NOVEL MUTATION DETECTION IN *RPOB* OF RIFAMPICIN-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* USING PYROSEQUENCING

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Abstract. Tuberculosis (TB) remains a major global public health problem particularly severe in parts of Asia and Africa, where often it is present in HIV-AIDS patients. Although rifampicin-resistant (RIF^r) TB is slow to emerge due to the low rate of mutation of its target leading to RIF^r being a marker of TB that is already resistant to other anti-TB drugs, and such cases are prone to treatment failure. More than 95% of rifampicin resistance is associated with mutations in *Mycobacterium tuberculosis* (MTB) *rpoB*, with 97% of mutations occurring within the 81 bp rifampicin-resistant determining region (RRDR) of this gene. In this study, we employed pyrosequencing technique to identify mutations in RRDR and 5 codons beyond of 39 MTB strains, comprising of 14 multi-drug resistance TB (MDRTB) and 3 RIF susceptible (RIF^s) MTB from the Center of Disease Control (CDC), Ratchaburi Province, and 19 mono RIF^r MTB, 1 MDRTB and 2 poly-drug resistant MTB from the Chest Institute, Ministry of Public Health, Thailand. Mutations in 8/22 samples from the Chest Institute and 13/14 from CDC were able to be identified. Six point mutations were detected, with Ser531Leu mutation accounting for 13, the silent mutation at Gly536 for 4, deletion of Gly523 for 2, combination of His526Cys and novel Leu533Arg for 1, and a novel Leu538Arg for 1. Mutation analysis of the 81 bp fragment and 5 codons beyond in MTB *rpoB* using pyrosequencing provides a useful approach in predicting RIF^r phenotype allowing early diagnosis and appropriate drug therapy.

Keywords: *Mycobacterium tuberculosis,* mutation, pyrosequencing, rifampicin, *rpoB*, tuberculosis

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INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB). Even though it mostly affects the lungs, it can occur in other parts of body. TB is spread from person to person by inhaling TB germs in air propelled from people with pulmonary TB through coughing, sneezing or spitting. In 2012, 8.6 million people suffered from TB and of these, 1.3 million died (WHO, 2013). The largest number of new TB cases occurs in Asia, accounting for 60% of new cases globally due to subjects co-infected with HIV-AIDS, with the risk of being infected being 21 to 34 times greater in HIV-AIDS infected subjects. In 2012, there were an estimated 1.1 million new HIV-positive TB cases and 75% of whom were living in Africa (WHO, 2013). A Global Plan to Stop TB: 2006-2015 was created by a network of organizations with the aim of reducing prevalence by 50% and mortality by 2015, followed by elimination of TB by 2050 (Young *et al*, 2008). However, a number of obstacles were needed to be addressed in the tuberculosis control program, including drug resistant tuberculosis (Young *et al*, 2008).

Standard anti-TB drugs used for decades, such as isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB), and streptomycin (SM), still play an important role but resistance to these drugs is growing (WHO, 2010). Multi-drug resistant (MDR)-TB is defined as bacteria that do not respond to at least INH and RIF, the two most powerful, first line (or standard) anti-TB drugs, with or without resistance to other anti-TB drugs (CDC, 2012). About 450,000 people worldwide have developed MDR-TB in 2012 (WHO, 2013). Currently, not only the emergence of MDR-TB imposes a burden to public health; but rifampicin resistant (RIF^r) MTB has become of importance as such cases are prone to treatment failure leading to mortality.

RIF is a semisynthetic derivative of rifamycin (discovered in 1972). RIF has a sterilizing effect on slow metabolized organisms and plays a pivotal role in tuberculosis treatment (Rattan *et al*, 1998). RIF^r is more difficult to occur than any other anti-tuberculosis drugs due to its slow rate of mutation induced by RIF (10^{-8}). However, the rate of RIF^r occurrence is increasing due to its wide use (Long, 2000). According to the Third National Drug Resistance Survey in Thailand, mono-RIF^r was found in 6.4% of new cases and 35.1% in previously treated cases (Migliori *et al*, 2008). More than 95% of RIF^r are associated with *rpoB* mutation despite the fact that RIF acts on *rpoB* encoding RNA polymerase β subunit (Lai *et al*, 2002). The majority (97%) of the mutations are harbored within an 81bp region of the RIF-resistance determining region (RRDR) in *rpoB*, which is considered as a mutation hot spot region. Among the different types of mutations, substitutions rather than indels are the most common, with more than 70% of the mutations are found in codons 526 and 531 (Rienthong *et al*, 2009).

