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Molecular Detection of Rifabutin-Susceptible *Mycobacterium tuberculosis*

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Rapid assays are still needed to detect rifabutin (RFB) susceptibility for proper tuberculosis treatment. To assess the use of the GenoType MTBDR*plus* assay and subsequent *rpoB* gene sequencing on detection of RFB susceptibility, we analyzed 800 multidrug-resistant *Mycobacterium tuberculosis* isolates, and 13% (104/800) were RFB susceptible. Of the 104 RFB-susceptible isolates, 71 (68.3%) isolates were rapidly identified using two molecular assays, while the remaining isolates could be determined using conventional drug-susceptibility testing according to the clinician's decision.

Rifamycins are a group of antibiotics that belong to a subclass of the large family ansamycin. They interact with the β-subunit of the bacterial RNA polymerase encoded by the *rpoB* gene, inhibit DNA-dependent RNA synthesis, and are particularly effective against mycobacteria. Two potent rifamycin derivatives, rifampin (RIF) and rifabutin (RFB), are used as effective antibiotics for treatment of tuberculosis (TB). The cross-resistance rate between the two rifamycin derivatives is high (1, 10, 11, 12). However, RFB might be still potent against certain multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains (with MDR defined as resistant to at least isoniazid [INH] and RIF) and is an alternative to RIF for TB patients with serious side effects during treatment or coinfected with HIV (8).

RFB is not included in a routine conventional first-line drug susceptibility testing (DST) for *M. tuberculosis* isolates, and subsequent RFB susceptibility testing of MDR isolates needs an additional 3 to 4 weeks. Several molecular tests have been applied to

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TABLE 1 Correlations between specific mutations of the *rpoB* genes and patterns of the GenoType MTBDR*plus* assay for identification of RFB-susceptible isolates[a]

Mutation codon no.	Codon	Amino acid change	No. of isolates	No. (%) of RFB-resistant isolates	Pattern by GenoType MTBDR*plus* assay											
					wt1	wt2	wt3	wt4	wt5	wt6	wt7	wt8	mut1	mut2	mut3	mut4
143	CGT/TGT	R→C	1	0 (0)												
146	GTC/TTC	V→F	6	6 (100)												
511	CTG/CCG	L→P	3	0 (0)	■		■	■	■	■	■	■				
513	CAA/AAA	Q→K	10	10 (100)	■			■	■	■	■	■				
	CAA/CTA	Q→L	4	4 (100)	■			■	■	■	■	■				
	CAA/GAA	Q→E	1	1 (100)	■			■	■	■	■	■				
	CAA/CCA	Q→P	7	7 (100)	■			■	■	■	■	■				
516	GAC/TAC	D→Y	14	0 (0)	■	■			■	■	■	■				
	GAC/GTC	D→V	22	0 (0)	■	■			■	■	■	■	■			
	GAC/TTC	D→F	8	0 (0)	■	■			■	■	■	■				
522	TCG/TTG	S→L	5	0 (0)	■	■	■	■			■	■				
526	CAC/CGC	H→R	18	18 (100)	■	■	■	■	■	■		■				
	CAC/TAC	H→Y	53	53 (100)	■	■	■	■	■	■		■		■		
	CAC/GAC	H→D	32	32 (100)	■	■	■	■	■	■		■			■	
	CAC/CAA	H→Q	2	2 (100)	■	■	■	■	■	■		■				
	CAC/CCC	H→P	1	1 (100)	■	■	■	■	■	■		■				
	CAC/TGC	H→C	2	0 (0)	■	■	■	■	■	■		■				
	CAC/CTC	H→L	11	0 (0)	■	■	■	■	■	■		■				
	CAC/ACC	H→T	1	0 (0)	■	■	■	■	■	■		■				
	CAC/AAC	H→N	4	0 (0)	■	■	■	■	■	■		■				
529	CGA/CTA	R→L	1	0 (0)	■	■	■	■	■	■		■				
531	TCG/TTG	S→L	491	491 (100)	■	■	■	■	■	■	■					■
	TCG/TGG	S→W	16	16 (100)	■	■	■	■	■	■	■					
533	CTG/CCG	L→P	27	8 (29.6)	■	■	■	■	■	■	■					

[a] Shading highlights mutations that confer both RIF and RFB resistance.

