

Rapid Detection of Isoniazid and Rifampin Resistance Mutations in *Mycobacterium tuberculosis* Complex from Cultures or Smear-Positive Sputa by Use of Molecular Beacons

S.-Y. Grace Lin, William Probert, Melanie Lo, and Edward Desmond*

Microbial Diseases Laboratory, California Department of Health Services, Richmond, California 94804

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The slow-growing nature of *Mycobacterium tuberculosis* complex hinders the improvement of turnaround time for phenotypic drug susceptibility testing. We designed a set of molecular beacons for the detection of isoniazid and rifampin resistance mutations in *M. tuberculosis* complex organisms from cultures or from *N*-acetyl-L-cysteine-NaOH-treated, smear-positive specimens. The performance of the molecular beacons was characterized by studying a total of 196 clinical isolates (127 drug-resistant isolates and 69 drug-susceptible isolates). For detection of isoniazid resistance, the sensitivity and specificity of the assay were 82.7 and 100%, and the positive predictive value (PPV) and negative predictive value (NPV) at a resistance prevalence of 10% were 100 and 98.11%, respectively. For detection of rifampin resistance, the sensitivity and specificity of the assay were 97.5 and 100%, and the PPV and NPV at a resistance prevalence of 2.0% were 100 and 99.95%, respectively.

Isoniazid (INH) and rifampin (RIF) are the backbone of the standard regimen for treating tuberculosis (TB), and resistance to these drugs indicates a necessity for alteration of the standard regimen (19). In order to treat patients properly and to reduce further spread of drug-resistant TB, it is clinically important to provide drug susceptibility results to health care providers as soon as possible. With the slow growth rate of *Mycobacterium tuberculosis* complex, the average turnaround time of drug susceptibility results obtained with the current phenotypic methods is 4 weeks or more from specimen receipt (4). A more rapid alternative to the current phenotypic drug susceptibility testing is greatly needed.

Since the early 1990s, genes associated with INH and RIF resistance have been studied and identified (16, 17, 20, 26). While over 95% of RIF resistance is due to mutations in the 81-bp core region of *rpoB* (6, 22, 23), only 80 to 90% of INH resistance has been found to be associated with mutations in *kaiG*, *inhA*, *ahpC*, and *ndh* (7, 10, 13, 23), collectively. The molecular beacon (MB), with its unique stem-loop structure, has proven to be an excellent tool for detection of single nucleotide polymorphisms (1, 11, 24, 25). When coupled with the real-time PCR technology, MB probes provide a method for rapid detection of drug resistance mutations without the risk of amplicon-carryover contamination.

The primary goal of this study was to develop a procedure using a minimal number of MBs for detection of INH and RIF resistance and improve the turnaround time of INH and RIF susceptibility testing. Here, we describe a procedure with a set of five MBs capable of detecting the most prevalent mutations associated with INH and RIF resistance in *M. tuberculosis* complex. For INH resistance, an MB targeting a region encompassing codon 315 of *kaiG* and an MB targeting the promoter region of *inhA* were designed. For RIF resistance, we

adopted one of the MBs designed by Piatek et al. (15) and designed two new MBs targeting the core region of *rpoB*. The performance of these MBs was characterized by studying 196 cultures from solid media. We also evaluated this assay for testing positive cultures grown in Mycobacterium Growth Indicator Tubes (MGIT), since the time to detection of a positive *M. tuberculosis* complex culture from liquid media is shorter than that from solid media. In addition, this assay was applied for testing smear-positive clinical specimens with the purpose of obtaining INH and RIF susceptibility results within 24 h of specimen receipt, thus maximizing the benefit of employing this method.

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MATERIALS AND METHODS

Archived strains for characterization of MB performance. One hundred twenty-seven drug-resistant isolates and 69 drug-susceptible isolates from 196 different TB patients were studied. These isolates were either cultured at the Microbial Diseases Laboratory in the California Department of Health Services or submitted to our laboratory by the California County Public Health Laboratories and Pacific islands laboratories during the period 1998 to 2001. Among the 127 resistant isolates, 22 were resistant to only INH, 24 were resistant to at least INH but not RIF, and 81 were resistant to at least INH and RIF. The phenotypic drug susceptibility assays were performed either by using the BACTEC radiometric method (21) or the agar proportion method (8).