The development of new tools for diagnosis of drug resistant TB is an important component of the Global Plan to Stop TB. Phenotypic and genotypic methods generally cannot detect a number of cases, especially those having less infectious forms of TB (Tang *et al*, 1997). However, molecular test techniques have become more popular as they (I) are highly sensitive and specific, (II) allow high throughput assays that are inexpensive and capable of being conducted in the field, (III) give rapid results, and (IV) are widely available and require less training

and infrastructure (Long, 2000). Most of the methods are based on nucleic acid amplification followed by electrophoresis, hybridization or sequencing, such as in loop mediated isothermal amplification (LAMP), Line probe and Xpert MTB/RIF assay, respectively (Ahmadian *et al*, 2006; Laurenzo and Mousa, 2011).

Pyrosequencing is a real-time sequencing technique based on detection of the released pyrophosphate during DNA elongation (Ronaghi *et al*, 1996). Single-strand DNA (ssDNA) template was hybridized with sequencing primer into pyrosequencing reaction and mixed with enzymes: DNA polymerase, ATP sulfurylase, luciferase, apyrase; other substrates: adenosine 5′-phosphosulfate (APS) and luciferin; and nucleotides. Specific nucleotide is individually dispensed in a defined order. After the reaction was generated, pyrophosphate (PPi) is released upon nucleotide incorporation in the elongated DNA strand. Then, ATP-sulfurylase converts PPi in the presence of APS to generate ATP, which then allows luciferase to convert luciferin to oxyluciferin, producing a visible light signal in amounts that are proportional to the amounts of the incorporated nucleotide. The light signal is detected by a charge coupled device (CCD) camera. Finally, apyrase, which is a nucleotide-degrading enzyme, degrades remaining ATP and unincorporated nucleotide (dNTPs) in the reaction mixture (Royo *et al*, 2007; Petrosino *et al*, 2009). As the pyrosequencing method is semi-automated, has high discriminatory power and throughput capability, and is rapid, we have employed this method to identify the mutation patterns in RIF^r MTB.

MATERIALS AND METHODS

Sample collection

DNA samples were obtained as follows: 14 MDRTB and 3 RIF^r MTB were from Ratchaburi Province, Center for Disease Control, Thailand; 19 RIF mono-resistant TB, 1 MDRTB and 2 poly-drug resistant MTB from the Chest Institute, Ministry of Public Health, Thailand. Drug susceptibity of samples obtained from Ratchaburi and the Chest Institute was confirmed by mycobacterial growth indicator tube test (MGIT) and proportional method using Lowenstein Jensen egg slants, respectively. H37Rv strain, which is stable and absent of virulent factors (Engström *et al*, 2012) was used as reference strain (GenBank, accession no. NC_000962).

This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (No. MUTM 2012-049-01).

PCR amplification of *rpoB* for pyrosequencing

PCR and sequencing primers of the 81 bp RRDR of *rpoB* and 5 codons beyond (Fig 1) were designed by PyroMark Assay Design software version 2.0 and sequence alignment was conducted using Bioedit and Clustal W. PCR in a volume of 25μl contained 20 μM each forward primer (5′-AAAACCAGATCCGGGTCG-3′) and reverse primer (5′-biotin-labeled GGTTTCGATCGGGCACAT-3′), 2.5 µl of 10X buffer, 0.4 mM each dNTP, 0.5 U *Taq* DNA polymerase and 100 ng of DNA template. The thermocycling (C1000, Biorad, Hercules, CA) conditions were as follows: 95°C for for 3 minutes; 45 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds; and a final step at 72°C for 7 minutes. The expected amplicon of 308 bp was confirmed by 2% agarose gel-electrophoresis (data not shown).

