Rapid Detection for Early Appearance of Rifampin and Isoniazid Resistance in *Mycobacterium Tuberculosis*

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Thailand, a middle-income country in Southeast Asia, ranks 18th on the list of 22 "high-burden" TB countries in the world, with an estimated 91,374 new TB cases occurring in 2005.[1] Although case notifications have declined in Thailand throughout most of the 20th century, notifications began rising in the 1990s co-incident with an explosive epidemic of HIV. In 1996, Thailand officially adopted the internationally-recommended TB control strategy known as DOTS, which includes a package of policies and practices to detect and cure TB patients, particularly those that are most infectious, i.e., sputum acid-fast bacilli (AFB)-positive. After initial piloting of the strategy in selected sites, Thailand declared in 2001 that at least one public health care facility implementing DOTS had been established in all districts The most effective treatment of drug-susceptible TB involves taking four first line anti-TB drugs including Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), and Ethambutol (EMB) for two months and continues with INH and RIF for four additional months. Patients with *Mycobacterium tuberculosis* (MTB) strains resistant to these "first-line" drugs may require treatment with "second-line" anti-TB drugs, which are less effective, more toxic, and more expensive than "first-line" medications. The most worrisome forms of drug-resistant TB are multi-drug resistant MDR-TB and extensively-drug resistant (XDR-TB). MDR-TB is defined as an MTB strain resistant to INH and RIF; because RIF is the most effective anti-TB drug, RIF-resistance is often used as a surrogate marker for MDR-TB. XDR-TB is defined as an MDR strain that is also resistant to fluoroquinolones and one of an injectable anti-TB medication (kanamycin, amikacin, or capreomycin).

There is concrete evidence that Thailand has a very high percentage of drug resistance *Mycobacterium tuberculosis* (MTB) around the country. The third national drug resistance survey showed 1.65% and 34.54% for multi-drug resistant (MDR-TB) among new smear positive and previously treated pulmonary tuberculosis, respectively. The combined multi-drug resistance was 6.40%. In new smear positive cases any resistance of INH and RMP was 9.70% and 6.40% respectively while among previously treated was 44.30% and 35.10% for INH and RMP.[2] It is alarming that the level of resistance in the previously treated patients for re-treatment in particular for the failure, relapse and MDR suspected cases reveals a high opportunity to become worst treatment outcomes. These evidences reflect that it might be the MTB strains may develop XDR-TB. Little is known about XDR-TB in Thailand. In 2006, XDR-TB was reported in all regions of the world and classified rapidly by WHO as a serious emerging threat to public health, and included 3 XDR-TB cases from Thailand. This raises concerns of a XDR-TB epidemic in the country. The XDR-TB based on laboratory diagnosis would lead to better understanding of the magnitude and trends of drug-resistance and must address the threat.

Preventing XDR-TB requires prompt diagnosis of multidrug-resistant TB (MDR-TB), correct treatment of MDR-TB, and prevention of all forms of drug resistance through strong TB control programs. Methods for determining resistance to anti-TB medications through drug-susceptibility testing (DST) take at least 8-12 weeks using solid media and 4-8 weeks using newer modern methods, Bactec MGIT 960 System, with liquid media. Because modern TB diagnostic methods are expensive, they are not used routinely in high-burden, resource-limited settings. Delays in MDR-TB diagnosis result in three primary adverse consequences: patients remain on inadequate treatment longer than appropriate, increasing the risk of treatment failure or death. regimens inadequate to kill TB bacilli amplify resistance to drugs to which their isolates were previously susceptible, and patients remain infectious, allowing for transmission of MDR-TB to close contacts.