MGIT cultures. A total of 31 cultures consisting of 26 naturally occurring positive MGIT cultures inoculated from *N*-acetyl-L-cysteine (NALC)-NaOH-treated clinical specimens (8) and 5 seeded INH-RIF-resistant cultures were studied. For each seeded culture, a 1:20,000 dilution of a McFarland 0.5 cell suspension was prepared in sterile water, 0.5 ml of which was used for seeding each MGIT. The average time to detection by the MGIT 960 for the seeded cultures was 12 days, which was equivalent to that for naturally occurring positive cultures inoculated with routine clinical specimens. When MGIT cultures are flagged positive by the MGIT 960, the organism loads in seeded cultures resemble those in naturally occurring positive cultures. Upon the confirmation of acid-fast bacilli with Ziehl-Neelsen acid-fast staining, positive MGIT cultures were immediately subjected to DNA extraction or refrigerated at 2 to 8°C until the time of DNA extraction.

* Corresponding author. Mailing address: Microbial Diseases Laboratory, CDHS, 850 Marina Bay Pkwy., Richmond, CA 94804. Phone: (510) 412-3781. Fax: (510) 412-3706. E-mail: edesmond@dhs.ca.gov.

TABLE 1. MB sequences

MB	Target (codons)	5' stem	Loop	3' stem
MBkatG	<i>katG</i> (312–317)	CCCCTT	GCGATCACCAGCGGCA	AACGGG
MBinhA	<i>inhA</i> promoter	CGCACT	CCCACAACTATCGTCTCG	AGTGCG
MB516	<i>rpoB</i> (511–518)	CGCGA	GAGCCAATTCATGGACCAGAAC	TCGCG
SW112 ^a	<i>rpoB</i> (522–527)	CCACG	CTTGTGGGTCAACCCC	CGTGG
MB531	<i>rpoB</i> (529–534)	CCGAGG	CCCCAGCGCCGACAGT	CCTCGG

^a This MB was designed by Piatek et al. (15).

Smear-positive specimens. Sixteen smear-positive specimens tested in the study were sediments of NALC-NaOH-treated clinical specimens. Twelve specimens were processed at our laboratory, three specimens were from the Alameda County Public Health Laboratory, and one specimen was from the Monterey County Public Health Laboratory. Ten of these specimens were graded 3+ or 4+ in auramine-rhodamine-stained smears, and the remaining six specimens were graded 1+ or 2+ (8).

DNA extraction. A 10- μ l loopful of mycobacterial growth from Lowenstein-Jensen slants of the 196 archived isolates was emulsified in 500 μ l of LAL reagent water (Cambrex, Rockland, Maine) in a 1.5-ml microcentrifuge tube and heated at 95 to 100°C for 20 min to lyse the bacteria. The heated cell suspensions were centrifuged at 10,000 rpm for 2 min using an Eppendorf 5415C microcentrifuge, and the supernatants were stored at –70°C until MB testing.

For positive MGIT cultures, 1 ml of the medium with bacterial growth was transferred from the bottom of an undisturbed, positive MGIT into a 1.5-ml microcentrifuge tube and centrifuged at 14,000 rpm for 5 min using an Eppendorf 5415C microcentrifuge. The pellet was resuspended in 100 μ l of LAL reagent water and subjected to the heat extraction procedure described above.

For NALC-NaOH-treated, smear-positive specimens, the sediment remaining after inoculation for mycobacterial culture was transferred into a 1.5-ml microcentrifuge tube and centrifuged at 14,000 rpm for 5 min in an Eppendorf 5415C microcentrifuge. The pellet was resuspended in 60 μ l of LAL reagent water and subjected to the heat extraction procedure described above.

Purified DNA from strain H37Rv was used in experiments for determining analytical sensitivity. The DNA purification procedure was performed with the DNeasy tissue kit (QIAGEN Inc., Valencia, Calif.) following the manufacturer's instructions.

