCHAI

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บทคัดย่อ

วัณโรคคือโรคติดต่อชนิดหนึ่งมีสาเหตุจากการติดเชื้อแบคทีเรีย *Mycobacterium tuberculosis* ในปี 2017 วัณโรคถูกจัดอยู่ในอันดับหนึ่งของโรคติดเชื้อแบคทีเรียที่ทำให้มีผู้เสียชีวิตจำนวนมากทั่วโลก วัณโรคดื้อยาเป็นปัญหาสำคัญมีผลต่อการรักษาและควบคุมโรค สาเหตุหลักที่ทำให้เชื้อวัณโรคดื้อยา คือ เกิดการกลายพันธุ์ภายในยีนที่เกี่ยวข้องกับการออกฤทธิ์ของยา การวิเคราะห์ลำดับสารพันธุกรรมสมรรถนะสูงเป็นวิธีที่ตรวจสอบความไวของเชื้อวัณโรคดื้อยาได้อย่างรวดเร็ว งานวิจัยนี้มีจุดประสงค์เพื่อพัฒนาวิธีเพิ่มปริมาณยีนดื้อยาด้วยมัลติเพล็กซ์พีซีอาร์ และตรวจหาลำดับนิวคลีโอไทด์ของยีนดื้อยาในเชื้อวัณโรคที่แยกได้จากผู้ป่วย ในงานวิจัยนี้ ไพโรมอร์จำนวน 14 คู่ถูกออกแบบให้จำเพาะกับยีนจำนวน 13 ยีนที่เกี่ยวข้องกับการดื้อยาของเชื้อวัณโรคโดยใช้ดีเอ็นเอของเชื้อ *M. tuberculosis* H37Rv เป็นดีเอ็นเอต้นแบบ ทำการจัดกลุ่มไพโรมอร์และหาสภาวะที่เหมาะสมในการทำมัลติเพล็กซ์พีซีอาร์ จากนั้น นำตัวอย่างดีเอ็นเอเชื้อวัณโรคจากผู้ป่วยจำนวน 10 ตัวอย่างมาเพิ่มปริมาณยีนดื้อยาด้วยมัลติเพล็กซ์พีซีอาร์ที่สภาวะเหมาะสม แล้วนำผลิตภัณฑ์ไปตรวจหาลำดับนิวคลีโอไทด์ด้วยการวิเคราะห์ลำดับสารพันธุกรรมสมรรถนะสูงโดยใช้ระบบ Nextseq 500 ผลการทดลองพบว่า ไพโรมอร์ที่ออกแบบนำมาจัดกลุ่มให้เหมาะสมกับการทำมัลติเพล็กซ์พีซีอาร์ได้เป็น 4 กลุ่ม กลุ่มละ 3-4 ไพโรมอร์ สภาวะที่เหมาะสมคือ ความเข้มข้นของไพโรมอร์ 0.05-0.6 มิลลิโมลาร์ อุณหภูมิ annealing ที่ 60 องศาเซลเซียส นาน 1 นาที และอุณหภูมิ extension 72 องศาเซลเซียส นาน 2 นาที ความเข้มข้นของดีเอ็นเอต้นแบบไม่ต่ำกว่า 40 นาโนกรัม วิธีมัลติเพล็กซ์พีซีอาร์สามารถเพิ่มจำนวนยีนดื้อยาของตัวอย่างดีเอ็นเอเชื้อวัณโรคจากผู้ป่วยได้ทั้ง 10 ตัวอย่างและมีความจำเพาะสูง ผลการตรวจหาการดื้อยาจากลำดับนิวคลีโอไทด์เป็นไปในทิศทางเดียวกับผลการทดสอบด้วยวิธีมาตรฐาน สรุปได้ว่าเทคนิคมัลติเพล็กซ์พีซีอาร์สามารถเพิ่มจำนวนยีนดื้อยาของเชื้อวัณโรคด้วยความจำเพาะสูง สะดวก และรวดเร็ว สำหรับใช้เตรียมตัวอย่างเพื่อวิเคราะห์ลำดับนิวคลีโอไทด์ของยีนดื้อยาในเชื้อวัณโรค

คำสำคัญ : วัณโรค *Mycobacterium tuberculosis* ยีนดื้อยา มัลติเพล็กซ์พีซีอาร์

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Abstract

Tuberculosis (TB) is a contagious disease caused by infection of a bacterium called *Mycobacterium tuberculosis*. In 2017, TB is a lead cause of death among the infectious diseases worldwide. Nowadays, drug resistance TB is an important problem for TB treatment and transmission control. A major cause of drug resistance in *M. tuberculosis* usually caused by a mutation occurred in genes involved in drug binding or drug activation. Targeted next-generation sequencing (NGS) is a rapid method to determine drug susceptibility. This work aims to develop a multiplex PCR method for amplification of drug-resistant genes in *M. tuberculosis* clinical strains and subsequently determine their nucleotide sequences by the targeted NGS. In this study, 14 primer pairs specific to 13 *M. tuberculosis* drug resistant genes were designed using *M. tuberculosis* H37Rv as DNA template. Primer clustering and amplification condition of the multiplex PCR were optimized. Drug resistant genes of 10 *M. tuberculosis* clinical strains were amplified by the optimized multiplex PCR. Nucleotide sequences of amplified products were analyzed by next-generation sequencing using the Nextseq 500 platform. Results showed that the designed primers were clustered into 4 sets each containing 3-4 primer pairs for multiplex PCR amplification. Optimal conditions included primer concentration at 0.2-0.6 mM, annealing temperature at 60°C for 1 minute, extension temperature at 72°C for 2 minutes and concentration of DNA template was not less than 40 ng. Drug resistant genes of all strains could be amplified by the multiplex PCR with high specificity. Drug susceptibility results determined by the targeted NGS were concordant with the results by the conventional drug susceptibility testing. In conclusion, the multiplex PCR was able to amplify *M. tuberculosis* drug-resistant genes with high specificity; it is simple and rapid method for preparing DNA samples to further analyze by the next-generation sequencing.

Keywords : Tuberculosis, *Mycobacterium tuberculosis*, Drug resistant gene, Multiplex PCR

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Chapter 1

Introduction

1.1 Research Motivation

Tuberculosis (TB) is one of infectious diseases that causes the death in millions of people each year. According to the World Health Organization (WHO) report, there were an estimated 10.4 million new TB cases and approximately 1.4 million TB deaths worldwide in 2015 (World Health Organization, 2016). Most of TB high-burden countries are in Asia and South Africa. In Africa, there are a large number of TB cases associated with HIV infection. Human immunodeficiency virus (HIV) is a virus that attacks a human immune system. It causes the acquired immunodeficiency syndrome (AIDS) disease which has been already spread around the world. People with HIV-infection were 26 to 31 times more likely to become sick with TB than normal people (World Health Organization, 2016). Thailand is one of TB high-burden countries harboring an estimated 117,000 new TB cases and 8,400 TB deaths in 2015 (World Health Organization, 2016).

TB is caused by an infection of the obligated aerobic slow-growing mycobacterium, *Mycobacterium tuberculosis* complex (MTBC). The bacilli are rod-shaped and non-motile. They do not produce any pigments and do not form endospores. Acid-fast staining is used to identify mycobacteria under a light microscope because of their high lipid content, particularly mycolic acid, in cell wall. Members of *M. tuberculosis* complex comprise *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium canettii*, *Mycobacterium mungi*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *Mycobacterium orygis* and *Mycobacterium microti*. *Mycobacterium tuberculosis* (MTB) is the major cause of human TB while the other species usually cause TB in animals.

Laboratory diagnosis of TB is depending on sputum smear microscopy and culture methods. Recently, molecular-based methods have been introduced and shown to be an alternative method for TB diagnosis. People, who develop active TB, will be treated with multiple TB drug regimen. The antituberculous drugs are divided into two major groups, first-line drugs and second-line drugs. First-line drugs comprise rifampicin, isoniazid, pyrazinamide, streptomycin and ethambutol. These drugs have the greatest activity against susceptible TB bacteria and are generally used for TB patients who have no history of TB treatment. Second-line drugs are used for TB patients who are suspected to infect with drug-resistant *M. tuberculosis*. The

examples of these drugs are aminoglycosides, fluoroquinolones and capreomycin. Drug resistance is an important problem for TB treatment. In 2015, there were an estimated 480,000 new cases of multidrug-resistant TB worldwide (World Health Organization, 2016). There are two main types of drug resistant TB, multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). MDR-TB is the type of drug resistant TB, of which the bacteria are resistant to at least rifampicin and isoniazid. XDR-TB is MDR-TB which additionally resists to any fluoroquinolones and one of the second line injectable TB drugs (amikacin, kanamycin, and capreomycin). Identification of drug-resistant *M. tuberculosis* strain is still based on cultivation of a pathogen and performing drug susceptibility testing. However, conventional drug susceptibility testing requires several weeks, maybe months, until reporting the results. Rapid method that could reduce time for identifying drug-resistant strains would be very useful for rapid diagnosis and treatment of drug-resistant TB, making the control of TB more efficiently.

In general, resistance to antituberculous drugs is caused by a chromosomal DNA mutation either by point mutation, insertion, or deletion of genes associated with drug binding or drug action (Zhang and Yew, 2015). Several genes have been reported to be involved in drug resistance in *M. tuberculosis*, such as *rpoB*, *katG*, *inhA*, *ahpC*, *oxyR*, *kasA*, *furA*, *ndh*, *pncA*, *rrs*, *rpsL*, *gidB*, *embCAB*, *rrs*, *gyrA* and *gyrB* (Campbell et al., 2011; Zhang and Yew, 2015). Detection of mutations within these genes could help to identify drug-resistant strains. Molecular-based methods have been developed and commercialized to use for detection of the resistance genes; however, most of them is used for detection only in rifampicin and isoniazid resistance (Morgan et al., 2005; Dorman et al., 2012; Bunsow et al., 2013). The molecular test for detecting second-line drug resistance such as Genotype®MTBDRsl can detect fluoroquinolones and aminoglycosides resistance (Theron et al., 2016). However, the equipment of this test is expensive and the technique used is very complex, requires a lot of expertise. Recently, high-throughput sequencing platforms have been introduced and used for performing whole-genome sequencing (WGS) of *M. tuberculosis* (Koser et al., 2013). Based on the genomic information, WGS could provide a rapid drug susceptibility result of all currently used anti-TB drugs, of which the mechanisms of resistance have been known, at the same time (Cole et al., 1998, Koser et al., 2013 Walker et al., 2015). Because of the high cost of performing WGS, the high-throughput sequencing method targeted only the genes associated with drug resistance has been proposed to rapidly determine antituberculous drug susceptibility and to reduce the cost of sequencing as well. In this research, the multiplex PCR method for targeted amplification of 13 drug resistant genes related to first-line and second-line antituberculous drugs will be developed and the amplified

products will be subsequently sequenced by next-generation sequencing (NGS) using Nextseq 500 platform.

1.2 Objectives of the study

The objectives of this study are

1) to optimize multiplex PCR condition for amplification of 13 MTB drug resistant genes with *M. tuberculosis* H37Rv.

2) to perform the targeted NGS with 10 DNA samples for *M. tuberculosis* clinical isolates.

3) to evaluate the accuracy of the targeted NGS for rapid determination of drug susceptibility comparing with the conventional phenotypic drug susceptibility testing.

1.3 Scopes of the study

In this study, 13 MTB genes associated with resistance to 5 first-line and 3 second-line drugs are selected; (1) *rpoB* for rifampicin resistance, (2) *katG*, *inhA* and *ahpC* for isoniazid resistance, (3) *rpsL* for streptomycin resistance, (4) *embB* for ethambutol resistance, (5) *pncA* and *rpsA* for pyrazinamide resistance, (6) *rrs* and *eis* for aminoglycoside resistance, (7) *gyrA* and *gyrB* for fluoroquinolone resistance, and (8) *tlyA* for capreomycin resistance. The scopes of this study are described as following;

1) PCR primers of drug resistant genes will be designed using the genome sequence of *M. tuberculosis* H37Rv (GenBank accession no. NC_000962) as a reference. Primer specificity will be tested by a simplex PCR using DNA thermal cycler (Biometra, Germany). MTB drug resistant genes will be clustered and amplified by a multiplex PCR using DNA thermal cycler. To optimize multiplex PCR condition, the parameters included gene cluster, extension time, annealing temperature, and primer concentration will be optimized. The sensitivity of multiplex PCR will be test by decreasing of template concentration and primer concentration. PCR products of drug resistant genes will be analyzed by 1.5 % (w/v) agarose gel electrophoresis.

2) MTB drug resistant genes will be amplified using genomic DNA from 10 MTB clinical isolates collected by Drug Resistant Tuberculosis (DR-TB) Research Fund at Siriraj hospital as DNA templates. PCR products will be sequenced by next-generation sequencing using the Nextseq 500 machine (Illumina, USA) at the Medical Genetics Center, Medical Life Science Institute, Ministry of Public Health, Nonthaburi, Thailand.

1.4 Benefits of the study

In this study, the developed multiplex PCR combination with targeted next-generation sequencing for 13 drug-resistant genes could provide drug susceptibility result of all important antituberculous drugs through the genetic information at the same time. It would help to rapidly detect drug-resistant MTB strains and provide critical information for selection of appropriate treatment regimen, making control of drug-resistant TB more effectively.

Chapter 2

Theory and Literature Reviews

2.1 Tuberculosis

Tuberculosis (TB) is one of infectious diseases caused by *Mycobacterium tuberculosis*. TB normally infects human lungs and is called “Pulmonary TB”. But TB can also infect other organs and is called “Extrapulmonary TB”. This disease can spread through the air when people who are sick with pulmonary TB cough or sneeze. In general, only small proportion (5–15%) of the estimated 2–3 billion people infected with *M. tuberculosis* will develop and become TB disease. However, the probability of developing TB disease is much higher in HIV infected patients. In 2015, there were an estimated 10.4 million new TB cases worldwide, dividing into 5.9 million men (56%), 3.5 million women (34%) and 1.0 million children (10%). Among them, 1.2 million people infected with HIV (11%) were found in new TB cases (World Health Organization, 2016). Thailand is one of TB high-burden countries harboring an estimated 117,000 new TB cases and 8,400 TB deaths in 2015 (World Health Organization, 2016).

2.1.1 Symptoms

In general, TB can be divided into 2 types depending on the symptoms; latent TB and active TB. Latent TB or inactivated TB is a type of TB in which bacteria remains in the host but clinical symptoms and obvious adverse effects do not appear. The activation of latent TB to become active TB occurs in 5–10 % of infected people by immunosuppression due to age, malnutrition, or infection with the human immunodeficiency virus (HIV) (Flynn, 2004). In the other hand, active TB is a type of TB in which the immune system is unable to stop the bacterial growing, leading to an infection in the lungs or other organs, and finally symptoms appear. In patients with active TB, bacteria can spread and infect to other normal people. The most important symptom suggestive of pulmonary TB, is a cough persisting for more than 3 weeks (Long, 2002). Other general symptoms include coughing up blood, excessive sweating especially at night, fatigue, fever, weight loss, breathing difficulty, chest pain and wheezing. The symptoms of extrapulmonary TB vary according to the infected organs; for example, TB of the kidney may cause blood in the urine, TB meningitis may cause headache or confusion, TB of the spine may cause back pain, and TB of the larynx can cause hoarseness.

2.1.2 Diagnosis of tuberculosis

Diagnosis of TB is performed by various kinds of techniques including chest radiograph, sputum smear microscopy, molecular test and cultivation method.

2.1.2.1 Chest radiograph

The chest radiograph is useful for diagnosis of pulmonary TB disease. TB-related chest abnormalities can be detected by a posterior-anterior radiograph of the chest (Fig. 2.1) and suggesting to be pulmonary TB disease. In some cases, a lateral view of the chest is helpful for TB diagnosis. For more information, a computerized tomography (CT) is used to provide more detailed images of parts of the body that cannot easily be seen on a standard chest radiograph. In infected person, the radiographic abnormalities are often seen in the apical and posterior segments of the upper lobe or in the superior segments of the lower lobe. However, lesions may appear anywhere in the lungs and may differ in size, shape, density, and cavitation, especially in HIV infected and other immunosuppressed persons. The chest radiograph might be used to diagnose pulmonary TB disease in an HIV-negative person who has a positive *M. tuberculosis* infection test and who has no symptoms or signs of TB disease. However, smear microscopy and culture methods of bacteria must be used to confirm diagnostic of TB.

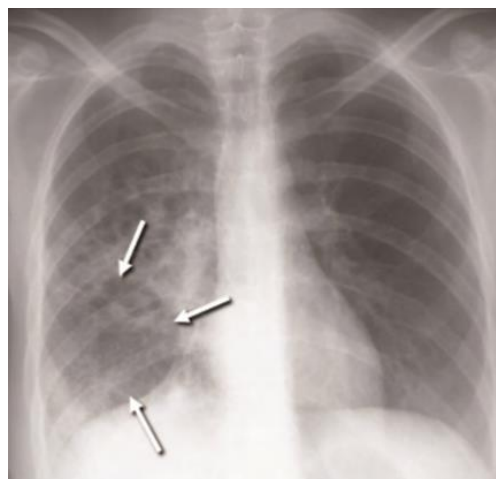


Figure 2.1 Chest Radiograph with Lower Lobe Cavity

Source: Center of Disease Control, 2013

2.1.2.2 Sputum smear microscopy (SMS)

Sputum smear microscopy or SMS was developed for more than 100 years ago. Sputum specimens are acid-fast stained and examined under a microscope to see if bacteria are present (Fig. 2.2). There are 2 procedures commonly used for

bacteria acid-fast staining consisting of Carbol-fuchsin procedure which includes the Ziehl-Neelsen and Kinyoun methods (direct microscopy) and Fluorochrome procedure using auramine-O or auramine-rhodamine dyes (fluorescent microscopy) (Center of Disease Control, 2013). SMS is a quick technique whose result should be available within 24 hours after specimen collection. However, SMS is only the postulated diagnosis of TB disease because other microorganisms not only *M. tuberculosis* might be stained by acid-fast staining. Furthermore, many TB patients can show negative by SMS method but a positive culture because there must be 5,000 to 10,000 bacterial cells per milliliter of sputum to allow the positive of TB bacteria in SMS. In contrast, only 10 to 100 bacterial cells are needed for a positive culture.

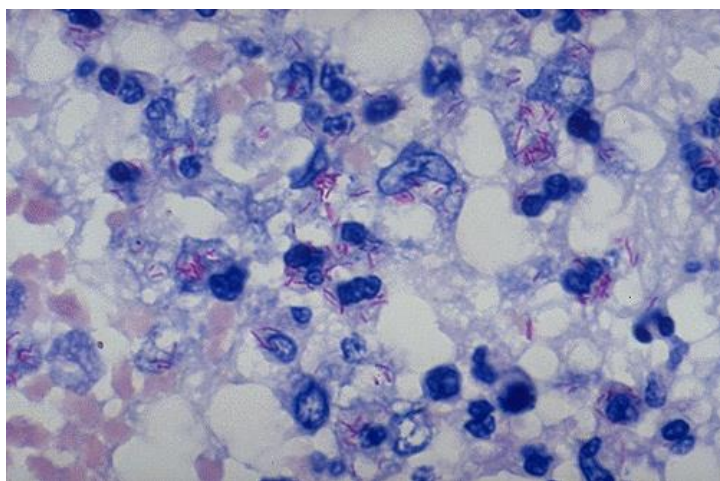


Figure 2.2 Acid fast staining of *Mycobacterium tuberculosis*; the red rods are *M. tuberculosis* cells in histologic sections or smears.

Available: <http://library.med.utah.edu/WebPath/INFEHTML/INFEC033.html>

2.1.2.3 Molecular test for tuberculosis detection

Molecular test is direct method for TB detection in clinical specimens using nucleic acid amplification (NAA). The result of DNA or RNA amplification by this method can be available in hours compared to 1 week or longer for cultivation method. The advantages for using NAA tests are an earlier laboratory confirmation of TB disease, improvement of patient treatments and interruption of transmission by early diagnosis. A single negative NAA result should not be used as a definitive result to exclude TB disease. Rather, the negative NAA result should be used as additional information in making clinical decisions, to expedite testing for an alternative diagnosis, or to prevent unnecessary TB disease treatment. The only rapid test for diagnosis of TB currently recommended by WHO is the Xpert® MTB/RIF assay (Cepheid, Sunnyvale USA) (WHO, 2014). It was initially recommended in 2010 for

diagnosis of pulmonary TB in adults (World Health Organization, 2014). Since 2013, it has also been recommended for diagnosis of pulmonary TB in children and for detection specific forms of extrapulmonary TB (World Health Organization, 2014). Xpert MTB/RIF assay is an automated polymerase chain reaction (PCR) test that uses a disposable cartridge with the GeneXpert Instrument System. A sputum sample is collected from the patient with suspected TB. The sputum is mixed with the reagents that are provided with the assay, and a cartridge containing this mixture is placed in the GeneXpert machine. All processing from this point on is fully automated. The test has much better accuracy than microscopy method.

2.1.2.4 Cultivation method

Mycobacterial cultivation is one of the reference standard methods for laboratory confirmation of TB disease. The growing bacteria need confirmation by performing drug-susceptibility testing and genotyping. Culture examinations should be done on all diagnostic specimens, without regard of Acid Fast Bacillus (AFB) testing smear or NAA results. The specimens are added to the culture medium and cultivated at 37°C. There are various kinds of commercially available culture medium systems such as BACTEC, MGIT, VersaTREK and MBBACT. Mycobacterial cultivation is the current reference standard method but it requires more developed laboratory capacity and can take up to 12 weeks to provide results. (World Health Organization, 2016).

2.1.3 Treatment for tuberculosis patients

An effective drug treatment for TB was first developed in the 1940s (Mitchison and Davies, 2012). The currently recommended treatment for new cases of drug-susceptible TB is a 6-month regimen of four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide. Treatment success rates of at least 85% for new cases of drug-susceptible TB are regularly reported (World Health Organization, 2016). Treatment for rifampicin-resistant TB (RR-TB) and multidrug-resistant TB (MDR-TB) is longer and requires more expensive and more toxic drugs. Patients with MDR-TB are treated with a different combination of second-line drugs, usually for 18 months or more. To reduce the length of conventional MDR-TB, in 2016, WHO recommended a standardized shorter MDR-TB regimen of 9–12 months for all patients (excluding pregnant women) with pulmonary MDR/ RR-TB that is not resistant to second-line drugs regimens (World Health Organization, 2016). Seven antituberculous drugs are used for shorter MDR-TB regimen including Kanamycin, Moxifloxacin, Prothionamide, Clofazimide, Pyrazinamide, Ethambutol and high dose of Isoniazid (World Health Organization, 2016). In addition, new TB drugs have begun to emerge from the

pipeline, and combination of regimens including new compounds are being tested in clinical trials.

2.1.4 Action of antituberculous drugs

2.1.4.1 Streptomycin (STR)

In 1946, streptomycin (STR) was first used as the first clinical trial with a random allocation of patients to the regimens, undertaken by the Tuberculosis Research Unit (Director Philip Hart) of the British Medical Research Council (BMRC) (Mitchison, 2000). STR inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome causing misreading of the mRNA message during translation (Hong et al., 2014). The site of action of STR is the small 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA (Hill, 1990). STR is thought to kill actively growing tubercle bacilli at neutral or alkaline pH conditions with an MIC of 2-8 mg/ml (Kenney and Churchward, 1994), but it is inactive against non-growing or intracellular bacilli. STR resistance occur and develop quickly since STR is often given alone for TB treatment.

2.1.4.2 Isoniazid (INH)

Isoniazid (INH) was started to use as an antituberculous drug in 1952 (Mitchison, 2000). It is converted to an isonicotinyl-NAD adduct by the bacterial peroxidase encoded by *katG*. The isonicotinyl-NAD inhibits InhA, a key bacterial enzyme of mycolic acid production via the fatty acid biosynthesis (FAS II) (Mitchison and Davies, 2012). The mycolic acid is a major component of *M. tuberculosis* cell wall. INH is highly bactericidal against the dividing bacterial cells, with an MIC of 0.05 mg/ml (Suo et al., 1988) and a high therapeutic margin but it has much slower action against the non-multiplying cells (Mitchison and Davies, 2012). At high dosage, it can cause peripheral neuritis since it quantitatively removes vitamin B6 from the body by combination in the urine (Biehl, 1954). The initial rapid part of the kill is due to the action of INH on an initially multiplying part of the bacterial population. After the initial period of bacterial kill, INH has little further bactericidal effect. However, it probably plays an important role in maintenance of a post-antibiotic inhibition of bacterial growth. (Awaness, 1973).

2.1.4.3 Rifampicin (RIF)

Rifampicin or rifampin (RMP) is used as an antituberculous drug in a short-course (6 months) chemotherapy with streptomycin and isoniazid (East African British Medical Research Council, 1972). It is produced by a fermentation process from *Streptomyces mediteranei*, renamed as *Amycolatopeus rifamycinica*. It binds to the

β -subunit of the bacterial polymerase, thereby preventing RNA synthesis and protein formation (Da Silva and Palomino, 2011). It is highly bactericidal against *M. tuberculosis* throughout treatment, but its therapeutic margin is low. Nevertheless, RMP is responsible for the major part of the bactericidal activity of the regimen (Mitchison, 2000).

2.1.4.4 Pyrazinamide (PZA)

Pyrazinamide (PZA) is also used as an antituberculous drug in a short-course (6 months) chemotherapy with rifampicin, streptomycin and isoniazid (East African British Medical Research Council, 1972). It is a synthetic pro-drug which is converted by the amidase enzyme of *M. tuberculosis*, a product of the *pncA* gene, to the active pyrazinoic acid (Zang and Mitchison, 2003). Pyrazinoic acid reaches the exterior of the bacterial cells, where it is reabsorbed by passive diffusion in a highly pH-dependent manner. However, inside the bacterial cells, pyrazinoic acid can be excreted by an inefficient efflux pump that requires energy. As a result, the pyrazinoic acid is accumulated within the bacterial cells, leading to an acidifying the interior. This is probably lethal by membrane damage or by inhibiting translation in persisting cells (Shi et al., 2011). The bactericidal activity of PZA will increase when the energy resource of the cell decreases. So, PZA is particularly effective in killing dormant or near dormant bacterial populations which are tolerant to other antibacterial drugs such as rifampicin.

2.1.4.5 Ethambutol (EMB)

Ethambutol (EMB) is only active against growing bacilli with an MIC of 1-5 mg/ml (Inderlied and Salfinger, 1999), but it has no activity against non-growing bacilli. EMB inhibits the polymerization of arabinan in arabinogalactan and lipoarabinomannan, causing rapid accumulation of decaprenyl-P-arabinose, an intermediate in the arabinan biosynthesis of cell wall; arabinogalactan and arabinomannan (Deng et al., 1995). By genetic studies, it is indicated that the target of EMB is EmbB, arabinosyl transferase, involved in synthesis of arabinogalactan. The Emb proteins including EmbA, EmbB, and EmbC are about 65% identical and are predicted to be integral membrane proteins (Telenti et al., 1997). EMB is often recommended to use when INH resistant strains are prevalent although there is no evidence to suggest that it reduces the risk of failure.

2.1.4.6 Fluoroquinolones (FQs)

Fluoroquinolones are important second-line drugs used for the treatment of drug resistant TB. Fluoroquinolones are quite active against *M. tuberculosis* with

MICs of 0.25-3 mg/ml (Kenney and Churchward, 1994). Fluoroquinolones used nowadays are ciprofloxacin, ofloxacin, levofloxacin, and more recently gatifloxacin and moxifloxacin. These agents target DNA gyrase, which is responsible for DNA supercoiling. Gyrase is consisted of two A and two B subunits encoded by *gyrA* and *gyrB*, respectively (Da Silva and Palomino, 2011). Fluoroquinolones bind to DNA gyrase, thereby inhibiting DNA supercoiling. Fluoroquinolones are particularly useful in the treatment of MDR-TB.

2.1.4.7 Amikacin (AMK) and Kanamycin (KM)

Amikacin and Kanamycin are the second-line drugs for the treatment of tuberculosis. They belong to the family of aminoglycoside antibiotics that target cellular RNA to inhibit bacterial replication. Kanamycin binds to four nucleotides of 16S rRNA and it interferes with decoding site in the vicinity of nucleotide 1400 in 16S rRNA of 30S subunit. This region interacted with the anticodon of tRNA, leads to interference with the initiation complex, misreading of mRNA so incorrect amino acids are inserted into the polypeptide leading to nonfunctional or toxic peptides and the breakup of polysomes into nonfunctional monosomes (Song, 2004).

2.1.4.8 Capreomycin (CAP)

Capreomycin is classified in aminoglycosides group as streptomycin. It is a macrocyclic peptide antibiotic produced by *Saccharothrix mutabilis* subspecies *capreolus* (Sutton et al., 1966). Capreomycin is cyclic peptide antibiotics and it inhibit bacterial growth by blocking protein synthesis on the ribosome. It appears to interfere with translation in mycobacteria. Capreomycin can inhibit phenylalanine synthesis in *in vitro* translation assay using mycobacterial ribosomes (Trnka and Smith, 1970). Similar inhibition was seen whether or not ribosomes were preincubated with mRNA, suggesting that capreomycin does not interfere with mRNA binding to the ribosome.

2.2 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is classified in kingdom Monera, phylum Actinobacteria, order Actinomycetales, family Mycobacteriaceae and genus *Mycobacterium*. It belongs to *M. tuberculosis* complex (MTC) that has at least 8 *Mycobacterium* species: *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canetti*, *Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii* and *Mycobacterium mungi* (van Ingen, 2012). These MTC species cause TB both in human and animals.

2.2.1 Characteristics of *M. tuberculosis*

M. tuberculosis is a small bacillus with 2-4 μm in length and 0.2-0.5 μm in width. It does not produce spores, capsule and is non-motile. Characteristics of *M. tuberculosis* colonies are colorless and rough surface (Fig. 2.3). It has an unusual, waxy coating on its surface primarily because the major component of *M. tuberculosis* cell wall is a lipid mycolic acid. This coating makes the cells impervious to Gram staining since *M. tuberculosis* can appear as either Gram-negative or Gram-positive (Fu and Fu-Liu, 2002). Therefore, identification of *M. tuberculosis*, an acid-fast bacterium, can be performed by acid-fast staining technique. *M. tuberculosis* requires oxygen to grow and divides every 15–20 hours, which is extremely slow compared with other bacteria. It can withstand weak disinfectants and can survive in a dry state for weeks (van Ingen et al. 2012).

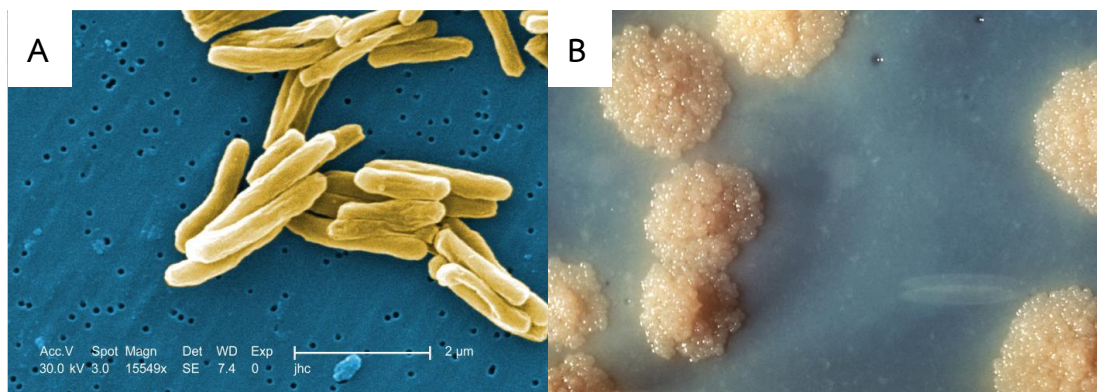


Figure 2.3 *M. tuberculosis* cells by scanning electron micrograph (A) and culture of *M. tuberculosis* on Lowenstein Jensen medium (B)

Source : Center of Disease Control, 1976

2.2.2 Virulence and disease process

Disease process of *M. tuberculosis* infection in human can be divided into 2 events; early event and later event.

2.2.2.1 Early event

M. tuberculosis usually enters the alveolar passages of exposed humans in an aerosol droplet, and it can infect and grow in alveolar cells *ex vivo* (Bermudez and Goodman, 1996). When the bacteria enter human body, human defense mechanisms will be stimulated to kill bacteria. *M. tuberculosis*-macrophage interaction occurs after infection. The bacteria are destroyed in a phagocytosis process that is initiated by bacterial contact with macrophage mannose receptors (Schlesinger, 1993). Surfactant protein A found on alveolar surfaces can enhance the

binding and uptake of *M. tuberculosis* by upregulating mannose receptor activity (Gaynore et al., 1995). On the other hand, surfactant protein D, similarly located in alveolar, inhibits phagocytosis of *M. tuberculosis* (Ferguson et al., 1999). Cholesterol in cell plasma membranes is thought to be important for this process since removal of cholesterol from human neutrophils decreases the phagocytosis of *M. kansasii* (Parson et al., 1998). On entry into a host macrophage, *M. tuberculosis* initially resides in an endocytic vacuole called the phagosome. If the normal phagosomal maturation cycle or phagosome-lysosome fusion occurs, these bacteria can encounter a hostile environment that includes acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides. Pathogenic mycobacteria are evolved by many ways to avoid this hostile vacuolar microenvironment by inhibition of phagosome maturation (Frehel et al., 1986).