Diagnosis of Drug-Resistant TB

Effective control of drug-resistant TB relies on timely and accurate identification of cases and the prompt initiation of appropriate therapy. In Thailand, as in most resource-limited settings, TB cases are routinely diagnosed by microscopic examination of sputum specimens stained for AFB and by chest radiography. Unfortunately, these methods do not provide any data about whether an MTB strain is drug-resistant. To diagnose drug resistance, a laboratory must process a sputum specimen, attempt to culture MTB from that specimen, and, if the culture grows, then continue to drug-susceptibility testing (DST)whether the culture continues to grow while in the presence of anti-TB drugs. In Thailand, the best performing laboratory can return DST results in a median of 42 days (6 weeks) after specimen collection, but other laboratories do not report results until 60 – 70 days, due to delays in transportation and batching of specimens for testing.[3] Because of this problem, the World Health Organization (WHO) is now recommending that countries immediately scale up their capacity for culture and DST, and begin evaluating the routine implementation of new, molecular-based assays for diagnosing drug-resistance.

Rapid Assays for Diagnosis of MDR-TB/Drug Susceptibility Testing

Substantial progress has been made over the past few years to enhance our understanding of the molecular mechanisms underlying resistance to anti-tuberculosis drugs, specifically INH and RIF. Most RIF-resistant strains have mutations in the *rpoB* gene.[4] A published case series of TB patients in Thailand confirmed that mutations in the *rpoB* gene were specific to rifampin resistance.[5] Resistance to INH has been demonstrated to be significantly associated with mutations in the *katG*, *inhA*, and *ahpC* genes.[6] Because of these scientific advances, diagnostic tests have now been developed to diagnose MDR-TB within 24 hours. The GenoType® MTBDR test is a deoxyribonucleic acid (DNA) strip assay which uses polymerase chain reaction (PCR) and hybridization to detect genetic mutations in *rpoB* and *katG* genes from MTB culture isolates and AFB-positive smear specimens. A newer second generation assay, the GenoType® MTBDR*plus* includes an amplification control replacing a universal control, a probe for *inhA* mutations' regions of the *inhA* gene to increase detection of INH-resistance and three additional *rpoB* probes to increase the sensitivity for detecting mutations that confer RIF-resistance. For simplicity, the original and enhanced assays are referred together as the Hain test.

Rapid identification of resistant strains is crucial for the early administration of appropriate therapy, for prevention of development of further resistance, and to curtail the spread of MDR strains, and it is imperative for optimal treatment of drug-resistant TB patients. Use of the Hain test for identification and detection of INH and RIF resistance directly from AFB-positive sputum specimens and from culture-positive isolates could greatly accelerate the reporting of drug-resistant TB to clinicians and TB program managers, compared with routine methods for culture and DST.

The aim of this study was to assess the performance and feasibility of the Genotype MTBDR*plus* assay for rapid detection of RIF and INH resistance in Thai *Mycobacterium tuberculosis* isolates. In the first step, all culture isolates were determined by the indirect drug susceptibility testing (DST) of first line drugs using the proportion method on Lowensteine-Jeensen (LJ) egg media.

METERIALS AND METHODS

This study took place in the National Tuberculosis Reference Laboratory (NTRL), Department of Disease Control in Bangkok, Thailand. Testing was performed on 475 *Mycobacterium tuberculosis* (MTB) strains which were cultivated samples grown on Lowenstein-Jensen slants and partially from the residual portion of routine pulmonary smear-positive direct patient specimens submitted for culture and drug susceptibility testing (DST). Results were de-linked from patient identifiers, and no patient information was collected. Therefore, informed consent was not required for the study.

Sputum specimens

All manipulation with potentially infectious clinical specimens was performed in a biosafety cabinet (BSC) class II in a BSL3 laboratory. Sputum specimens were decontaminated with N-acetyl-L-cysteine-sodium hydroxide. After centrifugation, the pellet was cultured on two Lowenstein-Jensen slants and incubated at 37°C for 8 weeks. The mycobacterail growth was observed every week. Positive cultures were confirmed as *Mycobacterium tuberculosis* complex (MTBC) using Ziehl-Neelsen staining and a biochemical test including the p−nitrobenzoic acid test, and then they were sub-mitted to conventional drug susceptibility testing (DST). The remained residual portions of routine pulmonary smear-positive direct patient specimens were kept at 4°C for further evaluation with the Genotype MTBDR*plus* detection from the direct clinical samples.

