JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2004, p. 4850–4852 Vol. 42, No. 10
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.10.4850–4852.2004

Multiplex Detection of Mutations in Clinical Isolates of Rifampin-Resistant *Mycobacterium tuberculosis* by Short Oligonucleotide Ligation Assay on DNA Chips

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Received 23 March 2004/Returned for modification 28 April 2004/Accepted 8 June 2004

A new approach, short-oligonucleotide-ligation assay on DNA chip (SOLAC), is developed to detect mutations in rifampin-resistant *Mycobacterium tuberculosis*. The method needs only four common probes to detect 15 mutational variants of the *rpoB* gene within 12 h. Fifty-five rifampin-resistant *M. tuberculosis* isolates were analyzed, resulting in 87.3% accuracy and 83.6% concordance relative to DNA sequencing.

Tuberculosis (TB) is one of the major public health problems worldwide, and it has been more serious since the reemergence of multidrug-resistant *Mycobacterium tuberculosis* (2, 6).

Rifampin was believed to target the RNA polymerase of *M. tuberculosis* and thereby kill the organism by interfering in the transcription process (13). More than 95% of rifampin-resistant (Rif^r) *M. tuberculosis* strains carry mutations in an 81-bp rifampin resistance-determining region (RRDR) in the *rpoB* gene (9, 14, 15), making it a good target for molecular diagnosis.

Early diagnosis of TB and rapid testing of rifampin resistance are important to efficient treatment and control of drug-resistant TB. Conventional culture-based methods for drug susceptibility testing usually take more than 1 month. To detect drug-resistant *M. tuberculosis* strains more rapidly, methods based on molecular diagnosis have been developed (3, 7, 12, 14, 17), with which drug resistance detection could be completed in 1 day or in hours.

In a previous study, we designed a DNA chip using the principle of an oligonucleotide ligation assay for multiplex detection of single-nucleotide polymorphism (4). Based on this approach, here we report a new protocol, short-oligonucleotide-ligation assay on chip (SOLAC), for multiplex detection of DNA mutations in rifampin-resistant *M. tuberculosis*. The method needs only four biotin-labeled common probes to detect 15 mutational variants in the RRDR of the *rpoB* gene. Another advantage of the method is that the discrimination result is visualized directly through enzyme-linked assay. The method was evaluated by using a panel of *M. tuberculosis* isolates obtained from local clinical patients in Wuhan who were suspected of infection with rifampin-resistant *M. tuberculosis*.

***M. tuberculosis* strains and drug susceptibility test.** The 60 *M. tuberculosis* isolates were recovered from 60 unrelated patients with newly diagnosed pulmonary TB. They originated from the Wuhan region of China and were admitted to the hospitals of the Wuhan Tuberculosis Prevention and Cure Institute between 1998 and 2003. Species identification of the isolates was based on standard microbiological tests: colony morphology, acid-fast staining, and biochemical tests (18, 19). All clinical isolates were grown on Löwenstein-Jensen medium. Rifampin resistance tests were performed by the absolute concentration method described previously (10).

Preparation of DNA samples from *M. tuberculosis* cultures and PCR amplification. DNA from cultured cells was extracted as described previously (11). Two-milliliter aliquots of clear supernatant were used for PCR. A 130-bp segment of the *rpoB* gene that contains the RRDR region was amplified by PCR (forward primer, 5′-GCCGCGATCAAGGAGTTCTTC-3′; reverse primer, 5′-GCACGTTCACGTGACAGACC-3′). The amplification was carried out as follows: 5 min at 94°C; 30 cycles of 45 s at 94°C, 45 s at 57°C, 30 s at 72°C; and 5 min at 72°C. The PCR products were purified with E.N.Z.A cycle-pure kits purchased from Omega Bio-tek (Doraville, Ga.).

Oligonucleotide design and chip preparation. Twenty-five oligonucleotides were designed (Table 1), including four common (Com) probes, three extra (Ex) probes, and 18 allele-specific (A) probes. They were divided into three groups (groups 16, 26, and 31). DNA chips were prepared as described previously (4).