MBs and primers. Five MBs (Table 1) were employed for detecting INH and RIF resistance mutations, two MBs for INH and three MBs for RIF. Four of these MBs (MBkatG, MBinhA, MB516, and MB531) were designed by our laboratory, and one MB (SW112) was previously designed by Piatek et al. (15). Wild-type sequences of *M. tuberculosis* complex were used in designing the loop portion of the MBs. The stem portion of the MBs consisted of 5 to 7 nucleotides that were self-complementary but not complementary to the wild-type sequence. DNA folding structures for each MB were evaluated using the DNA folding server program provided by M. Zuker (www.bioinfo.rpi.edu/~zukerm). The MBs, modified with 5'-fluorescein and 3'-dabcyl were manufactured by Integrated DNA Technologies, Inc. (Coralville, Iowa). The primers for the target sequences of *katG*, *rpoB*, and the *inhA* promoter region were designed by other investigators (14, 15) and manufactured by QIAGEN Operon, Inc. (Alameda, Calif.).

Real-time PCR. The MB experiments were performed with an iCycler iQ real-time detection system (Bio-Rad, Hercules, Calif.) using the 96-well format and the iCycler iQ software, version 2.3. For each 50- μ l reaction mixture, in addition to 5 μ l of DNA template, the final concentration of each reagent was as follows: 1 \times AmpliTaq Gold PCR buffer, 1.25 mM deoxynucleoside triphosphates, 4.0 mM MgCl₂, 2.5 U of AmpliTaq Gold enzyme (Applied Biosystems,

Foster City, Calif.), a 0.5 μ M concentration of each primer, and a 0.3 μ M concentration of an MB. To improve the discriminatory power of MB531, dimethyl sulfoxide was added to a final concentration of 8%. The amplification parameters included an initial activation of the AmpliTaq Gold enzyme at 95°C for 10 min, followed by 45 cycles (or 50 cycles for testing smear-positive specimens) of 95°C for 15 s, 62°C for 30 s, and 72°C for 20 s. The charge-coupled device in the iCycler recorded the fluorescence generated in each cycle during the 30-s annealing period at 62°C.

DNA sequencing. DNA sequencing was performed to resolve discrepant results between the MB assay and the phenotypic drug susceptibility testing and for confirmation of dual mutations associated with either INH or RIF resistance as detected by multiple MBs. In addition, DNA sequencing was also performed for investigation of mutations in *ndh* and the *oxyR-ahpC* intergenic region on INH-resistant isolates in which no mutations in *katG* or the *inhA* promoter region were demonstrated. Following amplification for the sequences of interest, cycle sequencing was performed using dRhodamine dye terminators and the sequences were analyzed on an ABI 377 sequencer (Applied Biosystems) as previously described (5). The primers for amplification and sequencing of *rpoB*, *katG*, *ndh*, the *inhA* promoter region, and the *oxyR-ahpC* intergenic region are listed in Table 2.

RESULTS

Sensitivity, specificity, PPV, and NPV. A total of 196 archived clinical isolates, including 46 INH-resistant isolates, 81 multidrug-resistant isolates, and 69 susceptible isolates, were tested. Mutations were detected by MBkatG in 78 (61.24%) isolates, by MBinhA in 29 (22.83%) isolates, by MB516 in 10 (12.34%) isolates, by SW112 in 23 (28.40%) isolates, and by MB531 in 48 (59.26%) isolates. Of the 127 INH-resistant isolates, dual mutations were detected in two isolates, while no mutations were detected in 22 isolates, by both MBkatG and MBinhA. These findings were verified by DNA sequencing of a 395-bp fragment of *katG* and a 451-bp fragment of the *inhA* promoter region. For detection of RIF resistance, of the 81 RIF-resistant isolates dual mutations were detected in two isolates, one by MB516 and MB531 and the other by MB531 and SW112. No mutations were detected in two isolates by the three MBs, MB516, MB531, and SW112. DNA sequencing of a 342-bp fragment of the *rpoB* core region confirmed the lack of a mutation in one isolate and revealed a 3-nucleotide deletion within codons 519 and 520 for the other isolate. In sum-

TABLE 2. Primer sequences for DNA sequencing

Gene	Forward primer	Reverse primer
<i>ahpC</i> ^a	AGCAGTGGCATGACTCTC	CGGCCGGCTAGCACCTCT
<i>inhA</i>	CGAGCGTAACCCAGTGCGAAAGT	CCCCGGTGAGGTTGGCGTTGAT
<i>katG</i>	TCGGCGGTCACACTTTCGGTAAGA	GCGACGCGTGATCCGCTCATAG
<i>ndh</i>	AGAAGCTGGGCGTGGAATC	GGGTACCCGGGAATGGACA
<i>rpoB</i> ^b	CGACCACTTCGGCAACCG	TCGATCGGGCACATCCGG

^a The primers were designed by Piatek et al. (14).