Culture specimens

The stock of MTBC in the mycobacterial bank of NTRL, after the confirmation by microscopical examination for acid-fast bacilli, were inoculated onto Lowenstein-Jensen media slants by standard procedures, testing of susceptibility to first line drugs was performed on the conventional DST using the proportional method on Lowenstein-Jensen (LJ) egg media.

Conventional Indirect DST using the proportion method

A representative portion of the culture was obtained by sampling as many colonies as possible. The sample is homogenized in a sterile screw-capped tube containing 25-50 µl 0.05% Tween80 and 5-7 glass beads 3 mm. in diameter. The mixture is homogenized on a vortex mixer for up to a minute, 7 ml of sterile distilled water (SDW) are added slowly under continuous shaking, and then the suspension is allowed to settle for about 30 minutes. The opacity of the bacterial suspension is then adjusted by the addition of SDW to a standard suspension containing 1 mg/ml of tubercle bacilli (or BCG). The serial dilutions of 10−1 mg/ml to 10−5 mg/ml of the standard suspension are prepared by diluting sequentially 0.5 ml of the bacterial suspension (1 mg/ml) in a 12 ml 3 dram vial containing 4.5 ml of SDW. 0.1 ml of dilutions of 10−3 mg/ml and 10−5

mg/ml are inoculated on each slope of the panel of LJ medium and drug containing LJ medium. The culture bottles are incubated at 37°C and the results are read on the 28th day. The colonies are counted only on the slopes seeded with the inoculums that have produced exact readable counts or actual counts (up to 100 colonies on the slope). The results are reported as "susceptible" and "resistant". The standard criteria percentage by the proportional method for classifying a strain as resistant is the ratio of the number of colonies obtained on drug-containing medium to the number of colonies obtained on drug-free medium (growth of ≥1% of colonies) were used.[15]

Rapid drug susceptibility testing

The Genotype MTBDR*plus* (Hain Lifescience, Germany), a reverse hybridization line probe assay, is based on the **DNA•STRIP®** technology and permits the molecular genetic identification of the *M. tuberculosis* complex and its resistance to rifampicin and/or isoniazid from cultivated samples or pulmonary smear-positive direct patient material. The identification of rifampicin resistance is enabled by the detection of the most significant mutations of the *rpoB* gene (coding for the b-subunit of the RNA polymerase). For testing of high level isoniazid resistance, the *katG* gene (coding for the catalase peroxidase) is examined and for testing of low level isoniazid resistance, the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase) is examined. The whole procedure is divided into three steps: DNA isolation, from cultured material (culture plates/liquid medium) or direct materials (pulmonary, smear-positive, and decontaminated), a multiplex amplification with biotinylated primers using thermostable DNA polymerase, and a reverse hybridization. The hybridization includes the following steps: chemical denaturation of the amplification products, hybridization of the single-stranded, biotin-labelled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction. A template ensures the easy and fast interpretation of the banding pattern obtained. The turn around time of this performance is about eight hours including the interpretable results.

Briefly, the crude cell lysates (one loopful of cells) were suspended in 300 µl of distilled water, heat killed at 95°C for 20 mins., in Thermoblock (peQLab, Biotechnologie GmbH, Nehren, Germany), then sonification for 15 mins., at the highest speed in an ultrasonic bath, followed by spinning the samples in a standard centrifuge with an aerosol-tight rotor at approximately 10,000 x g for 5 mins. The supernatant was used for PCR amplification. The amplification procedure consists of preparation of the master mix [35µl of primer-nucleotide mix (provided with the kit), amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U of Hot Start *Taq* polymerase (QIAGEN, Hilden, Germany)], and addition of 5 µl of a chromosomal DNA in a final volume of 50 µl were used. These steps were carried out in separate rooms with restricted access and unidirectional workflow. The amplification protocol consisted of 1 cycle of 15 min., of denaturing at 95°C; followed by 10 cycles of 30 secs., at 95°C and 120 secs., at 58°C; followed by 20 additional cycles of 25 secs., at 95°C, 40 secs., at 53°C, and 40 secs., at 70°C; with a final extension at 70°C for 8 mins., for 1 cycle. Hybridization was performed with the GT Blot 48 (Hain Lifescience), which is an automated hybridization machine. 10 The program was started after 20 µl of the amplification products was mixed with 20 µl of denaturing reagent (provided with the kit) for 5 min in separate trough wells of a tray, and then the tray was fixed into the machine. Automatically, 1.5 ml of prewarmed hybridization buffer was added, followed by a stop to put the membrane strips into each trough. The hybridization procedure was performed at 45°C for 30 mins., followed by two washing steps. For colorimetric detection of hybridized amplicons, streptavidin-conjugated with alkaline phosphatase and substrate buffer was added. After final washing, strips were removed, allowed to air dry and then fixed on paper.