In other way, *M. tuberculosis* prevents an increase of Ca^{2+} level to avoid host defense mechanisms since Ca^{2+} can stimulate many host responses to an infection including the respiratory burst as well as NO and cytokine production and the elevated Ca^{2+} level are associated with phagolysosome formation (Smith, 2003). An interesting finding is Ca^{2+} signaling that is inhibited when *M. tuberculosis* enters human macrophages but is not inhibited when killed *M. tuberculosis* or antibody-opsonized *M. tuberculosis* cells are destroyed by phagocytosis (Malik et al., 2000). It has also been postulated that a selective advantage to *M. tuberculosis* of staying in an early endosome would be less host immunosurveillance by CD4+ T cells. *M. tuberculosis* produces the secreted or surface-exposed *M. tuberculosis* 19-kDa lipoprotein. A decrease of the antigen presentation to CD4+ T cell called major histocompatibility complex class II (MHC-II) protein is induced and the MHC-II presentation of bacterial antigens in macrophages after *M. tuberculosis* infection is reduced (Noss et al., 2001).

2.2.2.2 Later event

In later stage, the bacterium survives and grows in the lung. Infected macrophages in the lung attract inactivated monocytes, lymphocytes, and neutrophils (van Crewel et al, 2002), none of which kill the bacteria very efficiently. Then, granulomatous focal lesions will be formed by macrophage derived from giant cells and lymphocytes. As cellular immunity develops, macrophages loaded with bacilli are killed, and this results in the formation of the caseous center of the granuloma, surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes (Dannenbergs and Rook, 1994). Although *M. tuberculosis* cells are unable to multiply within this caseous tissue due to its acidic pH, the low availability of oxygen, and the presence of toxic fatty acids. Some of them may remain dormant

but alive for decades. Whether an infection is arrested here or progresses to the next stages are regulated by the strength of the host cellular immune response. This TB stage is called latent or persistent TB that can persist throughout a person's life in a non-transmissible state. If a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied and then bacteria can use the liquor as a medium to replicate. At this point, viable bacterial cells can spread within the lungs (active pulmonary TB) or even to other tissues via the lymphatic system and the blood (extrapulmonary TB). When this happens, the person becomes infectious and requires antibiotic therapy to survive (Dannenberget al., 1994).

2.2.3 Genome description

The *M. tuberculosis* H37Rv genome consists of 4.4×10^6 bp and contains approximately 4,000 genes (Cole et al., 1998). The *M. tuberculosis* genome shows some unique features. Over 200 genes are annotated as encoding enzymes for the metabolism of fatty acids, comprising 6% of the total. Among them, approximately 100 genes are predicted to function in the β -oxidation of fatty acids. By comparison with other bacteria such as *E. coli*, they only have 50 enzymes involving in fatty acid metabolism. This large number of *M. tuberculosis* enzymes that putatively use in fatty acid metabolism might be related to the ability of this pathogen to grow in the tissues of the infected host, where fatty acids are the major carbon source. Another unique feature of the *M. tuberculosis* genome is the presence of the unrelated proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) families of acidic and glycine-rich proteins. The 172 genes comprising 104 genes of the PE class and 68 genes of the PPE variety, are over 4% of the genes in *M. tuberculosis*, and similar levels of abundance are noted in other members of the *M. tuberculosis* complex (Brosch et al., 2000). In *M. tuberculosis*, 104 PE genes encode 29 proteins with the PE region alone, 8 proteins in which the PE region is followed by unrelated C-terminal sequences, and 67 proteins that form the PE-PGRS family. The PE-PGRS family proteins have the conserved PE domain followed by C-terminal extensions with multiple repeats of Gly-Gly-Ala or Gly-Gly-Asn that are in the polymorphic GC-rich repetitive sequences (PGRS) domains. The size variation of these large families has been observed in members of the PE-PGRS family in clinical TB strains, and many of these proteins have been localized in the cell wall and cell membrane (Banu et al., 2002). These data and the antigenicity of these proteins have led to the hypothesis that at least some of these proteins may be involved in antigenic variation of *M. tuberculosis* during infection.

2.3 Drug resistant tuberculosis

Drug resistance is an important problem for TB treatment. In 2015, there were an estimated 480,000 new cases of multidrug-resistant TB (MDR-TB) and an additional 100,000 people with rifampicin-resistant TB (World Health Organization, 2016) who were also newly eligible for MDR-TB treatment (World Health Organization, 2016). Patients in India, China and the Russian Federation accounted for 45% of the combined total of 580,000 cases (World Health Organization, 2016). Drug resistance in bacteria can occur by using a variety of mechanisms such as a decreased uptake or an impermeability, an increased efflux, an enzymatic inactivation, a modification of the antibiotic targets and a reduced pro-drug-activating enzyme activity (Wade and Zang, 2004). These mechanisms of resistance are a result of intrinsic (natural) resistance and acquired resistance. Intrinsic or natural resistance refers to resistance that is not caused by any genetic alteration, while acquired resistance is due to a mutation or a genetic material transfer. *M. tuberculosis* has several meanings of intrinsic resistance. For example, *M. tuberculosis* has a very hydrophobic cell surface, which provides a permeability barrier for some antibiotics (Somoskovi et al., 2001). *M. tuberculosis* also possesses some enzymes such as beta-lactamase which can inactivate penicillin activity (Kwon et al., 1995). Aside from natural resistance mechanisms, *M. tuberculosis* has acquired resistance mechanisms to anti-tuberculosis drugs by spontaneous mutations in chromosomal genes (Zhang and Telenti, 2000). There are two main types of drug resistant TB, Multidrug-resistant tuberculosis (MDR-TB) and Extensively drug-resistant tuberculosis (XDR-TB). MDR-TB is the type of drug resistant TB, of which the bacteria are resistant to at least rifampicin and isoniazid. XDR-TB is MDR-TB which additionally resists to any fluoroquinolones and one of the second line injectable TB drugs.

2.3.1 Drug resistant genes and mechanism of resistance

2.3.1.1 Streptomycin resistance

Streptomycin (STR) is often given alone and as a result, the resistance to this drug is quickly developed. STR inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome causing misreading of the mRNA message during translation (Davies and Davis, 1968). Resistance to STR is typically through point mutations in S12 (*rpsL*) and 16S rRNA (*rrs*). The most common mutation in *rpsL* gene is a substitution in codon 43 from lysine to arginine, causing high-level resistance to STR. Mutations in codon 88 (K88Q) is also common. Mutations of the *rrs* gene occur in the loops of the 16S rRNA and are clustered in two regions around nucleotides 530 and 915 (Finken et al., 1993; Nair et al., 1993). Mutations in the *rrs* gene usually account for low-level resistance. Overall, mutations in the *rpsL* and *rrs* genes account

for approximately 75% of all STR-resistant isolates. Therefore, alternate mechanisms of STR resistance must exist in the remaining 25% of STR-resistant strains. A possible mechanism might be an alteration in drug uptake, which have been thought to contribute to this drug resistance (Cooksey et al., 1996).

2.3.1.2 Isoniazid resistance

Isoniazid (INH) is an important first-line anti-tuberculosis drug. In 1992, Zhang and his colleagues firstly showed that a mutation in *katG* gene resulted in INH resistance in clinical isolates of *M. tuberculosis*. Susceptibility could be restored in *katG* mutant strains (Zhang et al., 1993). Upon restoring the *katG* gene, INH sensitivity and virulence could be active (Wilson et al., 1995). In the previous study, various mutations in the *katG* gene have been reported, but the most common mutation is the S315T mutation (Zhang and Telenti, 2000). The gene *inhA*, encoding an NADH-dependent enoyl acyl carrier protein (ACP) reductase, has also been shown to be involved in INH resistance (Banerjee et al., 1994). The *inhA* mutations confers low level resistance of INH whereas *katG* mutations are more commonly associated with high level resistance of INH (Zhang and Telenti, 2000). Besides the above genes, *kasA* encoding a beta-keto-acyl ACP synthase, an enzyme involving in mycolic acid synthesis, was identified based on its induction by a low-level concentration of INH (Mdluli et al., 1998). In addition, mutations in the promoter region of *ahpC*, encoding an alkylhydroperoxide reductase, have been observed as a compensation for the lack of catalase-peroxidase activity (Wilson and Collins, 1996). The overexpression of *aphC* did not associate with INH resistance mechanism but can be a marker for INH resistance (Telenti et al., 1997).

2.3.1.3 Rifampicin resistance

Rifampicin (RIF) is one of important first-line anti-tuberculosis drugs that can kill *M. tuberculosis* at log phase and stationary phase. RIF resistance in *M. tuberculosis* is due to a single mutation at position 81 bp region in the *rpoB* gene, encoding the DNA-dependent RNA polymerase beta subunit (Telenti et al., 1993). The most common mutations found in this region are associated with codons Ser531 and His526 (Zhang and Telenti, 2000). Mutations in these codons result in high-level resistance to RIF (Zhang and Yew, 2015). Mutations in amino acid residues 511, 516, 518 and 522 are associated with low-level resistance to RIF and rifapentine (Williams et al., 1998). The single point mutations in *rpoB*, leading to the RIF resistance and the cross-resistance to other rifampicin derivatives, account for about 96% of RIF-resistant *M. tuberculosis* strains (Telenti et al., 1993).

2.3.1.4 Pyrazinamide resistance

Pyrazinamide (PZA) is an important first-line anti-tuberculosis drug. The mechanism involved in PZA resistance in *M. tuberculosis* has been reported to associate with the loss of pyrazinamidase (PZase) activity (Konno et al., 1967; Miller et al., 1995). Mutations in the *pncA* encoding results in resistance to PZA. These mutations are highly diverse and scattered along the *pncA* gene, with some degree of clustering in three regions of amino acid residues; 3-17, 61-85, 132-142 (Scorpio et al., 1997; Lemaitre et al., 1999). These regions are likely to contain catalytic sites of the PZase enzyme (Raynaud et al., 1999). Mutations at C138, D8, K96, D49, H51 and H71 modify the active site and metal binding site of PZAase (Shi et al., 2011). Mutations at Q10 and T142 involved in chelating the metal atom are predicted to cause loss of enzyme activity (Stoffels et al., 2012). The basis for high diversity of *pncA* mutation might be that *pncA* gene is not essential gene there is no selective pressure for specific mutation (Cheng et al., 2000).

2.3.1.5 Ethambutol resistance

Ethambutol (EMB) is a first-line anti-tuberculosis drug used for TB treatment. EMB inhibits synthesis of arabinogalactan, a major cell wall component of mycobacteria. Mechanisms of resistance to this anti-tuberculosis agent are primarily associated with point mutations in the *embCAB* operon (Sreevatsan et al., 1997), encoding various arabinosyl transferase enzymes necessary for cell wall biosynthesis. One of the most common mutations in this operon is associated with amino acid substitutions at codon M306 of the *embB* gene (Sreevatsan et al., 1997). Additional mutations in the *embC-embA* intergenic region have also been found. The mutations locate at position -8, -11, -12, -27 and -41 of upstream *embA*. These intergenic region mutations of gene might play a secondary or compensatory role in EMB resistance. In addition, mutations in *rmlD* and *rmlA2*, both of which are involved in modification of rhamnose, essential protein for the growth of mycobacteria were also found to be associated with EMB resistance (Ramaswamy et al., 2000).

2.3.1.6 Fluoroquinolones resistance

Fluoroquinolones (FQs) are second-line anti-tuberculosis group of drugs containing many various kinds of drugs. The target of these drugs is DNA gyrase, which is responsible for DNA supercoiling. DNA gyrase is consisted of two subunits; subunit A and subunit B which are encoded by *gyrA* and *gyrB*, respectively (Drlica and Malik, 2003). In *M. tuberculosis*, resistance to fluoroquinolones is associated with mutations in a 40-amino acid region in *gyrA*, which accounts for 40-70% of fluoroquinolone resistant isolates (Gupta and Gupta, 2012). In addition, a mutation at

codon position 90, 91 and 94 of GyrA has also been reported (Singh et al., 2015); however, fluoroquinolone resistance can be found in strains lacking of these mutations, suggesting that there are other alternative mechanisms of fluoroquinolone resistance (Singh et al., 2015). A mutation in both *gyrA* and *gyrB* causes higher levels of fluoroquinolone resistance (Kocagoz et al., 1996).

2.3.1.7 Amikacin and Kanamycin resistance

Amikacin and Kanamycin are inhibitors of protein synthesis through a modification of ribosomal structures at the 16S rRNA. A mutation at position 1400 of *rrs* is associated with high-level resistance to kanamycin in *M. tuberculosis* (Taniguchi et al., 1997). However, isolates with low-level kanamycin resistance usually do not have changes in the *rrs* gene. The most common molecular mechanism of AMK and KM resistance has been associated with an A1401G mutation in the *rrs* gene coding for 16S rRNA.

2.3.1.8 Capreomycin resistance

Capreomycin is one of a second-line antituberculous drug, typically used clinically after failure of therapy with first-line drugs. Capreomycin resistance in *M. tuberculosis* is due to the loss of the ability of capreomycin to bind to and inhibit ribosomes because of the lack of methylation of rRNA (*tlyA* mutation) or changes in the 16S rRNA (*rrs* mutation). Mutations in the gene *tlyA* have been implicated in resistance to capreomycin. This gene codes an rRNA methyltransferase specific for 2'-O-methylation of ribose in rRNA. When mutated, it determines an absence of methylation activity. The loss of rRNA methyltransferase or TlyA function has reduced the ability of capreomycin to interact with its target (Maus et al., 2005).

2.3.2 Diagnosis of drug resistance

In general, *M. tuberculosis* isolated from TB patients should be tested for resistance to the first-line anti-TB drugs: isoniazid, rifampin, ethambutol, and pyrazinamide. The results of drug-susceptibility tests are useful to choose the appropriate drugs for treatment of each patient.

2.3.2.1 Drug susceptibility testing

First-line drug susceptibility testing should be done in all patients and repeat in patients who do not respond to antituberculous drugs as expected or who have positive culture results despite 3 months of adequate treatment. Second-line drug susceptibility testing should be done only in reference laboratories and generally be limited to specimens from patients who are prior TB treatment, contact

with a patient with known anti-TB drug resistance, do not respond to first-line anti-TB drugs, or have positive-culture results after more than 3 months of treatment. The methods for detection of drug-susceptibility are liquid-culture based method and agar proportion method. Liquid culture method requires instrumentation system, such as BACTEC MGIT 960 system or Trek diagnostics system (Fig. 2.4). Agar proportion method requires a calculation of drug resistance by performing colony counts on drug-containing agar compared to drug-free agar. If there is $\geq 1\%$ growth on the drug-containing medium compared to drug-free medium, the organism is considered to be resistant to that drug (Hall et al., 2011).

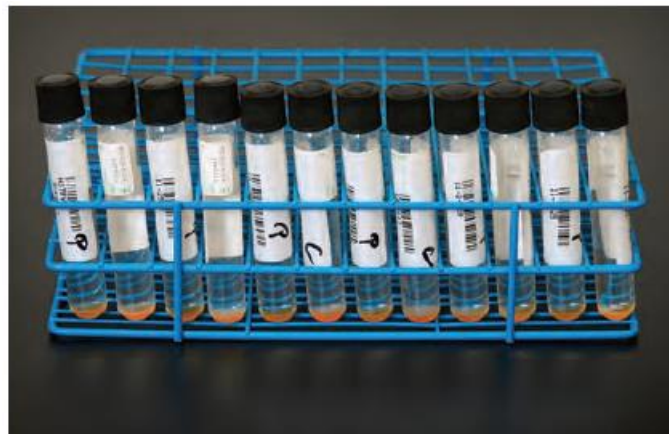


Figure 2.4 Culture tubes of BACTEC MGIT 960 system for first-line drug susceptibility testing

Source: Center of Disease Control, 2013

2.3.2.2 Molecular detection of drug resistance

The drug resistance of clinical isolates determined by conventional methods (broth-based and agar proportion) is due to the presence of mutations in specific *M. tuberculosis* genes. These mutations are often single base pair changes in the DNA sequence of the bacteria. There are various kinds of method that can detect mutations associated with drug resistance such as Line-probe assays, PCR amplification and DNA Sequencing.

2.4 Molecular methods for TB drug resistant detection

There are many molecular tests used for drug resistance detection of TB. They are (1) Xpert MTB/RIF assay which is a TB detection test simultaneously with rifampicin resistance detection (rifampicin is the most effective first-line anti-TB drug), (2) Rapid line probe assays (LPAs) which are divided into the first-line LPA and the second-line LPA. The first-line LPA is a resistance test to rifampicin and isoniazid whereas the second-line LPA is a resistance test to fluoroquinolones and injectable

anti-TB drugs, and (3) DNA sequencing technologies. In addition, culture-based methods currently remain the reference standard for drug susceptibility testing (World Health Organization, 2016).

2.4.1 Xpert MTB/RIF assay

The Xpert MTB/RIF assay is a commercial nucleic acid amplification (NAA) test that uses a disposable cartridge with the GeneXpert instrument system. It is an automated diagnostic test for detection of *Mycobacterium tuberculosis* complex (MTBC) and resistance to rifampin (RIF) within less than 2 hours. A sputum sample is collected from suspected TB patients. It is mixed with the reagents that are provided with the assay. Then, a cartridge containing this mixture is placed in the GeneXpert machine (Center of Disease Control, 2016). The Xpert MTB/RIF assay is recommended to use as an initial diagnostic test in each individual who is infected to contain suspected HIV-associated TB or multidrug-resistant TB. In addition, it is also an initial diagnostic test for cerebrospinal fluid specimens from patients who are presumed to contain TB meningitis (World Health Organization, 2016).

2.4.2 Rapid line probe assays

The line probe assay technology is based on DNA hybridization. DNA is extracted from *M. tuberculosis* isolates or directly extracted from clinical specimens. The gene resistance-determining region was amplified by polymerase chain reaction using biotinylated primers to create labeled PCR products. Then the products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled hybrids are detected by colorimetric development, enabling detection of both wild type and mutant of *M. tuberculosis*. Mutations are detected by lack of binding to wild-type probes as well as by binding to specific probes for the most commonly occurring mutations. The post-hybridization reaction leads to the development of color bands on the strip at the site of probe binding and is observed by eye (World Health Organization, 2008).

2.4.3 DNA sequencing

DNA sequencing is a method for determining the order of the nucleotides bases; adenine, guanine, cytosine and thymine, in a molecule of DNA. Drug resistance TB is mostly due to mutations in the bacterial genome. DNA sequencing has been developed for the simultaneous detection and identification of *M. tuberculosis* and for the common mutations causing resistance to specific antituberculous drugs. DNA sequencing is considered the gold standard among molecular techniques for the detection of mutations associated with drug resistance (World Health Organization,

2016). Its advantages include rapidity, and high accurate determination of the location and type of the mutations (Mani et al., 2003).

2.5 Related techniques of this research

2.5.1 Multiplex PCR

Multiplex polymerase chain reaction (Multiplex PCR) is a molecular method based on polymerase chain reaction (PCR). PCR is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. In diagnostic laboratories, the use of PCR is limited by the availability of test sample volume. To increase the diagnostic capacity of PCR, a multiplex PCR has been described. In a multiplex PCR reaction, more than one target sequence can be amplified by using more than one pair of primers. Multiplex PCR has a potential to produce considerable products with a time safe (Elnifro et al., 2000). It can be applied in many areas of nucleic acid diagnostics, including gene deletion analysis (Chamberlain et al., 1989), mutation and polymorphism analysis (Rithidech et al., 1997) and DNA quantitative analysis (Zimmermann et al., 1996). In the field of infectious diseases, this technique has been shown to be a valuable method for identification of viruses, bacteria, fungi, and parasites.

The factors influencing the multiplex analysis results include:

1. Primers

Sequence information for primer sites at the selected segment of DNA is important because nonspecific amplification may occur at other sites with similar sequences (Chamberlain et al., 1991). Otherwise, Failure or reduced amplification may occur because of primer-template mismatched sites (Zazzi et al., 1993). Primers for exon-amplifying multiplexes are ideally placed in intron sequences nearby the exons. This provides some margin for adjustment of fragment length or amplification quality as well as possible information about alterations affecting splice sites (Chamberlain et al., 1988). If the multiplex PCR products are resolved by electrophoresis, fragment sizes should be separated easily from each other. The range of band sizes should not be so wide that all fragments cannot be resolved well on the same gel.

2. PCR conditions

Conditions for each set of primers should be developed individually and modified if necessary when the primer sets are added. However, the possibility of

nonspecific priming is increased with each additional primer. The use of "Hot-start" PCR or high annealing temperature may solve the primer pairs that produce nonspecific bands in multiplex PCR but give a high signal of expected band (Zazzi et al., 1993). If equimolar primer concentrations do not yield uniform amplification signals for all fragments, the concentration of some primer pairs can be reduced in relation to others (Sunzeri et al., 1991). An inverse relation between the required primer concentration in multiplex PCR and its A/T content, but not its length or melting temperature, has been suggested (Vandenvelde et al., 1990). When all primer pairs are not compatible, it might be necessary to subgroup them in smaller multiplexes.

3. Concentrations of reaction components

The requirement of Mg^{2+} and dNTPs concentrations generally increases with the number of products in the multiplex PCR, but their concentrations must be optimized because each primer pair might be required at different concentrations (Chamberlain et al., 1991). Likewise, the requirement of *Taq* DNA polymerase generally increases with the size of the multiplex PCR products (Chamberlain et al., 1991). Dimethylsulfoxide is a beneficial ingredient or an inhibitor in different multiplex PCR systems (Uggozoli and Wallace, 1992; Vandenvelde et al., 1990; Zazzi et al., 1993). Other additives that minimize nonspecific binding in multiplex PCR are tween 20 and triton X-100, β -13-mercaptoethanol and tetramethylammonium chloride (Levinson et al., 1992; Chamberlain et al., 1988; Uggozoli and Wallace, 1992).

2.5.2 Next-generation sequencing (NGS)

Next-generation sequencing (NGS) method, also referred as deep, high-throughput, or massively parallel sequencing, comprises several sequencing technologies. The various platforms, which are different in their sequencing chemistry, read length and throughput capability, are currently available in the market (Table 2.1). These platforms have become particularly attractive to clinical microbiology laboratories depending on molecular methods for pathogen identification and characterization. NGS studies of infectious microorganisms typically follow one of two general strategies; targeted amplicon sequencing or whole genome sequencing (WGS) (Fig. 2.5). The first approach uses target-specific primers for PCR amplification, so that the genomic regions of interest are enriched and selectively sequenced. This approach is often performed to investigate well-characterized genomic regions. On the other hand, whole genomes sequencing relies on non-targeted library preparation. This is often performed when microorganisms are unknown or the goal is to define the genomic content and functional potential of the organisms under investigation. The use of NGS in clinical microbiology

laboratories is currently infrequent, although its role in the diagnosis and management of infectious diseases is anticipated to grow as standardized operational protocols, automation, and data analysis pipelines emerge.

Table 2.1 Information of current NGS platforms

Platform	Manufacturer	Sequencing chemistry	Amount of result data	Runtime
MiSeq/MiSeqDx	Illumina	Sequencing by synthesis-reversible terminator	13.2-15 Gb	~ 56 hours
NextSeq 500	Illumina	Sequencing by synthesis-reversible terminator	100-120 Gb	29 hours
Hiseq 2500	Illumina	Sequencing by synthesis-reversible terminator	900-1,000 Gb	6 days
Hiseq 3000	Illumina	Sequencing by synthesis-reversible terminator	650-750 Gb	<1-3.5 days
Hiseq 4000	Illumina	Sequencing by synthesis-reversible terminator	1,300-1,500Gb	<1-3.5 days
Hiseq X	Illumina	Sequencing by synthesis-reversible terminator	1.6-1.8 Tb	< 3 days
Ion PGM	Life Technologies	Sequencing by synthesis-hydrogen ion detection	30 Mb–2 Gb	2-7 hours
Ion proton	Life Technologies	Sequencing by synthesis-hydrogen ion detection	10 Gb	2-4 hours
SoLiD 5500xl W System	Life Technologies	Fluorescent probe ligation	~ 320 Gb	~ 5 days
PacBio RS II	Pacific Biosciences	Single-molecule real-time sequencing	275-375 Mb	30-180 minutes
454 GS Junior	Roche	Pyrosequencing	35-53 Mb	10 hours
454 GS FLX	Roche	Pyrosequencing	~ 600 Mb	23 hours
Titanium XL				

Source: Lefterova et al., 2015

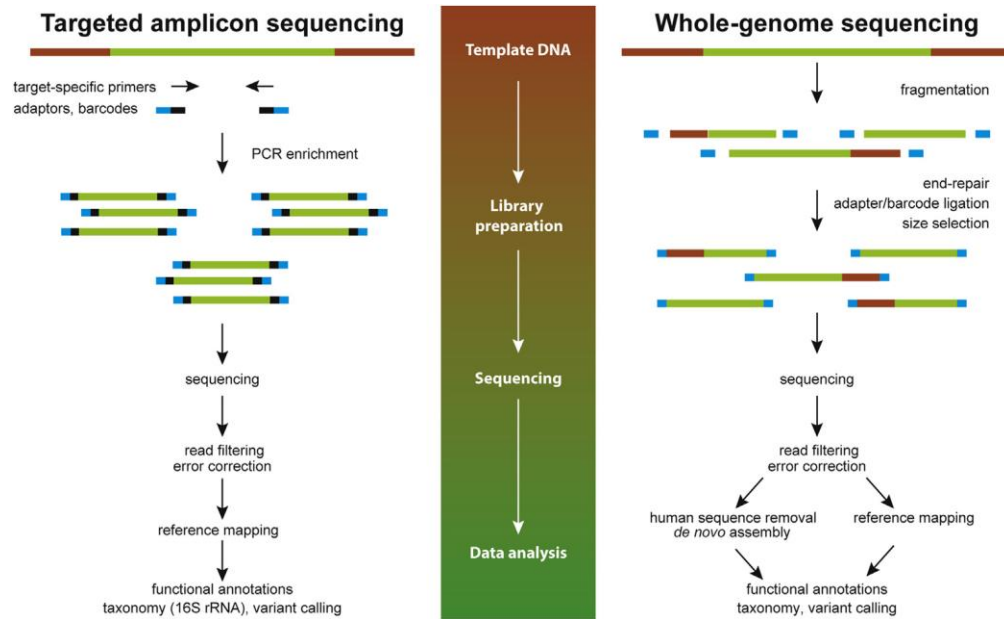


Figure 2.5 Overview of two general strategies for sequencing diagnosis of infectious microorganisms by NGS; targeted amplicon sequencing (left) and whole genome sequencing (WGS) (right)

Source: Lefterova et al., 2015

The Nextseq 500 system sequencer was released by Illumina incorporation at the early of 2014 (Reuter et al., 2015). This sequencer is based on next-generation sequencing using a high-throughput sequencing and microarray scanning technology (Reuter et al., 2015). The system utilizes sequencing by synthesis new DNA strand complimented with single stranded DNA template through DNA polymerization. Each bases of nucleotide are labelled with a different fluorescent dye to be as reversible terminator nucleotides and special DNA polymerase enzyme able to incorporate them. Illumina sequencing works on the principle of reversible termination with each sequencing cycle involving the addition of DNA polymerase and a mixture of four differently colored reversible dye terminators are then unblocked and the reporter dye cleaved and wash away. Following sequencing from a single end of the template, pair-end sequencing can be achieved by sequencing from an alternate primer on the reverse stand of the template molecule. the NextSeq 500 is capable producing 120 Gb, or a single 30x genome, in less than 30 hours.

The process of Illumina sequencing is according to three following steps:

1. Sample preparation

Genomic DNA or PCR product is fragmented and ligated to adaptors by transposomes to create tagmented libraries. Then, the libraries are amplified with indexes by PCR amplification. The pooled library contained multiple templates with

indexes is purified through a magnetic bead-based process. The template is denatured to be single stranded DNA and combined with PhiX control. The single strand templates are immobilized on four-channel flow cell. The cluster generation on solid-supported flow cell is performed by PCR amplification using Nextseq 500 system (Fig. 2.6). PCR is performed by the repeated thermal cycles of denaturation, annealing and extension. During amplification, each single stranded template that is immobilized at one end on the surface of flow cell creates a bridge structure by hybridizing with its free end to the free primer complement with adapters on the surface of flow cell. After several PCR cycles, a surface-bound DNA cluster is formed on each flow cell (Pareek, 2014).

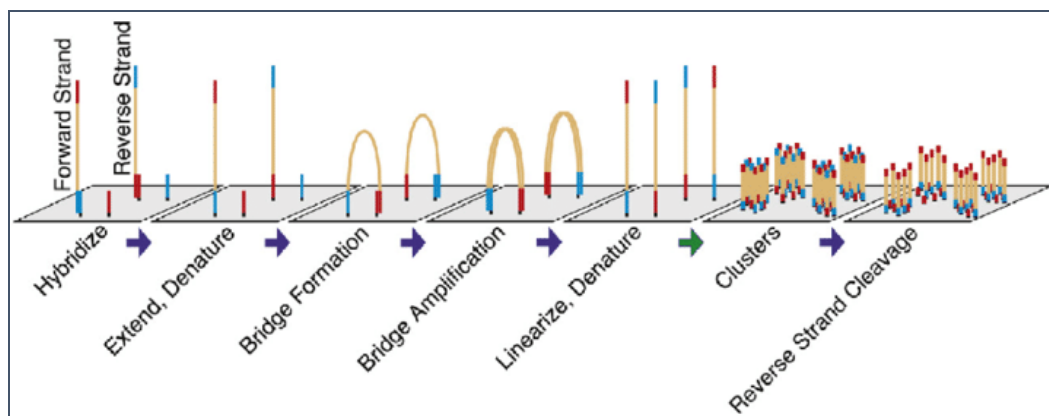


Figure 2.6 Drawing of DNA cluster generation on flow cell surface

Source: Chaitankar et al., 2016

2. Sequencing by synthesis

Sequencing by synthesis technology uses four fluorescently labeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel (Fig. 2.7). DNA templates are first clonally amplified on the surface of a glass flow cell. The first sequencing cycle begins by adding four labeled reversible deoxynucleotide triphosphate (dNTP), sequencing primer and DNA polymerase. During each sequencing cycle, a single labeled deoxynucleotide triphosphate is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the laser excitation is performed and the emitted fluorescence from each cluster is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Sequencing occurs via successive rounds of base incorporation, washing, and imaging. A cleavage step after image acquisition removes the fluorescent dye and regenerates

the 3' OH for the next cycle. Analysis of four-color images is used to determine base composition.

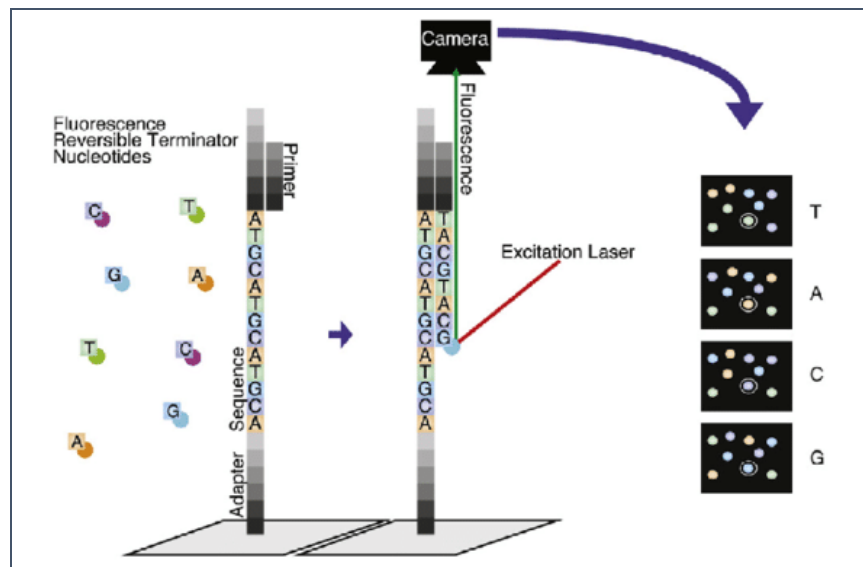


Figure 2.7 Illumina's four-color reversible termination sequencing method

Source: Chaitankar et al., 2016

3. Base calling and data analysis

Since all four reversible terminator-bound dNTPs (A, C, T, G) are present as single, separate molecules, natural competition minimizes incorporation bias. Base calls are made directly from signal intensity measurements during each cycle, which greatly reduces raw error rates compared to other technologies. During a sequencing run, a quality score is assigned to each base call for every cluster (Pareek, 2014). The end result is highly accurate base-by-base sequencing and poor-quality sequences are eliminated. Finally, the data are aligned and compared to a reference and sequencing differences are identified.

2.6 Literature reviews

There are many articles studying on detection of drug resistance of tuberculosis. The articles are divided according to the different topics as follows:

2.6.1 Articles related to first line drug resistance of tuberculosis

Boehme et al. (2010) used Xpert MTB/RIF assay, an automated molecular test to detect *Mycobacterium tuberculosis* (MTB) and resistance to rifampin in 1,730 sputum samples from Peru, Azerbaijan, South Africa and India, compared to culture test and DNA sequencing. Among patients with culture-positive tuberculosis, the

overall sensitivity of the MTB/RIF test was 97%. There was no significant difference in sensitivity between untreated sputum and treated sputum tests. For rifampin resistance, the MTB/RIF test was correctly detected with 99.1% sensitivity. Sixteen different mutations were identified, especially in codons 516, 526 and 531, in almost all resistant strains.