Pyrosequencing assay

SQA mode was chosen in order to

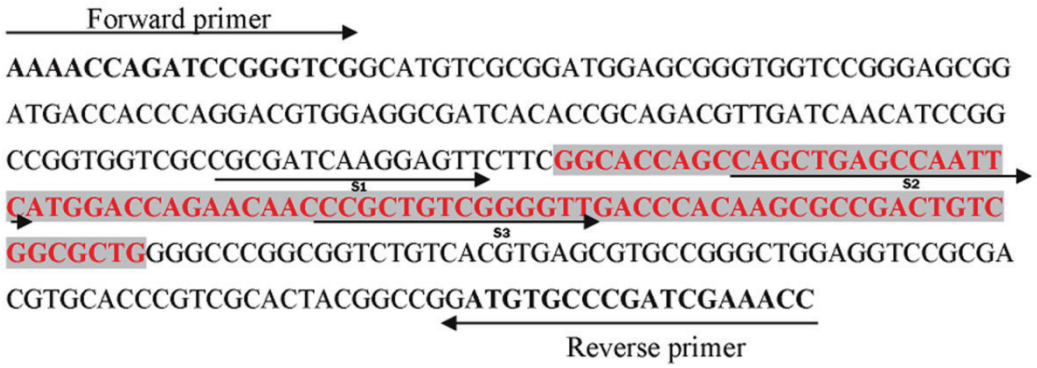

Fig1–PCR primer sequences, sequencing primers and rifampicin resistance determining region (RRDR) in *rpoB* (81bp region) of *Mycobacterium tuberculosis* H37Rv strain. Forward primer, 5′-AAAACCAGATCCGGGTCG-3′, 5′ biotin-labeled reverse primer, 5′-GGTTTCGATCGGGCACAT; S1 primer, 5′-GCGATCAAGGAGTTCT-3′; S2 primer, 5′-AGCTGAGCCAATTCAT-3′; S3, primer, 5′-CGCTGTCGGGGTTGA3′. Gray mark defines the RRDR.

detect all nucleotide changes within the target region. Biotinylated PCR products were immobilized by streptavidin-coated Sepharose beads in PyroMark Binding buffer solution (Qiagen, Hilden, Germany). The beads were washed with 70% ethanol. Then bead-bound double-strand DNA was denatured to produce single-strand (ss) DNA and the beads were washed once more with washing buffer. As the pyrosequencing procedure can sequence 50-60 bases, the Sepharose bead-attached biotinylated ssDNA strands were sequenced using three sequencing primers (S1: 5′-GCGATCAAGGAGTTCT-3′; S2: 5′-AGCTGAGCCAATTCAT-3′; S3: 5′-CGCTGTCGGGGTTGA-3′) to identify the whole RRDR. S1 primer allows sequence determination from codons 506 to 523 (53 nt), S2 primer from codons 515 to 536 (66 nt), and S3 primer from codons 526 to 549 (46 nt) (Fig 1). Beads were heated to 80°C for 2 minutes and then allowed to cool to room temperature. During the cooldown process, the sequencing primer was annealed to the ssDNA templates for pyrosequencing. The enzyme, substrates and nucleotides (dATP, dCTP, dGTP and dTTP) were added into PyroMark Q24 cartridge. During the reaction, the PCR products were then subjected to pyrosequencing using an automatically generated nucleotide dispensation order for the sequence to be analyzed. Cycling dispensation order was 20, 20 and 25 rounds of C, then T, then A and then G for S1, S2 and S3 pyrosequencing, respectively. If the dispensed nucleotide was incorporated into the elongating DNA strand, it generated the light signal detected by CCD sensors. The data from the CCD sensors were converted into a Pyrogram. The Pyrogram of each well is recorded as peaks of different heights, which indicate the numbers of nucleotides incorporated.

Data analysis

Bioedit and Clustal W alignment software were used to identify nucleotide changes. The results then were compared with those from the phenotypic drug tests, and sensitivity and specificity were calculated.