Quality control

Each strip contains 27 reaction zones (bands) including six controls (conjugate, amplification, *M. tuberculosis* complex, *rpoB*, *katG*, and *inhA* controls) to verify the test procedures, eight *rpoB* wild-type (WT) and four mutant (MUT) probes, one *katG* wild-type and two mutant probes, and two *inhA* wild-type and four mutant probes, (Figure 1, 2).

In conclusion, when all wild type probes of a gene stained positive and all of the mutation within the

Fig 1. *rpoB* wild type probes: WT 1 to WT 8, *rpoB* mutation probes: MUT D516V, H526Y, H526D, S531L. Detection of mutations through missing of wild type signals and detection of mutations through presence of mutation signals.

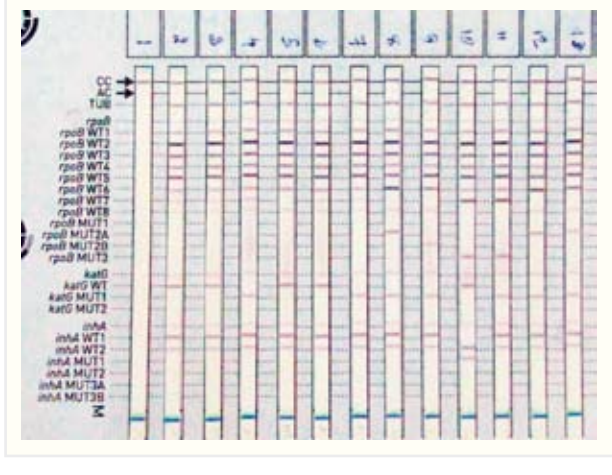

Fig 2. Examples of Genotype MTBDRplus strips ((Hain Lifescience, Germany). (lane 1) Negative control, (lane 2, 3, 5, 6) *Mycobacterium tuberculosis*, susceptible to isoniazid (INH) and rifampicin (RIF). (lane 4, 7) *M. tuberculosis*, INH monoresistant *katG* MUT1 (S315T1) mutation. (lane 8, 12, 13) Multidrug-resistant tuberculsis (MDB-TB), *rpoB* MUT2A (H526Y) mutation and *katG* MUT1 (S315T1) mutation. (lane 9) MDR-TB *rpoB* MUT2B (H526D) mutation and *katG* MUT1 (S315T1) mutation. (lane 10) MDR-TB *rpoB* MUT3 (S531L) mutation and *inhA* MUT1 (C15T) mutation. (lane 11) MDR-TB *rpoB* MUT3 (S531L) mutation and *katG* MUT1 (S315T1) mutatiON.

examined regions probes reacted negatively, the strain tested was considered susceptible to RIF and INH. In the case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes hence indicates a resistance of the tested strain to the respective antibiotic. Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone are to be considered. Each pattern that deviates from the wild type pattern indicates resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allows you to draw a conclusion about a rifampicin resistance of the strain tested, the banding pattern obtained with the *katG* probes allows you to draw a conlusion about a high level isoniazid resistance, the banding pattern obtained with the *inhA* probes allows you to draw a conclusion about a low level isoniazid resistance of the strain tested, respectively.