Feuerriegel et al. (2012) investigated mutations in 9 genes of *Mycobacterium tuberculosis* by DNA sequencing for first-line drug resistance detection. In this research, 9 genes including *katG*, *rpoB*, *rrs*, *rpsL*, *gidB*, *embB*, *pncA*, *inhA*, and *ahpC* were amplified by PCR and sequencing. For 97 *M. tuberculosis* strains isolated from patients in high burden country, Sierra Leone, prevalence isoniazid and rifampicin resistance mutations were found at *katG*315 and *rpoB*531. The streptomycin resistant strains had mutation in *rpsL* but did not have in *rrs*. The ethambutol resistant strains had mutation in *embB* and the pyrazinamide resistant strains had mutation in *pncA*.

Kapur et al. (1994) evaluated resistance of *Mycobacterium tuberculosis* strains to rifampicin. A 69-bp region of the *rpoB* gene was amplified by single PCR amplification and sequenced by automated DNA sequencing. The result revealed that higher than 90% of rifampin-resistant strains had sequence alterations in *rpoB* and showed that most were missense mutations. The analysis identified several mutant *rpoB* alleles which was not previously found to associate with resistant organisms and also identified one short region of *rpoB* that had an unusually high frequency of insertion and deletion.

Kiepiela et al. (2000) studied the mutation of isoniazid resistant genes, *katG* encoding catalase–peroxidase, *inhA* encoding the enoyl–acyl carrier protein involved in mycolic acid biosynthesis and *ahpC* encoding the alkyl hydroperoxide reductase involved in the cellular response to oxidative stress reductase. Three methods consisting of PCR-single strand conformation polymorphism (SSCP), DNA sequencing, and restriction fragment length polymorphism (PCR RFLP) using *Msp1* restriction enzyme were analyzed in 79 *M. tuberculosis* isolates collected from South African. The sensitivity of PCR-SSCP in the detection of mutations for INH resistance in *katG*, *inhA* and *ahpC* using DNA sequencing method as the gold standard was 100%, 98.7% and 100%, respectively, with 100% specificity. The most frequent mutation was found in *katG* region with *S315T* mutation identified by PCR-RFLP. Mutations in *inhA* had nucleotide substitutions (T→A; C→A) on the 3' end of the presumed ribosome binding site located in the region upstream of *mabA* in the *inhA* locus. Mutations in the *ahpC* promoter region were located in the 105 bp *oxyR-ahpC* intergenic region.

Kim et al. (2003) analyzed the mutation in *katG*, *inhA* and *ahpC*, associated with isoniazid resistance in *M. tuberculosis*. The genetic mutation of 97 isolates from

patients in South Korea was detected by DNA sequencing and Restriction fragment length polymorphism (RFLP) analysis with *MspI* restriction enzyme digestion. Mutations in *katG315* alone (54.9%), *katG315* plus *inhA* (2.8%), *katG315* plus *ahpC* (1.4%), *katG309* alone (1.4%), *katG309* plus *inhA* (1.4%), *inhA* alone (19.7%), and *ahpC* alone (5.6%) were detected in the 71 INH-resistant isolates. Mutations in *katG* at codon 315 were associated with the high-level of INH resistance whereas the mutation in the *inhA* promoter region was associated with the low-level of INH resistance. The mutations in these regions have highly sensitivity and specificity for INH resistance detection in South Korea.

Miotto et al. (2014) developed rapid molecular methods for identification of Pyrazinamide (PZA) resistance in *M. tuberculosis*. DNA sequencing of full length product of *pncA* was analyzed. The results of *pncA* sequencing were correlated with phenotype, enzymatic activity and structural to adjust for possible errors in phenotypic drug susceptibility testing. Of 1,950 clinical isolates, there were 280 genetic variants dividing into four classes: (i) very high confidence resistance mutations found only in PZA resistant strains, (ii) high-confidence resistance mutations found in more than 70% of PZA resistant strains, (iii) mutations with an unclear role found in less than 70% of PZA resistant strains, and (iv) mutations which were not associated with phenotypic resistance. Based on the result, the accuracy of any molecular diagnostic assay would be in the range of 89.5-98.8%.

Ramaswamy et al. (2000) investigated mutations in 12 genes associated with ethambutol resistance of *M. tuberculosis* by automated DNA sequencing. Twelve involving genes comprising *embA*, *embB*, *embC*, *embR*, Rv3124, Rv0340, *iniA*, *iniB*, *iniC*, *iniD*, *wbbL* and *rmLA2* of clinical isolates of *M. tuberculosis* strains were sequenced. The results showed that most of EMB resistant organisms had a molecular alteration. Fifty-one percent (51%) of EMB resistant strains contained a resistance-associated mutation in only 1 gene whereas rest of them (49%) showed resistance-associated mutation in two or more genes. Most mutations were found in amino acid replacements at position 306 or 406 of *EmbB*. The mutations of *iniA* operon were T143M replacement located in Rv0340 gene.

Rindi et al. (2005) investigated mutations involving in isoniazid (INH) resistance in *M. tuberculosis* by DNA sequencing. Five genes comprising *katG*, *kasA*, *ndh*, *inhA* and *ahpC* of *M. tuberculosis* isolated from Italian patients were amplified by PCR and sequencing. The result showed that INH resistance mutation was frequently found at codon 315 of *katG* and at position -15 of *inhA*. Following by a mutation at codon 269 of *kasA*. Another mutation points were found in position -24 of *inhA* and position -54 and -74 of *ahpC*.

Musser et al. (1996) amplified isoniazid resistant genes, *katG* and *inhA*, by single PCR amplification using genomic DNA of 152 strains of *M. tuberculosis* isolated from patients in different countries and 16 strains of *M. bovis* and *M. microti* as templates. These genes were sequenced by automated DNA sequencing. The result showed that most of resistant isolates appeared amino acid changes in KatG or nucleotide substitutions at upstream of *inhA*. The alteration in residues *S315* and *R463* of KatG was frequently found in most resistant strains.

Tan et al. (2014) investigated pyrazinamide resistance in 161 *M. tuberculosis* isolated from Southern China by Bactec MGIT 960 system and also investigated the mutation of *pncA* and *rpsA* plus flanking regions by DNA sequencing. Of 161 *M. tuberculosis* clinical isolates, 109 isolates were PZA-susceptible whereas 52 isolates were PZA-resistant. No *pncA* mutation was found in all PZA-susceptible isolates, but 84.6% of 52 PZA-resistant isolates showed *pncA* mutations containing 10 new mutation points. For *rpsA* mutation, 3 novel mutation points were observed in pyrazinamide-resistant strains.

Unissa et al. (2008) analyzed mutations in specific regions of *katG*, *inhA*, *oxyR-ahpC* and *kasA* associated with isoniazid (INH) resistance in clinical isolates of *M. tuberculosis* from Indian patients by DNA sequencing. In *katG*, point mutation at codon 315 (64.3%) and codon 138 (4%) was found. Mutation at *inhA* and *oxyR-ahpC* promoter regions occurred in 11.4% and 35.0% of isolates, respectively. No mutation in structural gene regions of *kasA* and *inhA* was found. This is the first comprehensive molecular analysis of INH resistance in India, suggesting that the major cause of INH resistance is a point mutation rather than deletional and insertional mutation.

Williamson et al. (2012) evaluated sensitivity and specificity of *M. tuberculosis* detection by the Xpert MTB/RIF assay and false-positive rifampicin resistance. The Xpert MTB/RIF assay was used to analyze 169 specimens and the results were compared with culture method results. The results showed that the assay was highly sensitive and specific for the detection of *M. tuberculosis*. However, the assay was less reliable for the detection of rifampicin resistance, by producing 31% of false-positive results.

2.6.2 Articles related to second line drug resistance of tuberculosis

Disratthakit et al. (2016) determined the mutations in the entire sequence of the gyrase genes to distinguish the relationships between mutation positions and quinolone resistance in *M. tuberculosis*. The *gyrA* and *gyrB* genes were amplified from DNA of 27 quinolone-susceptible isolates, 71 pre-XDR-TB and 30 XDR-TB. The minimum inhibitory concentration (MICs) were determined in some selected isolates

by agar dilution method. The quinolone resistance-associated mutations in *gyrA* gene are D94G, A90V, and D94A. The isolates carrying D94G exhibited higher levels of resistance to quinolone than isolates carrying A90V or D94A. The E540D mutation in *gyrB* gene caused high-level resistance to moxifloxacin, gatifloxacin and sparfloxacin, but caused low-level resistance to ofloxacin, levofloxacin and ciprofloxacin.

Von Groll et al. (2009) evaluated resistance of *M. tuberculosis* to second line drug fluoroquinolones (ofloxacin, moxifloxacin and gatifloxacin). Genes associated with fluoroquinolones resistance, *gyrA* and *gyrB*, were amplified by single PCR amplification and sequenced by automated DNA sequencing. The major mutation point is A90V mutation in *gyrA*. From 41 strains, 40 strains shared the same resistance results for the three fluoroquinolones. Twenty-five strains resistant to three fluoroquinolones and fifteen strains were susceptible. However, one strain, with an *N533T* mutation in *gyrB*, was susceptible to ofloxacin but resistant to moxifloxacin and gatifloxacin.

2.6.3 Articles related to first and second line drug resistance of tuberculosis

Campbell et al. (2011) used DNA sequencing to detect the gene resistance to first-line and second-line drugs. Nine genes including *rpoB*, *katG*, *inhA*, *pncA*, *embB*, *gyrA*, *rrs*, *eis* and *tlyA*, of 314 clinical *M. tuberculosis* complex isolates were sequenced. The results showed that molecular data by DNA sequencing of the first-line drug resistant genes can detect drug resistance with high sensitivity and specificity in comparison with phenotypic data.

Daum et al. (2012) investigated the mutation of genes associated with first- and second-line drug resistance in *M. tuberculosis* by next-generation sequencing. Five drug resistant genes, *rpoB* (resistance to rifampicin), *katG* (resistance to isoniazid), *pncA* (resistance to pyrazinamide), *gyrA* (resistance to ofloxacin/fluoroquinolone) and *rrs* (resistance to aminoglycoside) of 26 clinical isolates from South African patients were amplified by PCR and sequenced by Ion Torrent sequencing. The results were compared to those obtained by genotypic in line probe assay (LPA) and phenotypic Bactec MGIT960 analysis. Of the 26 *M. tuberculosis* clinical isolates, DNA sequencing result shown that 14 (54%) were MDR, 7 (27%) were XDR, and 5 (19%) were susceptible to drugs similar to phenotypic resistance obtained by MGIT960 culture and genotypic *rpoB* and *katG* results by LPA. In several rifampin-resistant isolates, Ion Torrent sequencing revealed uncommon substitutions (H526R and D516G) in *rpoB* gene that were not detected by LPA.

Köser et al. (2013) used rapid whole genome sequencing to investigate the mutations with resistance to 14 antibiotic drugs in two clinical isolates of extensively drug-resistant (XDR) *M. tuberculosis*. Phenotypic results were available found in 9 drugs including ethambutol, isoniazid, moxifloxacin, rifampin, streptomycin, ofloxacin, kanamycin, prothionamide and pyrazinamide but phenotypic results were not available found in 5 drugs including amithiozone, gatifloxacin, levofloxacin, rifapentine and rifabutin.

Lin et al. (2014) developed a pyrosequencing (PSQ) for a rapid and accurate detection of extensively drug resistance mutations in *M. tuberculosis* clinical isolates and clinical specimens. In a total of 130 clinical isolates, a correlation between PSQ results and phenotypic drug susceptibility testing (DST) results was high for all isoniazid, rifampin, quinolones, amikacin, capreomycin and kanamycin. In 129 clinical specimens testing, PSQ assay also had a high sensitivity for both detection of *Mycobacterium tuberculosis* complex (MTBC) and drug resistance mutation.

Walker et al. (2015) used whole-genome sequencing to characterize common and rare mutations for all first-line and second-line antituberculous drugs in predicted drug resistance and drug susceptibility. To characterized genetic mutations, 2,099 *M. tuberculosis* genomes were sequenced across 23 genes including *ahpC*, *fabG1*, *inhA*, *katG*, *ndh*, *rpoB*, *embA*, *embB*, *embC*, *embR*, *iniA*, *iniC*, *manB*, *rmlD*, *pncA*, *rpsA*, *gyrA*, *gyrB*, *rpsL*, *gidB*, *rrs*, *tlyA* and *eis* and the genomes were clustered into 3 groups including drug susceptibility (benign), drug resistance (resistance determinants), or uncharacterized. These characterized mutations can predict drug resistance and drug susceptibility of 1552 isolates with 92.3% sensitivity and 98.4% specificity.

Chapter 3

Research Methodology

3.1 DNA

3.1.1 Genomic DNA of *M. tuberculosis* H37RV (ATCC 27294) obtained from Tuberculosis Research Laboratory, Nation Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand

3.1.2 Crude DNA from 10 *M. tuberculosis* clinical isolates and crude DNA from sputum collected by Drug Resistant Tuberculosis (DR-TB) Research Fund under the Patronage to Her Royal Highness Princess Galayani Vadhana Krom Luang Naradhiwas Rajanagarindra, Mahidol University, Thailand

3.2 Chemical reagents

3.2.1. Chemicals and enzyme for PCR amplification

3.2.1.1 Deoxynucleoside triphosphate (Takara, Japan)

3.2.1.2 Longamp Hotstart *Taq* DNA polymerase (New England Biolabs, USA)

3.2.1.3 Nuclease-free water (Thermo Scientific, USA)

3.2.1.4 PCR buffer (New England Biolabs, USA)

3.2.2 Chemicals for gel electrophoresis

3.2.2.1 Boric acid (Merck, Germany)

3.2.2.2 Ethylenediaminetetraacetic acid (EDTA) (Bio Basic, USA)

3.2.2.3 Ethidium bromide (Biorad, USA)

3.2.2.4 Generuler 100 bp Plus DNA Ladder (Thermo Scientific, USA)

3.2.2.5 Loading dye (Thermo Scientific, USA)

3.2.2.6 Sodium hydroxide (Labscan, Ireland)

3.2.2.7 Tris(hydroxymethyl)aminomethane (Tris base) (USB, USA)

3.2.3 Chemicals for next-generation sequencing

The following chemicals are in Nextera XT DNA Library Prep kit (Illumina, USA)

3.2.3.1 Amplicon tagment Mix (Illumina, USA)

3.2.3.2 AMPure XP beads (Illumina, US)

3.2.3.3 Index i7 adapters (Illumina, USA)

3.2.3.4 Index i5 adapters (Illumina, USA)

3.2.3.5 Library Normalization additive (Illumina, USA)

- 3.2.3.6 Library Normalization beads (Illumina, USA)
- 3.2.3.7 Library Normalization wash (Illumina, USA)
- 3.2.3.8 Library Normalization storage buffer (Illumina, USA)
- 3.2.3.9 Nextera PCR master mix (Illumina, USA)
- 3.2.3.10 Neutralize tagment buffer (Illumina, USA)
- 3.2.3.11 PhiX stock (Illumina, USA)
- 3.2.3.12 Resuspension buffer (Illumina, USA)
- 3.2.3.13 Sodium hydroxide (Illumina, USA)
- 3.2.3.14 Tagment DNA buffer (Illumina, USA)

3.3 Instruments

- 3.3.1 Autoclave (Hiclave HV-50, Hirayama, Japan)
- 3.3.2 DNA thermal cycler (Biorad, USA)
- 3.3.3 DNA thermal cycler (TProfessional, Biometra, Germany)
- 3.3.4 Documentation gel analysis (InGenius, Syngene, Germany)
- 3.3.5 Qubit fluorometer (Invitrogen, USA)
- 3.3.6 Electrophoresis system (Mupid-EXU, Japan)
- 3.3.7 Fragment Analyzer (Life Technologies, USA)
- 3.3.8 Glass Wares (Pyrex, Germany)
- 3.3.9 Laminar air flow cabinet (International Scientific Supply, Thailand)
- 3.3.10 Magnetic stand (Biorad, USA)
- 3.3.11 Microplate shaker (Illumina, USA)
- 3.3.12 Microplate centrifuge (Kubota, Japan)
- 3.3.13 pH meter (Cyberscan 2000, Eutech Cybernetics, Singapore)
- 3.3.14 Sequencer (Nextseq 500; Illumina, USA)
- 3.3.15 Spectrophotometer (Nanodrop 2000; Thermo Scientific, USA)
- 3.3.16 Vortex (Genie 2, Scientific Industries, USA)

3.4 Methods

In this study, 13 MTB genes associated with resistance to 5 first-line and 3 second-line drugs were selected; (1) *rpoB* for rifampicin resistance, (2) *katG*, *inhA* and *ahpC* for isoniazid resistance, (3) *rpsL* for streptomycin resistance, (4) *embB* for ethambutol resistance, (5) *pncA* and *rpsA* for pyrazinamide resistance, (6) *rrs* and *eis* for aminoglycoside resistance, (7) *gyrA* and *gyrB* for fluoroquinolone resistance and (8) *tlyA* for capreomycin resistance. The multiplex PCR for targeted amplification of drug resistant genes was developed and the amplified products were sequenced by the next-generation sequencing using illumina platform.

3.4.1 Primer design for PCR amplification of drug resistant genes

Thirteen oligonucleotide primer pairs of drug resistant genes were designed from nucleotide sequences of *M. tuberculosis* H37Rv derived from the Genbank database (National Center for Biotechnology Information) (Accession No. NC_000962). The melting temperature, GC content and potential dimer formation of primers were determined using Primer3 and Oligocal tools. All oligonucleotides were ordered and purchased from Bio Basic Incorporation, Canada. The nucleotide sequences, melting temperatures and GC contents of primers of drug resistance are shown in Table 3.1.

Table 3.1 Nucleotide sequences, melting temperature and GC content of primers for targeted drug resistant gene amplification

Resistance to drug	Genes	Primers	Nucleotide sequences (5'→3')	Tm (°C)	GC content (%)	PCR product size (bp)
Rifampicin	<i>rpoB</i>	rpoBIF	TCCTCTAAGGGCTCTCGTT	55	53	1,652
		rpoBIR	GTCAGGTACACGATCTCG T	55	53	
		rpoBIIF	GACATCGACCACTTCGGCAA	60	55	2,532
		rpoBIIR	TCCTCCGCGGTAGCAAGAC	61	63	
Isoniazid	<i>katG</i>	katGF	ACACCAACTCCTGGGAAGGAAT	55	50	2,447
		katGR	TGATCGCACATCCAGCACATTT	55	45	
	<i>inhA</i>	inhAF	GCCACTGACACAACAAGG	60	55	1,185
		inhAR	TGTGTGCAGCTCGAGTAACC	60	55	
	<i>ahpC</i>	ahpCF	CTGCGGAATCAGTGTACC	60	58	1,215
		ahpCR	ATACCTGCGGATTTCTGTGTC	60	50	
Streptomycin	<i>rpsL</i>	rpsLF	CGAATACACCGCTGCGACTA	62	55	1,000
		rpsLR	ACAATGCGCTCGGCCAGCG	62	68	
Ethambutol	<i>embB</i>	embBF	CGACGCCGTGGTGATATTCG	62	60	863
		embBR	CCACGCTGGGAATTCGCTTG	62	60	
Pyrazinamide	<i>pncA</i>	pncAF	GACGGATTTGTCGCTCACTAC	61	52	960
		pncAR	GCCGGAGACGATATCCAGAT	60	55	
	<i>rpsA</i>	rpsAF	GACGAGCTGCTGTTTCAAAT	60	50	1,779
		rpsAR	ACGTGAACGCGATCAGCTGC	62	60	
Kanamycin	<i>rrs</i>	rrsF	TTCTAAATACCTTTGGCTCCCT	55	41	1,680
		rrsR	TGGCCAACCTTTGTTGTCATGCA	55	45	
	<i>eis</i>	eisF	GGATCAAGATGGGACAAACC	60	50	1,521
		eisR	GCCAGACACTGTCGTCGTAA	58	55	
Fluoro quinolone	<i>gyrA</i>	gyrAF	AAGGATGTTTCGGTTCCTGGAT	55	48	2,664
		gyrAR	TAACACTCGTACCCGGCT	55	56	
	<i>gyrB</i>	gyrBF	CGCGGTTAGATGGGTAAAAA	55	45	2,266
		gyrBR	CGCTGCATCTCCTGCTCGA	60	63	
Capreomycin	<i>tlyA</i>	tlyAF	CTCGTTACACCAGGACAGCA	60	55	1,395
		tlyAR	GCGAAGCGCAATTTTATTGT	55	40	

3.4.2 PCR amplification

3.4.2.1 Simplex PCR amplification of drug resistant genes of *M. tuberculosis* H37Rv

For primer specificity testing, drug resistant genes were amplified with each designed primer pairs (as described in 3.4.1) using genomic DNA of *M. tuberculosis* H37Rv as a template. Amplification was carried out in a 50- μ l final volume of reaction mixture containing nuclease-free water, PCR buffer containing 2 mM of MgSO₄, 800 μ M of dNTPs mixture, 2.5 units of Longamp Hotstart *Taq* DNA polymerase, 0.2 μ M of each primer and 50 ng of genomic DNA. PCR amplification was performed using thermal cycler (TProfessional, Biometra, Germany) under this condition; initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 65°C for 2 minutes, followed by final extension at 65°C for 10 minutes. The PCR products were analyzed using 1.5% (w/v) agarose gel electrophoresis.

3.4.2.2 Multiplex PCR amplification of drug resistant genes of *M. tuberculosis* H37Rv

Multiplex PCR amplification reactions for all *M. tuberculosis* gene targets were optimized and performed using 4 primer sets. Each primer set contained 3-4 primer pairs and amplified PCR product with different sizes. In addition, the concentration of each primer pair in one set was adjusted in order to gain the similar intensity bands. Multiplex PCR amplification was carried out in a 50- μ l final reaction volume using thermal cycler (TProfessional, Biometra, Germany). The amplified reagents and thermal cycling conditions were similar to simplex PCR amplification condition (as described in 3.4.2.1). The PCR products were analyzed using 1.5% (w/v) agarose gel electrophoresis. The sensitivity of multiplex PCR amplification was determined by varying concentrations of DNA template and primers. The specificity of multiplex PCR amplification was determined by existence of non-specific bands of multiplex PCR products.

3.4.2.3 Multiplex PCR amplification of drug resistant genes of 10 MTB clinical isolates

Multiplex PCR amplification reactions for 4 primer sets containing 13 targeted drug resistant genes were performed using crude DNA from 10 *M. tuberculosis* clinical isolates collected by Drug Resistant Tuberculosis Research Fund at Siriraj hospital, Mahidol University, Thailand. The multiplex PCR amplification

condition was used as described in 3.4.2.2 and optimized again. PCR products were analyzed using 1.5% (w/v) agarose gel electrophoresis.

3.4.3 DNA analysis by Agarose gel electrophoresis

PCR products of drug resistant genes were added with nuclease-free water and loading dye. DNA in PCR products was separated on 1.5% (w/v) agarose gel. Electrophoretic separation of products was carried out for 40 minutes at 8 V/cm in Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM borate and 2 mM EDTA, pH 7.4) using Electrophoresis system (Mupid-EXU, Japan). After that, gel was stained with TBE buffer containing 0.5 mg/l of ethidium bromide. Nucleic acids were visualized under UV light using gel documentation, and size estimation was performed using a Generuler 100 bp Plus DNA Ladder.

3.4.4 Purification of PCR products

PCR products were purified using the Nucleospin gel and PCR cleanup kit (Macherey-Nagel, Germany). Briefly, 2 volumes of NTI buffer were added to 1 volume of PCR products, and the mixture was mixed. A column was placed into a provided 2 ml collection tube. The mixture was loaded to the column, and the column containing the mixture was centrifuged at 11,000 xg for 30 seconds. Flow-through was discarded. The column was placed back into the collection tube. Centrifugation was repeatedly carried out after adding 700 µl of NT3 Buffer into the column to wash the PCR product, and the flow-through was discarded. For removing residual NT3 Buffer, the column was placed back into the collection tube and centrifuged for an additional 1 minute. The column was placed into a new 1.5-ml microcentrifuge tube. Purified PCR products were eluted from the column by adding 20 µl of NE buffer to the center of the membrane and then column was centrifuged for 1 minute. An aliquot of purified PCR products was analyzed by gel electrophoresis.

3.4.5 Nucleotide Sequencing

Purified PCR products of drug resistant genes were sequenced by Sanger sequencing to detect primer specificity or sequenced by next-generation sequencing to detect gene mutation and drug susceptibility.

3.4.5.1 Sanger sequencing

Simplex PCR products of drug resistant genes of *M. tuberculosis* H37Rv were sequenced with the Big-Dye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) and ABI PRISM® 3700 DNA analyzer at First BASE Laboratories (Malaysia). Primers used for sequencing were similar to primers used for PCR amplification shown

in Table 3.1. Nucleotide sequences of PCR products were analyzed by comparison with *M. tuberculosis* H37Rv sequence available in Genbank database (Accession No. NC_000962).

3.4.5.2 Next-generation sequencing

Multiplex PCR products of drug resistant genes of 10 *M. tuberculosis* clinical strains were sequencing and the sequencing result were analyzed comparing with *M. tuberculosis* H37Rv sequence deposited in Genbank database (Accession No. NC_000962). There are 7 steps for sample preparation and sequencing shown in flowchart below (Fig. 3.1). Next-generation sequencing was performed with Nextseq 500 machine (Illumina, USA) at the Medical Life Sciences Institute, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.

3.4.5.2.1 DNA tagmentation

Concentration of purified multiplex PCR products were analyzed by Qubit fluorometer (Biorad, USA) and each of multiplex PCR products concentration were adjusted at 0.4 ng/ μ l. 5 μ l of multiplex PCR products, 10 μ l of Tagment DNA buffer and 5 μ l of Amplicon tagment mix contained transposomes and adapter sequences were added into 96-well PCR plate and mixed by pipetting. The 96-well PCR plate containing the mixture was centrifuge at 280 xg for 1 minute. After that, the 96-well PCR plate containing the mixture was incubated at 55°C for 5 minutes and hold at 10°C by DNA thermal cyler (Biorad, USA). Multiplex PCR products were fragmented into 300 bp blunt-ended DNA fragments and tagged with adapter sequences. After incubation, 5 μ l of Neutralize tagment buffer were added immediately. The 96-well PCR plate contained 25 μ l tagmented DNA was used in DNA libraries amplification.

3.4.5.2.2 DNA libraries amplification

To amplify the tagmented DNA, 5 μ l of each index i7 adapter, 5 μ l of each index i5 adapter and 15 μ l Nextera PCR master mix contained DNA polymerase, deoxynucleotide and Mg^{2+} were added to the 96-well PCR plate carrying tagmented DNA, and the mixture was mixed by pipetting. The 96-well PCR plate was centrifuged at 280 xg for 1 minute and placed on DNA thermal cyler. The libraries amplification was performed under this condition; extension at 72°C for 3 minutes, initial denaturation at 95°C for 30 seconds, 12 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds and finally extension at 72°C for 5 minutes.

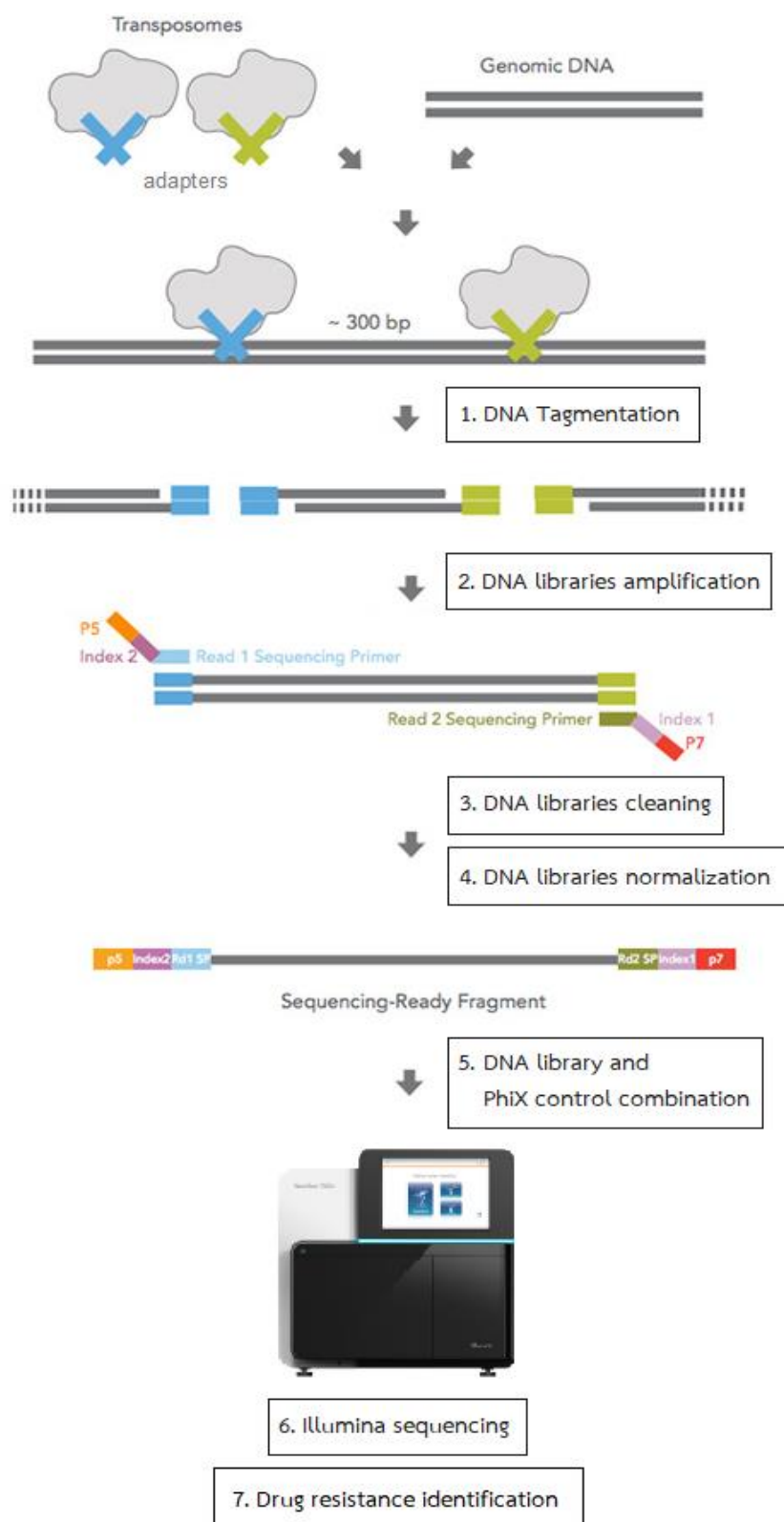


Figure 3.1 Overview protocol of sequencing by Nextseq 500 sequencer

Source: Adapted from Illumina Inc., 2016

3.4.5.2.3 DNA libraries cleaning

To clean up the DNA libraries from DNA libraries amplification (3.4.5.2.2), 50 μl of each DNA libraries were transferred to new 96-well PCR plate. The 90 μl of Ampure XP beads (Illumina, USA) were added to each well. The plate contained the mixture was shake at 1800 rpm for 2 minutes and incubated at room temperature for 5 minutes. The plate was placed in magnetic stand for 2 minutes and then the supernatant was discarded. For DNA washing, 200 μl of 80% (v/v) ethanol were added into each well. The plate was incubated on magnetic stand for 30 seconds and the supernatant was discarded. After DNA washing was repeatedly performed, the plate was incubated on magnetic stand for 15 minutes to remove ethanol. The 52.5 μl of Resuspension buffer (Illumina, USA) was added to each well. the plate contained the mixture was shaken for 2 minutes by microplate shaker (Illumina, USA) and incubated at room temperature for 2 minutes. The plate was placed back to magnetic rack. DNA libraries was eluted and 20 μl of DNA libraries transferred to a new 96-well PCR plate.

3.4.5.2.4 DNA libraries normalization

Normalization of DNA libraries was performed by mixing of Library normalization additives (Illumina, USA) and Libraries normalization beads (Illumina, USA) at ratio 11:2. The 45 μl of mixture were added into the plate contained 20 μl of eluted DNA libraries. The plate was shaken at 1800 rpm for 30 minutes by microplate shaker (Illumina, USA). The plate was placed on magnetic stand for 2 minutes and then the supernatant was discarded. For DNA washing, 45 μl of Library normalization wash (Illumina, USA) were added into each well. After shaking at 1800 rpm for 5 minutes, the plate was placed on magnetic stand for 2 minutes and the supernatant was discarded. DNA washing was repeatedly performed. the 30 μl of Library normalization storage buffer (Illumina, USA) were added into each well. After incubation at room temperature for 5 minutes, the plate was placed back to magnetic stand. Normalized DNA libraries was eluted.

3.4.5.2.5 DNA Library and PhiX control combination

The 5 μl of each Normalized DNA libraries from DNA libraries normalization (3.4.5.2.4) were pooled into new 1.5-ml microcentrifuge tube. The PhiX mixture was prepared by adding 5 μl of 0.2 N sodium hydroxide and 5 μl of Resuspension buffer into new 1.5-ml microcentrifuge tube contained 4 nM of PhiX (10 μl of 10nM PhiX and 15 μl of resuspension buffer). For total volume 1300.2 μl , 1299 μl of pooled DNA libraries were combined with 1.2 μl of the PhiX mixture in new 1.5-ml microcentrifuge tube. The mixture was loading to reagent cartridge (Illumina, USA).

3.4.5.2.6 Illumina sequencing

The reagent cartridge containing DNA Library and PhiX and Mid output flow cell cartridge (Illumina, USA) were added into Nextseq 500 machine and the sequencing was performed on Nextseq 500 system. Quality control of raw sequencing data and trimming was inspected with Prinseq program. The sequence reads were mapped to the reference *M. tuberculosis* H37Rv strain (GenBank accession no. NC_000962) by Burrows-Wheeler Aligner program (BWA) (Li and Durbin, 2009). Then, identification of the sequence variations was performed using Genome Analysis Toolkit program (GATK) (Broad institute, 2018). Finally, the sequence variations were matched with selected tuberculosis drug resistant gene by Variant Call Format tools program (VCFtools) (Danecek et al., 2011).