Multiplex detection of point mutations in the *rpoB* gene. All of the allele-specific probes were immobilized into the wells. Three sets of oligonucleotides corresponding to the three groups of allele-specific probes were prepared in tubes. The first set contained 1.0 μM 516Com1. The second set included 0.75 μM 526Com, 1 μM 531Ex1, and 0.5 μM 526Ex. The third set included 1.0 μM 516Com2, 1.5 μM 531Com, and 1.5 μM 531Ex2. The 130-bp PCR products were denatured at 100°C for 5 min and then cooled on ice for 5 min. After denaturation, PCR products (about 200 ng per 10 μl of reaction mixture) were mixed with other components (10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and T4 phage DNA ligase [3 U per 10 μl of reaction mixture]) and three sets of oligonucleotides. Ligation reactions were performed at three temperatures. Optimal reaction temperatures for the reactions containing the first, second, and third set of oligonucleotides were 26, 30, and

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TABLE 1. Probes designed to detect mutations in the *rpoB* gene

Amino acid position and probe[a]	Size	Group	Sequence (5′→3′)[b]
516			
516Com1	9		Biotin-GTTGTTCTG
516Com2	15		Biotin-CAGCGGGTTGTTCTG
516Wt	15	16	$_{p}$GTCCATTTTTTTTT*T*
516A1	15	16	$_{p}$G**A**CCATTTTTTTTT*T*
516A2	16	31	$_{p}$GTACAATTTTTTTTT*T*
516A3	15	16	$_{p}$G**C**CCATTTTTTTTT*T*
516A4	15	16	$_{p}$**C**ACCATTTTTTTTT*T*
526			
526Com	15		Biotin-CGACAGTCGGCGCTT
526Wt	15	26	$_{p}$GTGGGATTTTTTTT*T*
526A1	15	26	$_{p}$GT**A**GGTTTTTTTTT*T*
526A2	15	26	$_{p}$GT**C**GGTTTTTTTTT*T*
526A3	16	26	$_{p}$GT**T**GGTTTTTTTTT*T*
526A4	16	26	$_{p}$G**A**GGGTTTTTTTTT*T*
526A5	16	26	$_{p}$G**C**GGGTTTTTTTTT*T*
526A6	16	26	$_{p}$**T**TGGGTTTTTTTTT*T*
526A7	16	26	$_{p}$**C**TGGGTTTTTTTTT*T*
526Ex	15		TCAACCCCGACAGCG
531			
531Com	15		Biotin-CCCCAGCGC
531Wt	15	31	$_{p}$CGACATTTTTTTTT*T*
531A1	15	31	$_{p}$C**A**ACATTTTTTTTT*T*
531A2	15	31	$_{p}$C**C**ACATTTTTTTTT*T*
531A3	15	31	$_{p}$CG**G**CATTTTTTTTT*T*
531A4	16	31	$_{p}$**A**GACAGTTTTTTTTT*T*
531Ex1	11		AGACCGCCGGG
531Ex2	15		CGCTTGTGGGTCAA

[a] Com, common probe; Wt, wild-type probe; A, allele-specific probe; Ex, extra probe.
[b] The italic "*T*" was modified $(CH_2)_6$-S-S-$(CH_2)_6$-(PO_4) on the 3′ end. The common probes contained biotin labels on their 5′ ends. The allele-specific probes contained 5′-phosphate groups and the discriminating bases near their 5′ ends (in bold). Subscript p, 5′-phosphate group.

21°C, respectively. Ligation products were visualized by avidin-alkaline phosphatase conjugates as previously described (4).

DNA sequencing of the *rpoB* gene. A 442-bp fragment including the RRDR region of the *rpoB* gene was sequenced after PCR amplification in order to analyze the mutations associated with rifampin resistance. The PCR was performed by using another pair of primers: forward primer, 5′-CAGAC CACGATGACCGTTCC-3′; reverse primer, 5′-GAGCCGAT CAGACCGATGTT-3′. The amplification products were directly sequenced by Shanghai CASarray Co., Ltd.

The optimal ligation temperatures of short allele-specific oligonucleotides cannot be estimated by biological analysis software. To optimize the reaction temperature for multiplex ligation, three common mutants (with 516GAC→GTC, 526CAC→TAC, and 531TCG→TTG substitutions) were constructed. In addition, DNA samples from 15 Rif^r strains with known sequences (obtained from Wuhan Tuberculosis Prevention and Cure Institute) were also used in the optimization process. The G+C content of the nucleotides, the length of allele-specific oligonucleotides, and the length of the common probes were the main factors being adjusted in optimization. After optimization, the allele-specific probes were divided into three groups according to their optimal ligation temperatures.

A total of 25 probes were designed, including 18 allele-specific short oligonucleotides, four common probes, and three extra probes. In a previous study, we found that sometimes extra probes were needed to enhance ligation efficiency (4).