TABLE 2 Multidrug-resistant *Mycobacterium tuberculosis* isolates with triple mutations, double mutations, or deletion in the *rpoB* gene, their patterns of the GenoType MTBDR*plus* assay, and possibility of misinterpretation as patterns for single mutation

Mutated codons	No. of MDR isolates	No. of RFB-susceptible isolates	Pattern of the GenoType MTBDR*plus* assay												Misinterpretation of mutation site(s)
			wt1	wt2	wt3	wt4	wt5	wt6	wt7	wt8	mut1	mut2A	mut2B	mut3	
Triple mutations															
A501T, H526R, R529Q	1		■	■	■	■	■	■		■					526 or 529 only
Double mutations															
V146A, L533P	3		■	■	■	■	■	■	■						531 or 533 only
T480I, S531L	3		■	■	■	■	■	■	■						531 or 533 only
L533P, E562A	2	1	■	■	■	■	■	■	■						531 or 533 only
D444V, S531L	1		■	■	■	■	■	■	■						531 or 533 only
S531L, I561V	1		■	■	■	■	■	■	■						531 or 533 only
Q513E, M558K	2		■			■	■	■	■	■					513 only
E458K, Q513L	1		■			■	■	■	■	■					513 only
L511P, M515L	1	1	■			■	■	■	■	■					513 only
D516Y, Q148R	2		■	■			■	■	■	■					516 only
D516G, I572F	1		■	■			■	■	■	■					516 only
S164P, H526N	1		■	■	■	■	■	■		■					526 only
L511P, S512G	1		■		■	■	■	■	■	■					511 only
H526N, L533P	4		■	■	■	■	■	■							Direct ID[a]
N518S, H526P	1		■	■	■		■	■		■					Direct ID
D516Y, T525I	1		■	■			■	■		■					Direct ID
D516G, L533P	2		■	■			■	■	■						Direct ID
D516Y, L533P	1		■	■			■	■	■						Direct ID
D516N, H526D	2		■	■			■	■		■					Direct ID
D516Y, H526D	1		■	■			■	■							Direct ID
D516G, S522W	2	2	■	■					■	■					Direct ID
L511 silent, H526Y	1		■		■	■	■	■		■		■			Direct ID
L511P, H526N	2		■		■	■	■	■		■					Direct ID
L511R, D516A	1		■		■		■	■	■	■					Direct ID
L511P, F505L	1	1			■	■	■	■	■	■					Direct ID
V144A, V146F	1														None
V146F, S164P	1														None
Deletion															
509–511 (or 510–512)	2	2	■		■	■	■	■	■	■					511 only
517	2	2	■	■			■	■	■	■					516 only
9 bp at 513–516	1						■	■	■	■					Direct ID
518 (or 519)	1	1	■	■	■			■	■	■					Direct ID
Total	47	10 (21.3%)													

[a] Direct ID, isolates with multiple mutations or deletions could be identified directly by the GenoType MTBDR*plus* assay.

detect RIF resistance, such as the GenoType MTBDR*plus* assay and the INNO-LiPA Rif assay (3, 7). The GenoType MTBDR*plus* assay, a line-probe assay endorsed by World Health Organization in 2008, can detect *M. tuberculosis* complex and resistance to INH and RIF (5). The GenoType MTBDR*plus* assay has been successfully applied to detection of culture samples since 2007 and clinical specimens since 2010 in Taiwan. Moreover, D516V mutation of the *rpoB* gene has been confirmed to confer resistance specifically to RIF in the commercial INNO-LiPA Rif assay (11, 13). The GenoType MTBDR*plus* assay also has a probe targeting D516V that might be used for identification of RFB susceptibility. However, the feasibility of the GenoType MTBDR*plus* assay to simultaneously identify RFB-susceptible isolates was not known and was thus evaluated in this study.

