Evaluation of the TB-Biochip Oligonucleotide Microarray System for Rapid Detection of Rifampin Resistance in *Mycobacterium tuberculosis*

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The TB-Biochip oligonucleotide microarray system is a rapid system to detect mutations associated with rifampin (RIF) resistance in mycobacteria. After optimizing the system with 29 laboratory-generated rifampin-resistant mutants of *Mycobacterium tuberculosis*, we evaluated the performance of this test using 75 clinical isolates of *Mycobacterium tuberculosis*. With this small sample set, the TB-Biochip system displayed a sensitivity of 80% and a specificity of 100% relative to conventional drug susceptibility testing results for RIF resistance. For these samples (~50% tested positive), the positive predictive value was 100% and the negative predictive value was 85%. Four of the seven observed discrepancies were attributed to rare and new mutations not represented in the microarray, while three of the strains with discrepant results did not carry mutations in the RIF resistance-determining region. The results of this study confirm the utility of the system for rapid detection of RIF resistance and suggest approaches to increasing its sensitivity.

The emergence of multidrug-resistant tuberculosis (MDR TB) presents a threat to global TB control efforts (15, 19, 20). MDR TB is defined as resistant to at least isoniazid and rifampin (RIF), the two most active first-line antimicrobial agents against TB (19, 20). Rapid and reliable drug susceptibility testing is essential for the prompt initiation of appropriate therapy, which rapidly renders patients noninfectious and hence facilitates infection control measures to halt transmission (1).

Rifampin is the mainstay of short-course chemotherapy for TB, and the loss of rifampin as an effective drug leads to a need for a longer duration of therapy and often to a lower cure rate (19, 20). In *Mycobacterium tuberculosis*, resistance to RIF results from mutations in the β subunit of RNA polymerase, which is encoded by the *rpoB* gene (2, 15). Approximately 95% of RIF-resistant strains carry mutations within the RIF resistance-determining region (RRDR), an 81-bp region carrying codons 507 through 533 of the *rpoB* gene (9, 10, 15). In this study, the TB-Biochip oligonucleotide microarray system (10) (Engelhardt Institute of Molecular Biology, Moscow, Russia) was used to detect RIF resistance among TB clinical isolates within a 24-hour period.

The TB-Biochip oligonucleotide microarray system is designed to detect and identify 29 codon substitutions and 1 codon deletion distributed over 10 codon positions within the RRDR (10) (Fig. 1). These 30 mutations are found in >90% of RIF-resistant strains that have mutations in the RRDR (3–6, 10, 15). Each element (an acrylamide gel pad) of the microarray contains an immobilized oligonucleotide whose sequence matches that of either a wild-type or mutated segment of the RRDR. The use of acrylamide gel pads reduces the cost of each microarray to less than five U.S. dollars and increases

the amount of oligonucleotide target that is present on the microarray, which increases the robustness of the hybridization reaction. Hybridization of the microarray with fluorescently labeled target DNA produces a spatial pattern of fluorescence intensities corresponding to the efficiencies of hybridization of the labeled target DNA to the various oligonucleotide probes. In the TB-Biochip system, the fluorescence intensities are recorded using a charge-coupled device camera, and the relative intensities of fluorescence for the elements representing wild-type sequences and mutant sequences for each codon are compared using imaging software and automated computer-assisted interpretation of hybridization results (3, 10). The isolate is designated RIF susceptible if the fluorescence of each of the wild-type elements is greater than the fluorescence of any of the corresponding mutant elements. The isolate is designated RIF resistant if the fluorescence of any one of the mutant elements is greater than the fluorescence of its corresponding wild-type element.

This study evaluated the performance of the TB-Biochip system in detecting RIF resistance in previously characterized laboratory-generated RIF-resistant mutants and in clinical isolates that were RIF resistant or RIF susceptible by routine drug susceptibility testing (DST). The presence of mutations in the *rpoB* gene among the RIF-resistant clinical isolates was confirmed by automated DNA sequencing.

MATERIALS AND METHODS

***M. tuberculosis* isolates.** A set of 29 in vitro-selected RIF-resistant mutants of *M. tuberculosis* strain H37Rv identified in a previous study (12), each containing a mutation represented in the TB-Biochip system, was obtained from the Mycobacteriology Laboratory Branch, Centers for Disease Control and Prevention (CDC). A total of 75 clinical *M. tuberculosis* isolates for which RIF resistance was tested by the modified agar proportion method (7) were obtained from the culture collection of the CDC Mycobacteriology Laboratory Branch. The isolates were selected randomly from those received by CDC for drug susceptibility testing and included 19 RIF-monoresistant isolates, 16 MDR TB isolates, and 40 susceptible isolates.