^b The primers were designed by Kim et al. (9).

TABLE 3. Summary of MB results

Group	Phenotypic results		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	No. resistant	No. susceptible				
INH						
Mutations detected	105	0	82.67	100	100	98.11 ^a
No mutations detected	22	69				
RIF						
Mutations detected	79	0	97.53	100	100	99.95 ^b
No mutations detected	2	69				

^a Calculated for a prevalence of 10% INH resistance.

^b Calculated for a prevalence of 2% RIF resistance.

mary, the MB assay correctly identified 105 out of the 127 INH-resistant isolates and 79 out of the 81 RIF-resistant isolates, and no mutations were detected in the 69 susceptible isolates. The sensitivity and specificity of the assays were 82.67 and 100% for INH and 97.53 and 100% for RIF, respectively. In California, the INH resistance prevalence has been at approximately 10% for the past decade, at which the calculated positive predictive value (PPV) and negative predictive value (NPV) were 100 and 98.11%, respectively. As for RIF resistance, the prevalence rate has been below 2%, at which the calculated PPV and NPV were 100 and 99.95%, respectively (Table 3).

Mutations in *ndh* and the *oxyR-ahpC* intergenic region associated with INH resistance. We explored the possibility of mutations in *ndh* and the *oxyR-ahpC* intergenic region for the 22 INH-resistant isolates in which no mutations in the target sequences of MBkatG or MBinhA were demonstrated. For these isolates, we sequenced a 235-bp fragment of *ndh* and a 136-bp fragment of the *oxyR-ahpC* intergenic region where predominant mutations associated with INH resistance have been reported (10, 14). We found one isolate possessing a point mutation at codon 263 (GAC→TAC) of *ndh* and six isolates possessing point mutations in the *oxyR-ahpC* intergenic region at positions -48 to -54, relative to the ATG start codon of *ahpC*.

Analytical sensitivity. Experiments for analytical sensitivity were performed by testing a series of 10-fold dilutions of purified H37Rv DNA, from 1 ng/μl to 1 fg/μl, with each MB. The analytical sensitivity revealed the detection limit of the assay and the feasibility of detecting mutations from samples containing lower organism loads, such as growth from liquid media or NALC-NaOH-treated specimen sediments. The results showed that the analytical sensitivity was 100 fg/μl for MBkatG and MB531 and 10 fg/μl for MBinhA, MB516, and SW112.

Positive MGITs. The concentration of DNA extracted from a fresh, positive MGIT culture was estimated as 10 to 100 pg/μl by comparison to the C_T value (threshold-crossing cycles) from a titration of purified H37Rv DNA (data not shown). This estimated DNA concentration was above the analytical sensitivity (100 fg/μl) of the MB assay. To evaluate the assay for testing positive MGITs, we studied 31 samples, including 5 seeded, resistant cultures and 26 positive routine cultures with unknown drug susceptibility. The MB assay detected mutations in all five seeded, resistant cultures and identified one culture resistant to INH, one culture resistant to INH and RIF, and 24 cultures susceptible to both INH and RIF from the 26 un-

knowns. These results were verified with BACTEC drug susceptibility tests.

Smear-positive sediments. The applicability of the MB assay for detection of INH and RIF drug resistance on smear-positive specimens was evaluated by testing 16 clinical specimen sediments processed with the NALC-NaOH procedure. For these specimens, 10 of which were graded ≥3+ and 6 of which were graded 1+ or 2+ in auramine-rhodamine-stained smears, no mutations were detected by the MB assay. These results were confirmed in 15 out of 15 specimens by BACTEC drug susceptibility testing. One specimen failed to grow, and the MB results could not be confirmed.