RESULTS AND DISCUSSION

The study population, 475 *Mycobacterium tuberculosis* strains, represents 330 clinical isolated strains from the mycobacterium bank of NTRL, 50 new clinical isolated strains including residual portions of routine pulmonary smear-positive direct patient specimens, and 95 strains from an external quality assurance program of the SRL network. The DNA strip test is based on a multiplex PCR in combination with reverse hybridization with membrane strips coated with target-specific oligonucleotides. The conjugate control bands were visible in all cases and the amplification control bands showed a strong positive signal in all tests. Furthermore, the results of Genotype MTBDR*plus* assay were compared with phenotypic DST results, as shows in Table 1-3.

Table 1 shows results for detection of rifampicin-monoresistance, isoniazid-monoresistance and multidrug resistance by Genotype MTBDRplus compared with conventional Lowenstein-Jensen proportion drug susceptibility testing. Of the specimens with conventional DST results, 65.26% (310/475) were MDR-TB, 2.74% (13/475) were RIF-monoresistant strains, 8.21% (39/475) were INH-monoresistant strains and 23.79% (113/475) were RIF and INH susceptible by conventional DST. Overall of the Genotype MTBDR*plus* testing, 48% (228/475) had mutation in the *rpoB*, *katG* and *inhA* genes, 7.58% (36/475) of RIF-monoresistant had a mutation in the *rpoB* gene, 17.68% (84/475) of INH-monoresistant had both mutation in the *katG* and *inhA* genes and 26.74% (127/475) did not have all mutation genes.

Table 2 shows the results of detecting rifampicin and isoniazid resistance, eighty four percent (270/323 = 83.59%) of MDR strains had a mutation in the *rpoB* gene and were detected as RFP resistant by a mutation in the *rpoB* gene. Ninety percent (313/349 = 89.69%) INH-resistant MTBDR*plus* result compared with conventional DST, out of these, ninety five percent (296/313 = 94.57%) had a mutation in the *katG* gene and were detected as INH resistant by a mutation in the *katG* gene. Eight percent (26/313 = 8.31%) did not have a mutation in the *katG* gene and were detected by the presence of a mutation in *inhA* only. Three percent of INH-resistant strains (9/313 = 2.88%) had mutations in both the *katG* and *inhA* genes.

Table 3 shows the sensitivity and specificity for detection of rifampicin, isoniazid and multidrug resistance of Genotype MTBDR*plus* against the proportion

TABLE 1. Summary of results of Genotype MTBDR*plus* test and LJ proportion DST method for detection of Rifampicin and Isoniazid susceptibility among the 475 *M. tuberculosis isolates.*

Susceptibility		No. of isolates	
		Genotype MTBDR*plus*	LJ proportion DST method
Rifampicin	Monoresistant	36	13
Isoniazid	Monoresistant	84	39
MDR-TB	Resistant to RIF & INH	228	310
Susceptible	Susceptible to RIF & INH	127	113

TABLE 2. Performance of Genotype MTBDR*plus* in detecting rifampicin and isoniazid resistance against the conventional proportional susceptibility test (PST) on LJ media

	No. of positive strains / No. of strains tested (%)						
	RIF resistance		INH resistance		INH resistance		
	rpoB	PST	Hain MTBDR*plus*	PST	*katG*	*inhA*	*katG& inhA*
475 *M.tuberculosis strains*	270 (83.59)	323	313 (89.69)	349	296/313 (94.57)	26/313 (8.31)	9/313 (2.88)

TABLE 3. Performance of the Genotype MTBDRplus in detection of rifampicin, isoniazid and multidrug resistance from clinical samples smear-positive sputum specimens and *Mycobacterium tuberculosis* isolates.