3.4.5.2.7 Drug resistance identification

The single nucleotide polymorphisms (SNPs) were matched with selected antituberculous drug resistant genes by VCFtools program. Before identification of drug resistance, low-quality SNPs were filtered out at read depth (DP) ≤ 10 and quality value ≤ 95 . The mutations found within or upstream of the resistant genes were compared with reference mutations in database to interpret as mutations conferring resistance phenotype based on Tuberculosis Drug Resistance Mutation Database (TBDreaMDB) (Sandgren et al., 2009).

Chapter 4

Main Results

In order to simultaneously detect drug resistance-associated mutations in *M. tuberculosis* by the next generation sequencing, 13 MTB genes associated with resistance to 5 first-line, namely rifampicin, isoniazid, streptomycin, ethambutol, and pyrazinamide, and 3 second-line drugs, including fluoroquinolones, aminoglycosides, and capreomycin were selected for analysis as summarized in Table 3.1. Multiplex PCR for targeted amplification of drug-resistant genes was developed and optimized using purified DNA of *M. tuberculosis* H37Rv as template. Multiplex PCR was conducted with 10 crude DNA samples isolated from cultured *M. tuberculosis* clinical strains and amplified products were subsequently sequenced by the next generation sequencing (NGS) using Nextseq 500 platform. Drug susceptibility was interpreted from sequencing results and compared with those obtained by the standard drug susceptibility testing.

4.1 Primer design for PCR amplification of drug resistant genes

Fourteen oligonucleotide primer pairs were designed for targeted amplification of 13 genes associated with drug resistance. Two pairs of primers were used to amplify the entire *rpoB* gene, a longest gene in this study. Of 14 primer pairs, 5 primer pairs targeting the first-half of *rpoB*, *katG*, *pncA*, *gyrA*, and *rrs* were previously described by Daum et al. (2012) and one pair for *embB* were reported by Bakuta et al. (2013). The remaining 8 primer pairs targeting the second-half of *rpoB* (*rpoBII*), *inhA*, *rpsL*, *pncA*, *rpsA*, *eis*, *gyrB*, and *tlyA* gene were designed based on the nucleotide sequence of *M. tuberculosis* H37Rv (GenBank accession no. NC_000962). All primers were tested individually by simplex PCR amplification for their specificity using DNA from *M. tuberculosis* H37Rv as PCR template. Specific PCR products of each gene were analyzed using 1.5% agarose gel electrophoresis and sizes of each amplified product were matched their expected sizes (Fig. 4.1 and Table 3.1). Non-specific products were not detected for all genes. The amplified genes were submitted for nucleotide sequencing and compared with those of *M. tuberculosis* H37Rv deposited in GenBank database using the Nucleotide Basic Local Alignment Search Tool (BLAST: Altschul et al., 1990). Result showed that all 13 amplified genes had correct nucleotide sequences, indicating the specificity of the designed primers (Appendix A).

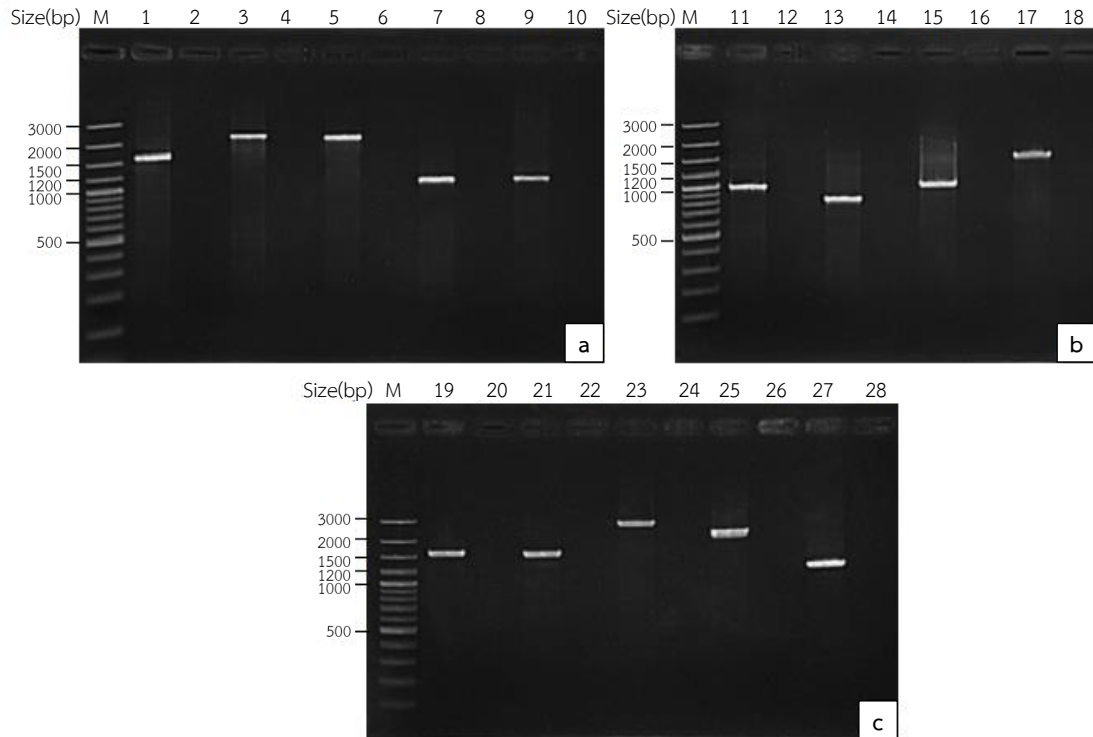


Figure 4.1 Simplex PCRs of 13 drug-resistant genes. PCR products were analyzed using 1.5% agarose gel electrophoresis.

- | | |
|------------------------------|-----------------------------|
| Lane M: 100 bp DNA ladder | Lane 15: PCR of <i>pncA</i> |
| Lane 1: PCR of <i>rpoBI</i> | Lane 16: negative control |
| Lane 2: negative control | Lane 17: PCR of <i>rpsA</i> |
| Lane 3: PCR of <i>rpoBII</i> | Lane 18: negative control |
| Lane 4: negative control | Lane 19: PCR of <i>rrs</i> |
| Lane 5: PCR of <i>katG</i> | Lane 20: negative control |
| Lane 6: negative control | Lane 21: PCR of <i>eis</i> |
| Lane 7: PCR of <i>inhA</i> | Lane 22: negative control |
| Lane 8: negative control | Lane 23: PCR of <i>gyrA</i> |
| Lane 9: PCR of <i>ahpC</i> | Lane 24: negative control |
| Lane 10: negative control | Lane 25: PCR of <i>gyrB</i> |
| Lane 11: PCR of <i>rpsL</i> | Lane 26: negative control |
| Lane 12: negative control | Lane 27: PCR of <i>tlyA</i> |
| Lane 13: PCR of <i>embB</i> | Lane 28: negative control |
| Lane 14: negative control | |

4.2 Optimization of multiplex PCR amplification

A condition of multiplex PCR was optimized based on protocol guided by Henegariu et al. (1997). The multiplex PCR of 13 drug-resistant genes was established and optimized to yield the best amplification results by adjusting various parameters including primer sets, extension times, annealing temperatures, and primer concentrations.

4.2.1 Primer clustering

Initially, primers were clustered into 3 sets by type of drugs (Fig. 4.2). Set 1 and 2 were consisted of primer pairs of gene associated with first-line drug resistance, set 1 include primers of *rpoB* (*rpoBI* + *rpoBII*) *rpsL*, and *embB* genes (associated with rifampicin, streptomycin and ethambutol resistance respectively), set 2 include primers of *katG*, *inhA*, *ahpC*, *pncA*, and *rpsA* gene (isoniazid and pyrazinamide resistance respectively), and set 3 were consisted of primers of *rrs*, *eis*, *gyrA*, *gyrB*, and *tlyA* genes associated with second-line drug resistance (fluoroquinolones, aminoglycosides, and capreomycin resistance). PCR products of *rpsL*, *embB*, *katG*, *inhA*, and *rpsA* were clearly detected. However, PCR products of *rpoBI*, *rpoBII* gene fragments were not seen and PCR products of *ahpC* and *pncA* were not separated on an agarose gel (Fig. 4.2a, lane 1 and 3). For primer set 3, all specific PCR products could not be detected and non-specific products were also generated (Fig. 4.2a, lane 5). The results suggested clustering primers by type of drugs were not appropriated for the developed multiplex PCR. The resistant genes, thus, were re-clustered into 3 sets based on size of PCR products (Fig. 4.2b). PCR products generated from primers in the same set should have different size enough to clearly separate by the agarose gel electrophoresis. With these criteria, primers for *katG*, *rpoBI*, *inhA*, *rpsL*, and *embB* were selected as primer set 1 whereas set 2 comprised of primers for *gyrA*, *rpsA*, *eis*, *ahpC*, and *pncA*, and consisting of Primers for *ropBII*, *gyrB*, *rrs*, and *tlyA* were included into set 3. Results of multiplex PCR revealed that PCR products of *katG* (Fig. 4.2b, lane 1) and *gyrA* (Fig. 4.2b, lane 3) were not detected and the amount of long PCR products in all primer sets was very low. To solve these problems, it might be necessary to subgroup the primers into a smaller group. Therefore, the primers were re-clustered into 4 sets based on the size of PCR products (Table 4.1). The multiplex PCR results showed all specific PCR products and non-specific PCR products were not detected. Sizes of products were matched their predicted size and different sizes of PCR products in each primer set visualized on an agarose gel were clearly seen for all 14 gene fragments. However, the amount of long PCR products was still low (Fig.

4.2c). To increase amount of long PCR products, PCR condition including extension time, annealing temperature, and primer concentration were adjusted.

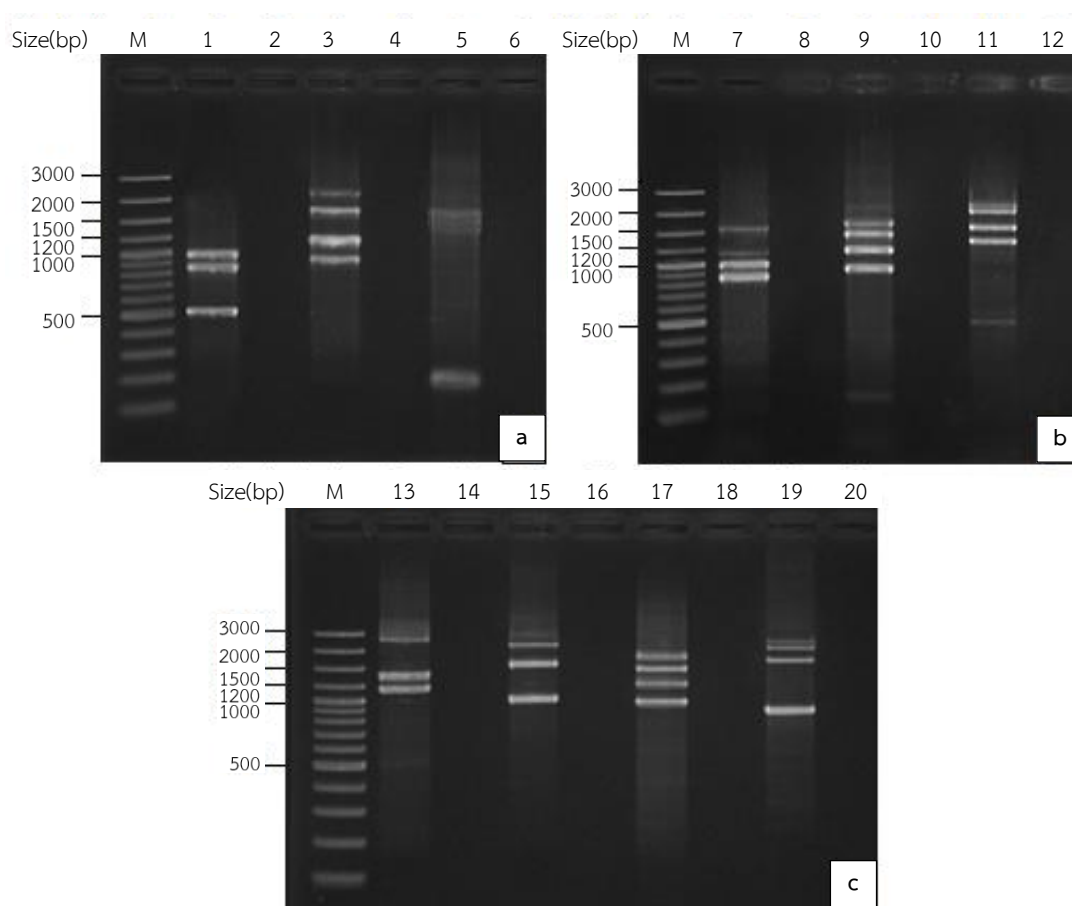


Figure 4.2 Multiplex PCR with different primer sets. Amplified products were analyzed using 1.5% (w/v) agarose gel electrophoresis. (a) Primer clustering based on type of drugs; (b) Primers clustering based on sizes of products; (c) Primers re-clustering based on sizes of products and using 4 reaction tubes.

Lane M: 100 bp DNA ladder

Lane 1: PCR of *rpoBI*, *rpoBII*, *rpsL*, *embB*

Lane 2: negative control

Lane 3: PCR of *katG*, *inhA*, *ahpC*, *pncA*, *rpsA*

Lane 4: negative control

Lane 5: PCR of *rrs*, *eis*, *gyrA*, *gyrB*, *tlyA*

Lane 6: negative control

Lane 7: PCR of *katG*, *rpoBI*, *inhA*, *rpsL*, *embB*

Lane 8: negative control

Lane 9: PCR of *gyrA*, *rpsA*, *eis*, *ahpC*, *pncA*

Lane 10: negative control

Lane 11: PCR of *rpoBII*, *gyrB*, *rrs*, *tlyA*

Lane 12: negative control

Lane 13: PCR of *gyrA*, *tlyA*, *inhA*

Lane 14: negative control

Lane 15: PCR of *katG*, *rpoBI*, *rpsL*

Lane 16: negative control

Lane 17: PCR of *rpsA*, *eis*, *ahpC*, *pncA*

Lane 18: negative control

Lane 19: PCR of *rpoBII*, *gyrB*, *rrs*, *embB*

Lane 20: negative control

Table 4.1 Primer sets and size of PCR products for multiplex PCR amplification

Set 1		Set 2		Set 3		Set 4	
Gene	PCR product size (bp)	Gene	PCR product size (bp)	Gene	PCR product size (bp)	Gene	PCR product size (bp)
<i>gyrA</i>	2,664	<i>katG</i>	2,447	<i>rpsA</i>	1,779	<i>rpoBII</i>	2,535
<i>tlyA</i>	1,395	<i>rpoBI</i>	1,625	<i>eis</i>	1,521	<i>gyrB</i>	2,266
<i>inhA</i>	1,185	<i>rpsL</i>	1,000	<i>ahpC</i>	1,215	<i>rrs</i>	1,680
				<i>pncA</i>	960	<i>embB</i>	863

4.2.2 Optimization by adjusting extension times

Multiplex PCR was performed with primer sets described in Table 4.1 under the following condition; initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minutes, and extension at 65°C varied from 1.5 to 3.5 minutes (1.5, 2, 2.5, 3, and 3.5 minutes), followed by final extension at 65°C for 10 minutes. For primer set 1, amounts PCR products of *gyrA* and *inhA* increased when increasing extension time from 1.5 to 2 minutes but almost stable at 2, 2.5, 3, and 3.5 minutes (Fig. 4.3). In the same way, the amount of amplified *rpsA*, the longest PCR products generated by primer set 3, increased when increasing extension time from 1.5 to 2 minutes but hardly differentiated at 2, 2.5, 3, and 3.5 minutes (Fig. 4.3). In contrast, the amount of shorter PCR products *rpsL* decreased when increasing extension time while no difference was observed among other gene products (Fig 4.3). For primer set 4, increasing the extension time slightly increased amounts of the longer PCR products but decreased those of smaller products (Fig. 4.3). The best result was obtained when using extension time of 2 minutes.

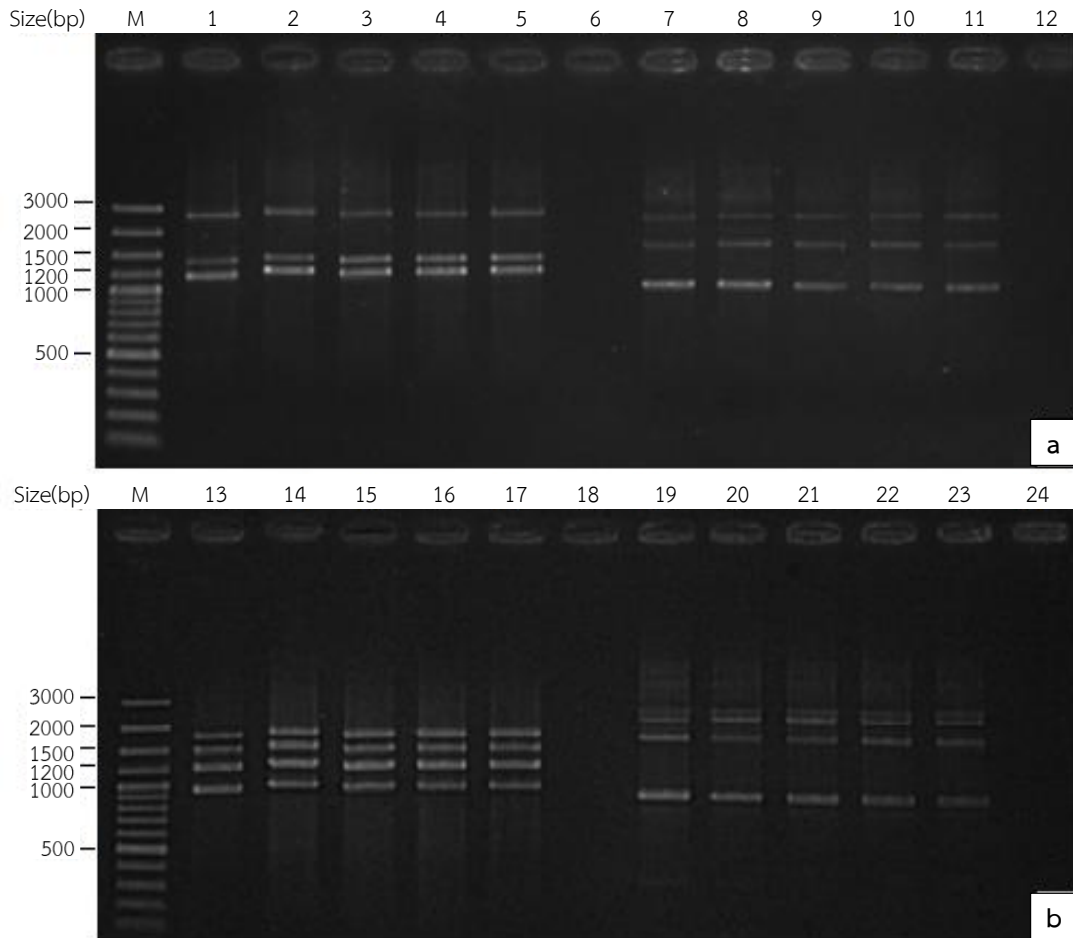


Figure 4.3 Multiplex PCR amplification under different extension time (1.5, 2, 2.5, 3 and 3.5 minutes) and analyzed using 1.5% (w/v) agarose gel electrophoresis. (a) multiplex PCR of primer set 1 (*gyrA*, *tlyA*, *inhA*) and 2 (*katG*, *rpoBI*, *rpsL*); (b) multiplex PCR of primer set 3 (*rpsA*, *eis*, *ahpC*, *pncA*) and 4 (*rpoBII*, *gyrB*, *rrs*, *embB*)

Lane M: 100 bp DNA ladder

Lane 1: primer set 1, 1.5 min

Lane 2: primer set 1, 2 min

Lane 3: primer set 1, 2.5 min

Lane 4: primer set 1, 3 min

Lane 5: primer set 1, 3.5 min

Lane 6: negative control

Lane 7: primer set 2, 1.5 min

Lane 8: primer set 2, 2 min

Lane 9: primer set 2, 2.5 min

Lane 10: primer set 2, 3 min

Lane 11: primer set 2, 3.5 min

Lane 12: negative control

Lane 13: primer set 3, 1.5 min

Lane 14: primer set 3, 2 min

Lane 15: primer set 3, 2.5 min

Lane 16: primer set 3, 3 min

Lane 17: primer set 3, 3.5 min

Lane 18: negative control

Lane 19: primer set 4, 1.5 min

Lane 20: primer set 4, 2 min

Lane 21: primer set 4, 2.5 min

Lane 22: primer set 4, 3 min

Lane 23: primer set 4, 3.5 min

Lane 24: negative control

4.2.3 Optimization by adjusting annealing temperature

The optimal annealing temperature was determined by performing the multiplex PCR with the following condition; initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing varied from 56°C to 64°C (56, 58, 60, 62 and 64°C) for 1 minutes, and extension at 65°C for 2 minutes, followed by final extension at 65°C for 10 minutes. Results revealed non-specific PCR products from amplification of primer set 1 and 2 at annealing temperature of 56°C and 58°C (Fig. 4.4a). When increasing the annealing temperature, the amounts of longest amplified genes, *gyrA* and *katG* for primer set 1 and 2 respectively, increased with the highest amount at the annealing temperature of 64°C. In contrast to other smaller amplified genes, the amounts of amplified products decreased when increasing the annealing temperature. (Fig. 4.4a). Similar results were obtained from amplification of primer set 4, the amount of the longest product, *rpoBII*, increased whereas amounts of the shorter products, *rrs* and *embB* genes, decreased when increasing the annealing temperature (Fig. 4.4b). For primer set 3, all PCR products were barely different when using annealing temperatures at 56°C, 58°C, and 60°C but they decreased when increasing the annealing temperatures to 62°C and 64°C (Fig. 4.4b). The annealing temperature at 60°C was selected for optimal multiplex PCR amplification for all primer sets. At this temperature non-specific product was not detected and yields of each amplified gene looked similar and consistent.

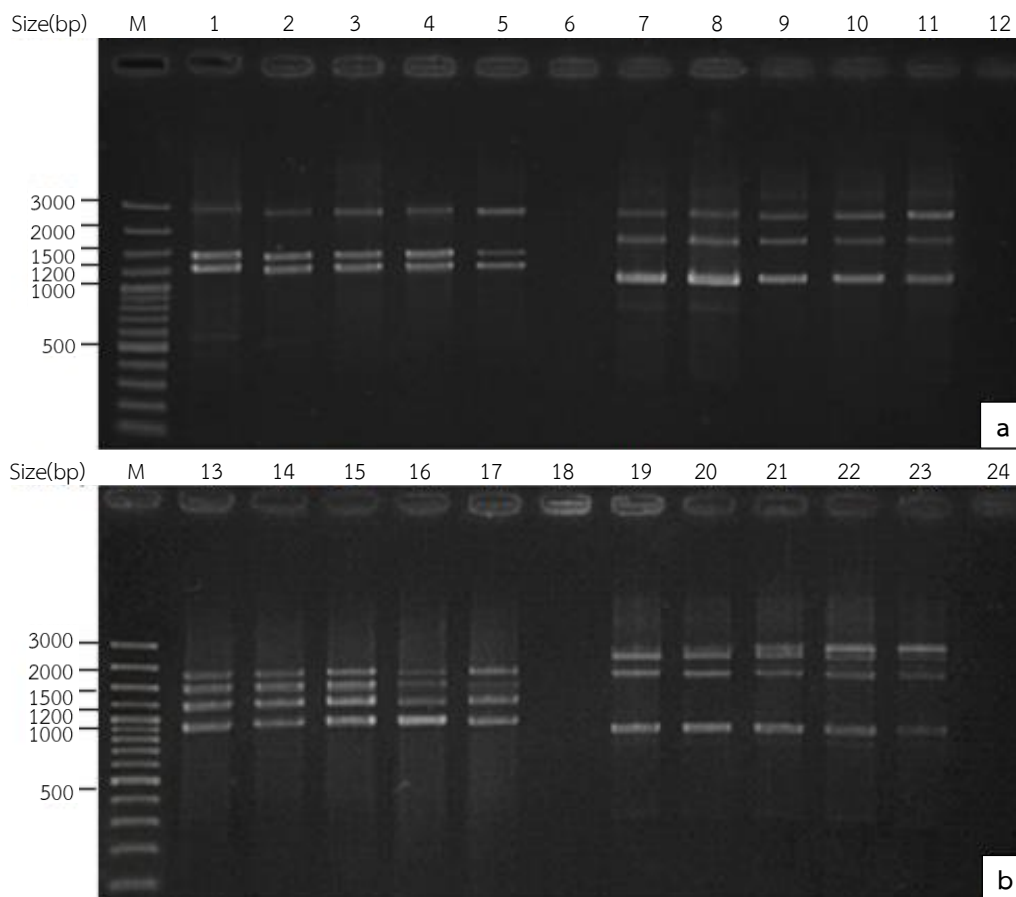


Figure 4.4 Multiplex PCR amplification under different annealing temperatures (56, 58, 60, 62, and 64°C). PCR products were analyzed using 1.5% (w/v) agarose gel electrophoresis. (a) multiplex PCR of primer set 1 (*gyrA*, *tlyA*, *inhA*) and 2 (*katG*, *rpoBI*, *rpsL*); (b) multiplex PCR of primer set 3 (*rpsA*, *eis*, *ahpC*, *pncA*) and 4 (*rpoBII*, *gyrB*, *rrs*, *embB*)

Lane M: 100 bp DNA ladder

Lane 1: primer set 1, 56°C

Lane 2: primer set 1, 58°C

Lane 3: primer set 1, 60°C

Lane 4: primer set 1, 62°C

Lane 5: primer set 1, 64°C

Lane 6: negative control

Lane 7: primer set 2, 56°C

Lane 8: primer set 2, 58°C

Lane 9: primer set 2, 60°C

Lane 10: primer set 2, 62°C

Lane 11: primer set 2, 64°C

Lane 12: negative control

Lane 13: primer set 3, 56°C

Lane 14: primer set 3, 58°C

Lane 15: primer set 3, 60°C

Lane 16: primer set 3, 62°C

Lane 17: primer set 3, 64°C

Lane 18: negative control

Lane 19: primer set 4, 56°C

Lane 20: primer set 4, 58°C

Lane 21: primer set 4, 60°C

Lane 22: primer set 4, 62°C

Lane 23: primer set 4, 64°C

Lane 24: negative control

4.2.4 Optimization by adjusting primer concentrations

Initially, all primers were used with the concentration of 0.2 μM for all primer sets in the multiplex PCR reactions. With this primer concentration the primer set 3 yielded the same amount of PCR products for all amplified genes but multiplex PCR of primer set 1, 2, and 4 generated low amounts of some PCR products, particularly the longest products, yield even after optimizing the extension time and annealing temperature. Therefore, the concentrations of primers in primer set 1, 2, and 4 were optimized by varying the concentration from 0.05-0.6 μM in order to yield the same amount of PCR products as summarized in Table 4.2. It was showed that the best amplification results obtained when the concentrations of primers for *gyrA*, *katG*, *rpoBII*, and *rrs* were increased whereas the concentrations of primers used for amplifying *inhA*, *rpsL*, and *embB* were decreased (Table 4.2 and Fig. 4.5). The optimized PCR condition and primer concentrations were applied to amplify all selected drug-resistant genes by the multiplex PCR using *M. tuberculosis* H37Rv as DNA template. PCR products were analyzed by using 1.5% (w/v) agarose gel electrophoresis. The specific PCR products of all amplified genes were detected with the expected sizes for each corresponding gene and non-specific PCR products were not found (Fig. 4.6).

Table 4.2 Final concentration of primer for Multiplex PCR amplification

Primer set	Gene	Primer concentration (μM)						
		M1	M2	M3	M4	M5	M6	M7
1	<i>gyrA</i>	0.2	0.2	0.2	0.4	0.6		
	<i>tlyA</i>	0.2	0.2	0.2	0.2	0.2		
	<i>inhA</i>	0.2	0.1	0.05	0.05	0.05		
2	<i>katG</i>	0.2	0.2	0.2	0.4	0.6		
	<i>rpoBI</i>	0.2	0.2	0.2	0.2	0.2		
	<i>pncA</i>	0.2	0.1	0.05	0.05	0.05		
4	<i>rpoBII</i>	0.2	0.2	0.2	0.2	0.2	0.4	0.6
	<i>gyrB</i>	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	<i>rrs</i>	0.2	0.2	0.2	0.4	0.6	0.4	0.4
	<i>embB</i>	0.2	0.1	0.05	0.05	0.05	0.05	0.05

Remark : Bold characters show optimized concentration for multiplex PCR reaction

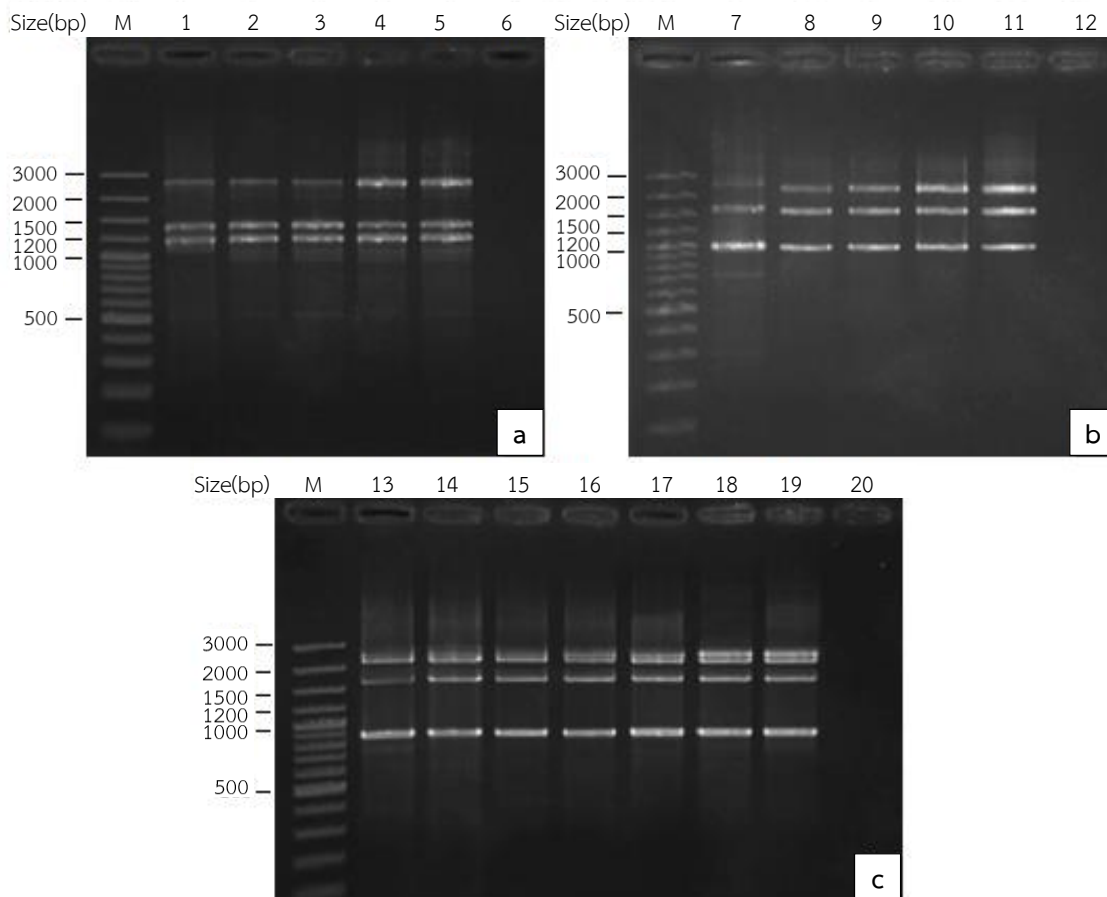


Figure 4.5 Multiplex PCR of primer set 1 (a), set 2 (b), and set 3 (c) with various primer concentrations (Table 4.2). PCR products were analyzed using 1.5% (w/v) agarose gel electrophoresis. (a) multiplex PCR of primer set 1 (*gyrA*, *tlyA*, *inhA*); (b) multiplex PCR of primer set 2 (*katG*, *rpoBI*, *rpsL*); (c) multiplex PCR of primer set 4 (*rpoBII*, *gyrB*, *rrs*, *embB*)

Lane M: 100 bp DNA ladder

Lane 1: primer set 1, M1

Lane 2: primer set 1, M2

Lane 3: primer set 1, M3

Lane 4: primer set 1, M4

Lane 5: primer set 1, M5

Lane 6: negative control

Lane 7: primer set 2, M1

Lane 8: primer set 2, M2

Lane 9: primer set 2, M3

Lane 10: primer set 2, M4

Lane 11: primer set 2, M5

Lane 12: negative control

Lane 13: primer set 4, M1

Lane 14: primer set 4, M2

Lane 15: primer set 4, M3

Lane 16: primer set 4, M4

Lane 17: primer set 4, M5

Lane 18: primer set 4, M6

Lane 19: primer set 4, M7

Lane 20: negative control

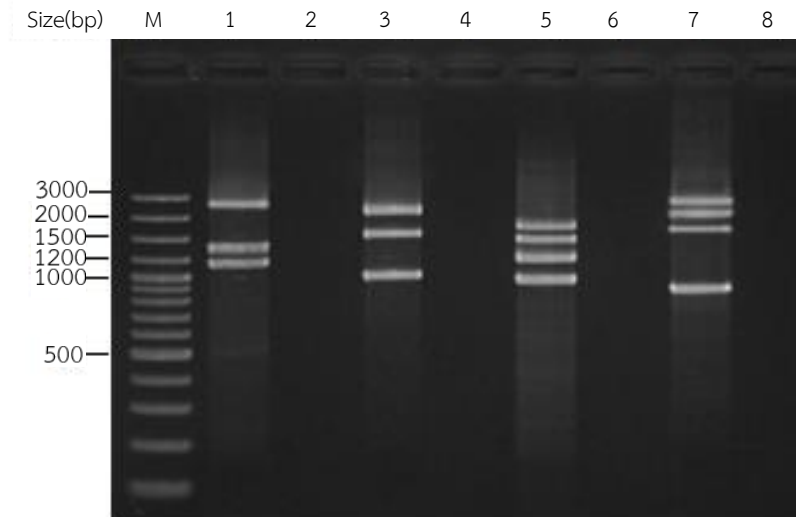


Figure 4.6 PCR product of optimized multiplex PCR amplification analyzed using 1.5% (w/v) agarose gel electrophoresis.