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507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533
Gly	Thr	Ser	Gln	Leu	Ser	Gln	Phe	Met	Asp	Gln	Asn	Asn	Pro	Leu	Ser	Gly	Leu	Thr	His	Lys	Arg	Arg	Leu	Ser	Ala	Leu
GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TGG	GCG	CTG
Del				Pro	Thr	Leu		Ile	Val					Leu					Tyr					Leu		Pro
GGC				CCG	ACC	CTA		ATA	GTC					TTG					TAC					TTG		CCG
				Arg	Arg	Lys			Tyr										Asp					Trp		
				CGG	CGC	AAA			TAC										GAC					TGG		
						Pro			Glu										Arg					Cys		
						CCA			GAG										CGC					TGT		
									Gly										Leu					Gln		
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FIG. 1. Codon substitutions and codon deletion in the RRDR region represented in the TB-Biochip.

Genomic DNA isolation. Briefly, 1.5 ml of a mid-log-phase broth culture was added to 150 μ l of buffered phenol (Invitrogen, Carlsbad, CA) and approximately 100 μ l zirconium beads in a 2-ml screw-top tube. The suspension was mixed by repeated inversion of the tube, left to settle for 10 min, and centrifuged at $20,800 \times g$ for 5 min at room temperature. The supernatant was discarded, and the pellet was resuspended in 400 μ l of extraction buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The resuspended cells were mechanically disrupted in a FastPrep instrument (Q-Biogene, Montreal, Canada) at setting 4 for 20 seconds. A volume of 400 μ l of chloroform-isoamyl alcohol (24:1) (Amresco, Solon, Ohio) was added to the lysate, and the mixture was repeatedly inverted for 15 seconds and then centrifuged at $20,800 \times g$ for 5 min at room temperature. The aqueous layer was transferred to a 1.5-ml microcentrifuge tube and stored at 4°C.

TB-Biochip analysis. DNA samples were analyzed according to the protocol provided by the manufacturer (Engelhardt Institute of Molecular Biology, Moscow, Russia). Briefly, the target DNA sequence containing the RRDR was amplified by a two-stage PCR. The presence of PCR products was confirmed by agarose gel electrophoresis after each reaction. The first-stage PCR amplified a 212-bp fragment of the *rpoB* gene, using 3 μ l of genomic DNA in a total reaction volume of 30 μ l, of which 1 μ l in a total reaction volume of 60 μ l was used for the second-stage PCR to amplify a 126-bp internal sequence containing the RRDR. The reaction mixture for the second-stage PCR contained a 10-fold higher concentration of fluorescently labeled forward primer to yield predominantly single-stranded DNA, which was subsequently hybridized with immobilized oligonucleotide probes on a microarray chip by dispensing 12 μ l of the labeled PCR product in hybridization buffer into the hybridization chamber and incubating the chip for 18 h at 37°C. The chip was washed with deionized water and air dried. The presence and nature of mutation in the RRDR were determined by analysis of the fluorescence intensity pattern on the chip, using a Chipdetector portable fluorescence analyzer equipped with Imageware software (Biochip-IMB, Moscow, Russia).

Sequencing of the *rpoB* gene. Sequencing of the RRDR was performed for all clinical isolates that were RIF resistant by conventional DST. A 688-bp *rpoB* gene fragment was amplified from genomic DNA by using the forward primer 5'-TCAGACCACGATGACCGTTCC-3' and the reverse primer 5'-GTCCATG TAGTCCACCTCAGACG-3'. The PCR product was purified and concentrated using DNA Clean and Concentrator-5 (Zymo Research, Orange, CA). Cycle sequencing was performed using a CEQ DTCS-Quick Start kit (Beckman Coulter, Fullerton, CA) in conjunction with either of two oligonucleotide primers, i.e., a forward sequencing primer (5'-TCGGCATGTCGCGGATGGAG-3') or a reverse sequencing primer (5'-GTACACGATCTCGTCTAACC-3'), to cover a 419-bp *rpoB* sequence containing the RRDR. The CleanSEQ sequencing

reaction clean-up system with SPRI paramagnetic beads (Agencourt, Beverly, MA) was utilized for removal of unincorporated dye terminator. Automated DNA sequencing was then performed using a CEQ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA). The BioEdit software package (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html> [version 7.0.5]) was used for sequence alignment.