DISCUSSION

The initial objective of this study was to verify the performance of the MBs designed by other investigators (14, 15) for detection of INH and RIF resistance. We were unable to generate satisfactory results with those MBs. Since it was unclear whether the quality of the MBs or differences in instrumentation were the causes for the unsatisfactory results, we decided to design new MBs for this study.

MB probes can be an excellent tool for detection of drug resistance when genes associated with drug resistance are well established and the mutations in each of those genes are localized within a short stretch of nucleotide sequence. For detection of RIF resistance, due to the fact that over 95% of mutations are localized to the 81-bp core region of *rpoB* and the excellent performance of the MBs used in this study, the sensitivity, specificity, PPV, and NPV for the detection of RIF resistance were very high. Unlike RIF resistance, mechanisms for INH resistance are more complex and not fully understood. As a consequence, the sensitivity of a molecular assay for detection of mutations conferring INH resistance is determined not only by how well an assay can detect mutations in the genes of interest but also by the collective prevalence of mutations present in those genes among the test strains. In our study, the two MBs, MBkatG and MBinhA, detected mutations in 105 out of the 127 INH-resistant isolates and no mutations in 22 INH-resistant isolates. As evidenced by DNA sequencing, the failure of detecting mutations in these 22 isolates was due to the absence of mutations in the target sequences of these MBs. Even though the two MBs were able to detect all mutations that were present in their target sequences, the sensitivity of the MB assay for detection of INH resistance was only 82.67% (105 of 127). Thus, the sensitivity

(82.67%) reflects the collective prevalence of mutations in the target sequences of *katG* and the *inhA* promoter region of the strains tested. It also represents the ultimate sensitivity this assay could achieve for testing strains in our region. In areas where the prevalence of mutations in these target sequences is higher, such as Russia, in which 94% of INH-resistant strains contain mutations in codon 315 of *katG* (12), the sensitivity of the MB assay would be expected to increase.

As for NPV, it is influenced by the sensitivity of an assay and the resistance prevalence in a population (2). In California, the INH resistance prevalence is approximately 10%, and the calculated NPV is 98.11%. At the current resistance prevalence, the NPV is high enough to make this assay applicable for diagnostic usage in testing the general population of California. However, the NPV would be lower when testing a population with a higher INH resistance rate.

Mutations in sequences other than *katG* and *inhA*, in particular those found in *ndh* and the *oxyR-ahpC* intergenic region, have been associated with INH resistance (10, 14). For the 22 INH-resistant isolates in which no mutations were detected by MBkatG and MBinhA, we found only one isolate possessing a point mutation in *ndh*, although a mutation rate of 9.5% in *ndh* has been reported for INH-resistant isolates in Singapore (10). This may indicate that mutations in *ndh*, independent of mutations in *katG* and the promoter region of *inhA*, are rare in our region. In addition, we found six isolates possessing point mutations within a short stretch of 7 nucleotides of the *oxyR-ahpC* intergenic region. The addition of an MB for this intergenic region may have increased the sensitivity of the assay from 82.67 to 87.40%, with a slight improvement of the NPV from 98.11 to 98.62%.

For detection of RIF resistance, instead of using five MBs as reported by other investigators (15), we employed three MBs, MB516, SW112, and MB531, which were able to detect 97.53% of mutations conferring RIF resistance. With the addition of an MB encompassing codons 519 to 521, where the existing MBs failed to detect mutations in one of the RIF-resistant isolates, the sensitivity may have increased from 97.53 to 98.77%, with a negligible improvement of the NPV from 99.95 to 99.97%. This would not yield a significant impact on the performance of this assay.

Of the 81 RIF-resistant isolates, dual mutations associated with RIF resistance were detected in two isolates by multiple MBs. In one isolate, DNA sequencing confirmed mutations associated with the target sequences of MB516 and MB531. For the other isolate in which mutations were detected by MB531 and SW112, DNA sequencing revealed a 4-nucleotide mutation in codons 525, 526, and 527 within the target sequence of SW112, but no mutations were found within that of MB531. One possible explanation for this discrepancy might be the formation of a GC-rich, 25-nucleotide self-complementary segment resulting from the 4-nucleotide mutation. The secondary structure associated with the mutated region may be thermodynamically favored and may have prevented efficient hybridization of MB531 to its target sequence. In spite of the aberrant performance of MB531 with this particular isolate, resistance to RIF was correctly identified by SW112. Therefore, the sensitivity and the specificity of the assay were not affected.