Lane M: 100 bp DNA ladder

Lane 1: primer set 1 (*gyrA*, *tlyA*, *inhA*)

Lane 2: negative control

Lane 3: primer set 2 (*katG*, *rpoBI*, *rpsL*)

Lane 4: negative control

Lane 5: primer set 3 (*rpsA*, *eis*, *ahpC*, *pncA*)

Lane 6: negative control

Lane 7: primer set 4 (*rpoBII*, *gyrB*, *rrs*, *embB*)

Lane 8: negative control

4.3 Detection limit of multiplex PCR amplification

A limit of detection (LOD) of the multiplex PCR was determined by performing the multiplex PCR with various concentrations of DNA templates. The concentrations of DNA were determined by spectrophotometry using spectrophotometer (Nanodrop 2000; Thermo Scientific, USA). For total volume 50 μ l per reaction, different concentrations of *M. tuberculosis* H37Rv purified DNA ranging from 20-50 ng/ μ l (20, 30, 40, and 50 ng/ μ l) were amplified by the multiplex PCR. Decreasing of template DNA concentration resulted in the decreased amount of PCR products, especially the longer PCR products, for all primer sets (Fig. 4.7). Therefore, the lowest template concentration that could be amplified and detected was 40 ng/ μ l of DNA template.

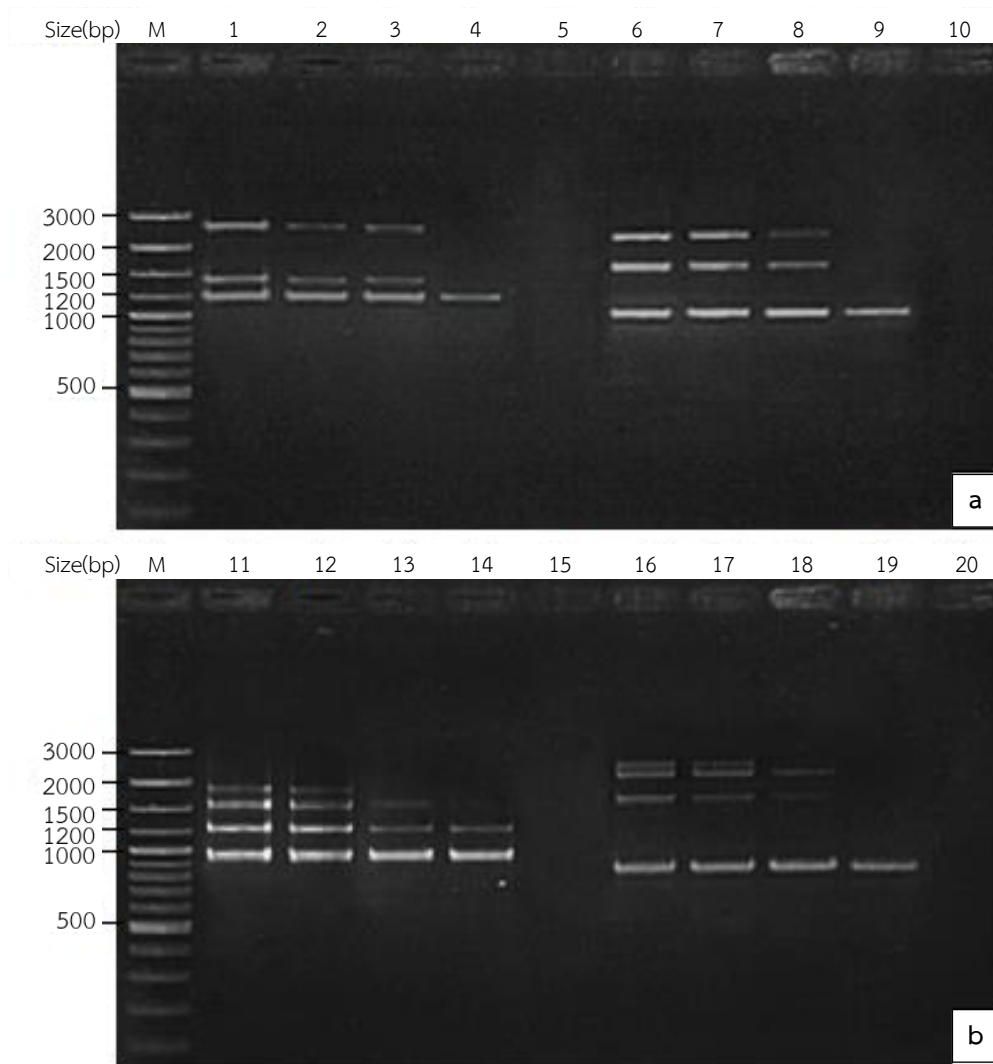


Figure 4.7 Multiplex PCR amplification with different DNA concentrations (50, 40, 30, 20 ng/ μ l). PCR products were analyzed using 1.5% (w/v) agarose gel electrophoresis.

Lane M: 100 bp DNA ladder

lane 1: primer set 1, 50 ng DNA

lane 2: primer set 1, 40 ng DNA

Lane 3: primer set 1, 30 ng DNA

Lane 4: primer set 1, 20 ng DNA

Lane 5: negative control

Lane 6: primer set 2, 50 ng DNA

Lane 7: primer set 2, 40 ng DNA

Lane 8: primer set 2, 30 ng DNA

Lane 9: primer set 2, 20 ng DNA

Lane 10: negative control

lane 11: primer set 3, 50 ng DNA

Lane 12: primer set 3, 40 ng DNA

Lane 13: primer set 3, 30 ng DNA

Lane 14: primer set 3, 20 ng DNA

Lane 15: negative control

Lane 16: primer set 4, 50 ng DNA

Lane 17: primer set 4, 40 ng DNA

Lane 18: primer set 4, 30 ng DNA

Lane 19: primer set 4, 20 ng DNA

Lane 20: negative control

4.4 Multiplex PCR amplification of *M. tuberculosis* clinical isolates

Ten *M. tuberculosis* clinical strains with known phenotypic drug susceptibility information were selected and provided by Drug Resistant Tuberculosis (DR-TB) Research Fund, Siriraj Foundation. Crude DNAs were prepared and determined the concentration by the spectrophotometric method using spectrophotometer (Nanodrop 2000; Thermo Scientific, USA). Drug resistant genes were amplified by the multiplex PCR with the optimal condition described in Section 4.2. Results revealed that all drug resistant genes were specifically amplified and detected in all *M. tuberculosis* clinical strains (Fig. 4.8). Primer set 1 could generate amplified *gyrA* (2,664 bp), *tlyA* (1,395 bp), and *inhA* (1,185 bp), with their predicted sizes (Fig. 4.8a). Primer set 2 could correctly amplified *katG* (2,447 bp), *rpoBI* (1,652 bp), and *rpsL* (1,000 bp) (Fig. 4.8b). Four resistant genes, including *rpsA* (1,779 bp), *eis* (1,521 bp), *ahpC* (1,215 bp), and *pncA* (960 bp), were specifically amplified by primer set 3 (Fig. 4.8c) whereas *rpoBII* (2,532 bp), *gyrB* (2,266 bp), *rrs* (1,680 bp), and *embB* (863 bp) were correctly amplified by primer set 4 (Fig. 4.8d). Non-specific PCR product was not founded in all primer sets. The result clearly showed high specificity of the multiplex PCR for amplifying the drug-resistant genes.

4.5 Multiplex PCR amplification of sputum samples

Acid-fast bacilli (AFB) positive sputum samples were obtained from the Drug Resistant Tuberculosis Research Fund, Siriraj Foundation. Concentrations of DNA extracted from sputum samples were measured by spectrophotometric method using spectrometer (Nanodrop; Thermoscientific, USA). Multiplex PCR was conducted with 15 sputum samples using the optimal condition and 50 ng of template DNA. Drug resistant genes were inconsistently amplified by all primer sets (Fig. 4.9). Almost all samples showed either low amount or unequal amount of amplified products. Some samples could not generate the amplified products. Since the multiplex PCR performing with sputum samples generated low quality and quantity of PCR products, it was, therefore, not suitable for further sequencing by the next generation sequencing.

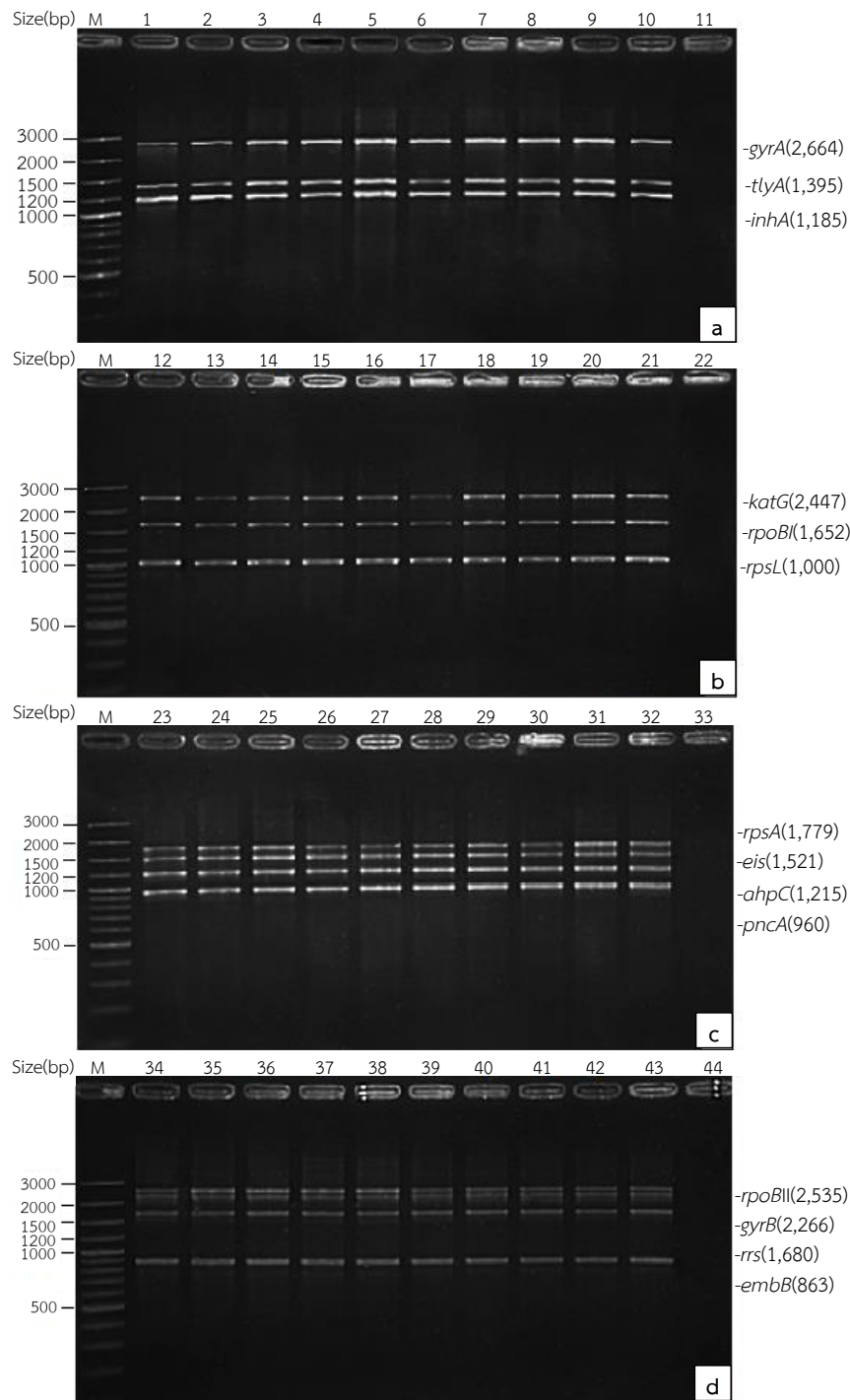


Figure 4.8 Multiplex PCR of *M. tuberculosis* clinical isolates analyzed on agarose gel 1.5 % (w/v) electrophoresis.

Lane M: 100 bp DNA marker

Lane 1-10: multiplex PCR of *M. tuberculosis* clinical isolates by primer set 1

Lane 12-21: multiplex PCR of *M. tuberculosis* clinical isolates by primer set 2

Lane 23-32: multiplex PCR of *M. tuberculosis* clinical isolates by primer set 3

Lane 34-43: multiplex PCR of *M. tuberculosis* clinical isolates by primer set 4

Lane 11, 22, 33, 44: negative control

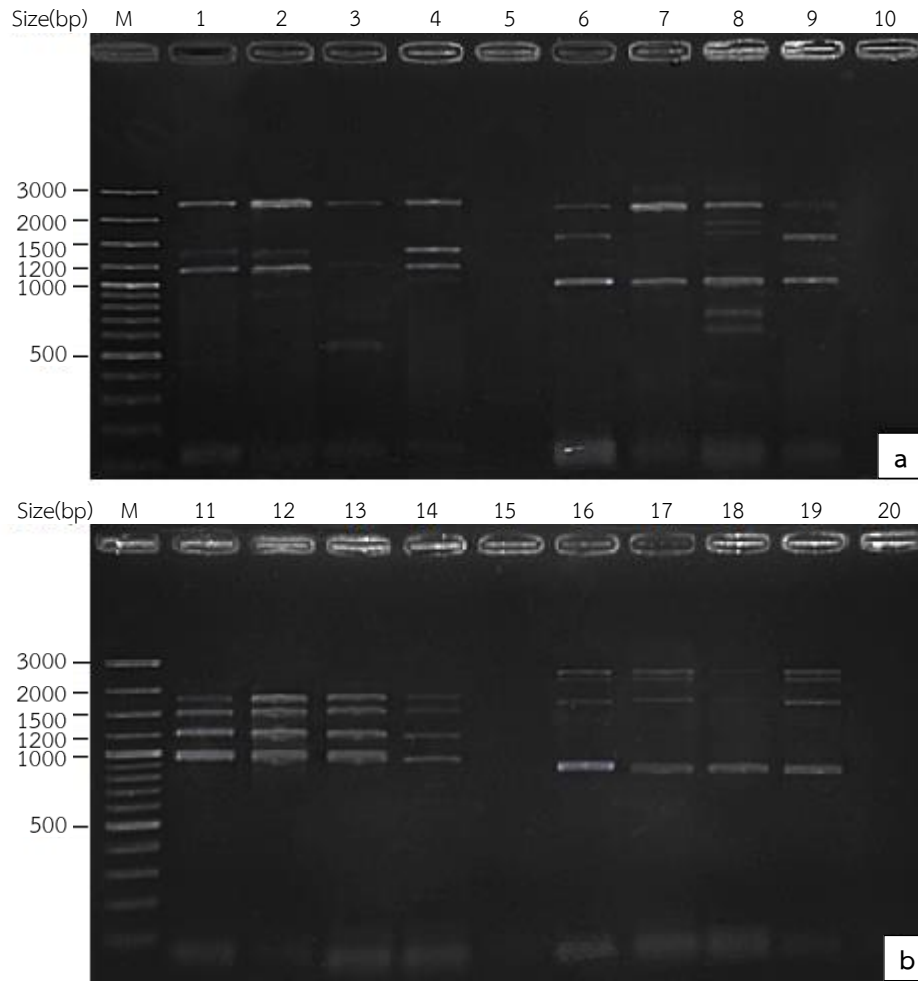


Figure 4.9 Multiplex PCR of DNA extracted from sputum samples analyzed on agarose gel 1.5 % (w/v) electrophoresis.

Lane M: 100 bp DNA marker

Lane 1-4: multiplex PCR of DNA extracted from sputum by primer set 1

Lane 6-9: multiplex PCR of DNA extracted from sputum by primer set 2

Lane 11-14: multiplex PCR of DNA extracted from sputum by primer set 3

Lane 16-19: multiplex PCR of DNA extracted from sputum by primer set 4

Lane 5, 10, 15, 20: negative control

4.6 Rapid drug susceptibility by targeted next-generation sequencing

Amplified products of multiplex PCR targeted drug-resistant genes from 10 *M. tuberculosis* clinical strains were submitted for nucleotide sequence analysis by the next-generation sequencing using the Nextseq 500 platform. The sequence reads were mapped to the reference *M. tuberculosis* H37Rv strain (GenBank accession no. NC_000962). Nucleotide substitutions differed from the reference sequence were called by the vcftools program. Mutations found within or upstream of the resistant genes were interpreted as mutations conferring resistance phenotype based on the TBDreaMDB. All mutation result of *M. tuberculosis* clinical strains by next-generation sequencing shown in Appendix B. Results obtained by targeted NGS were compared with those obtained by the conventional phenotypic drug susceptibility testing (DST). Single nucleotide polymorphisms (SNPs) causing amino acid changes in all targeted genes of 10 *M. tuberculosis* clinical strains were listed in Table 4.3.

Table 4.3 Drug resistance results of 10 *M. tuberculosis* clinical isolates from Thailand by targeted NGS and conventional phenotypic DST

Genes	Related drug	No. of strains	Amino acid substitutions	Results	
				NGS	DST
<i>rpoB</i>	Rifampicin (RIF)	2	S450L, L731P, A1075A	R	R
		4	S450L, A1075A	R	R
		4	H445D, A1075A	R	R
<i>katG</i>	Isoniazid (INH)	10	S315T, R463L	R	R
<i>rpsL</i>	Streptomycin (STR)	7	K43R	R	R
		2	K88R	R	R
<i>embB</i>	Ethambutol (EMB)	1	I489T	S	S
		2	G406D	R	R
		2	M306I	R	R
		3	M306V	R	ND
		1	Q497R	R	ND
<i>pncA</i>	Pyrazinamide (PZA)	1	I133T	R	ND
		3	I31T	S	ND
		1	Y41*	R	ND
<i>rpsA</i>	Pyrazinamide (PZA)	9	R212R (636A>C)	S	ND
<i>gyrA</i>	Ofloxacin (OFX)	8	E21Q, S95T, G668D	S	S
		1	E21Q, S95T, G668D	S	R
		1	E21Q, D94A, S95T, G668D	R	S
<i>tlyA</i>	Capreomycin (CAP)	10	L11L (33A>G)	S	ND

Remark : S=Susceptible, R=Resistant, ND=Not determined

The phenotypic DST identified 9 of 10 strains as multidrug-resistant tuberculosis (MDR-TB) strains and one strain as extensively drug-resistant tuberculosis (XDR-TB) strain. The Nextseq 500 sequencing method; however, identified 9 strains as MDR-TB strains and one strain as pre-XDR-TB strain (Table 4.4). Discordant results were found in two strains. One was identified as ofloxacin-resistant strain by the phenotypic DST but the sequencing result interpreted as susceptible strain due to the absence of mutation conferring resistance in both *gyrA* and *gyrB* genes. In addition, this strain was identified as a kanamycin-resistant strain by the phenotypic DST where none of any mutation was found in the *rrs*, the well-known gene associated with aminoglycoside resistance. Another strain having the well-known mutation conferring fluoroquinolone resistance, GyrA94 D>A, was interpreted as FQs-resistant strain but the DST identified this strain as FQs-susceptible strain (Table 4.4).

Table 4.4 Drug susceptibility profiles of each *M. tuberculosis* clinical isolates

Strain no.	Drug susceptibility result by NGS								Drug susceptibility result by DST							
	RIF	INH	STR	EMB	PZA	OFX	KM	CAP	RIF	INH	STR	EMB	PZA	OFX	KM	CAP
1	R	R	R	R	S	S	S	S	R	R	R	ND	ND	R	R	ND
2	R	R	R	R	S	S	S	S	R	R	R	ND	ND	S	S	ND
3	R	R	R	R	S	S	S	S	R	R	R	ND	ND	S	S	ND
4	R	R	R	R	S	S	S	S	R	R	R	ND	ND	S	S	ND
5	R	R	R	R	R	S	S	S	R	R	R	ND	ND	S	S	ND
6	R	R	S	S	S	S	S	S	R	R	S	ND	ND	S	S	ND
7	R	R	R	R	S	S	S	S	R	R	R	ND	ND	S	S	ND
8	R	R	R	R	S	R	S	S	R	R	R	R	ND	S	S	ND
9	R	R	R	R	S	S	S	S	R	R	R	R	ND	S	S	ND
10	R	R	R	S	R	S	S	S	R	R	R	S	ND	S	S	ND

Remark : S=Susceptible, R=Resistant, ND=Not determined, RIF=Rifampicin, INH=Isoniazid, STR=Streptomycin, EMB=Ethambutol, PZA=Pyrazinamide, OFX=Ofloxacin, KM=Kanamycin, CAP=Capreomycin

Chapter 5

Discussion

Conventional drug susceptibility testing is still a gold standard for detection of *M. tuberculosis* drug-resistant strain. Since the method relies on bacterial growth, it takes, therefore, several weeks or months until obtaining the results. Genotypic or molecular methods are alternative method based on detection of mutations associated with drug resistance and used to identify drug-resistant strain. However, only few tests have been approved and recommended to use as TB diagnostic tests. In addition, almost all available tests could detect only rifampicin resistance like the Xpert MTB/RIF (Cepheid) or both of rifampicin and isoniazid resistance like the GenoType MTBDRplus (Hain Lifescience, Germany). Recently, high-throughput sequencing platforms have been introduced and used for performing whole-genome sequencing (WGS) of *M. tuberculosis* (Koser et al., 2013). A rapid drug susceptibility of all currently used antituberculous drugs were simultaneously conducted based on the bacterial genomic information and mechanisms of resistance. Since the cost of performing WGS is still high, a high-throughput sequencing method targeted only the genes associated with drug resistance has been proposed to rapidly determine antituberculous drug susceptibility and could reduce the cost of sequencing as well. The targeted sequencing of drug resistant genes has been previous described using the next generation Ion Torrent sequencing (Daum et al., 2012). Five *M. tuberculosis* genes including *rpoB*, *katG*, *pncA*, *gyrA*, and *rrs* associated with first- and second-line drug resistance were separately amplified by simplex PCR and the amplified products were sequenced by next generation sequencing using Ion Torrent Personal Genome Machine (PGM). The result showed 100% concordance to the phenotypic drug susceptibility result by MGIT 960 culture system (Daum et al., 2012). Simplex PCR amplification is a simple method to amplify drug resistant genes for further sequencing. However, it is laborious to prepare several reaction tubes, if we have to investigate all resistant genes associated with most currently used antituberculous drugs. gene. Alternatively, multiplex PCR, a method that could amplify several genes in the same reaction tube, is a method of choice for this propose. In a multiplex PCR reaction, more than one target sequences can be amplified using several pairs of primers. The expense of reagents and preparation time is reduced and also cost-effective for investigating high numbers of samples.

In this study, amplification of MTB drug-resistant genes was developed using the multiplex PCR method. Fourteen primer pairs targeting 13 *M. tuberculosis* genes were designed and clustered in to 4 primer sets. Optimization of the method was achieved

by varying various parameters. Primers were designed not only to be specific to their targets but also to avoid potential formation of primer cross-dimers and melting temperatures needed to be balanced for all primers to achieve an even performance. Size of PCR products was different enough to clearly separate by agarose gel electrophoresis (Chamberlain et al., 1988; Zazzi et al., 1993), and was also appropriate for further analysis by the NGS. In addition, primer clustering was considered to give the best amplification results. Since additional primers increases the risk of cross-reactivity between primers and amplification of non-targeted DNA fragments, the optimal group of primers could reduce primer dimer formation during a multiplex PCR reaction (Elnifro et al., 2000). Extension time and annealing temperature also affected the multiplex PCR reaction. Our results demonstrated that increasing extension time caused decreased amount of shorter PCR products while increased amount of longer PCR products, it came from the fact that the amount of enzyme and nucleotides was limited in the reaction and more time was necessary for the polymerase enzyme to complete synthesis of all the products (Henegariu et al., 1997). Annealing temperature was optimized to avoid nonspecific amplification and to ensure that each of the selected targets was correctly amplified at about equal intensity (Thong et al., 2011). For amplicons to be obtained from the targeted regions of DNA, PCR primer pairs need to be compatible with similar annealing temperatures. Annealing temperature at 60°C proved to be best suited for the developed multiplex PCR. Decreasing the annealing temperature from simplex PCR condition was required for amplification of the same loci in multiplex PCR method (Henegariu et al., 1997) but the results showed non-specific PCR products at low annealing temperature in this study. The purity and yield of the products depend on several parameters, one of which is the annealing temperature. At both lower and higher optimal temperature, non-specific products may be formed (Rychlick et al., 1990) and the yield of products is reduced because of a limited supply of enzyme and nucleotides (Henegariu et al., 1997). The primer concentration was also optimized to ensure an equal amount of amplified products. Primers that work together in one reaction is very likely that they will differ in efficiency. In multiplex PCR reaction, many specific genes are simultaneously amplified, the more efficiently amplified loci will negatively influence the yield of product from the less efficient loci. This is due to PCR reaction has a limited of enzyme and nucleotides, and all products competed in the same reaction (Henegariu et al., 1997). Therefore, primer concentration in each primer set have to be adjusted for each primer pair to compensate for varying amplification efficiency (Sint et al., 2010). The sensitivity of amplification in the multiplex PCR assays has been shown to depend upon the template concentration (Meng et al., 2001). Lower template concentrations will give

negative result. The detection limit of this method was higher than other method. The detection limit of multiplex PCR for mutations in pyrethroid-resistant *Aedes aegypti* was 2 ng (Saingamsook, 2017) and detection limit of multiplex allele-specific PCR method for *rpoB* mutation in MDR-TB strains was 0.1 ng (Prammananan et al., 2008). Multiplex PCR has a potential to produce several amplified products with a time safe (Elnifro et al., 2000). It can be applied in many areas of nucleic acid diagnostics, including gene deletion analysis, mutation and polymorphism analysis and DNA quantitative analysis (Zimmermann et al., 1996).

To perform genotypic drug susceptibility testing by detection of mutations associated with drug resistance using the Nextseq 500 platform, DNAs extracted from 10 *M. tuberculosis* clinical strains and 10 from sputum samples were selected for amplification by the multiplex PCR. The 13 drug-resistant genes of all DNA samples were amplified with 4 primer sets under the optimal condition. All resistant genes could be amplified in all *M. tuberculosis* clinical strains by the multiplex PCR with high specificity but almost all sputum samples could not successfully generate the amplified products. The results indicated that our multiplex PCR was sensitive enough for amplifying the target genes from crude DNA strains extracted from cultured bacteria. Earlier studies demonstrated an important role of multiplex PCR as a powerful tool in clinical microbiology and has been widely applied to detect bacteria and genes of interest (Khan et al., 1999, Cerna et al., 2003 and Thong et al., 2011). In contrast, the multiplex PCR was not sensitive enough to amplify the target genes directly from sputum samples. DNA directly extracted from sputum certainly contained both DNA from bacteria (MTB and other bacteria) and from human, resulting in overestimating the concentration of DNA from *M. tuberculosis* and also affected the performance of the method. Adjustments of the amount of actual template are impossible. Although the same amount of total DNA is present for each target, the available number of template molecules is also different (Sint et al., 2012).

Only the amplified products from 10 *M. tuberculosis* clinical strains were used for rapid drug susceptibility by next-generation sequencing. Nucleotide sequences of 14 amplified products were sequenced using the Nextseq 500 platform. Of 13 drug resistant genes, mutations were detected in 8 genes including *rpoB*, *katG*, *rpsL*, *embB*, *pncA*, *rpsA*, *gyrA*, and *tlyA* whereas none of any mutations were found in *inhA*, *ahpC*, *rrs*, *eis*, and *gyrB*.

The *rpoB* gene of *M. tuberculosis* encodes the beta subunit for a DNA-dependent RNA polymerase enzyme. Rifampicin resistance is mainly due to a

mutation within an 81-bp “hot-spot region” of the gene. Mutations occurred within this region are responsible for approximately 95% of rifampin resistance in *M. tuberculosis* strains and also result in high-level rifampicin resistance (Telenti et al., 1993). The S450L and H445D mutations mainly associated with rifampicin resistance amongst mutations conferring resistance (Kuma and Jena, 2014). Similar to our results, S450L mutation was the most common mutation (60%) found in this study while H445D was found in the remaining strains. There has been an additional nucleotide polymorphism resulted in a silence mutation at codon 1075, which has been identified as a phylogenetic marker without any role as a compensatory mutation (Black et al., 2015).

The *katG* gene encodes the bacterial catalase-peroxidase enzyme that used to convert Isoniazid into the active form, isonicotinyl-NAD. The isonicotinyl-NAD inhibits InhA, a key bacterial enzyme of mycolic acid production via the fatty acid biosynthesis (FAS II). The S315T mutation in the KatG is most commonly found in isoniazid resistant *M. tuberculosis* strain. The S315T mutation produces a functional catalase-peroxidase (KatG) with enzymatic activity while highly defective in its ability to form an isoniazid-nicotinamide adenine dinucleotide adduct, reducing the isoniazid toxicity. All of the strains investigated in this study had this mutation. In addition, the R463L mutation was also found in all strains This mutation is known to be a phylogenetic marker and is not related with isoniazid resistance (Torres et al., 2015).

Streptomycin inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome causing misreading of the mRNA message during translation (Davies and Davis, 1968). Resistance to streptomycin is typically through a point mutation in the *rpsL* gene encoding the ribosomal S12 protein. The most common mutation is a substitution at codon 43 from lysine to arginine and mutations at codon 88 are also reported to confer streptomycin resistance (Islam et al., 2017). Since *rpsL* gene encode ribosomal S12 protein involving with stabilization of the pseudoknot structure formed by 16S rRNA, amino acid substitution in the *rpsL* gene affect the higher-order structure of 16S rRNA (Allen and Noller, 1989). Our finding showed the similar result; the K43R mutation was detected in 7 of 10 (70%) strains and the remainder had the K88R mutation.

Ethambutol is a first-line anti-tuberculosis drug. It inhibits the synthesis of arabinogalactan, a major cell wall component of mycobacteria. The *embB* gene is located as the *embCAB* operon encoding arabinosyl transferase enzymes necessary for cell wall biosynthesis. Ethambutol resistance is associated primarily with

mutations within the ethambutol resistance determining region (ERDR) of the *embB* gene. The most commonly mutations occur at EmbB codon 306, with 50 to 70% of clinical strains (Stark et al., 2009). The G406D and Q497R mutation also increased the ethambutol resistance (Lee et al., 2004). Our results identified the same mutations. Half of the strains investigated had mutations within EmbB codon 306 (3 strains with M306V and 2 with M306I) whereas the remainder had G406D and Q497R mutations. However, in this study we had the DST result of ethambutol in only five strains and the NGS results showed 100% concordant with those of the DST.

Pyrazinamide is a synthetic pro-drug, which is converted by *M. tuberculosis* pyrazinamidase, the enzyme encoded by the *pncA* gene, to the active pyrazinoic acid (Zang and Mitchison, 2003). The pyrazinoic acid is accumulated within the bacterial cells, leading to an acidifying the interior. This is probably lethal by membrane damage or by inhibiting trans-translation in persisting cells (Shi, 2011). Due to the mutation in *pncA* gene, pyrazinamidase activity is lost and bacteria becomes incapable of converting prodrug to its bactericidal form. No specific mutation predominates in the pyrazinamide-resistant strains and almost all mutations occurred along the gene have been shown to involve in pyrazinamide resistance (Cheng et al., 2000; Zhang and Mitchison, 2003) Although the information of pyrazinamide susceptibility of all strains in this study was not available, three mutations, I31T, Y41* and I133T, were detected in five strains. Based on the TBDreaMDB the mutations Y41* and I133T were resistant markers for pyrazinamide resistance (Sandgren et al., 2009). The mutation I133T has been previously reported and found in 12% of pyrazinamide resistant strains (Sekigushi et al. 2007). In addition, substitution of amino acid I31T was identified in three strains. This mutation has also been reported in pyrazinamide-susceptible strains, indicating the unrelated of this mutation to the resistant phenotype (Jonmalung et al., 2010). However, amino acid change at this codon was found to affect the distance between active site and metal ion position of the enzyme (Ahmadi et al., 2016). the mutation Y41* was single nucleotide polymorphisms founded in *M. tuberculosis* pyrazinamide resistant strain. Mutations in the *rpsA* have been recently shown to be involved in pyrazinamide resistance but they were not the main mechanism of resistance (Alexander et al., 2012). None of any mutations within this gene was found in this study.