The NI, NII, and cluster II and III regions of the *rpoB* genes of the three isolates lacking mutations in the RRDR were sequenced similarly, using the primers shown in Table 1.

RESULTS

As an initial step to characterize the performance of the TB-Biochip system, a set of 29 laboratory-generated mutants were identified that each contained a mutation that should be detected by the TB-Biochip system. The RRDR was efficiently amplified from each of the 29 strains by using the manufacturer's protocol, as judged by agarose gel electrophoresis of the first- and second-stage PCR products (data not shown). The hybridization, washing, and reading steps required little hands-on time, but reliable distinguishing of the fluorescence signals from positive and negative spots and the computer-assisted recognition of mutations often required manual adjustment of the exposure time. Once optimized, the TB-Biochip system was able to correctly identify all mutations in the RRDR among the 29 laboratory-generated mutants analyzed in the study. Including an overnight hybridization step, all steps, from PCR amplification through report generation, usually required less than 24 h.

To characterize the performance of the TB-Biochip system, a set of 75 clinical *M. tuberculosis* isolates were selected randomly from isolates received by CDC for drug susceptibility testing. The set included 35 RIF-resistant and 40 RIF-susceptible isolates. The results for the clinical isolates are summarized in Table 2. Mutations were detected by the TB-Biochip

TABLE 1. Primers used for this study

Region	Sequence (5'-3')		<i>rpoB</i> position ^a
	Forward	Reverse	
NI ^b	CTTCTCCGGGTCGATGTCGTTG	CGCGCTTGTCGACGTCAAACCTC	276–640
NII ^b	CGGTCACCGTGCTGCTCAAG	GCTGGCTGGTGCCGAAGAAC	680–1288
RRDR CII(p) ^c	TCAGACCACGATGACCGTTCC	GTCCATGTAGTCCACCTCAGACG	1035–1722
RRDR CII(s) ^d	TCGGCATGTCGCGGATGGAG	GTACACGATCTCGTCTAACC	1154–1572
CIII ^b	ACGGCGTGTTAGCGACGAG	GGTGCGCTCGTCTGCTC	1544–1792

^a Sequence numbering is based on *M. tuberculosis rpoB* (Rv0667; GenBank accession no. NC_000962).

^b For PCR amplification and sequencing.

^c For PCR amplification of cluster II.

^d For sequencing of cluster II.

TABLE 2. Results of conventional drug susceptibility testing and TB-Biochip testing

TB-Biochip result	No. of isolates with conventional DST result		
	Resistant	Susceptible	Total
Mutation present	28	0	28
Wild type	7	40	47
Total	35	40	75

system in 28 of the 35 RIF-resistant isolates. The locations of the most frequently detected mutations were, in order of decreasing frequency, codons 531, 516, and 526 (Table 3). No mutations were detected in 47 clinical isolates, which included all 40 susceptible isolates as well as 7 RIF-resistant isolates. Using this small sample set, the TB-Biochip system displayed a sensitivity of 80% and a specificity of 100% compared to conventional DST. For these samples (~50% tested positive), the positive predictive value was 100% and the negative predictive value was 85%.

To investigate the discrepant results, the RRDRs were sequenced for all 35 RIF-resistant isolates. Sequence analysis confirmed that the TB-Biochip Imageware software correctly identified the RRDR mutation in 27 of 28 RIF-resistant/TB-Biochip mutation-positive isolates, including one isolate bearing two mutations (Leu511→Arg and Asp516→Val). However, one RIF-resistant/TB-Biochip mutation-positive isolate yielded a hybridization pattern that was interpreted by Imageware software as the mutation Asp516→Val (GAC→GTC), with low discrimination at Met515, yet sequencing revealed instead the substitution Asp516→Phe (GAC→TTC) (6), which is not represented on the TB-Biochip. Of the seven RIF-resistant/TB-Biochip mutation-negative isolates, three contained no mutations in the RRDR and four contained mutations in the RRDR which were not represented on the TB-Biochip (Table 3). Of the four unrepresented mutations, one was a rare, previously reported deletion (5, 6), and three have not been described in the published literature. The NI, NII, and cluster II and III regions of the *rpoB* genes of the three isolates lacking mutations in the RRDR were sequenced, and no mutations were found in these regions.