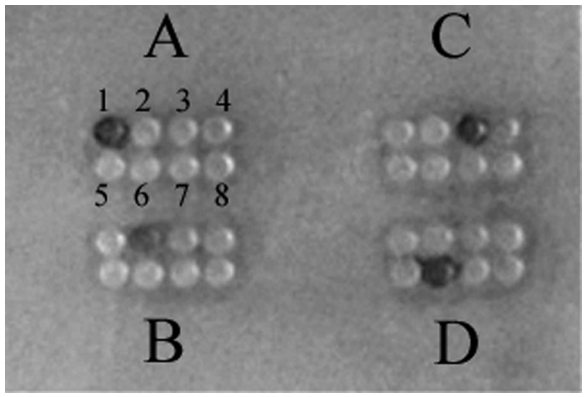

FIG. 1. Multiplex detection of point mutations in codon 526 of the *rpoB* gene in rifampin-resistant *M. tuberculosis*. (A) Probes 526Wt, 526A1, 526A2, 526A3, 526A4, 526A5, 526A6, and 526A7 were immobilized onto loci 1, 2, 3, 4, 5, 6, 7, and 8, respectively. Mutations in codon 526 of the *rpoB* gene in four rifampin-resistant *M. tuberculosis* clinical isolates (1, 8, 13, and 54) were detected at four separate arrays (A, B, C, and D). Reactions were performed at 30°C for 2 h. No mutation in codon 526 was found in one isolate, and three substitutions in codon 526 (C→T, C→G, and A→G) were detected in the other three isolates (B, C, and D, respectively). All the results were verified by DNA sequencing.

The three extra probes were designed for this purpose. The 18 allele-specific probes were covalently immobilized on the glass slides to form DNA chips, with which 15 mutational variants of the *rpoB* gene could be detected within 12 h. These variants are found in nearly 85% of all mutants whose rifampin resistance is caused by mutations in the RRDR of the *rpoB* gene. The 130-bp PCR products from 60 clinical isolates of *M. tuberculosis*, 55 known to be rifampin resistant and 5 known to be rifampin sensitive, were scanned for mutations by the SOLAC assay. Among the 55 rifampin-resistant isolates, 47 were found to harbor point mutations belonging to seven types (Fig. 1). In addition, a deletion mutation in one isolate was also found by this method (data not shown). Subsequent DNA sequencing of

TABLE 2. Detection of *rpoB* gene mutations of *M. tuberculosis* in Wuhan by SOLAC compared with DNA sequencing results

Number of strains	Rifampin resistance[a]	Mutations[b]	
		SOLAC assay	DNA sequencing
26	R	531 TCG→TTG	531 TCG→TTG
3	R	531 TCG→TGG	531 TCG→TTG
7	R	526 CAC→TAC	526 CAC→TAC
3	R	526 CAC→GAC	526 CAC→GAC
3	R	526 CAC→CGC	526 CAC→CGC
2	R	516 GAC→TAC	516 GAC→TAC
3	R	516 GAC→GTC	516 GAC→GTC
1	R	531 TCG→TTG	531 TCG→TTG, 511 CTG→TTG
1	R	526 negative[c]	526 CAC→AC, one base deletion
1	R	WT	511 CTG→CGG
2	R	WT	513 CAA→CTA
1	R	WT	533 CTG→CCG
3	R	WT	WT
1 (H37Rv)	S	WT	WT
5	S	WT	WT

[a] R, resistant; S, sensitive.
[b] WT, wild type.
[c] Negative, no results of ligation could be seen.

the RRDR in the *rpoB* gene confirmed mutations within these isolates (Table 2). Thus, the accuracy of the SOLAC assay was 87.3% (48 of 55), and the concordance between the SOLAC results and those of DNA sequencing was 83.6% (46 of 55). No mutations were found in six rifampin-sensitive isolates, including H37Rv, by SOLAC; the results were fully concordant with those of DNA sequencing.

Seven Rif^r isolates that were verified to be rifampin resistant by conventional susceptibility testing gave false wild-type results by the SOLAC assay, which implies that this method has limitations in the following situations: (i) some mutations, e.g., L511N, Q513L, and L533P, are outside the detection range; and (ii) sometimes no mutation exists in the RRDR of the *rpoB* gene even though the isolates are resistant to rifampin (other resistance mechanisms, such as a permeability barrier or drug efflux pumps, probably exist). Similar results were reported from independent investigations using different methods (1, 5, 8, 16).

In spite of its limitations, the SOLAC assay obviously has a number of advantages. First, the ligation condition is nonstringent, enabling the typing of multiple nucleotide substitutions in a single assay yet requiring specificity at the site of interest. Second, the assay is highly adaptable: additional oligonucleotides can be easily incorporated to detect more mutations in the target DNA. Third, the SOLAC method needs much less common probe than the conventional oligonucleotide ligation assay in detection of multiple DNA mutations. This feature further facilitates multiplex detection of DNA mismatches in a single reaction in combination with DNA chips. Furthermore, this assay is simple to perform and interpret and does not require expensive equipment or technical expertise. Finally, either enzyme labeling or fluorescent labeling can be incorporated into the common probes to generate positive signals, allowing the sensitive and automatic scanning of a large number of clinical samples with or without specific equipment.

Support from the Ministry of Science and Technology (project number 2001BA756C) and Chinese Academy of Sciences is acknowledged.

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