Fluoroquinolones are second-line anti-tuberculosis group of drugs containing many generations. The target of these drugs is DNA gyrase, which is responsible for DNA supercoiling. DNA gyrase comprised of two subunits; subunit A and subunit B that are encoded by *gyrA* and *gyrB*, respectively (Drlica and Malik, 2003). In *M. tuberculosis*, resistance to fluoroquinolones is associated with mutations in a 40-

amino acid region in *gyrA*, which account for 40-70% of fluoroquinolone-resistant strains (Gupta and Gupta, 2012). Amino acid substitutions at codon 94 located within a short region of the *gyrA* known as the quinolone resistance-determining region (QRDR) were the most common mutations occurred in fluoroquinolone-resistant *M. tuberculosis* strains. However, discordant results between NGS and DST was found in two strains. One ofloxacin-susceptible *M. tuberculosis* strain (no.8) identified by the phenotypic DST had the D94A mutation in the *gyrA* gene. Amino acid substitutions in the *gyrA* at codon 94 were associated with different level of resistance. D94G and D94N mutations were correlated with high-level fluoroquinolones resistance, while the D94A was associated with low-level resistance (Li et al., 2014, Disratthakit et al., 2016). The strain containing the D94A mutation conferring low-level of resistance might be able to grow at the drug concentration similar to the concentration used as the critical concentration in the conventional DST, resulting in that the DST identified this strain as drug-susceptible strain. Another strain (no.1) was the ofloxacin-resistant strain but NGS result showed a wild type sequence of both *gyrA* and *gyrB*. One explanation is that this strain comprised of two subpopulations between ofloxacin susceptible and resistant (approximately 25%) subpopulations. During the subcultivation without drug pressure, the resistant subpopulation might be lost during the process. Another might come from that there are other alternative mechanisms causing fluoroquinolone resistance (Singh et al., 2015).

Kanamycin is an inhibitors of protein synthesis through a modification of ribosomal structures at the 16S rRNA. A mutation at position 1401 of *rrs* is associated with high-level resistance to kanamycin in *M. tuberculosis* (Taniguchi et al., 1997). Our results showed the wild type sequence of this gene in all strains. However, the strain no. 1 was also reported as kanamycin resistant strain by the conventional DST, by which there were 15% resistant subpopulation. This strain did not contain any mutations in both *rrs* and *eis* genes, supporting the earlier explanation that the resistant subpopulation might be lost during subcultivation without drug pressure.

Capreomycin is one of a second-line injectable drug. Capreomycin resistance in *M. tuberculosis* is due to the loss of the ability of capreomycin to bind to and inhibit ribosomes because of the lack of methylation of rRNA. The *tlyA* gene codes an rRNA methyltransferase specific for 2'-O-methylation of ribose in rRNA. Mutations within the *tlyA* and subsequent loss of methylation activity have been shown to confer capreomycin resistance (Maus et al., 2005). In this study all *M. tuberculosis* strains carried the mutation in *tlyA* at codon 11. Although we did not have DST result of capreomycin, the L11L mutation is a silent mutation and has been found in both

capreomycin-resistant and susceptible clinical strains, confirming that this mutation did not involve in capreomycin resistance (Sowajassatakul et al., 2014)

Drug susceptibility results obtained by the targeted NGS were 100% concordant with those obtained by the phenotypic DST for the first-line antituberculous drugs. The discordant results occurred with fluoroquinolones and aminoglycosides. However, the targeted gene sequencing using Nextseq 500 platform reported here could provide full-length characterization of drug-resistant genes, making possible discovery of amino acid substitutions and rapidly determine antituberculous drug susceptibility. This targeted gene sequencing method also reduces the cost of sequencing comparison with whole genome sequencing.

Chapter 6

Conclusions and Suggestions

6.1 Conclusions

1) In this research, the multiplex PCR method for targeted amplification of 13 drug resistant genes related to first-line and second-line antituberculous drugs was developed. The 14 oligonucleotide primers specific to drug resistant genes were designed from the nucleotide sequences of *M. tuberculosis* H37Rv reference strain. For primer specificity testing, the target genes of *M. tuberculosis* H37Rv were amplified with each primer pair individually by simplex PCR amplification. All primers showed high specificity that could amplify specific target genes without any nonspecific products.

2) For multiplex PCR amplification, 14 primer pairs were clustered into 4 sets based on sizes of the PCR product. The condition for multiplex PCR amplification were optimized by adjusting the extension time, annealing temperature, and primer concentration using DNA of *M. tuberculosis* H37Rv as template. Optimal PCR condition was initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 65°C for 1.5-3.5 minutes, followed by final extension at 65°C for 10 minutes. The multiplex PCR could amplify all resistant genes and clearly generate the amplified products with high specificity. The limit of detection (LOD) of the method was 40 ng of DNA template.

3) In order to perform drug susceptibility by targeted NGS, the multiplex PCR was conducted with crude DNA extracted from 10 cultured *M. tuberculosis* clinical strains. Drug resistant genes were successfully amplified under an optimal multiplex PCR condition in all clinical strains. For next-generation sequencing, nucleotide sequences of all amplified resistant genes were determined using the Nextseq 500 platform. The sequence reads were mapped to the reference *M. tuberculosis* H37Rv strain (GenBank accession no. NC_000962). Nucleotide polymorphisms causing amino acid changes were analyzed for mutations conferring resistance and drug susceptibility was interpreted from such mutations based on the TBdreaMDB database. Comparison the NGS results with the phenotypic DST results, 9 of 10 (90%) strains were concordantly identified as MDR-TB strains. The discordant results were found in two strains. One MDR-TB strain was discordantly identified as pre-XDR-TB and one XDR-TB strain was misidentified by the NGS.

6.2 Suggestion

The targeted NGS using the selected amplified resistant genes generated from the multiplex PCR developed in this study showed high sensitivity and specificity when performing with DNA extracted from cultured *M. tuberculosis* strains. Although the method can deliver rapid and complete drug susceptibility results, it needs to culture the bacteria prior to perform the test, making it not an ideal test that should be promptly performed when receiving the samples. In addition, this method was still not appropriate to use directly with clinical samples because of its low sensitivity. Selected capture the MTB cells before amplification or increasing the amplification signal or both would be helpful to use this developed multiplex PCR method directly with clinical samples.

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Appendices

Appendix A

Nucleotide sequences of PCR products comparison with drug resistant gene of *M. tuberculosis* H37Rv sequence available in Genbank database (Accession No. NC_000962)

1. PCR product of *rpoB* I and *rpoB* II *rpoB* gene

rpoB_gene_Rv0667	-----TTGGCAGATTCCCGCCAGAGCAAAACAGCCGCTAGTCCTA	40
rpoBI_PCR_product	TTTGGGGGGTGGAGAGCATCTTGGCAGATTCCCGCCAGAGCAAAACAGCCGCTAGTCCTA	60
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	GTCCGAGTCGCCGCAAAGTTCCCTCGAATAACTCCGTACCCGGAGCGCAAACCCGGGTCT	100
rpoBI_PCR_product	GTCCGAGTCGCCGCAAAGTTCCCTCGAATAACTCCGTACCCGGAGCGCAAACCCGGGTCT	120
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	CCTTCGCTAAGCTGCGCGAACCACCTTGAGGTTCCGGGACTCCTTGACGTCCAGACCGATT	160
rpoBI_PCR_product	CCTTCGCTAAGCTGCGCGAACCACCTTGAGGTTCCGGGACTCCTTGACGTCCAGACCGATT	180
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	CGTTCGAGTGGCTGATCGGTTCCGCCGCTGGCGCGAATCCGCCGCCGAGCGGGGTGATG	220
rpoBI_PCR_product	CGTTCGAGTGGCTGATCGGTTCCGCCGCTGGCGCGAATCCGCCGCCGAGCGGGGTGATG	240
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	TCAACCCAGTGGGTGGCCTGGAAGAGGTGCTCTACGAGCTGTCTCCGATCGAGGACTTCT	280
rpoBI_PCR_product	TCAACCCAGTGGGTGGCCTGGAAGAGGTGCTCTACGAGCTGTCTCCGATCGAGGACTTCT	300
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	CCGGGTCGATGTCGTTGTCGTTCTGACCCCTCGTTTCGACGATGTCAAGGCACCCGTCG	340
rpoBI_PCR_product	CCGGGTCGATGTCGTTGTCGTTCTGACCCCTCGTTTCGACGATGTCAAGGCACCCGTCG	360
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	ACGAGTGCAAAGACAAGGACATGACGTACGCGGCTCCACTGTTGTCACCCGCCGAGTTCA	400
rpoBI_PCR_product	ACGAGTGCAAAGACAAGGACATGACGTACGCGGCTCCACTGTTGTCACCCGCCGAGTTCA	420
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	TCAACAACAACACCCGGTGAGATCAAGAGTCAGACGGTGTTCATGGGTGACTTCCCGATGA	460
rpoBI_PCR_product	TCAACAACAACACCCGGTGAGATCAAGAGTCAGACGGTGTTCATGGGTGACTTCCCGATGA	480
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	TGACCGAGAAGGGCACGTTTCATCATCAACGGGACCGAGCGTGTGGTGGTCAGCCAGCTGG	520
rpoBI_PCR_product	TGACCGAGAAGGGCACGTTTCATCATCAACGGGACCGAGCGTGTGGTGGTCAGCCAGCTGG	540
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	TGCGGTCGCCCGGGGTGACTTCGACGAGACCATTGACAAGTCCACCGACAAGACGCTGC	580
rpoBI_PCR_product	TGCGGTCGCCCGGGGTGACTTCGACGAGACCATTGACAAGTCCACCGACAAGACGCTGC	600
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	ACAGCGTCAAGGTGATCCCGAGCCGCGGCGCGTGGCTCGAGTTTGACGTGACAAGCGCG	640
rpoBI_PCR_product	ACAGCGTCAAGGTGATCCCGAGCCGCGGCGCGTGGCTCGAGTTTGACGTGACAAGCGCG	660
rpoBII_PCR_product	-----	0

rpoB_gene_Rv0667	ACACCGTCGGCGTGCGCATCGACCGCAAACGCCGCAACCGGTACCCTGCTGCTCAAGG	700
rpoBI_PCR_product	ACACCGTCGGCGTGCGCATCGACCGCAAACGCCGCAACCGGTACCCTGCTGCTCAAGG	720
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	CGCTGGGCTGGACCAGCGAGCAGATTGTCGAGCGGTTCCGGTTCCTCCGAGATCATGCGAT	760
rpoBI_PCR_product	CGCTGGGCTGGACCAGCGAGCAGATTGTCGAGCGGTTCCGGTTCCTCCGAGATCATGCGAT	780
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	CGACGCTGGAGAAGGACAACACCGTCGGCACCGACGAGGCGCTGTTGGACATCTACCGCA	820
rpoBI_PCR_product	CGACGCTGGAGAAGGACAACACCGTCGGCACCGACGAGGCGCTGTTGGACATCTACCGCA	840
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	AGCTGCGTCCGGGCGAGCCCCGACCAAGAGTCAGCGCAGACGCTGTTGGAAAATTGT	880
rpoBI_PCR_product	AGCTGCGTCCGGGCGAGCCCCGACCAAGAGTCAGCGCAGACGCTGTTGGAAAATTGT	900
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	TCTTCAAGGAGAAGCGCTACGACCTGGCCCCGCTCGGTGCTATAAGGTCAACAAGAAGC	940
rpoBI_PCR_product	TCTTCAAGGAGAAGCGCTACGACCTGGCCCCGCTCGGTGCTATAAGGTCAACAAGAAGC	960
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	TCGGGCTGCATGTCGGCGAGCCATCACGTCGTCGACGCTGACCGAAGAAGACGTCGTGG	1000
rpoBI_PCR_product	TCGGGCTGCATGTCGGCGAGCCATCACGTCGTCGACGCTGACCGAAGAAGACGTCGTGG	1020
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	CCACCATCGAATATCTGGTCCGCTTGACAGAGGGTCAGACCACGATGACCGTCCGGGCG	1060
rpoBI_PCR_product	CCACCATCGAATATCTGGTCCGCTTGACAGAGGGTCAGACCACGATGACCGTCCGGGCG	1080
rpoBII_PCR_product	-----	0
rpoB_gene_Rv0667	GCGTCGAGGTGCCGGTGGAAACCGACGACATCGACCACTTCGGCAACCGCCGCTGCGTA	1120
rpoBI_PCR_product	GCGTCGAGGTGCCGGTGGAAACCGACGACATCGACCACTTCGGCAACCGCCGCTGCGTA	1140
rpoBII_PCR_product	-----TGCCTA *****	6
rpoB_gene_Rv0667	CGGTCGGCGAGCTGATCCAAAACAGATCCGGGTCGGCATGTCGCGGATGGAGCGGGTGG	1180
rpoBI_PCR_product	CGGTCGGCGAGCTGATCCAAAACAGATCCGGGTCGGCATGTCGCGGATGGAGCGGGTGG	1200
rpoBII_PCR_product	CGGTCGGCGAGCTGATCCAAAACAGATCCGGGTCGGCATGTCGCGGATGGAGCGGGTGG *****	66
rpoB_gene_Rv0667	TCCGGGAGCGGATGACCACCCAGGACGTGGAGGCGATCACACCGCAGACGTTGATCAACA	1240
rpoBI_PCR_product	TCCGGGAGCGGATGACCACCCAGGACGTGGAGGCGATCACACCGCAGACGTTGATCAACA	1260
rpoBII_PCR_product	TCCGGGAGCGGATGACCACCCAGGACGTGGAGGCGATCACACCGCAGACGTTGATCAACA *****	126
rpoB_gene_Rv0667	TCCGGCCGGTGGTCCGCCGATCAAGGAGTTCTTCGGCACAGCCAGCTGAGCCAATTCA	1300
rpoBI_PCR_product	TCCGGCCGGTGGTCCGCCGATCAAGGAGTTCTTCGGCACAGCCAGCTGAGCCAATTCA	1320
rpoBII_PCR_product	TCCGGCCGGTGGTCCGCCGATCAAGGAGTTCTTCGGCACAGCCAGCTGAGCCAATTCA *****	186
rpoB_gene_Rv0667	TGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTGGGGC	1360
rpoBI_PCR_product	TGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTGGGGC	1380
rpoBII_PCR_product	TGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTGGGGC *** *****	246
rpoB_gene_Rv0667	CCGGCGGTCTGTACGTCGAGCGTCCGGGCTGGAGGTCGCGACGTGCACCCGTCGCACT	1420
rpoBI_PCR_product	CCGGCGGTCTGTACGTCGAGCGTCCGGGCTGGAGGTCGCGACGTGCACCCGTCGCACT	1440
rpoBII_PCR_product	CCGGCGGTCTGTACGTCGAGCGTCCGGGCTGGAGGTCGCGACGTGCACCCGTCGCACT *****	306
rpoB_gene_Rv0667	ACGGCCGGATGTGCCGATCGAAACCCCTGAGGGGCCAACATCGTCTGATCGGCTCGC	1480
rpoBI_PCR_product	ACGGCCGGATGTGCCGATCGAAACCCCTGAGGGGCCAACATCGTCTGATCGGCTCGC	1500
rpoBII_PCR_product	ACGGCCGGATGTGCCGATCGAAACCCCTGAGGGGCCAACATCGTCTGATCGGCTCGC *****	366

rpoB_gene_Rv0667	TGTCGGGTACGCGCGGGTCAACCCGTTTCGGGTTTCATCGAAACGCCGTACCGCAAGGTGG	1540
rpoB_PCR_product	TGTCGGGTACGCGCGGGTCAACCCGTTTCGGGTTTCATCGAAACGCCGTACCGCAAGG---	1557
rpoBII_PCR_product	TGTCGGGTACGCGCGGGTCAACCCGTTTCGGGTTTCATCGAAACGCCGTACCGCAAGGTGG *****	426
rpoB_gene_Rv0667	TCGACGGCGTGGTTAGCGACGAGATCGTGTACCTGACCGCCGACGAGGAGGACCGCCACG	1600
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TCGACGGCGTGGTTAGCGACGAGATCGTGTACCTGACCGCCGACGAGGAGGACCGCCACG	486
rpoB_gene_Rv0667	TGGTGGCACAGGCCAATTCGCCGATCGATGCGGACGGTCGCTTCGTGAGCCGCGCGTGC	1660
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TGGTGGCACAGGCCAATTCGCCGATCGATGCGGACGGTCGCTTCGTGAGCCGCGCGTGC	546
rpoB_gene_Rv0667	TGGTCCGCCCAAGCGGGCGAGGTGGAGTACGTGCCCTCGTCTGAGGTGGACTACATGG	1720
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TGGTCCGCCCAAGCGGGCGAGGTGGAGTACGTGCCCTCGTCTGAGGTGGACTACATGG	606
rpoB_gene_Rv0667	ACGTCTCGCCCCGCCAGATGGTGTTCGGTGGCCACCGCGATGATCCCTTCTGGAGCACG	1780
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	ACGTCTCGCCCCGCCAGATGGTGTTCGGTGGCCACCGCGATGATCCCTTCTGGAGCACG	666
rpoB_gene_Rv0667	ACGACGCCAACCGTGCCCTCATGGGGGCAAACATGCAGCGCCAGGCGGTGCCGCTGGTCC	1840
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	ACGACGCCAACCGTGCCCTCATGGGGGCAAACATGCAGCGCCAGGCGGTGCCGCTGGTCC	726
rpoB_gene_Rv0667	GTAGCGAGGCCCGCTGGTGGGCACCGGATGGAGCTGCGCGCGGCGATCGACGCCGGCG	1900
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GTAGCGAGGCCCGCTGGTGGGCACCGGATGGAGCTGCGCGCGGCGATCGACGCCGGCG	786
rpoB_gene_Rv0667	ACGTCGTTCGTCGCCAAGAAAGCGGCGTCATCGAGGAGGTGTCGGCCGACTACATCACTG	1960
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	ACGTCGTTCGTCGCCAAGAAAGCGGCGTCATCGAGGAGGTGTCGGCCGACTACATCACTG	846
rpoB_gene_Rv0667	TGATGCACGACAACGGCACCCGGCGTACCTACCGGATGCGCAAGTTTGCCCGGTCCAACC	2020
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TGATGCACGACAACGGCACCCGGCGTACCTACCGGATGCGCAAGTTTGCCCGGTCCAACC	906
rpoB_gene_Rv0667	ACGGCACTTGCGCCAACCAAGTGCCTTCATCGTGGACGCGGGCGACCGAGTCGAGGCCGGTC	2080
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	ACGGCACTTGCGCCAACCAAGTGCCTTCATCGTGGACGCGGGCGACCGAGTCGAGGCCGGTC	966
rpoB_gene_Rv0667	AGGTGATCGC-CGACGGTCCCTGTACTGACGACGGCGAGATGGCGCTGGGCAAGAACCTG	2139
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	AGGTGATCGCCCCGACGGTCCCTGTACTGACGACGGCGAGATGGCGCTGGGCAAGAACCTG	1026
rpoB_gene_Rv0667	CTGGTGGCCATCATGCCGTGGGAGGGCCACAACCTACGAGGACGCGATCATCTGTCCAAC	2199
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CTGGTGGCCATCATGCCGTGGGAGGGCCACAACCTACGAGGACGCGATCATCTGTCCAAC	1086
rpoB_gene_Rv0667	CGCCTGGTCGAAGAGGACGTGCTCACCTCGATCCACATCGAGGAGCATGAGATCGATGCT	2259
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CGCCTGGTCGAAGAGGACGTGCTCACCTCGATCCACATCGAGGAGCATGAGATCGATGCT	1146
rpoB_gene_Rv0667	-CGCGACACCAAGCTGGGTGCGGAGGAGATCACCCGCGACATCCCGAACATCTCCGACGA	2318
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TCGCGACACCAAGCTGGGTGCGGAGGAGATCACCCGCGAMATCCCGAACATCTCCGACGA	1206

rpoB_gene_Rv0667	CTCCGACGGTGACAAGCTGGCCGGCCGGCACGGCAACAAGGGCGTGATCGGCAAGATCCT	2678
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CTCCGACGGTGACAAGCTGGCCGGCCGGCACGGCAACAAGGGCGTGATCGGCAAGATCCT	1566
rpoB_gene_Rv0667	GCCGGTTGAGGACATGCCGTTCCCTTGCCGACGGCACCCCGGTGGACATTATTTTGAACAC	2738
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GCCGGTTGAGGACATGCCGTTCCCTTGCCGACGGCACCCCGGTGGACATTATTTTGAACAC	1626
rpoB_gene_Rv0667	CCACGGCGTGCCGCGACGGATGAACATCGGCCAGATTTTGGAGACCCACCTGGGTTGGTG	2798
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CCACGGCGTGCCGCGACGGATGAACATCGGCCAGATTTTGGAGACCCACCTGGGTTGGTG	1686
rpoB_gene_Rv0667	TGCCACAGCGGCTGGAAGGTCGACGCCCAAGGGGGTTCGGACTGGGCCGCCAGGCT	2858
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TGCCACAGCGGCTGGAAGGTCGACGCCCAAGGGGGTTCGGACTGGGCCGCCAGGCT	1746
rpoB_gene_Rv0667	GCCCCGACGAAGTCTCGAGGCGCAGCCGAACGCCATTGTGTCGACGCCGGTTCGACGG	2918
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GCCCCGACGAAGTCTCGAGGCGCAGCCGAACGCCATTGTGTCGACGCCGGTTCGACGG	1806
rpoB_gene_Rv0667	CGCCCAGGAGGCCGAGCTGCAGGGCCTGTTGTGTCGTCACGCTGCCAACCCGCGACGGTGA	2978
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CGCCCAGGAGGCCGAGCTGCAGGGCCTGTTGTGTCGTCACGCTGCCAACCCGCGACGGTGA	1866
rpoB_gene_Rv0667	GGTGCTCGCCGACCTGGATGAGCGGGGCATCGTGCGCATCGGTGCCGAGGTTTCGCGACGG	2378
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GGTGCTCGCCGACCTGGATGAGCGGGGCATCGTGCGCATCGGTGCCGAGGTTTCGCGACGG	1266
rpoB_gene_Rv0667	GGACATCCTGGTCGCAAGGTCAACCCGAAGGGTGAGACCGAGCTGACGCCGGAGGAGCG	2438
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GGACATCCTGGTCGCAAGGTCAACCCGAAGGGTGAGACCGAGCTGACGCCGGAGGAGCG	1326
rpoB_gene_Rv0667	GCTGCTGCGTGCCATCTTCGGTGAGAAGGCCCGCGAGGTGCGCGACACTTCGCTGAAGGT	2498
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GCTGCTGCGTGCCATCTTCGGTGAGAAGGCCCGCGAGGTGCGCGACACTTCGCTGAAGGT	1386
rpoB_gene_Rv0667	GCCGCACGGCGAATCCGGCAAGGTGATCGGCATTGGGTGTTTTCCCGCGAGGACGAGGA	2558
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GCCGCACGGCGAATCCGGCAAGGTGATCGGCATTGGGTGTTTTCCCGCGAGGACGAGGA	1446
rpoB_gene_Rv0667	CGAGTTGCCGGCCGGTGTCAACGAGCTGGTGCGTGTGTATGTGGCTCAGAAACGCAAGAT	2618
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CGAGTTGCCGGCCGGTGTCAACGAGCTGGTGCGTGTGTATGTGGCTCAGAAACGCAAGAT	1506
rpoB_gene_Rv0667	CTCCGACGGTGACAAGCTGGCCGGCCGGCACGGCAACAAGGGCGTGATCGGCAAGATCCT	2678
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CTCCGACGGTGACAAGCTGGCCGGCCGGCACGGCAACAAGGGCGTGATCGGCAAGATCCT	1566
rpoB_gene_Rv0667	GCCGGTTGAGGACATGCCGTTCCCTTGCCGACGGCACCCCGGTGGACATTATTTTGAACAC	2738
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GCCGGTTGAGGACATGCCGTTCCCTTGCCGACGGCACCCCGGTGGACATTATTTTGAACAC	1626
rpoB_gene_Rv0667	CCACGGCGTGCCGCGACGGATGAACATCGGCCAGATTTTGGAGACCCACCTGGGTTGGTG	2798
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CCACGGCGTGCCGCGACGGATGAACATCGGCCAGATTTTGGAGACCCACCTGGGTTGGTG	1686

rpoB_gene_Rv0667	TGCCCACAGCGGCTGGAAGGTCGACGCCCAAGGGGGTTCGGACTGGGCCGCCAGGCT	2858
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TGCCCACAGCGGCTGGAAGGTCGACGCCCAAGGGGGTTCGGACTGGGCCGCCAGGCT	1746
rpoB_gene_Rv0667	GCCCCGACGAACTGCTCGAGGCGCAGCCGAACGCCATTGTGTCGACGCCGGTGTTCGACGG	2918
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GCCCCGACGAACTGCTCGAGGCGCAGCCGAACGCCATTGTGTCGACGCCGGTGTTCGACGG	1806
rpoB_gene_Rv0667	CGCCCAGGAGGCCGAGCTGCAGGGCCTGTTGTCTGTCACGCTGCCCAACCGCGACGGTGA	2978
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CGCCCAGGAGGCCGAGCTGCAGGGCCTGTTGTCTGTCACGCTGCCCAACCGCGACGGTGA	1866
rpoB_gene_Rv0667	CGTGCTGGTTCGACGCCGACGGCAAGGCCATGCTCTTCGACGGGCGCAGCGGCGAGCCGTT	3038
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CGTGCTGGTTCGACGCCGACGGCAAGGCCATGCTCTTCGACGGGCGCAGCGGCGAGCCGTT	1926
rpoB_gene_Rv0667	CCCGTACCCGGTCACGGTTGGCTACATGTACATCATGAAGCTGCACCACCTGGTGGACGA	3098
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CCCGTACCCGGTCACGGTTGGCTACATGTACATCATGAAGCTGCACCACCTGGTGGACGA	1986
rpoB_gene_Rv0667	CAAGATCCACGCCCGCTCCACCGGGCCGTAICTGATGATCACCCAGCAGCCGCTGGGCGG	3158
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CAAGATCCACGCCCGCTCCACCGGGCCGTAICTGATGATCACCCAGCAGCCGCTGGGCGG	2046
rpoB_gene_Rv0667	TAAGGCGCAGTTCGGTGGCCAGCGGTTTCGGGGAGATGGAGTGCTGGGCCATGCAGGCCTA	3218
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	TAAGGCGCAGTTCGGTGGCCAGCGGTTTCGGGGAGATGGAGTGCTGGGCCATGCAGGCCTA	2106
rpoB_gene_Rv0667	CGGTGCTGCCTACACCCTGCAGGAGCTGTTGACCATCAAGTCCGATGACACCGTCGGCCG	3278
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CGGTGCTGCCTACACCCTGCAGGAGCTGTTGACCATCAAGTCCGATGACACCGTCGGCCG	2166
rpoB_gene_Rv0667	CGTCAAGGTGTACGAGGCGATCGTCAAGGGTGAGAACATCCCGAGCCGGGCATCCCCGA	3338
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	CGTCAAGGTGTACGAGGCGATCGTCAAGGGTGAGAACATCCCGAGCCGGGCATCCCCGA	2226
rpoB_gene_Rv0667	GTCGTTCAAGGTGCTGCTCAAAGAACTGCAGTCGCTGTGCCTCAACGTCGAGGTGCTATC	3398
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GTCGTTCAAGGTGCTGCTCAAAGAACTGCAGTCGCTGTGCCTCAACGTCGAGGTGCTATC	2286
rpoB_gene_Rv0667	GAGTGACGGTGCGGCGATCGAACTGCGCGAAGGTGAGGACGAGGACCTGGAGCGGGCCGC	3458
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GAGTGACGGTGCGGCGATCGAACTGCGCGAAGGTGAGGACGAGGACCTGGAGCGGGCCGC	2346
rpoB_gene_Rv0667	GGCCAACCTGGGAATCAATCTGTCCCACAACGAATCCGCAAGTGTCGAGGATCTTGCGTA	3518
rpoB_PCR_product	-----	1557
rpoBII_PCR_product	GGCCAACCTGGGAATCAATCTGTCCCACAACGAATCCGCAAGTGTCGAGGATCTTGCGTA	2406

2. PCR product of *katG* gene

katG_gene_Rv1908c	GTGCCCGAGCAACACCCACCCATTACAGAAACCACCACCGGAGCCGCTAGCAACGGCTGT	60
PCR_product	-----CAACACCCACCCATTACAGAAACCACCACCGGAGCCGCTAGCAACGGCTGT	51

katG_gene_Rv1908c	CCCGTCGTGGGTCATATGAAATACCCCGTCGAGGGCGGCGGAAACCAGGACTGGTGGCCC	120
PCR_product	CCCGTCGTGGGTCATATGAAATACCCCGTCGAGGGCGGCGGAAACCAGGACTGGTGGCCC	111

katG_gene_Rv1908c	AACCGGCTCAATCTGAAGGTACTGCACAAAACCCGGCCGTCGCTGACCCGATGGGTGCG	180
PCR_product	AACCGGCTCAATCTGAAGGTACTGCACAAAACCCGGCCGTCGCTGACCCGATGGGTGCG	171

katG_gene_Rv1908c	GCGTTCGACTATGCCGCGGAGGTCGCGACCATCGACGTTGACGCCCTGACGCGGGACATC	240
PCR_product	GCGTTCGACTATGCCGCGGAGGTCGCGACCATCGACGTTGACGCCCTGACGCGGGACATC	231

katG_gene_Rv1908c	GAGGAAGTGATGACCACCTCGCAGCCGTGGTGGCCCGCCGACTACGGCCACTACGGGCCG	300
PCR_product	GAGGAAGTGATGACCACCTCGCAGCCGTGGTGGCCCGCCGACTACGGCCACTACGGGCCG	291

katG_gene_Rv1908c	CTGTTTATCCGGATGGCGTGGCAGCTGCCGGCACCTACCGCATCCACGACGGCCGCGGC	360
PCR_product	CTGTTTATCCGGATGGCGTGGCAGCTGCCGGCACCTACCGCATCCACGACGGCCGCGGC	351

katG_gene_Rv1908c	GGCGCCGGGGCGGCATGCAGCGGTTGCGCCGCTTAACAGCTGGCCCGACAACGCCAGC	420
PCR_product	GGCGCCGGGGCGGCATGCAGCGGTTGCGCCGCTTAACAGCTGGCCCGACAACGCCAGC	411

katG_gene_Rv1908c	TTGGACAAGGCGCGCCGGCTGCTGTGGCCGGTCAAGAAGAAGTACGGCAAGAAGCTCTCA	480
PCR_product	TTGGACAAGGCGCGCCGGCTGCTGTGGCCGGTCAAGAAGAAGTACGGCAAGAAGCTCTCA	471

katG_gene_Rv1908c	TGGGCGGACCTGATTGTTTTCCGCCGGCAACTGCGCGCTGGAATCGATGGGCTTCAAGACG	540
PCR_product	TGGGCGGACCTGATTGTTTTCCGCCGGCAACTGCGCGCTGGAATCGATGGGCTTCAAGACG	531

katG_gene_Rv1908c	TTCGGGTTGCGCTTCGGCCGGTGCAGCAGTGGGAGCCCGATGAGGTCTATTGGGCAAG	600
PCR_product	TTCGGGTTGCGCTTCGGCCGGTGCAGCAGTGGGAGCCCGATGAGGTCTATTGGGCAAG	591

katG_gene_Rv1908c	GAAGCCACCTGGCTCGGCATGAGCGTTACAGCGGTAAAGCGGGATCTGGAGAACCCTGCTG	660
PCR_product	GAAGCCACCTGGCTCGGCATGAGCGTTACAGCGGTAAAGCGGGATCTGGAGAACCCTGCTG	651

katG_gene_Rv1908c	GCCGCGGTGCAGATGGGGCTGATCTACGTGAACCCGGAGGGGCCGAACGGCAACCCGGAC	720
PCR_product	GCCGCGGTGCAGATGGGGCTGATCTACGTGAACCCGGAGGGGCCGAACGGCAACCCGGAC	711

katG_gene_Rv1908c	CCCATGGCCGCGGGTGCACATTCGCGAGACGTTTCGGCGCATGGCCATGAACGACGTC	780
PCR_product	CCCATGGCCGCGGGTGCACATTCGCGAGACGTTTCGGCGCATGGCCATGAACGACGTC	771

katG_gene_Rv1908c	GAAACAGCGCGCTGATCGTCGGCGGTACACTTTCGGTAAGACCCATGGCGCCGGCCCG	840
PCR_product	GAAACAGCGCGCTGATCGTCGGCGGTACACTTTCGGTAAGACCCATGGCGCCGGCCCG	831

katG_gene_Rv1908c	GCCGATCTGGTCGGCCCGAACCCGAGGCTGCTCCGCTGGAGCAGATGGGCTTGGGCTGG	900
PCR_product	GCCGATCTGGTCGGCCCGAACCCGAGGCTGCTCCGCTGGAGCAGATGGGCTTGGGCTGG	891

katG_gene_Rv1908c	AAGAGCTCGTATGGCACCAGAACCCGTAAGGACGCGATACCAGCGGCATCGAGGTCGTA	960
PCR_product	AAGAGCTCGTATGGCACCAGAACCCGTAAGGACGCGATACCAGCGGCATCGAGGTCGTA	951

katG_gene_Rv1908c	TGGACGAACACCCCGACGAAATGGGACAACAGTTTCTCGAGATCCTGTACGGCTACGAG	1020
PCR_product	TGGACGAACACCCCGACGAAATGGGACAACAGTTTCTCGAGATCCTGTACGGCTACGAG	1011

katG_gene_Rv1908c	TGGGAGCTGACGAAGAGCCCTGCTGGCGCTTGGCAATACACCGCCAAGGACGGCGCCGGT	1080
PCR_product	TGGGAGCTGACGAAGAGCCCTGCTGGCGCTTGGCAATACACCGCCAAGGACGGCGCCGGT	1071

katG_gene_Rv1908c	GCCGGCACCATCCCGGACCCGTTTCGGCGGGCCAGGGCGCTCCCCGACGATGCTGGCCACT	1140
PCR_product	GCCGGCACCATCCCGGACCCGTTTCGGCGGGCCAGGGCGCTCCCCGACGATGCTGGCCACT	1131

katG_gene_Rv1908c	GACCTCTCGCTGCGGGTGGATCCGATCTATGAGCGGATCACGCGTCGCTGGCTGGAACAC	1200
PCR_product	GACCTCTCGCTGCGGGTGGATCCGATCTATGAGCGGATCACGCGTCGCTGGCTGGAACAC	1191

katG_gene_Rv1908c	CCCGAGGAATTGGCCGACGAGTTCGCCAAGGCCTGGTACAAGCTGA-TCCACCGAGACAT	1259
PCR_product	CCCGAGGAATTGGCCGACGAGTTCGCCAAGGCCTGGTACAAGCTGAATCCACCGAGACAT	1251
	***** *****	
katG_gene_Rv1908c	GGGTCCCGTTGCGAGATACCTTGGGCCGCTGGTCCCCAAGCAGACCCTGCTGTGGCAGGA	1319
PCR_product	GGGTCCCGTTGCGAGATACCTTGGGCCGCTGGTCCCCAAGCAGA-----	1295