Genotype MTBDR*plus* Assay	No. of positive strains / No. of strains tested (%)						Agreement rate
	RIF resistance		INH resistance		MDR-TB		
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	
475 MTB isolated strains	260/323 (80.50)	145/152 (95.40)	311/349 (89.11)	126/126 (100)	230/310 (74.19)	159/165 (96.36)	382/475 (80.42)

Definition of abbreviations: MTB = *Mycobacterium tuberculosis*, RIF = rifampicin, INH = isoniazid, MDR-TB = multidrug resistance

of DST on LJ media. Provided that conventional DST is the gold standard, among the 475 *M. tuberculosis* isolates included in this study, the sensitivity of the MTBDR*plus* assay were 81% (260/323) for rifampicin resistance and 90% (311/349) for isoniazid resistance. Specificity was 95% (145/152) for rifampicin resistance and 100% (126/126) for isoniazid resistance. Whereas, the sensitivity and specificity for detection of multidrug reistance were 74 and 96%, respectively. The genotypic result was 80% concordant with the proportional DST in 382 of 475 samples. Discordant results could be explained by the rifampicin and isoniazid resistance mutations being located elsewhere than the *rpoB* 81bp hot spot region leading to RMP resistance and codon of *katG* and *inhA* genes.

Table 4 (a) and 4 (b) show the distribution of different band patterns of mutation in drug-resistance isolates, including MDR-TB, rifampicin-monoresistant, and isoniazid-monoresistant strains. Typical banding patterns obtained on the MTBDR*plus* strips are shown

TABLE 4 (a). Pattern of gene mutations in resistant *Mycobacterium tuberculosis* strains using Genotype MTBDR*plus* assay

Gene	Band	Gene Region of mutation	MDR n = 228, (%)	RIF-Monoresistant n = 36, (%)	INH-Monoresistant n = 84, (%)
rpoB	MUT1	D516V	20 (8.9)	8 (22.22)	0 (0)
	MUT2A	H526DY	41 (17.89)	6 (16.67)	0 (0)
	MUT2B	H526D	23 (10.09)	1 (2.78)	0 (0)
	MUT3	S531L	146 (64.04)	21 (58.33)	0 (0)
katG	MUT1	S315T1	205 (89.91)	0 (0)	73 (86.9)
	MUT2	S315T2	1 (0.44)	0 (0)	1 (1.19)
inhA	MUT1	C15T	26 (11.4)	0 (0)	10 (11.9)
	MUT2	A16G	0 (0)	0 (0)	0 (0)
	MUT3A	T8C	0 (0)	0 (0)	0 (0)
	MUT3B	T8A	0 (0)	0 (0)	0 (0)

Definition of abbreviations: RIF = rifampicin; INH = isoniazid; MDR-TB = multidrug resistance. Values are numbers, with percentages in parentheses.

TABLE 4 (b). Pattern of gene mutations in resistant *Mycobacterium tuberculosis* strains using Genotype MTBDR*plus* assay

Pattern of gene mutations	Percentage (No. of mutation strains / No. of resistant strains)
All RIF-resistant strains, 264 strains	
rpoB MUT1	10.61% (28/264)
rpoB MUT2A	17.80% (47/264)
rpoB MUT2B	9.09% (24/264)
rpoB MUT3,	63.26% (167/264)
All INH-resistant strains, 312strains	
katG MUT1INH	91.03% (284/312)
katG MUT2	0.64% (2/312)
inhA MUT1	11.54% (36/312)
MDR-TB strains (228 strains)	
rpoB MUT3, *katG* MUT1	57.02% (130/228)
rpoB MUT3, *inhA* MUT1	5.26% (12/228)
rpoB MUT3, *katG* MUT1, *inhA* MUT1	1.75% (4/228)
rpoB MUT2B, *katG* MUT1	8.33% (19/228)
rpoB MUT2B, *katG* MUT2	0.44% (1/228)
rpoB MUT2B, *inhA* MUT1	0.88% (2/228)
rpoB MUT2A, *katG* MUT1	14.47% (33/228)
rpoB MUT2A, *inhA* MUT1	73.07% (7/228)
rpoB MUT1, *katG* MUT1	7.90% (18/228)
rpoB MUT1, *inhA* MUT1	0.44% (1/228)
rpoB MUT1, *rpoB* MUT2A, *rpoB* MUT2B, *katG* MUT1	0.44% (1/228)
RIF-Monoresistant strains (36 strains)	
rpoB MUT1	22.22% (8/36)
rpoB MUT2A	16.67% (6/36)
rpoB MUT2B	2.78% (1/36)
rpoB MUT3	58.33% (21/36)
INH-Monoresistant strains (84 strins)	
katG MUT1	86.91% (73/84)
katG MUT2	1.19% (1/84)
inhA MUT1	5.95% (5/84)
katG MUT1, *inhA MUT1*	5.95% (5/84)