The ultimate goal of this study was to test NALC-NaOH-

treated specimen sediments so that susceptibility results of INH and RIF could be obtained within 24 h of specimen receipt. Our results indicated that this assay was of sufficient sensitivity to test smear-positive sediments graded $\geq 1+$ in auramine-rhodamine-stained smears. We did not test sediments containing rare acid-fast organisms in this study. Given the analytical sensitivity determined in this study, conclusive test results for specimens with rare acid-fast organisms observed in auramine-rhodamine-stained smears may not be achievable.

In recent years a variety of new approaches (both phenotypic and genotypic assays) to improve the turnaround time of drug susceptibility testing of *M. tuberculosis* have been explored and investigated (18). The luciferase reporter phage assay, with its capability of providing drug susceptibility results within 2 to 4 days, is one of the fastest phenotypic assays (3). However, the procedure, involving several manipulations of rather high concentrations of actively growing *M. tuberculosis* organisms, creates a safety concern. In contrast the MB assay, a genotypic approach, does not require viable organisms. The risk of contracting tuberculosis for laboratory personnel may be reduced, since after DNA extraction by a simple heat extraction procedure specimens are no longer viable or infectious. In addition, the MB assay is rapid, sensitive, and highly specific. It can provide INH and RIF susceptibility results within 3 h, and it can be performed directly on NALC-NaOH-treated specimen sediments. Due to the primer specificity and the MB discriminatory power, it can be performed on contaminated cultures without the time-consuming procedure of obtaining pure cultures required for phenotypic methods.

Despite the excellent performance of this assay, a few issues may need to be considered when applying this assay in diagnostic or public health laboratories. (i) Mixed populations of *M. tuberculosis* complex: we tested preparations with different relative proportions of DNA from susceptible and resistant strains. The MB assay could detect a susceptible population when it constituted as little as 25 to 50% of a mixed population (data not shown). Therefore, a mixed population may be interpreted as susceptible. (ii) Silent mutations: this assay detects single-nucleotide mutations, but it does not discern missense mutations from silent mutations. A strain with a silent mutation will be interpreted as resistant. Although we did not encounter isolates containing silent mutations during this study, theoretically and perhaps remotely they may exist. (iii) Quality and stability of reagents: during the course of our study, we experienced the impact of lot-to-lot variations in MBs due to a change in the source of the fluorophore by the manufacturer. The consistency and quality of reagents may be one of the major factors affecting the long-term performance of the assay. We also experienced instability of the MB solutions, which may have been due to repeated thawing and freezing. Our remedy to this problem was to make only a few (five to seven) aliquots of MB working solutions at a time in small volumes from stock solutions (10 \times of the working solution) and to limit thawing and freezing to no more than five times for each aliquot. With this practice, the MB stock solutions have demonstrated sustained stability at -20°C for over 17 months and the working solutions over 6 months.

In conclusion, the MB probes described in this report recognize the most prevalent mutations associated with INH and

RIF resistance, and the assay provides a simple and rapid method for detection of these mutations. At present, with its limitation in detecting only INH and RIF resistance, this assay is not intended to replace the phenotypic drug susceptibility testing; rather, it can be used as a supplementary test for cases when rapid turnaround time of INH and RIF results is critical. We have worked closely with TB control personnel and effectively applied MB testing under the following conditions: (i) when a suspected multidrug-resistant TB case was smear positive, (ii) when a suspected TB case was smear positive and of significant epidemiological impact or of critical concern in a clinical setting, (iii) for a relapsed TB case or a TB case under treatment in which the smear remained positive, (iv) when a specimen from an untreated patient was smear positive but culture negative—this situation may occur when a specimen is delayed in transportation or not properly transported, and (v) when an *M. tuberculosis* complex culture was contaminated or mixed with non-TB mycobacteria. In these situations, rapid turnaround times for INH and RIF susceptibility results are provided, which enable early initiation of proper regimens for the TB patients and proper prophylaxis for their contacts, and better TB control is anticipated.

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