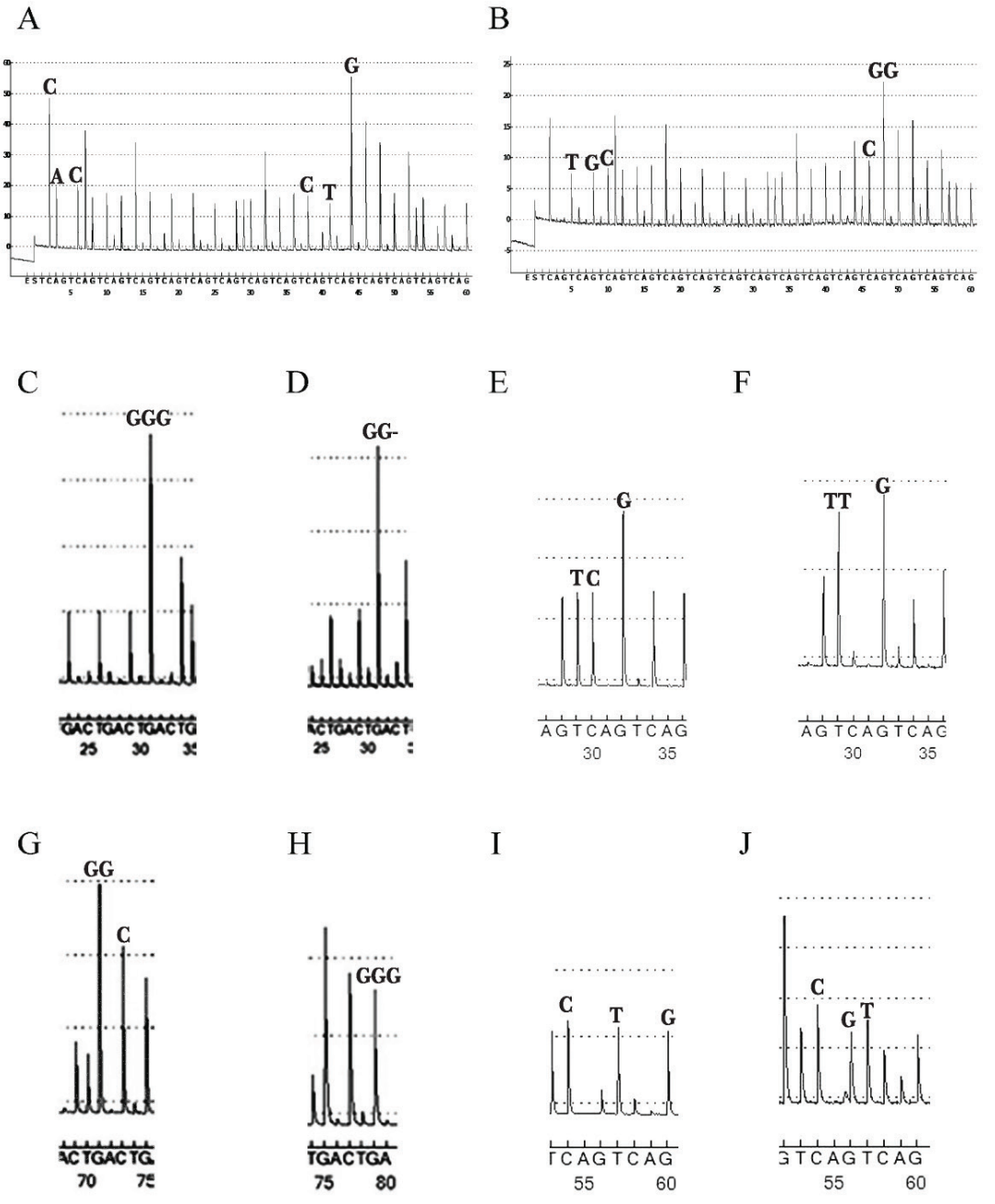

Fig 2–Pyrogram of *rpoB* mutations of 21 rifampicin-resistant *Mycobacterium tuberculosis* strains. (A) reference sequence of H37Rv at codon 526, CAC, and codon 533, CTG; (B) nucleotide mutation CAC526TGC and CTG533CGG; (C) reference sequence of H37Rv at codon 523, GGG; (D) nucleotide mutation GGG523GG-; (E) reference sequence of H37Rv at codon 531, TCG; (F) nucleotide mutation TCG531TTG; (G) reference sequence of H37Rv at codon 536, GGC; (H) nucleotide mutation GGC536GGG; (I) reference sequence of H37Rv at codon 538, CTG; (J) nucleotide mutation CTG538CGT.