Gene region of mutation: rpoB; MUT1 D516V, MUT2A H526Y, MUT2B H526D, MUT3 S531L. katG; MUT1 S315T1, MUT2 S315T2. inhA; MUT1 C15T, MUT2 A16G, MUT3A TBC, MUT3B TBA

TABLE 5. Summary results of Genotype MTBDR*plus* in detecting rifampicin and isoniazid resistance against the Bactec MGIT960 AST and proportional susceptibility test on LJ media.

	RIF resistance		INH resistance Hain		INH resistance		
	rpoB	PST	MTBDR*plus*	PST	*katG*	*inhA*	*katG&inhA*
45 MTB strains on *Bactec MGIT960* AST	17	20	29	29	28/29	4/29	3/29
45 MTB strains on *Proportional DST method on LJ media*	17	22	29	29	28/29	4/29	3/29

in Figure 2. For detection of rifampicin resistance, a S531L mutation (MUT3 band) was the most common mutation, with 63.26% (167/264) of all RIF-resistant strains (64% of MDR- and 58% of RIF-monoresistant strains). Most of MDR strains had only one position of mutation of RIF resistances in their genomes, and data were shown in table 4 (a). Other mutations in the 530-533 regions (Figure 1) were common, as detected by the lack of binding to the WT8 probe in the absence of a S531L mutation. A significantly higher proportion of RIF-monoresistant strains (22%) had a D516V mutation (MUT1 band) compared with MDR strains (9%). Other mutations occurred at *rpoB*526 (26.89% overall), and one MDR strain had almost deletion of the *rpoB* gene (D516V, H526Y, and H526D mutations). Of all INH-resistant strains, 88.10% (90.35% of MDR strains and 98.81% of INH-monoresistant strains) had a mutation in the *katG* gene, and 11.89% (11.4% of MDR strains and 11.9% of INH-monoresistant strains) had a mutation in the *inhA* gene. This difference in prevalence of mutations in MDR strains compared with INH-monoresistant strains was significant for *katG*, but not for *inhA*. Five strains had mutations in both the *katG* and *inhA* genes.

A second part of the study included additional testing of 45 *M. tuberculosis strains* for first line drug in the Bactec MGIT960 AST (Becton Dickinson) according to the manufacturer's instructions. The results of Genotype MTBDR*plus* were compared to those phenotypic DST results of the MGIT960 AST and conventional proportional DST. Table 5 shows results for detection of RIF, INH and multidrug resistance that were calculated from specimens for which rapid and conventional results were available. In the performance of MGIT960 AST, eighty five percent (17/20 = 85%) of drug resistant strains had a mutation in the *rpoB* gene while the proportional DST was seventy seven percent (17/22 = 77.27%) of drug resistance strains had a mutation in the *rpoB* gene and were detected as RFP resistant by a mutation in the *rpoB* gene. Of all INH-resistant strains, in both procedures these showed 100% concordance results, one hundred percent (29/29 = 100%) INH-resistant MTBDR*plus* result compared with conventional DST, out of these, ninety seven percent (28/29 = 96.55%) had a mutation in the *katG* gene and were detected as INH resistant by a mutation in the *katG* gene. Fourteen percent (4/29 = 13.79%) did not have a mutation in the *katG* gene and were detected by the presence of a mutation in inhA only. Ten percent of INH-resistant strains (3/29 = 10.35%) had mutations in both the *katG* and *inhA* genes.