3. PCR product of *inhA* gene (1674202..1675011)

inhA_gene_Rv1484	ATGACAGGACTGCTGGACGGCAAACGGATTCTGTTAGCGGAATCATCACCGACTCGTCG	60
PCR_product	-----ACTGCTGGACGGCAAACGGATTCTGTTAGCGGAATCATCACCGACTCGTCG *****	52
inhA_gene_Rv1484	ATCGCGTTTCACATCGCACGGGTAGCCAGGAGCAGGGCGCCCAGCTGGTGCTCACCGGG	120
PCR_product	ATCGCGTTTCACATCGCACGGGTAGCCAGGAGCAGGGCGCCCAGCTGGTGCTCACCGGG *****	112
inhA_gene_Rv1484	TTCGACCGGCTGCGGCTGATTACAGCGCATCACCGACGGCTGCCGGCAAAGGCCCGCTG	180
PCR_product	TTCGACCGGCTGCGGCTGATTACAGCGCATCACCGACGGCTGCCGGCAAAGGCCCGCTG *****	172
inhA_gene_Rv1484	CTCGAACTCGACGTGCAAAACGAGGAGCACCTGGCCAGCTTGGCCGGCGGGTGACCGAG	240
PCR_product	CTCGAACTCGACGTGCAAAACGAGGAGCACCTGGCCAGCTTGGCCGGCGGGTGACCGAG *****	232
inhA_gene_Rv1484	GCGATCGGGCGGGCAACAAGCTCGACGGGTGGTGCATTGATTGGGTTATGCCGCAG	300
PCR_product	GCGATCGGGCGGGCAACAAGCTCGACGGGTGGTGCATTGATTGGGTTATGCCGCAG *****	292
inhA_gene_Rv1484	ACCGGGATGGGCATCAACCCGTTCTTCGACGCGCCCTACGCGGATGTGTCCAAGGGCATC	360
PCR_product	ACCGGGATGGGCATCAACCCGTTCTTCGACGCGCCCTACGCGGATGTGTCCAAGGGCATC *****	352
inhA_gene_Rv1484	CACATCTCGGCTATTTCGTATGCTTCGATGGCCAAGGCGCTGCTGCCGATCATGAACCCC	420
PCR_product	CACATCTCGGCTATTTCGTATGCTTCGATGGCCAAGGCGCTGCTGCCGATCATGAACCCC *****	412
inhA_gene_Rv1484	GGAGGTTCCATCGTCGGCATGGACTTCGACCCGAGCCGGGCGATGCCGGCTACAACCTGG	480
PCR_product	GGAGGTTCCATCGTCGGCATGGACTTCGACCCGAGCCGGGCGATGCCGGCTACAACCTGG *****	472
inhA_gene_Rv1484	ATGACGGTCGCCAAGAGCGCGTTGGAGTCGGTCAACAGGTTCTGTGGCGCGGAGGCCGGC	540
PCR_product	ATGACGGTCGCCAAGAGCGCGTTGGAGTCGGTCAACAGGTTCTGTGGCGCGGAGGCCGGC *****	532
inhA_gene_Rv1484	AAGTACGGTGTGCGTTCGAATCTCGTTGCCGAGGCCCTATCCGGACGCTGGCGATGAGT	600
PCR_product	AAGTACGGTGTGCGTTCGAATCTCGTTGCCGAGGCCCTATCCGGACGCTGGCGATGAGT *****	592
inhA_gene_Rv1484	GCGATCGTCGGCGGTGCGCTCGGCGAGGAGGCCGGCGCCAGATCCAGCTGCTCGAGGAG	660
PCR_product	GCGATCGTCGGCGGTGCGCTCGGCGAGGAGGCCGGCGCCAGATCCAGCTGCTCGAGGAG *****	652
inhA_gene_Rv1484	GGCTGGGATCAGCGCGCTCCGATCGGCTGGAACATGAAGGATGCGACCGCGTCCGCAAG	720
PCR_product	GGCTGGGATCAGCGCGCTCCGATCGGCTGGAACATGAAGGATGCGACCGCGTCCGCAAG *****	712
inhA_gene_Rv1484	ACGGTGTGCGCGCTGCTGTCTGACTGGCTGCCGGGACACGGGTGACATCATCTACGCC	780
PCR_product	ACGGTGTGCGCGCTGCTGTCTGACTGGCTGCCGGGACACGGGTGACATCATCTACGCC *****	772
inhA_gene_Rv1484	GACGGCGGCGCGCACACCCAATTGCTCTAG-----	810
PCR_product	GACGGCGGCGCGCACACCCAATTGCTCTAGAACGCATGCAATTTGATGCCGTCCTGCTGC *****	832

4. PCR product of *rpsL* gene

rpsL_gene_Rv0682	-----ATGCCAACCATCCAGCAGCTGGT	23
PCR_product	ACAGAACGTGAAAGCGCCCAAGATAGAAAGCCGGTAGATGCCAACCATCCAGCAGCTGGT *****	480
rpsL_gene_Rv0682	CCGCAAGGGTCGTCGGGACAAGATCAGTAAGGTC AAGACCGCGGCTCTGAAGGGCAGCCC	83
PCR_product	CCGCAAGGGTCGTCGGGACAAGATCAGTAAGGTC AAGACCGCGGCTCTGAAGGGCAGCCC *****	540
rpsL_gene_Rv0682	GCAGCGTCGTGGTGTATGCACCCGCGTGTACACCACCACTCCGAAGAAGCCGAACTCGGC	143
PCR_product	GCAGCGTCGTGGTGTATGCACCCGCGTGTACACCACCACTCCGAAGAAGCCGAACTCGGC *****	600
rpsL_gene_Rv0682	GCTTCGGAAGGTTGCCCGCGTGAAGTTGACGAGTCAGGTCGAGGTCACGGCGTACATTCC	203
PCR_product	GCTTCGGAAGGTTGCCCGCGTGAAGTTGACGAGTCAGGTCGAGGTCACGGCGTACATTCC *****	660
rpsL_gene_Rv0682	CGGCGAGGGCCACAACCTGCAGGAGCACTCGATGGTGCTGGTGCGCGGGCGGGTCAA	263
PCR_product	CGGCGAGGGCCACAACCTGCAGGAGCACTCGATGGTGCTGGTGCGCGGGCGGGTCAA *****	720
rpsL_gene_Rv0682	GGACCTGCCTGGTGTGCGCTACAAGATCATCCGCGGTTTCGCTGGATACGCAGGGTGCAA	323
PCR_product	GGACCTGCCTGGTGTGCGCTACAAGATCATCCGCGGTTTCGCTGGATACGCAGGGTGCAA *****	780
rpsL_gene_Rv0682	GAACCGCAAACAGGCACGCAGCCGTTACGGCGCTAAGAAGGAGAAGGGCTGA-----	375
PCR_product	GAACCGCAAACAGGCACGCAGCCGTTACGGCGCTAAGAAGGAGAAGGGCTGATGCCACGC *****	840

5. PCR product of *embB* gene

embB_gene_Rv3795 PCR_product	GCCGTGGTGATATTCGGCTTCTGCTCTGGCATGTCATCGGCGGAATTCGTCGGACGAC -----ATCGGCGGAATTCGTCGGACGAC *****	900 24
embB_gene_Rv3795 PCR_product	GGCTACATCCTGGGCATGGCCCGAGTCGCCGACCACGCCGGCTACATGTCCAATATTTT GGCTACATCCTGGGCATGGCCCGAGTCGCCGACCACGCCGGCTACATGTCCAATATTTT *****	960 84
embB_gene_Rv3795 PCR_product	CGCTGGTTCGGCAGCCCGAGGATCCCTTCGGCTGGTATTACAACCTGCTGGCGCTGATG CGCTGGTTCGGCAGCCCGAGGATCCCTTCGGCTGGTATTACAACCTGCTGGCGCTGATG *****	1020 144
embB_gene_Rv3795 PCR_product	ACCCATGTCAGCGACGCCAGTCTGTGGATGCGCCTGCCAGACCTGGCCGCCGGGCTAGTG ACCCATGTCAGCGACGCCAGTCTGTGGATGCGCCTGCCAGACCTGGCCGCCGGGCTAGTG *****	1080 204
embB_gene_Rv3795 PCR_product	TGCTGGCTGCTGCTGTCGCGTGAGGTGCTGCCCGCCTCGGGCCGGCGTGGAGGCCAGC TGCTGGCTGCTGCTGTCGCGTGAGGTGCTGCCCGCCTCGGGCCGGCGTGGAGGCCAGC *****	1140 264
embB_gene_Rv3795 PCR_product	AAACCCGCTACTGGGCGCGGCCATGGTCTTGCTGACCGCGTGGATGCCGTTCAACAAC AAACCCGCTACTGGGCGCGGCCATGGTCTTGCTGACCGCGTGGATGCCGTTCAACAAC *****	1200 324
embB_gene_Rv3795 PCR_product	GGCCTGCGGCCGGAGGGCATCATCGCGCTCGGCTCGTGGTCACCTATGTGCTGATCGAG GGCCTGCGGCCGGAGGGCATCATCGCGCTCGGCTCGTGGTCACCTATGTGCTGATCGAG *****	1260 384
embB_gene_Rv3795 PCR_product	CGGTCCATGCGGTACAGCCGGCTCACACCGCGGCGCTGGCCGTCGTTACCGCCGCATTC CGGTCCATGCGGTACAGCCGGCTCACACCGCGGCGCTGGCCGTCGTTACCGCCGCATTC *****	1320 444
embB_gene_Rv3795 PCR_product	ACACTGGGTGTGACGCCACCGGCTGATCGCGGTGGCCGCGCTGGTGGCCGGCGGCCGC ACACTGGGTGTGACGCCACCGGCTGATCGCGGTGGCCGCGCTGGTGGCCGGCGGCCGC *****	1380 504
embB_gene_Rv3795 PCR_product	CCGATGCTGCGGATCTTGGTGCGCCGTATCGCCTGGTCGGCACGTTGCCGTTGGTGTG CCGATGCTGCGGATCTTGGTGCGCCGTATCGCCTGGTCGGCACGTTGCCGTTGGTGTG *****	1440 564
embB_gene_Rv3795 PCR_product	CCGATGCTGGCCGCCGGCACCGTATCCTGACCGTGGTGTTCGCCGACCAGACCCTGTCA CCGATGCTGGCCGCCGGCACCGTATCCTGACCGTGGTGTTCGCCGACCAGACCCTGTCA *****	1500 624
embB_gene_Rv3795 PCR_product	ACGGTGTGGAAAGCCACCAGGGTTCGCGCCAAAATCGGGCCGAGCCAGGCGTGGTATACC ACGGTGTGGAAAGCCACCAGGGTTCGCGCCAAAATCGGGCCGAGCCAGGCGTGGTATACC *****	1560 684
embB_gene_Rv3795 PCR_product	GAGAACCTGCGTTACTACTACCTCATCCTGCCACCGTCGACGGTTCGCTGTCGCGGCCGC GAGAACCTGCGTTACTACTACCTCATCCTGCCACCGTCGACGGTTCGCTGTCGCGGCCGC *****	1620 744
embB_gene_Rv3795 PCR_product	TTCGGCTTTTGTATACCGCGCTATGCCTGTTACCGCGGTGTTTCATCATGTTGCGGCCGC TTCGGCTTTTGTATACCGCGCTATGCCTGTTACCGCGGTGTT----- *****	1680 788

6. PCR product of *pncA* gene

pncA_gene_Rv2043c PCR_product	----- CTATATCTGTGGCTGCCGCGTCGGTAGGCAAACCTGCCGGGCAGTCGCCGAACGTATGG	0 120
pncA_gene_Rv2043c PCR_product	-----ATGCGGGCGTTGATCATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTC TGGACGTATGCGGGCGTTGATCATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTC *****	53 180
pncA_gene_Rv2043c PCR_product	GCTGGCGGTAACCGGTGGCGCCGCGCTGGCCCGGCCATCAGCGACTACCTGGCCGAAGC GCTGGCGGTAACCGGTGGCGCCGCGCTGGCCCGGCCATCAGCGACTACCTGGCCGAAGC *****	113 240
pncA_gene_Rv2043c PCR_product	GGCGGACTACCATCACGTCGTGGCAACCAAGGACTTCCACATCGACCCGGGTGACCACTT GGCGGACTACCATCACGTCGTGGCAACCAAGGACTTCCACATCGACCCGGGTGACCACTT *****	173 300
pncA_gene_Rv2043c PCR_product	CTCCGGCACACCGGACTATTCTCTGTCGTGGCCACCGCATTGCGTCAGCGTACTCCCGG CTCCGGCACACCGGACTATTCTCTGTCGTGGCCACCGCATTGCGTCAGCGTACTCCCGG *****	233 360
pncA_gene_Rv2043c PCR_product	CGCGGACTTCCATCCCAGTCTGGACACGTCGGCAATCGAGGCGGTGTTCTACAAGGGTGC CGCGGACTTCCATCCCAGTCTGGACACGTCGGCAATCGAGGCGGTGTTCTACAAGGGTGC *****	293 420
pncA_gene_Rv2043c PCR_product	CTACACCGGAGCGTACAGCGGCTTCGAAGGAGTCGACGAGAACGGCAGCCACTGCTGAA CTACACCGGAGCGTACAGCGGCTTCGAAGGAGTCGACGAGAACGGCAGCCACTGCTGAA *****	353 480
pncA_gene_Rv2043c PCR_product	TTGGCTGCGGCAACGCGGCGTCGATGAGGTCGATGTGGTGGTATTGCCACCGATCATTG TTGGCTGCGGCAACGCGGCGTCGATGAGGTCGATGTGGTGGTATTGCCACCGATCATTG *****	413 540
pncA_gene_Rv2043c PCR_product	TGTGCCAGACGGCCGAGGACGCGGTACGCAATGGCTTGCCACCGGGTCTGGTGGGA TGTGCCAGACGGCCGAGGACGCGGTACGCAATGGCTTGCCACCGGGTCTGGTGGGA *****	473 600
pncA_gene_Rv2043c PCR_product	CCTGACAGCGGGTGTGTCGGCCGATACCACCGTCGCCGCGCTGGAGGAGATGCGCACCGC CCTGACAGCGGGTGTGTCGGCCGATACCACCGTCGCCGCGCTGGAGGAGATGCGCACCGC *****	533 660
pncA_gene_Rv2043c PCR_product	CAGCGTCGAGTTGGTTTGCAGCTCCTGA----- CAGCGTCGAGTTGGTTTGCAGCTCCTGATGGCACCAGCCGAACCGGGATGAACTGTTGGCG *****	561 720

7. PCR product of *rpsA* gene

rpsA_gene_Rv1630 PCR_product	-----ATGCCGAGTCCCACCGTCACCTCGCCGCAAGTAGCCGTCAACGACAT GCAACCCAACAATATGCCGAGTCCCACCGTCACCTCGCCGCAAGTAGCCGTCAACGACAT *****	47 120
rpsA_gene_Rv1630 PCR_product	AGGCTCTAGCGAGGACTTTCTCGCCGCAATAGACAAAACGATCAAGTACTTCAACGATGG AGGCTCTAGCGAGGACTTTCTCGCCGCAATAGACAAAACGATCAAGTACTTCAACGATGG *****	107 180
rpsA_gene_Rv1630 PCR_product	CGACATCGTCGAAGGCACCATCGTCAAAGTGGACCGGGACGAGGTGCTCCTCGACATCGG CGACATCGTCGAAGGCACCATCGTCAAAGTGGACCGGGACGAGGTGCTCCTCGACATCGG *****	167 240
rpsA_gene_Rv1630 PCR_product	CTACAAGACCGAAGGCGTGATCCCCGCCGCGAACTGTCCATCAAGCACGACGTCGACCC STACAAGACCGAAGGCGTGATCCCCGCCGCGAACTGTCCATCAAGCACGACGTCGACCC *****	227 300
rpsA_gene_Rv1630 PCR_product	CAACGAGGTCGTTTCCGTGCGGTGACGAGGTCGAAGCCCTGGTGTCTACCAAGGAGGACAA CAACGAGGTCGTTTCCGTGCGGTGACGAGGTCGAAGCCCTGGTGTCTACCAAGGAGGACAA *****	287 360
rpsA_gene_Rv1630 PCR_product	AGAGGGCCGGCTCATCTCTCCAAGAAACGCGCGCAGTACGAGCGTGCCTGGGGCACCAT AGAGGGCCGGCTCATCTCTCCAAGAAACGCGCGCAGTACGAGCGTGCCTGGGGCACCAT *****	347 420
rpsA_gene_Rv1630 PCR_product	CGAGGCCTCAAGGAGAAGGACGAGGCCGTCAAGGGCACGGTCATCGAGGTCGTCGAAGGG CGAGGCCTCAAGGAGAAGGACGAGGCCGTCAAGGGCACGGTCATCGAGGTCGTCGAAGGG *****	407 480
rpsA_gene_Rv1630 PCR_product	TGGCCTGATCCTCGACATCGGGCTGCGCGGTTTCTGCCCGCCTCGCTGGTGGAGATGCG TGGCCTGATCCTCGACATCGGGCTGCGCGGTTTCTGCCCGCCTCGCTGGTGGAGATGCG *****	467 540
rpsA_gene_Rv1630 PCR_product	CCGGGTGCGCGACCTGCAGCCCTACATCGGCAAGGAGATCGAGGCCAAGATCATCGAGCT CCGGGTGCGCGACCTGCAGCCCTACATCGGCAAGGAGATCGAGGCCAAGATCATCGAGCT *****	527 600
rpsA_gene_Rv1630 PCR_product	GGACAAGAACCGAACAACGTTGCTGTCCCGTCGCGCCTGGTGGAGCAGACCCAGTC GGACAAGAACCGAACAACGTTGCTGTCCCGTCGCGCCTGGTGGAGCAGACCCAGTC *****	587 660
rpsA_gene_Rv1630 PCR_product	CGAGGTGCGCAGCGAGTTCTGAATAACTTGCAAAAAGGCACCATCCGAAAGGGTGTCTGT CGAGGTGCGCAGCGAGTTCTGAATAACTTGCAAAAAGGCACCATCCGAAAGGGTGTCTGT *****	647 720
rpsA_gene_Rv1630 PCR_product	GTCTCGATCGTCAACTTCGGCGGTTCTGTGATCTCGGCGGTGGACGGTCTGGTGA GTCTCGATCGTCAACTTCGGCGGTTCTGTGATCTCGGCGGTGGACGGTCTGGTGA *****	707 780
rpsA_gene_Rv1630 PCR_product	TGTCTCCGAGCTATCGTGAAGCACATCGACCACCCGTCGAGGTGGTCCAGGTTGGTGA TGTCTCCGAGCTATCGTGAAGCACATCGACCACCCGTCGAGGTGGTCCAGGTTGGTGA *****	767 840
rpsA_gene_Rv1630 PCR_product	CGAGGTCACCGTCGAGGTGCTCGACGTGACATGGACCGTGAGCGGGTTTCGTTGTCCT CGAGGTCACCGTCGAGGTGCTCGACGTGACATGGACCGTGAGCGGGTTTCGTTGTCCT *****	827 900
rpsA_gene_Rv1630 PCR_product	CAAGGCGACTCAGGAAGACCCGTGGCGGCACTTCGCCCGCACTCACGCGATCGGGCAGAT CAAGGCGACTCAGGAAGACCCGTGGCGGCACTTCGCCCGCACTCACGCGATCGGGCAGAT *****	887 960
rpsA_gene_Rv1630 PCR_product	CGTGCCGGGCAAGGTCACCAAGTTGGTTCCGTTCCGTTGCGATTGTCGCGCTCGAGGAGGG CGTGCCGGGCAAGGTCACCAAGTTGGTTCCGTTCCGTTGCGATTGTCGCGCTCGAGGAGGG *****	947 1020
rpsA_gene_Rv1630 PCR_product	TATCGAGGGCCTGGTGCACATCTCCGAGCTGGCCGAGCGTCACGTCGAGGTGCCGATCA TATCGAGGGCCTGGTGCACATCTCCGAGCTGGCCGAGCGTCACGTCGAGGTGCCGATCA *****	1007 1080

rpsA_gene_Rv1630 PCR_product	TATCGAGGGCCTGGTGCACATCTCCGAGCTGGCCGAGCGTCACGTCGAGGTGCCCGATCA TATCGAGGGCCTGGTGCACATCTCCGAGCTGGCCGAGCGTCACGTCGAGGTGCCCGATCA *****	1007 1080
rpsA_gene_Rv1630 PCR_product	GGTGGTTGCCGTCGGCGACGACGCGATGGTCAAGGTCATCGACATCGACCTGGAGCGCCG GGTGGTTGCCGTCGGCGACGACGCGATGGTCAAGGTCATCGACATCGACCTGGAGCGCCG *****	1067 1140
rpsA_gene_Rv1630 PCR_product	TCGGATCTCGTTGTCGCTCAAGCAAGCCAATGAGGACTACACCGAGGAGTTCGACCCGGC TCGGATCTCGTTGTCGCTCAAGCAAGCCAATGAGGACTACACCGAGGAGTTCGACCCGGC *****	1127 1200
rpsA_gene_Rv1630 PCR_product	GAAGTACGGCATGGCCGACAGTTACGACGAGCAGGGCAACTACATCTTCCCGAGGGCTT GAAGTACGGCATGGCCGACAGTTACGACGAGCAGGGCAACTACATCTTCCCGAGGGCTT *****	1187 1260
rpsA_gene_Rv1630 PCR_product	CGATGCCGAAACCAACGAATGGCTTGAGGGATTGAAAAGCAGCGCCGAATGGGAAGC CGATGCCGAAACCAACGAATGGCTTGAGGGATTGAAAAGCAGCGCCGAATGGGAAGC *****	1247 1320

8. PCR product of *rrs* gene

<i>rrs_gene_Rvnr01</i>	-----TTTTGTTGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCG	44
PCR_PRODUCT	AATTTGAAGGTTTGGGTTTTGTTGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCG *****	60
<i>rrs_gene_Rvnr01</i>	GCGTGCTTAACACATGCAAGTCGAACGGAAAGGTCTCTTCGGAGATACTCGAGTGGCGAA	104
PCR_PRODUCT	GCGTGCTTAACACATGCAAGTCGAACGGAAAGGTCTCTTCGGAGATACTCGAGTGGCGAA *****	120
<i>rrs_gene_Rvnr01</i>	CGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGT	164
PCR_PRODUCT	CGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGT *****	180
<i>rrs_gene_Rvnr01</i>	CTAATACCGGATAGGACCACGGGATGCATGTCTTGTGGTGGAAAGCGCTTTAGCGGTGTG	224
PCR_PRODUCT	CTAATACCGGATAGGACCACGGGATGCATGTCTTGTGGTGGAAAGCGCTTTAGCGGTGTG *****	240
<i>rrs_gene_Rvnr01</i>	GGATGAGCCCGCGGCCTATCAGCTTGTGGTGGGGTGACGGCCTACCAAGGCGACGACGG	284
PCR_PRODUCT	GGATGAGCCCGCGGCCTATCAGCTTGTGGTGGGGTGACGGCCTACCAAGGCGACGACGG *****	300
<i>rrs_gene_Rvnr01</i>	GTAGCCGGCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCAGACTCCTAC	344
PCR_PRODUCT	GTAGCCGGCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCAGACTCCTAC *****	360
<i>rrs_gene_Rvnr01</i>	GGGAGGCAGCAGTGGGAATATTGCAACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGT	404
PCR_PRODUCT	GGGAGGCAGCAGTGGGAATATTGCAACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGT *****	420
<i>rrs_gene_Rvnr01</i>	GGGGGATGACGGCCTTCGGGTTGTAACCTCTTTCACCATCGACGAAGGTCCGGGTTCTC	464
PCR_PRODUCT	GGGGGATGACGGCCTTCGGGTTGTAACCTCTTTCACCATCGACGAAGGTCCGGGTTCTC *****	480
<i>rrs_gene_Rvnr01</i>	TCGGATTGACGGTAGGTGGAGAAGAAGCACCGCCAACACTACGTGCCAGCAGCCGCGGTAA	524
PCR_PRODUCT	TCGGATTGACGGTAGGTGGAGAAGAAGCACCGCCAACACTACGTGCCAGCAGCCGCGGTAA *****	540
<i>rrs_gene_Rvnr01</i>	TACGTAGGGTGCAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGT	584
PCR_PRODUCT	TACGTAGGGTGCAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGT *****	600
<i>rrs_gene_Rvnr01</i>	CGCGTTGTTCTGTAATCTCACGGCTTAACCTGTGAGCGTGCGGGGATACGGGCAGACTA	644
PCR_PRODUCT	CGCGTTGTTCTGTAATCTCACGGCTTAACCTGTGAGCGTGCGGGGATACGGGCAGACTA *****	660
<i>rrs_gene_Rvnr01</i>	GAGTACTGCAGGGGAGACTGGAATCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAG	704
PCR_PRODUCT	GAGTACTGCAGGGGAGACTGGAATCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAG *****	720
<i>rrs_gene_Rvnr01</i>	GAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTG	764
PCR_PRODUCT	GAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTG *****	780
<i>rrs_gene_Rvnr01</i>	GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCGTAAACGGTGGGTAAGGTGT	824
PCR_PRODUCT	GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCGTAAACGGTGGGTAAGGTGT *****	840
<i>rrs_gene_Rvnr01</i>	GGGTTTCTTCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCTGGGGAGT	884
PCR_PRODUCT	GGGTTTCTTCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCTGGGGAGT *****	900
<i>rrs_gene_Rvnr01</i>	ACGGCCGCAAGGCTAAAACCTCAAAGGAATTGACGGGGCCCGCACAAAGCGGGGAGCATG	944
PCR_PRODUCT	ACGGCCGCAAGGCTAAAACCTCAAAGGAATTGACGGGGCCCGCACAAAGCGGGGAGCATG *****	960
<i>rrs_gene_Rvnr01</i>	TGGATTAATTCGATGCAACGCGAAGAACCCTTACCTGGGTTTGACATGCACAGGACGCGTC	1004
PCR_PRODUCT	TGGATTAATTCGATGCAACGCGAAGAACCCTTACCTGGGTTTGACATGCACAGGACGCGTC *****	1020

rrs_gene_Rvnr01 PCR_PRODUCT	TAGAGATAGGCGTTCCTTGTGCCCTGTGTGCAGGTGGTGCATGGCTGTCGTGAGCTCGT TAGAGATAGGCGTTCCTTGTGCCCTGTGTGCAGGTGGTGCATGGCTGTCGTGAGCTCGT *****	1064 1080
rrs_gene_Rvnr01 PCR_PRODUCT	GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTCATGTTGCCAGCA GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTCATGTTGCCAGCA *****	1124 1140
rrs_gene_Rvnr01 PCR_PRODUCT	CGTAATGGTGGGACTCGTGAGAGACTGCCGGGTCAACTCGGAGGAAGGTGGGGATGAC CGTAATGGTGGGACTCGTGAGAGACTGCCGGGTCAACTCGGAGGAAGGTGGGGATGAC *****	1184 1200
rrs_gene_Rvnr01 PCR_PRODUCT	GTCAAGTCATCATGCCCTTATGTCCAGGGCTTACACATGCTACAATGGCCGGTACAAA GTCAAGTCATCATGCCCTTATGTCCAGGGCTTACACATGCTACAATGGCCGGTACAAA *****	1244 1260
rrs_gene_Rvnr01 PCR_PRODUCT	GGGCTGCGATGCCGCGAGGTTAAGCGAATCCTTAAAAGCCGGTCTCAGTTCGGATCGGGG GGGCTGCGATGCCGCGAGGTTAAGCGAATCCTTAAAAGCCGGTCTCAGTTCGGATCGGGG *****	1304 1320
rrs_gene_Rvnr01 PCR_PRODUCT	TCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGG TCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGG *****	1364 1380
rrs_gene_Rvnr01 PCR_PRODUCT	TGAATACGTTCCCGGCCTTGTACACACCCGCCGTACGTCATGAAAGTCGGTAACACCC TGAATACGTTCCCGGCCTTGTACACACCCGCCGTACGTCATGAAAGTCGGTAACACCC *****	1424 1440
rrs_gene_Rvnr01 PCR_PRODUCT	GAAGCCAGTGGCCTAACCCCTCGGGAGGGAGCTGTGCAAGGTGGGATCGGCGATTGGGACG GAAGCCAGTGGCCTAACCCCTCGGGAGGGAGCTGTGCAAGGTGGGATCGGCGATTGGGACG *****	1484 1500
rrs_gene_Rvnr01 PCR_PRODUCT	AAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTTTCT----- AAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAGC *****	1537 1560

9. PCR product of *eis* gene

<i>eis</i> _gene_Rv2416c	-----GTGACTGTGACCTGTGTAGCCCGACCGAGG	31
PCR_product	GGCCGCGGCATATGCCACAGTCGGATTCTGTGACTGTGACCTGTGTAGCCCGACCGAGG	60

<i>eis</i> _gene_Rv2416c	ACGACTGGCCGGGATGTTCTACTGGCCGCGCCAGTTTCACCGATTCATCGGCCCTG	91
PCR_product	ACGACTGGCCGGGATGTTCTACTGGCCGCGCCAGTTTCACCGATTCATCGGCCCTG	120

<i>eis</i> _gene_Rv2416c	AATCAGCGACCGCTGGCGGACCTGGTGCCACCGACGGAGCGGTGGTCCGCGATG	151
PCR_product	AATCAGCGACCGCTGGCGGACCTGGTGCCACCGACGGAGCGGTGGTCCGCGATG	180

<i>eis</i> _gene_Rv2416c	GTGCCGGCCGGTTCTGAGGTGGTGGGATGGCGCTGTACATGGATCGCGTTGACGG	211
PCR_product	GTGCCGGCCGGTTCTGAGGTGGTGGGATGGCGCTGTACATGGATCGCGTTGACGG	240

<i>eis</i> _gene_Rv2416c	TGCCTGGTGAAGTGGTGTCTCCCGACCGCGGTCTCAGTTTCGTCGCGGTGGCGCCGACGC	271
PCR_product	TGCCTGGTGAAGTGGTGTCTCCCGACCGCGGTCTCAGTTTCGTCGCGGTGGCGCCGACGC	300

<i>eis</i> _gene_Rv2416c	ATCGCCGGCGCGGCTTGCTGCGCGCATGTGCGCCGAAGTGCACCGCCCATAGCCGATT	331
PCR_product	ATCGCCGGCGCGGCTTGCTGCGCGCATGTGCGCCGAAGTGCACCGCCCATAGCCGATT	360

<i>eis</i> _gene_Rv2416c	CCGCTATCCGGTCGCGGCACTGCATGCTAGCGAGGGCGGCATCTACGCCGGTTCCGGCT	391
PCR_product	CCGCTATCCGGTCGCGGCACTGCATGCTAGCGAGGGCGGCATCTACGCCGGTTCCGGCT	420

<i>eis</i> _gene_Rv2416c	ACGGGCCCGCTACCACCTTGATGAGCTGACGGTCGACCGACGCTTCGCGCGCTTTCACG	451
PCR_product	ACGGGCCCGCTACCACCTTGATGAGCTGACGGTCGACCGACGCTTCGCGCGCTTTCACG	480

<i>eis</i> _gene_Rv2416c	CCGACGCACCGGGCGCGGCTAGGTGGCAGCAGCGTCCGGTTGGTCAGACCCACCGAGC	511
PCR_product	CCGACGCACCGGGCGCGGCTAGGTGGCAGCAGCGTCCGGTTGGTCAGACCCACCGAGC	540

<i>eis</i> _gene_Rv2416c	ATCGCGCGAGTTTGAGGCGATCTACGAGCGATGGCGCCAGCAGGTGCCGGGCGGGCTGC	571
PCR_product	ATCGCGCGAGTTTGAGGCGATCTACGAGCGATGGCGCCAGCAGGTGCCGGGCGGGCTGC	600

<i>eis</i> _gene_Rv2416c	TACGCCCGCAGGTGCTCTGGGACGAGTCTGGCAGAATGCAAAGCCGCGCCCGTGGAG	631
PCR_product	TACGCCCGCAGGTGCTCTGGGACGAGTCTGGCAGAATGCAAAGCCGCGCCCGTGGAG	660