MDR isolates. We analyzed 800 MDR *M. tuberculosis* isolates collected from clinical mycobacteriology laboratories from January 2006 to December 2010. RFB and first-line DST were performed using the agar proportion method on either

TABLE 3 MDR *Mycobacterium tuberculosis* isolates with multiple mutations in the *rpoB* gene and the change in their resistance to RFB

Mutated codon	No. (%) of isolates with multiple mutations	No. (%) of isolates changing resistance to RFB[a]
146	5 (45.5)	0
511	7 (70.0)	5 (71.4)
513	3 (12.0)	0
516	13 (22.8)	11 (84.6)
526	13 (9.5)	7 (53.8)
531	5 (1.0)	0
533	12 (30.8)	ND
Total	58 (7.5)	

[a] All changes shown were from susceptible to resistant. ND, not determined.

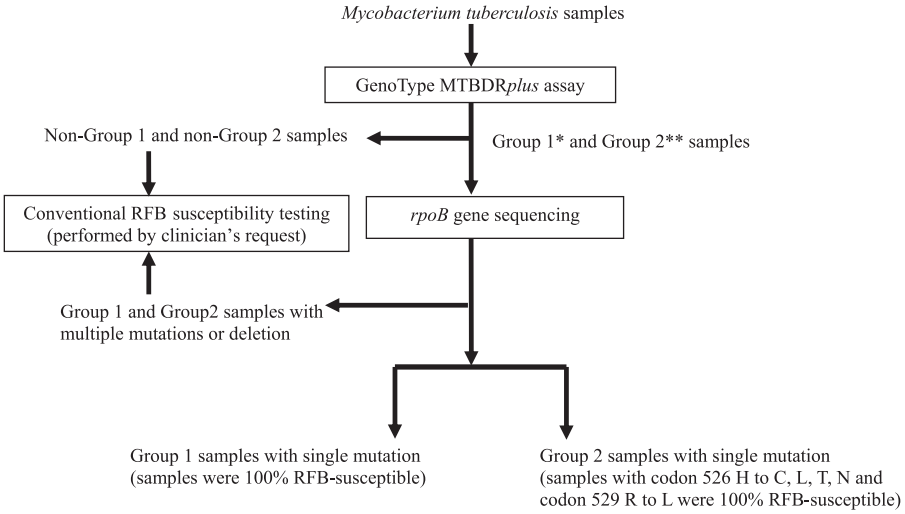

FIG 1 Proposed flowchart for identification of rifabutin (RFB) susceptibility. In this study, 104 rifabutin-susceptible isolates were identified among 800 multidrug-resistant *Mycobacterium tuberculosis* isolates. Shown is a suggested algorithm for identification of rifabutin susceptibility. *, group 1 represents samples missing only wt2, only wt3 and wt4, or only wt5 and wt6; **, group 2 represents samples simultaneously missing wt7, mut2A, and mut2B.

Middlebrook 7H10 or 7H11 according to standardized protocols (9).

***rpoB* gene sequencing.** The *rpoB* gene was amplified with primers *rpoB*-F (5′-TCG GCG AGC CCA TCACGT CG-3′) and *rpoB*-R (5′-GCG TAC ACC GAC AGC GAG CC-3′), which yielded a 541-bp fragment containing the hot spot region (6). In addition, A 365-bp fragment targeting the V146F mutation (V176F according to the *M. tuberculosis* numbering system) was amplified and sequenced with the primers TB-176-F (5′-CTT CTC CGG GTC GAT GTC GTT G-3′) and TB-176-R (5′-CGC GCT TGT CGA CGT CAA ACT C-3′) (4). PCR products were sequenced, and data were assembled and edited thereafter.