Table 1
Comparison of drug resistance profile with mutation in *rpoB* 81 bp rifampicin-resistant determining region and five codons beyond of *Mycobacterium tuberculosis* (MTB) isolates from the Chest Institute, Ministry of Public Health and the Center for Disease Control, Ratchaburi Province, Thailand.

Origin of samples	Drug resistant profile	Base change	Mutation	Number of strains
Chest Institute	RIF^r	GGG⟶GG-	Gly523-	2
	RIF^r	CAC⟶TGC CTG⟶CGG	His526Cys, Leu533Arg	1
	RIF^r, SM^r	TCG⟶TTG	Ser531Leu	1
	RIF^r	GGC⟶GGG	Silent Gly536	2
	RIF^r, SM^r	GGC⟶GGG	Silent Gly536	1
	RIF^r	CTG⟶CGT	Leu538Arg	1
	RIF^r	N	N	13
	MDRTB	N	N	1
Center for Disease Control	MDRTB	TCG⟶TTG	Ser531Leu	12
	MDRTB	GGC⟶GGG	Silent Gly536	1
	MDRTB	N	N	1
	RIF^s	N	N	3

RIF^r, rifampicin resistant ; RIF^s, rifampicin susceptible; SM^r, streptomycin resistant; $MDRTB^r$, multi-drug resistant; N, no mutation.

RESULTS

Mutations detected in *rpoB*

RRDR is an 81 bp (codons 507-533) region in *rpoB* (Fig 1). In this study of 39 MTB DNA samples, 22 were sequenced up to codon 538 and 3 to codon 536. Among 21 samples, 6 mutations were detected at codons 523, 526, 531, 533, 536 and 538 (Fig 2). Mutations at codons 526 and 531 were most frequent, and mutations at codons 533 and 538 were novel. Twelve of 21 (57%) strains from CDC and 1/21 (5%) from the Chest Institute showed missense mutation at Ser531Leu (Table 1). Silent mutation at Gly536 were seen in 3/21 (14%) from the Chest Institute and1/21 (5%) from CDC (Table 1). One of 21 (5%) strains from the Chest Institute showed missense mutation at Leu538Arg (Table 1). In total, there were 15/21 (73%) missense, 4/21 (18%) silent, and 2/21 (9%) were deletion mutations (Table 1). Double mutations, His526Cys and Leu533Arg, were present in 1/21 (5%) samples from the Chest Institute.

Specificity and sensitivity of pyrosequencing method

Compared with existing drug susceptibility test (DST), samples from Ratchaburi, CDC gave sensitivity and specificity of the pyrosequencing method of 93% and 100%, respectively (Table 2), whereas, 8/22 samples of phenotypic rifampicin resistant samples from the Chest Institute revealed mutations, giving rise to a sensitivity of the pyrosequencing method of 36% (data not shown).

Table 2

A) Dichotomous table for calculation of sensitivity, specificity, positive value and negative predictive value of total 17 samples from the Center for Disease Control, Ratchaburi Province, Thailand. B) Results of sensitivity, specificity, positive value and negative predictive value with 95% confidence interval (CI).

A)

		Drug susceptibility test	
		+	-
Mutation by pyrosequencing	+	13 (a)	0 (c)
	-	1 (b)	3 (d)

(a) mutation present in RIF^r phenotype; (b) no mutation in RIF^r phenotype;
(c) mutation present in RIF^s phenotype; (d) no mutation in RIF^s phenotype.