<i>eis</i> _gene_Rv2416c	ACCGTGAATCGTTCGCGTACTGCATCCCGACGGGTACGCGCTGTACCGGTGGATCGCA	691
PCR_product	ACCGTGAATCGTTCGCGTACTGCATCCCGACGGGTACGCGCTGTACCGGTGGATCGCA	720

<i>eis</i> _gene_Rv2416c	CCGATCTCAAGCTAGCGCGCTCAGCGAACTCAGGGCGGTAAACCGCAGATGCGCATTGTG	751
PCR_product	CCGATCTCAAGCTAGCGCGCTCAGCGAACTCAGGGCGGTAAACCGCAGATGCGCATTGTG	780

<i>eis</i> _gene_Rv2416c	CGTTGTGGCGGCCCTGATTGCGCTCGACTCCATGGAGCGAATCAGCATCATACCCATC	811
PCR_product	CGTTGTGGCGGCCCTGATTGCGCTCGACTCCATGGAGCGAATCAGCATCATACCCATC	840

<i>eis</i> _gene_Rv2416c	CACAGGACCCGTTACCCACCTGCTCACCGATACCCGACTGGCCCGCACTACCTGGCGCC	871
PCR_product	CACAGGACCCGTTACCCACCTGCTCACCGATACCCGACTGGCCCGCACTACCTGGCGCC	900

<i>eis</i> _gene_Rv2416c	AGGACGGCCTGTGGTTGCGCATCATGAACGTACCGCCGCACTCGAGGCGCGTGGTTACG	931
PCR_product	AGGACGGCCTGTGGTTGCGCATCATGAACGTACCGCCGCACTCGAGGCGCGTGGTTACG	960

<i>eis</i> _gene_Rv2416c	CTCACGAAGTTGGCGAGTTTCCACGGTCTCGAGGTATCCGATGGCGGCCGGTTCCGCGC	991
PCR_product	CTCACGAAGTTGGCGAGTTTCCACGGTCTCGAGGTATCCGATGGCGGCCGGTTCCGCGC	1020

1.10 PCR product of *gyrA* gene

<i>gyrA</i> _gene_Rv0006	-----ATGACAGACACGACGTTGCCGCTGACG	28
PCR_product	TTGGGGACCGGCGTCGATTGCAACGAGGATAGATGACAGACACGACGTTGCCGCTGACG *****	60
<i>gyrA</i> _gene_Rv0006	ACTCGCTCGACCGGATCGAACCAGTTGACATCGAGCAGGAGATGACAGCGCAGCTACATCG	88
PCR_product	ACTCGCTCGACCGGATCGAACCAGTTGACATCGAGCAGGAGATGACAGCGCAGCTACATCG *****	120
<i>gyrA</i> _gene_Rv0006	ACTATGCGATGAGCGTGATCGTGGCCGCGCTGCCGAGGTGCGCGACGGGCTCAAGC	148
PCR_product	ACTATGCGATGAGCGTGATCGTGGCCGCGCTGCCGAGGTGCGCGACGGGCTCAAGC *****	180
<i>gyrA</i> _gene_Rv0006	CCGTGCATCGCCGGTGCTCTATGCAATGTTTCGATCCGGCTTCCGCCCGACCGCAGCC	208
PCR_product	CCGTGCATCGCCGGTGCTCTATGCAATGTTTCGATCCGGCTTCCGCCCGACCGCAGCC *****	240
<i>gyrA</i> _gene_Rv0006	ACGCCAAGTCGGCCCGTTCGTTGCCGAGACCATGGGCAACTACCACCCGCACGGCGACG	268
PCR_product	ACGCCAAGTCGGCCCGTTCGTTGCCGAGACCATGGGCAACTACCACCCGCACGGCGACG *****	300
<i>gyrA</i> _gene_Rv0006	CGTCGATCTACGACAGCCTGGTGCATGGCCAGCCCTGGTCGCTGCGCTACCCGCTGG	328
PCR_product	CGTCGATCTACGACAGCCTGGTGCATGGCCAGCCCTGGTCGCTGCGCTACCCGCTGG *****	360
<i>gyrA</i> _gene_Rv0006	TGGACGGCCAGGGCAACTTCGGTCGCCAGGCAATGACCCACCGCGCGATGAGGTACA	388
PCR_product	TGGACGGCCAGGGCAACTTCGGTCGCCAGGCAATGACCCACCGCGCGATGAGGTACA *****	420
<i>gyrA</i> _gene_Rv0006	CCGAAGCCCGGCTGACCCCGTTGGCGATGGAGATGCTGAGGGAAATCGACGAGGAGACAG	448
PCR_product	CCGAAGCCCGGCTGACCCCGTTGGCGATGGAGATGCTGAGGGAAATCGACGAGGAGACAG *****	480
<i>gyrA</i> _gene_Rv0006	TCGATTCATCCCTAACTACGACGGCCGGTGCAAGAGCCGACGGTGTACCCAGCCGGT	508
PCR_product	TCGATTCATCCCTAACTACGACGGCCGGTGCAAGAGCCGACGGTGTACCCAGCCGGT *****	540
<i>gyrA</i> _gene_Rv0006	TCCCCAACCTGCTGGCCAACGGTCAAGGCGCATCGCGGTCGGCATGGCAACCAATATCC	568
PCR_product	TCCCCAACCTGCTGGCCAACGGTCAAGGCGCATCGCGGTCGGCATGGCAACCAATATCC *****	600
<i>gyrA</i> _gene_Rv0006	CGCCGCACAACCTGCGTGAGCTGGCCGACGCGGTGTTCTGGGCGCTGGAGAATCACGACG	628
PCR_product	CGCCGCACAACCTGCGTGAGCTGGCCGACGCGGTGTTCTGGGCGCTGGAGAATCACGACG *****	660
<i>gyrA</i> _gene_Rv0006	CCGACGAAGAGGAGACCCTGGCCGCGGTATGGGGCGGGTTAAAGGCCCGGACTTCCCGA	688
PCR_product	CCGACGAAGAGGAGACCCTGGCCGCGGTATGGGGCGGGTTAAAGGCCCGGACTTCCCGA *****	720
<i>gyrA</i> _gene_Rv0006	CCGCCGGACTGATCGTCGGATCCAGGGCACCGCTGATGCCTACAAAACCTGGCCGCGGCT	748
PCR_product	CCGCCGGACTGATCGTCGGATCCAGGGCACCGCTGATGCCTACAAAACCTGGCCGCGGCT *****	780
<i>gyrA</i> _gene_Rv0006	CCATTGGAATGCGCGGAGTTGTTGAGGTAGAAGAGGATCCCGCGGTCGTACCTCGCTGG	808
PCR_product	CCATTGGAATGCGCGGAGTTGTTGAGGTAGAAGAGGATCCCGCGGTCGTACCTCGCTGG *****	840
<i>gyrA</i> _gene_Rv0006	TGATCACCGAGTTGCCGATCAGGTCACCACGACAACCTCATCACTTCGATCGCCGAAC	868
PCR_product	TGATCACCGAGTTGCCGATCAGGTCACCACGACAACCTCATCACTTCGATCGCCGAAC *****	900
<i>gyrA</i> _gene_Rv0006	AGGTCCGAGACGGCAAGCTGGCCGGCATTTCGAACATTGAGGACCAGTCTAGCGATCGGG	928
PCR_product	AGGTCCGAGACGGCAAGCTGGCCGGCATTTCGAACATTGAGGACCAGTCTAGCGATCGGG *****	960
<i>gyrA</i> _gene_Rv0006	TCGGTTTACGCATCGTCATCGAGATCAAGCGCGATGCGGTGGCCAAGTGGTGATCAATA	988
PCR_product	TCGGTTTACGCATCGTCATCGAGATCAAGCGCGATGCGGTGGCCAAGTGGTGATCAATA *****	1020

gyrA_gene_Rv0006 PCR_product	ACCTTTACAAGCACACCCAGCTGCAGACCAGCTTTGGCGCCAACATGCTAGCGATCGTCTG ACCTTTACAAGCACACCCAGCTGCAGACCAGCTTTGGCGCCAACATGCTAGCGATCGTCTG *****	1048 1080
gyrA_gene_Rv0006 PCR_product	ACGGGGTGCCGCGCACGCTGCGGCTGGACCAGCTGATCCGCTATTACGTTGACCACCAAC ACGGGGTGCCGCGCACGCTGCGGCTGGACCAGCTGATCCGCTATTACGTTGACCACCAAC *****	1108 1140
gyrA_gene_Rv0006 PCR_product	TCGACGTATTGTGCGGCGCACACCTACCGGCTGCGCAAGGCAACAGAGCGAGCCACA TCGACGTATTGTGCGGCGCACACCTACCGGCTGCGCAAGGCAACAGAGCGAGCCACA *****	1168 1200
gyrA_gene_Rv0006 PCR_product	TTCTGCGCGGCTGGTTAAAGCGCTCGACGCGCTGGACGAGGTATTGCACTGATCCGGG TTCTGCGCGGCTGGTTAAAGCGCTCGACGCGCTGGACGAGGTATTGCACTGATCCGGG *****	1228 1260
gyrA_gene_Rv0006 PCR_product	CGTCGGAGACCGTCGATATCGCCGGGCGGACTGA - - TCGAGCTGCTCGACATCGACGA CGTCGGAGACCGTTCATATCGCCGGGCGGACTGAATTCGAGCTGCTCGACATTGACGA *****	1286 1320
gyrA_gene_Rv0006 PCR_product	GATCCAGGCCAGGCAATCCTGGACATGCAAGTTGCGGCGCTGGCCGCACTGGAACGCCA GATCCAGGCCAGGCAATCCTGGACATGCAAGTTGCGGCGCTGGCCGCACTGGAACGCCA *****	1346 1380
gyrA_gene_Rv0006 PCR_product	-GCGCATCATCGACGACCTGGCCAAAATCGAGGCCGAGATCGCCGATCTGGAAGACATCC AGGCCATCATCGACGACCTGGCCAAAATCGAGGCCGAGATCGCCGATCTGGAAGACATCC * *****	1405 1440
gyrA_gene_Rv0006 PCR_product	TGGCAAACCCGAGCGGCAGCGTGGGATCGTGCGGACGAACTCGCCGAAATCGTGGACA TGGCAAACCCGAGCGGCAGCGTGGGATCGTGCGGACGAACTCGCCGAAATCGTGGACA *****	1465 1500
gyrA_gene_Rv0006 PCR_product	GGCACGGCGACGACCGGCTACCGGATCATCGCGCCGACGGAGAGCTCAGCGACGAGG GGCACGGCGACGACCGGCTACCGGATCATCGCGCCGACGGAGAGCTCAGCGACGAGG *****	1525 1560
gyrA_gene_Rv0006 PCR_product	ATTTGATCGCCCGAGGACGCTGTTGTCACTATCACCGAAACGGGATACGCCAAGCGCA ATTTGATCGCCCGAGGACGCTGTTGTCACTATCACCGAAACGGGATACGCCAAGCGCA *****	1585 1620
gyrA_gene_Rv0006 PCR_product	CCAAGACCGATCTGTATCGCAGCCAGAAACGCGCGGCAAGGGCGTGCAGGGTGCAGGGT CCAAGACCGATCTGTATCGCAGCCAGAAACGCGCGGCAAGGGCGTGCAGGGTGCAGGGT *****	1645 1680
gyrA_gene_Rv0006 PCR_product	TGAAGCAGGACGACATCGTCGCGCACTTCTTGTGTGCTCCACCACGATTTGATCCTGT TGAAGCAGGACGACATCGTCGCGCACTTCTTGTGTGCTCCACCACGATTTGATCCTGT *****	1705 1740
gyrA_gene_Rv0006 PCR_product	TCTTACCACCCAGGGACGGGTTTATCGGGCCAAGGCCTACGACTTGCCCGAGGCTCCC TCTTACCACCCAGGGACGGGTTTATCGGGCCAAGGCCTACGACTTGCCCGAGGCTCCC *****	1765 1800
gyrA_gene_Rv0006 PCR_product	GGACGGCGCGGGCAGCACGTGGCCAACCTGTTAGCCTTCCAGCCGAGGAACGCATCG GGACGGCGCGGGCAGCACGTGGCCAACCTGTTAGCCTTCCAGCCGAGGAACGCATCG *****	1825 1860
gyrA_gene_Rv0006 PCR_product	CCCAGGTCATCCAGATTCGCGGCTACACCGACGCCCGTACCTGGTGTGCGCACTCGCA CCCAGGTCATCCAGATTCGCGGCTACACCGACGCCCGTACCTGGTGTGCGCACTCGCA *****	1885 1920
gyrA_gene_Rv0006 PCR_product	ACGGGCTGGTGAAAAAGTCCAAGCTGACCGACTTCGACTCCAATCGCTCGGGCGGAATCG ACGGGCTGGTGAAAAAGTCCAAGCTGACCGACTTCGACTCCAATCGCTCGGGCGGAATCG *****	1945 1980
gyrA_gene_Rv0006 PCR_product	TGGCGGTCAACCTGCGCGACAACGACGAGCTGGTGGTGGTGTGTGTTGCGCCGGCG TGGCGGTCAACCTGCGCGACAACGACGAGCTGGTGGTGGTGTGTGTTGCGCCGGCG *****	2005 2040
gyrA_gene_Rv0006 PCR_product	ACGACCTGCTGTGTTCTCGGCCAACGGGACGTCATCAGGTTCTCGGCGACCGACGAGG ACGACCTGCTGTGTTCTCGGCCAACGGGACGTCATCAGGTTCTCGGCGACCGACGAGG *****	2065 2100

gyrA_gene_Rv0006	CGCTGCGGCCAATGGGTCTGTGCCACCTCGGGTGTGCAGGGCATGCGGTTCAATATCGACG	2125
PCR_product	CGCTGCGGCCAATGGGTCTGTGCCACCTCGGGTGTGCAGGGCATGCGGTTCAATATCGACG	2160

gyrA_gene_Rv0006	ACCGGCTGCTGTGCTGAACGTCGTGCGTGAAGGCACCTATCTGCTGGTGGCGACGTCAG	2185
PCR_product	ACCGGCTGCTGTGCTGAACGTCGTGCGTGAAGGCACCTATCTGCTGGTGGCGACGTCAG	2220

gyrA_gene_Rv0006	GGGGCTATGCGAAACGTACCGCGATCGAGGAATACCCGGTACAGGGCCGCGCGGTAAAG	2245
PCR_product	GGGGCTATGCGAAACGTACCGCGATCGAGGAATACCCGGTACAGGGCCGCGCGGTAAAG	2280

gyrA_gene_Rv0006	GTGTGCTGACGGTCATGTACGACCGCCGGCGCGGCAGGTTGGTTGGGGCGTTGATTGTCG	2305
PCR_product	GTGTGCTGACGGTCATGTACGACCGCCGGCGCGGCAGGTTGGTTGGGGCGTTGATTGTCG	2340

gyrA_gene_Rv0006	ACGACGACAGCGAGCTGTATGCCGTCACTTCCGGCGGTGGCGTGATCCGCACCGCGGCAC	2365
PCR_product	ACGACGACAGCGAGCTGTATGCCGTCACTTCCGGCGGTGGCGTGATCCGCACCGCGGCAC	2400

gyrA_gene_Rv0006	GCCAGGTTTCGCAAGGCGGGACGGCAGACCAAGGGTGTTCGGTTGATGAATCTGGGCGAGG	2425
PCR_product	GCCAGGTTTCGCAAGGCGGGACGGCAGACCAAGGGTGTTCGGTTGATGAATCTGGGCGAGG	2460

gyrA_gene_Rv0006	GCGACACACTGTTGGCCATCGCGCGCAACGCCGAAGAAAGTGCGACGATAATGCCGTGG	2485
PCR_product	GCGACACACTGTTGGCCATCGCGCGCAACGCCGAAGAAAGTGCGACGATAATGCCGTGG	2520

gyrA_gene_Rv0006	ACGCCAACGGCGCAGACCAGACGGGCAATTAA-----	2517
PCR_product	ACGCCAACGGCGCAGACCAGACGGGCAATTAAATCAGGCTCGCCCGACGACGATGCGGATC	2580

11. PCR product of *gyrB* gene

<i>gyrB</i> _gene_Rv0005	-----GTGGCTGCCAGAAAAAGAAGG	22
PCR_product	ACCGCATGCACGCAACCCCTGAGGAGAGTATTCGGATCGTGGCTGCCAGAAAAAGAAGG *****	120
<i>gyrB</i> _gene_Rv0005	CCCAAGACGAATACGGCGCTGCGTCTATCACCATTCTCGAAGGGCTGGAGGCCGTCCGCA	82
PCR_product	CCCAAGACGAATACGGCGCTGCGTCTATCACCATTCTCGAAGGGCTGGAGGCCGTCCGCA *****	180
<i>gyrB</i> _gene_Rv0005	AACGTCCCGGCATGTACATTGGCTCGACCGGTGAGCGCGTTTACACCATCTCATTGGG	142
PCR_product	AACGTCCCGGCATGTACATTGGCTCGACCGGTGAGCGCGTTTACACCATCTCATTGGG *****	240
<i>gyrB</i> _gene_Rv0005	AGGTGGTCGACAACGCGGTGACGAGGGCGATGGCCGTTATGCAACCACAGTGAACGTAG	202
PCR_product	AGGTGGTCGACAACGCGGTGACGAGGGCGATGGCCGTTATGCAACCACAGTGAACGTAG *****	300
<i>gyrB</i> _gene_Rv0005	TGCTGCTTGAGGATGGCGGTGTCGAGGTCGCCGACGACGGCCGCGGCATTCCGGTCGCCA	262
PCR_product	TGCTGCTTGAGGATGGCGGTGTCGAGGTCGCCGACGACGGCCGCGGCATTCCGGTCGCCA *****	360
<i>gyrB</i> _gene_Rv0005	CCCACGCCTCCGGCATAACCGACCGTGCAGTGGTGATGACACAACACTACATGCCGGCGGCA	322
PCR_product	CCCACGCCTCCGGCATAACCGACCGTGCAGTGGTGATGACACAACACTACATGCCGGCGGCA *****	420
<i>gyrB</i> _gene_Rv0005	AGTTCGACTCGGACGCGTATGCGATATCTGGTGGTCTGCACGGCGTCGGCGTGTGCGTGG	382
PCR_product	AGTTCGACTCGGACGCGTATGCGATATCTGGTGGTCTGCACGGCGTCGGCGTGTGCGTGG *****	480
<i>gyrB</i> _gene_Rv0005	TTAACGCGCTATCCACCCGGCTCGAAGTCGAGATCAAGCGCGACGGGTACGAGTGGTCTC	442
PCR_product	TTAACGCGCTATCCACCCGGCTCGAAGTCGAGATCAAGCGCGACGGGTACGAGTGGTCTC *****	540
<i>gyrB</i> _gene_Rv0005	AGGTTTATGAGAAGTCGGAACCCCTGGGCCCTCAAGCAAGGGGCGCCGACCAAGAAGACGG	502
PCR_product	AGGTTTATGAGAAGTCGGAACCCCTGGGCCCTCAAGCAAGGGGCGCCGACCAAGAAGACGG *****	600
<i>gyrB</i> _gene_Rv0005	GGTCAACGGTGCGGTTCTGGGCCGACCCGCTGTTTTCGAAACCACGGAATACGACTTCG	562
PCR_product	GGTCAACGGTGCGGTTCTGGGCCGACCCGCTGTTTTCGAAACCACGGAATACGACTTCG *****	660
<i>gyrB</i> _gene_Rv0005	AAACCGTCGCCC GCCGGCTGCAAGAGATGGCGTTCTCAACAAGGGGCTGACCATCAACC	622
PCR_product	AAACCGTCGCCC GCCGGCTGCAAGAGATGGCGTTCTCAACAAGGGGCTGACCATCAACC *****	720
<i>gyrB</i> _gene_Rv0005	TGACCGACGAGAGGGTGACCCAAGACGAGGTCGTGACGAAGTGGTCAGCGACGTCGCCG	682
PCR_product	TGACCGACGAGAGGGTGACCCAAGACGAGGTCGTGACGAAGTGGTCAGCGACGTCGCCG *****	780
<i>gyrB</i> _gene_Rv0005	AGGCGCCGAAGTCGGCAAGTGAACGCGCAGCCGAATCCACTGCACCGCACAAAGTTAAGA	742
PCR_product	AGGCGCCGAAGTCGGCAAGTGAACGCGCAGCCGAATCCACTGCACCGCACAAAGTTAAGA *****	840
<i>gyrB</i> _gene_Rv0005	GCCGCACCTTTCACTATCCGGGTGGCCTGGTGGACTTCGTGAAACACATCAACCGCACCA	802
PCR_product	GCCGCACCTTTCACTATCCGGGTGGCCTGGTGGACTTCGTGAAACACATCAACCGCACCA *****	900
<i>gyrB</i> _gene_Rv0005	AGAACGCGATTATAGCAGCATCGTGGACTTTCCGGCAAGGGCACCGGGCACGAGGTGG	862
PCR_product	AGAACGCGATTATAGCAGCATCGTGGACTTTCCGGCAAGGGCACCGGGCACGAGGTGG *****	960
<i>gyrB</i> _gene_Rv0005	AGATCGGATGCAATGGAACGCCGGTATTCGGAGTCGGTGCACACCTTCGCCAACACCA	922
PCR_product	AGATCGGATGCAATGGAACGCCGGTATTCGGAGTCGGTGCACACCTTCGCCAACACCA *****	1020
<i>gyrB</i> _gene_Rv0005	TCAACACCCACGAGGGCGGCACCCACGAAG-AGGGCTCCGCGAGCGGCTGACGTCGGTG	981
PCR_product	TCAACACCCACGAGGGCGGCACCCACGAAG-AGGGCTCCGCGAGCGGCTGACGTCGGTG *****	1080

12. PCR product of *tlyA* gene

tlyA_gene_Rv1694	-----GTGGCACGACGTGCCCGCCTTGACGCCGA	29
PCR_product	TGGAGTTCGGCGGAGAAGGGTTGAGTGCGGGTGGCACGACGTGCCCGCCTTGACGCCGA *****	60
tlyA_gene_Rv1694	GCTAGTCCGGCGGGGCTGGCGCGATCACGTCAACAGG-CCGCGGAGTTGATCGGCGCCG	88
PCR_product	GCTAGTCCGGCGGGGCTGGCGCGATCACGTCAACAGG-CCGCGGAGTTGATCGGCGCCG *****	119
tlyA_gene_Rv1694	GCAAGGTGCGCATCGACGGGCTGCCGGCGGTCAAGCCGGCCACCGCCGTGTCCGACACCA	148
PCR_product	GCAAGGTGCGCATCGACGGGCTGCCGGCGGTCAAGCCGGCCACCGCCGTGTCCGACACCA *****	179
tlyA_gene_Rv1694	CCGCGCTGACCGTGGTGACCGACAGTGAACGCGCCTGGGTATCGCGCGGAGCGCACAAAC	208
PCR_product	CCGCGCTGACCGTGGTGACCGACAGTGAACGCGCCTGGGTATCGCGCGGAGCGCACAAAC *****	239
tlyA_gene_Rv1694	TAGTCGGTGCCTGGAGGCGTTCGCGATCGCGGTGGCGGGCCGGCGCTGTCTGGACGCGG	268
PCR_product	TAGTCGGTGCCTGGAGGCGTTCGCGATCGCGGTGGCGGGCCGGCGCTGTCTGGACGCGG *****	299
tlyA_gene_Rv1694	GCGCATCGACCGGTGGGTTCAACGAAGTACTGCTGGACCGTGGTGCCGCCACGTGGTGG	328
PCR_product	GCGCATCGACCGGTGGGTTCAACGAAGTACTGCTGGACCGTGGTGCCGCCACGTGGTGG *****	359
tlyA_gene_Rv1694	CCGCCGATGTCGGATACGGCCAGCTGGCGTGGTTCGTCGCAACGATCCTCGGGTGGTGG	388
PCR_product	CCGCCGATGTCGGATACGGCCAGCTGGCGTGGTTCGTCGCAACGATCCTCGGGTGGTGG *****	419
tlyA_gene_Rv1694	TCCTCGAGCGGACCAACGCACGTGGCCTCACACCGGAGGCGATCGGCGGTGCGTTCGACC	448
PCR_product	TCCTCGAGCGGACCAACGCACGTGGCCTCACACCGGAGGCGATCGGCGGTGCGTTCGACC *****	479
tlyA_gene_Rv1694	TGGTAGTGGCCGACCTGTCGTTATCTCGTTGGCTACCGTGTGCCCGCGCTGGTTGGAT	508
PCR_product	TGGTAGTGGCCGACCTGTCGTTATCTCGTTGGCTACCGTGTGCCCGCGCTGGTTGGAT *****	539
tlyA_gene_Rv1694	GCGCTTCGCGCGACGCCGATATCGTTCCACTGGTGAAGCCGAGTTTGAGGTGGGGAAAG	568
PCR_product	GCGCTTCGCGCGACGCCGATATCGTTCCACTGGTGAAGCCGAGTTTGAGGTGGGGAAAG *****	599
tlyA_gene_Rv1694	GTCAGGTCGGCCCCGGTGGGGTGGTCCATGACCCGCGAGTTGCGTGCAGGTCGGTGCTCG	628
PCR_product	GTCAGGTCGGCCCCGGTGGGGTGGTCCATGACCCGCGAGTTGCGTGCAGGTCGGTGCTCG *****	659
tlyA_gene_Rv1694	CGGTCGCGCGGGCGGACAGGAGCTGGGCTGGCACAGCGTCGGCGTCAAGGCCAGCCCGC	688
PCR_product	CGGTCGCGCGGGCGGACAGGAGCTGGGCTGGCACAGCGTCGGCGTCAAGGCCAGCCCGC *****	719
tlyA_gene_Rv1694	TGCCGGGCCATCGGGCAATGTCGAGTACTTCTGTGGTTGCGCACGACGACCGACCGGG	748
PCR_product	TGCCGGGCCATCGGGCAATGTCGAGTACTTCTGTGGTTGCGCACGACGACCGACCGGG *****	779
tlyA_gene_Rv1694	CATTGTCGGCCAAGGGATTGGAGGATGCGGTGCACCGTGCATTAGCGAGGGCCCGTAG-	807
PCR_product	CATTGTCGGCCAAGGGATTGGAGGATGCGGTGCACCGTGCATTAGCGAGGGCCCGTAG- *****	839

Appendix B

Mutation result of drug resistant gene in *M. tuberculosis* clinical isolates by next-generation sequencing

1. Result of *M. tuberculosis* strain no. 1 (DS21388)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8469	249
	284	G	C	S95T	8740	250
	2003	G	A	G668D	8728	250
<i>rpoB</i>	1349	C	T	S450L	8127	250
	2192	T	C	L731P	8365	250
	3225	T	C	A1075A	8013	250
<i>rpsL</i>	128	A	G	K43R	7547	250
<i>rpsA</i>	636	A	C	R212R	8140	250
<i>tlyA</i>	33	A	G	L11L	8626	250
<i>katG</i>	1388	G	T	R463L	8514	250
	944	G	C	S315T	8454	249
<i>pncA</i>	92	T	C	I31T	8740	250
<i>embB</i>	1217	G	A	G406D	9035	250

Remark : Ref=Referenced allele, Alt=Alternate allele

2. Result of *M. tuberculosis* strain no. 2 (DS21639)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8691	249
	284	G	C	S95T	8506	250
	2003	G	A	G668D	8487	249
<i>rpoB</i>	1349	C	T	S450L	8213	249
	3225	T	C	A1075A	7927	250
<i>rpsL</i>	263	A	G	K88R	8471	249
<i>rpsA</i>	636	A	C	R212R	8216	250
<i>tlyA</i>	33	A	G	L11L	8528	250
<i>katG</i>	1388	G	T	R463L	9006	250
	944	G	C	S315T	8521	249
<i>embB</i>	1490	A	G	Q497R	7930	250

Remark : Ref=Referenced allele, Alt=Alternate allele

3. Result of *M. tuberculosis* strain no. 3 (DS21808)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8658	250
	284	G	C	S95T	8691	250
	2003	G	A	G668D	8847	250
<i>rpoB</i>	1349	C	T	S450L	8010	249
	3225	T	C	A1075A	7935	250
<i>rpsL</i>	128	A	G	K43R	8007	250
<i>rpsA</i>	636	A	C	R212R	8535	250
<i>tlyA</i>	33	A	G	L11L	8585	250
<i>katG</i>	1388	G	T	R463L	8894	250
	944	G	C	S315T	8857	250
<i>embB</i>	916	A	G	M306V	8739	250

Remark : Ref=Referenced allele, Alt=Alternate allele

4. Result of *M. tuberculosis* strain no. 4 (DS21950)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8896	250
	284	G	C	S95T	9025	249
	2003	G	A	G668D	8792	250
<i>rpoB</i>	1333	C	G	H445D	8667	250
	3225	T	C	A1075A	7749	248
<i>rpsL</i>	128	A	G	K43R	8203	250
<i>rpsA</i>	636	A	C	R212R	8504	250
<i>tlyA</i>	33	A	G	L11L	8850	250
<i>katG</i>	1388	G	T	R463L	8713	248
	944	G	C	S315T	8760	250
<i>embB</i>	916	A	G	M306V	8934	250

Remark : Ref=Referenced allele, Alt=Alternate allele

5. Result of *M. tuberculosis* strain no. 5 (DS21995)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8926	250
	284	G	C	S95T	8476	250
	2003	G	A	G668D	8741	250
<i>rpoB</i>	1349	C	T	S450L	8086	250
	3225	T	C	A1075A	8123	249
<i>rpsL</i>	128	A	G	K43R	7661	250
<i>rpsA</i>	636	A	C	R212R	8299	250
<i>tlyA</i>	33	A	G	L11L	8487	249
<i>katG</i>	1388	G	T	R463L	9035	250
	944	G	C	S315T	8840	250
<i>pncA</i>	398	T	C	I133T	8802	249
<i>embB</i>	916	A	G	M306V	8215	249

Remark : Ref=Referenced allele, Alt=Alternate allele

6. Result of *M. tuberculosis* strain no. 6 (DS22016)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	9108	250
	284	G	C	S95T	8744	250
	2003	G	A	G668D	8439	250
<i>rpoB</i>	1349	C	T	S450L	8202	250
	3225	T	C	A1075A	7909	250
<i>rpsA</i>	636	A	C	R212R	8188	250
<i>tlyA</i>	33	A	G	L11L	8839	250
<i>katG</i>	1388	G	T	R463L	8715	250
	944	G	C	S315T	8551	250

Remark : Ref=Referenced allele, Alt=Alternate allele

7. Result of *M. tuberculosis* strain no.7 (DS22345)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8565	250
	284	G	C	S95T	8655	249
	2003	G	A	G668D	8736	250
<i>rpoB</i>	1349	C	T	S450L	7806	250
	3225	T	C	A1075A	7630	250
<i>rpsL</i>	128	A	G	L43R	7640	250
<i>rpsA</i>	636	A	C	R212R	8408	250
<i>tlyA</i>	33	A	G	L11L	8825	249
<i>katG</i>	1388	G	T	R463L	8525	250
	944	G	C	S315T	8584	250
<i>pncA</i>	92	T	C	I31T	8556	249
<i>embB</i>	1217	G	A	G406D	9027	250

Remark : Ref=Referenced allele, Alt=Alternate allele

8. Result of *M. tuberculosis* strain no. 8 (DS23004)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	9064	249
	281	A	C	D94A	4312	248
	284	G	C	S95T	8646	248
	2003	G	A	G668D	8521	249
<i>rpoB</i>	1349	C	T	S450L	7349	248
	3225	T	C	A1075A	8113	249
<i>rpsL</i>	128	A	G	K43R	7920	250
<i>rpsA</i>	636	A	C	R212R	8372	250
<i>tlyA</i>	33	A	G	L11L	8593	250
<i>katG</i>	1388	G	T	R463L	8605	250
	944	G	C	S315T	8638	250
<i>embB</i>	918	G	C	M306I	8774	250

Remark : Ref=Referenced allele, Alt=Alternate allele

9. Result of *M. tuberculosis* strain no. 9 (DS23415)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8895	250
	284	G	C	S95T	8705	249
	2003	G	A	G668D	8642	250
<i>rpoB</i>	1349	C	T	S450L	8220	250
	2192	T	C	L731P	8549	250
	3225	T	C	A1075A	7850	250
<i>rpsL</i>	128	A	G	K43R	8431	250
<i>rpsA</i>	636	A	C	R212R	8589	250
<i>tlyA</i>	33	A	G	L11L	8423	250
<i>katG</i>	1388	G	T	R463L	8925	250
	944	G	C	S315T	8899	250
<i>pncA</i>	92	T	C	I31T	8889	249
<i>embB</i>	1217	G	A	G406D	8993	249

Remark : Ref=Referenced allele, Alt=Alternate allele

10. Result of *M. tuberculosis* strain no. 10 (DS24188)

Genes	Nucleotide Substitution			Amino acid Substitution	Quality	Read depth (DP)
	Position	Ref	Alt			
<i>gyrA</i>	61	G	C	E21Q	8928	250
	284	G	C	S95T	8809	250
	2003	G	A	G668D	8735	250
<i>rpoB</i>	1349	C	T	S450L	8287	250
	3225	T	C	A1075A	7571	249
<i>rpsL</i>	263	A	G	K88R	8418	249
<i>rpsA</i>	636	A	C	R212R	8550	249
<i>tlyA</i>	33	A	G	L11L	8533	250
<i>katG</i>	1388	G	T	R463L	8654	250
	944	G	C	S315T	8766	250
<i>pncA</i>	123	C	G	Y41*	8323	250
<i>embB</i>	1466	T	C	I489T	8836	250

Remark : Ref=Referenced allele, Alt=Alternate allele

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