The GenoType MTBDR*plus* assay. The GenoType MTBDR*plus* assay was performed according to the instructions provided by the manufacturer (Hain Lifescience GmbH, Nehren, Germany) (2). Eight wild-type (wt) probes and four mutation (mut) probes were used to determine the resistance to RIF. The probes for detecting target sequences were as follows: wt1 (codons 505 to 509), wt2 (codons 510 to 513), wt3 (codons 513 to 517), wt4 (codons 516 to 519), wt5 (codons 518 to 522), wt6 (codons 522 to 525), wt7 (codons 526 to 529), wt8 (codons 530 to 533), mut1 (codon D516V), mut2A (codon H526Y), mut2B (codon H526D), and mut3 (codon S531L). Due to overlapping design of probes for detection of mutations at codons 513, 516, and 522, continuous absence of each of the wt2 and wt3, wt3 and wt4, and wt5 and wt6 pairs could represent a single mutation. To clarify the specific pattern and its usage as a marker for detecting RFB susceptibility, we defined group 1 isolates as those missing only wt2, only wt3 and wt4, or only wt5 and wt6, while group 2 was designated as containing isolates simultaneously missing wt7, mut2A, and mut2B (Table 1).

Of the 800 MDR *M. tuberculosis* isolates, 104 (13%) RIF-resistant but RFB-susceptible isolates were identified. In addition, we identified 740 isolates with a single mutation, 41 with multiple mutations, 6 with deletions, and 13 without mutation at the beginning region or at the 81-bp hot spot region of the *rpoB* gene. Of the 740 MDR *M. tuberculosis* isolates with a single mutation in the *rpoB* gene, 91 (12.3%) were susceptible to RFB. Of the 91 RFB-susceptible isolates, 52 (57.1%) isolates with single mutations at codons 511, 516, and 522 can be confirmed directly using the GenoType MTBDR*plus* assay (Table 1). However, isolates with multiple mutations or deletions might be incorrectly interpreted as RFB susceptible (Table 2). Of the 47 isolates with multiple mutations and deletions, 21 isolates would not be misinterpreted as isolates with only a single mutation, including 12 isolates that had an absence of multiple probes at a discontinuous position, four isolates that were missing probes wt7 and wt8, two isolates missing probes wt3 to wt6, one isolate missing probes wt1 and wt2, one isolate missing probes wt1 to wt4, and one isolate missing probes wt4 and wt5. Nevertheless, 24 isolates were misinterpreted as isolates with single mutations and two isolates, including one with V144A and V146F and another with V146F and S164P, were misinterpreted as wild-type isolates (Table 2).

Only 1% of isolates with codon 531 mutations have other concurrent mutations, while the percentages of isolates with codon 511 or codon 146 were 70% and 45.5%, respectively (Table 3). In addition, 71.4% and 84.6% of isolates with either codon 511 or codon 516 mutations changed their susceptibilities to RFB. With the high multiple-mutation rate and high proportion of the above-mentioned isolates changing their susceptibilities, confirmation of RFB susceptibility by *rpoB* gene sequencing was recommended.

Consequently, we proposed an algorithm for identification of RFB-susceptible *M. tuberculosis* (Fig. 1). For group 1 samples with a single mutation and group 2 samples with specific single mutations at either codon 526 or 529, 100% of the samples were RFB susceptible. In this study, of the 104 RFB-susceptible isolates, 50% and 18.3% were identified from group 1 samples with a single mutation and group 2 samples with a single mutation at codon 526 (H to C, L, T, or N) and codon 529 (R to L), respectively (Fig. 1). Furthermore, according to clinical decisions or the patient's status, determination of the remaining 31.7% RFB-susceptible isolates could be optionally performed using conventional RFB DST. We have thus demonstrated that a line-probe assay in combination of *rpoB* gene sequencing could simultaneously identify TB, resistance to INH and RIF, and susceptibility to RFB.

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