B)

Test	Formula	Percent	95% CI
Sensitivity	a/a+b	93	41-99
Specificity	d/c+d	100	30-100
Positive predictive value	a/a+c	100	75-100
Negative predictive value	d/b+d	75	20-96

DISCUSSION

Early diagnosis of drug-resistant tuberculosis is important so that appropriate drug treatment can be provided and also to prevent the spread of infection. A retrospective study conducted at Ramathibodi Hospital, Mahidol University, Bangkok revealed that the resistance rate of RIF MTB is 4.5%, and RIF resistance is correlated with the recurrent TB infection within 6 months after completion of TB chemotherapy (Boonsarngsuk *et al*, 2009). Although cultures and drug susceptibility tests are sensitive and specific; these techniques are labor intensive and time consuming, taking from 8 to 12 weeks before results become known (Engström *et al*, 2012; Senol, 2013). A number of rapid and sensitive molecular techniques to detect RIF^r MTB are currently available, but they have various limitations. For instance, INNO-LiPA Rif TB assay, a line probe assay, detects RIF^r MTB by reverse hybridization using DNA probes of the most common *rpoB* mutations. This assay has high sensitivity and specificity for sputum positive specimens; but is expensive and requires sophisticated laboratory infrastructure and technical expertise (Yue *et al*, 2003; Migliori *et al*, 2008). Xpert MTB/RIF assay is based on semi-nested quantitative PCR and results are obtained within a couple of hours. However, sensitivity of the assay is high for smear-positive MTB but sensitivity is low for smear-negative culture- samples positive; in addition, this method is costly and cannot differentiate mutation types (Boehme *et al*, 2010; WHO, 2011; Wilson, 2011). Oligonucleotide

microarrays detect RIF^r MTB with 80% sensitivity and 100% specificity, but detect common mutation and if more mutations have to be detected, more probes have to be included in the array leading to higher costs (Caoili *et al*, 2006).

DNA sequencing is recognized as a gold standard among molecular methods (Bakker, 2006). In this study we employed the pyrosequencing method known as alternative Sanger method by nucleic acid synthesis in order to detect nucleotides. Almost all *rpoB* mutations detected were single point mutations while only one sample contained double mutations at codons 526 and 533. His526Cys and Ser531Leu were the most common mutations, similar to other reports from various countries (Ramaswamy, 1998; Archaeos Projects, 1999; Sun *et al*, 2009). Deletion mutation at codon 523, rare mutation, was observed in two isolates, whereas the more common mutations reported are Gly523Ala and silent (Ramasoota *et al*, 2006; Bostanabad *et al*, 2007; Bahrmand *et al*, 2009). Previous study of RIF^r MTB mutations in Thailand reported nine silent mutations within the RRDR (Ramasoota *et al*, 2006), whereas this study found only 4 isolates with silent mutation at Gly536. Surprisingly, there were two new mutations, Leu533Arg and Leu538Arg, with the former appearing together with His526Cys mutation. Previous studies in Thailand, reported Leu533Pro mutation (Ramasoota *et al*, 2006; Sun *et al*, 2009). Leu538Arg was 5 codons beyond the 81 bp RRDR of *rpoB*. Another study from China reported Leu538Pro mutation (Yue *et al*, 2003). It is worth noting that the two new mutations were revealed in this study, indicating that there is considerable diversity in *rpoB*.

Because of the short DNA lengths that could be obtained using the pyrosequencing method employed in this study, three sequencing primers were required to cover the RRRDR region (codons 507 to 533) and beyond to codon 549. However, the S3 primer designed to obtain sequence from codon 526 to 549 produced low signals towards the 3′ end of the sequence. Thus reliable sequences could only be obtained from codons 526 to 538. However, pyrosequencing machines are automated and can handle a number of samples simultaneously, with sequencing results available with a few hours.

Sensitivity of detection of RIF^r MTM by pyrosequencing of samples from CDC was 93% but that of specimens from the Chest Institute was 36%. Previous study in Thailand showed that 39% of mutations in *rpoB* occur outside RRDR (codons 432 to 498 and from codons 550 to 564), and 14/22 (63%) samples from the Chest Institute with RIF resistance and MDRTB might have mutations outside RRDR. Sequencing by conventional method should be conducted on these samples in order to determine if they contain mutations outside RRDR. Nevertheless, pyrosequencing can be useful in the detection of RIF^r MTB as it can detect not only the common mutations but also rare and novel mutations.

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