Table 6 shows the sensitivity and specificity for detection of rifampicin, isoniazid and multidrug resistance of Genotype MTBDR*plus* against Bactec MGIT960 AST and the proportional susceptibility test on LJ media. The MGIT960 AST performance, sensitivity of the MTBDR*plus* assay were 85% for rifampicin resistance and 100% for isoniazid resistance. Specificity was 100% for rifampicin resistance and isoniazid resistance. The sensitivity and specificity for detection of multidrug reistance were 82 and 100%, respectively. The genotypic result was 93% (42 of 45 samples) concordant with MGIT960 AST. When consideration to the proportional DST performance, sensitivity of the MTBDR*plus* assay were 73% for rifampicin resistance and 100% for isonia-

TABLE 6. Comparison of the performance of the Genotype MTBDRplus in detection of rifampicin, isoniazid and multidrug resistance from *Mycobacterium tuberculosis* isolates against Bactec MGIT960 AST and proportional susceptibility test on LJ media.

	No. of positive strains / No. of strains tested (%)						
Genotype MTBDRplus Assay	RIF resistance Sensitivity	Specificity	INH resistance Sensitivity	Specificity	Sensitivity	MDR-TB Specificity	Agreement rate
45 MTB strains on *Bactec MGIT960 AST*	17/20 (85.0)	25/25 (100)	29/29 (100)	16/16 (100)	14/17 (82.35)	28/28 (100)	42/45 (93.33)
45 MTB strains on PST *method*	16/22 (72.73)	22/23 (95.65)	16/16 (100)	29/29 (100)	13/18 (72.22)	26/27 (96.30)	38/45 (84.44)

Definition of abbreviations: MTB = *Mycobacterium tuberculosis*, LJ = Lowenstein-Jeensen media, PST = proportional susceptibility test, RIF = rifampicin, INH = isoniazid, MDR-TB = multidrug resistance

zid resistance. Specificity was 96% for rifampicin resistance and 100% for isoniazid resistance. The sensitivity and specificity for detection of multidrug resistance were 72 and 96%, respectively. The rate of concordance between the results of Genotype MTBDR*plus* and those obtained with "in vitro" DST were 93% with MGIT960 AST and 84% with proportional DST.

The performance of the Genotype MTBDR*plus* test is that the test performs well on specimens both culture isolates and AFB-positive smear specimens. The detection of mutations of those genes in the recognition of RIF- and INH-resistance gives a high specificity value from 95 to 100 percent even though it is not so superior on sensitivity of the test. However, the sensitivity for RIF resistance may be low in other settings where mutations outside the 81-bp region of the *rpoB* gene, which are not detected by the assay, are responsible for RIF resistance (16). In agreement with most other studies, we found the most common mutations at codons 531, 526, and 516. The rate of mutations at codon 516 (10.61% in all refampin-resistant strains) was within the range reported elsewhere. Most of INH resistant strains in this setting showed the prevalence of mutations in the high level isoniazid resistance of the *katG* gene that agrees with many reports. The *katG* mutations were found in 95% (296/313) and inhA mutations in 8% (26/313) of INH-resistant isolates. Studies from other countries have confirmed this variability in the contribution of different mutations to INH resistance (17, 18). A high prevalence of *katG* mutations has been reported to account for a high proportion of INH resistance in high TB prevalence countries and for a much lower proportion in lower TB prevalence setting, presumably due to ongoing transmission of these strains in a high burden setting (18).

A higher proportion of concordant result was obtained from Genotype MTBDR*plus* against MGIT 960 AST (93%) than conventional LJ proportion DST (84%). This shows the high correlation of the Genotype MTBDR*plus* results with MGIT 960 AST results which reflects well on the performance of the test in this setting.

Although the Genotype MTBDR*plus* assay can produce results within eight hours from when the sample reaches the laboratory, all the performance procedure steps are required for the GLP, knowledgeable staff, and also good support from TB control programs are needed.

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