DISCUSSION

The results of this study are in agreement with those of two previous evaluations of TB-Biochip's performance, which tested 130 RIF-resistant *M. tuberculosis* strains (10) and 168 MDR TB strains (3), confirming the utility of the system for rapid detection of RIF resistance. The complete TB-Biochip system may be suitable for use in clinical laboratories with molecular biology expertise because it requires relatively little hands-on time for experimental manipulations or data analysis, tests can be run individually or in batches, and specialized training is not required. Also, the TB-Biochip system should be affordable because it uses an inexpensive charge-coupled device camera to record results, and the microarrays are anticipated to cost 5 to 10 dollars each. Much of the cost of the system lies in the PCR amplification steps.

The test displays excellent specificity and good sensitivity,

TABLE 3. *rpoB* mutations in 32 RIF-resistant isolates

Mutation	Codon change	No. (%) of isolates
Ser531→Leu	TCG→TTG	16 (50)
Asp516→Val	GAC→GTC	4 (12)
His526→Tyr	CAC→TAC	2 (6)
His526→Leu	CAC→CTC	2 (6)
Ser531→Trp	TCG→TGG	1 (3)
His526→Asp	CAC→GAC	1 (3)
Leu511→Arg and Asp516→Val	CTG→CGG and GAC→GTC	1 (3)
Asp516→Phe ^{a,b}	GAC→TTC	1 (3)
Insertion ^{a,b}	CAC, between codons 512 and 513	1 (3)
Deletion ^{a,c}	CCA, third base of codon 516 and first two bases of codon 517	1 (3)
	TGG, second and third bases of codon 515 and first base of codon 516	1 (3)
	TCATGG, second and third bases of codon 514, codon 515, and first base of codon 516	1 (3)

^a RIF-resistant isolates with no mutation detected by TB-Biochip.

^b Previously described, rare mutations not present in TB-Biochip.

^c Previously undescribed mutations not present in TB-Biochip.

similar to those of other genetic tests for RIF resistance, such as line probe or molecular beacon tests (8, 11, 14, 16, 17). The observed discrepancies between the results of conventional DST and the TB-Biochip system (all were falsely called susceptible with the TB-Biochip system) likely result from the large number of mutations found in RIF-resistant isolates and the limited range of mutations included on the biochip. The TB-Biochip was designed to detect a set of mutations that accounts for the vast majority (>90%, depending on the population tested) of clinical RIF-resistant isolates that contain mutations in the RRDR (3–6, 10, 15). For example, in Russia, the TB-Biochip has been shown to detect >95% of mutations that occur in the RRDR in RIF-resistant isolates (3, 10). Less common mutations are known to occur within the *rpoB* gene (5, 6), and these are not represented in the microarray. For example, two rare mutations within the RRDR observed in this study, namely, Asp516→Phe (6) and a TTG deletion overlapping codons 515 and 516 (5), have both been reported in the literature but are not represented in the TB-Biochip. In addition, three previously undescribed mutations were also identified in this study (Table 3), including a CAC insertion between codons 512 and 513, a CCA deletion overlapping codons 516 and 517, and a TCATGG deletion within and overlapping codons 514 to 516. The unusual number of rare and unreported RRDR mutations seen in this study may reflect the source of the isolates. The isolates were selected randomly from isolates that had been referred to the CDC laboratory and may not be representative of isolates in the community.

Although most RIF resistance arises due to mutations in the RRDR, a small percentage (approximately 5%) of RIF-resistant isolates do not have mutations in the RRDR (2, 4). The mutations responsible for RIF resistance in such isolates may lie in other regions of the *rpoB* gene (e.g., NI, NII, or cluster II or III) or in as yet unidentified loci elsewhere in the chromosome (4, 9). Such mutations could account for RIF-resistant isolates that exhibited both wild-type TB-Biochip hybridization

patterns and RRDR sequences. However, in our study, none of the three mutants lacking mutations in the RRDR had mutations in the NI, NII, or cluster II or III region of the *rpoB* gene.

The inclusion of probes for additional mutations within the microarray could, in principle, further increase the overall sensitivity of the system, and the biochip system does have the ability to accommodate another 50 to 100 elements. However, the associated increase in production costs and potential for compromised specificity must be weighed against any gain in sensitivity. Furthermore, the performance of the system, particularly the positive and negative predictive values, and modifications thereof must be validated in the context of the particular patient populations for which their use is intended. Although this assay cannot replace conventional susceptibility testing, given the strong correlation between RIF resistance and isoniazid resistance (13, 15, 18), the high specificity of the system for detecting RIF resistance can facilitate the early diagnosis and treatment of MDR TB, particularly for patients with a history of prior TB treatment (20).

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Use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the Public Health Services, or the Centers for Disease Control and Prevention.

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