Rapid Detection of Rifampin Resistance in Mycobacterium tuberculosis Isolates from India and Mexico by a Molecular Beacon Assay

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Received 15 April 2004/Returned for modification 21 May 2004/Accepted 2 August 2004

We assessed the performance of a rapid, single-well, real-time PCR assay for the detection of rifampin-resistant Mycobacterium tuberculosis by using clinical isolates from north India and Mexico, regions with a high incidence of tuberculosis. The assay uses five differently colored molecular beacons to determine if a short region of the M. tuberculosis rpoB gene contains mutations that predict rifampin resistance in most isolates. Until now, the assay had not been sufficiently tested on samples from countries with a high incidence of tuberculosis. In the present study, the assay detected mutations in 16 out of 16 rifampin-resistant isolates from north India (100%) and in 55 of 64 rifampin-resistant isolates from Mexico (86%) compared to results with standard susceptibility testing. The assay did not detect mutations (a finding predictive of rifampin susceptibility) in 37 out of 37 rifampin-susceptible isolates from India (100%) and 125 out of 126 rifampin-susceptible isolates from Mexico (99%). DNA sequencing revealed that none of the nine rifampin-resistant isolates from Mexico, which were misidentified as rifampin susceptible by the molecular beacon assay, contained a mutation in the region targeted by the molecular beacons. The one rifampin-susceptible isolate from Mexico that appeared to be rifampin resistant by the molecular beacon assay contained an S531W mutation, which is usually associated with rifampin resistance. Of the rifampin-resistant isolates that were correctly identified in the molecular beacon assay, one contained a novel L530A mutation and another contained a novel deletion between codons 511 and 514. Overall, the molecular beacon assay appears to have sufficient sensitivity (89%) and specificity (99%) for use in countries with a high prevalence of tuberculosis.

Multidrug-resistant tuberculosis (MDR-TB) is an increasing problem worldwide (13). MDR-TB is associated with significant mortality (12, 23) and has resulted in serious institutional outbreaks (5). Rapid diagnostic assays for MDR-TB should address these problems by enabling early isolation and treatment of patients with this disease (9, 17). Rifampin resistance is an excellent marker for multidrug-resistant Mycobacterium tuberculosis, as 90% of rifampin-resistant M. tuberculosis strains are also isoniazid resistant and, hence, are classified as multidrug resistant (20). Rifampin resistance is also amenable to detection by rapid genotypic assays, because approximately 95% of all rifampin-resistant strains contain mutations localized in an 81-bp core region of the bacterial RNA polymerase gene, rpoB (11, 17). Moreover, virtually all mutations that occur in this region result in rifampin resistance. By contrast, nearly all rifampin-susceptible M. tuberculosis isolates have the same wild-type nucleotide sequence in this region (11, 17, 19).

Various molecular methods have been developed to rapidly detect mutations in the M. tuberculosis rpoB core region, including the line probe assay (3), single-strand conformational polymorphism (SSCP) PCR (2, 20), and real-time PCR (6, 21, 22). Researchers developed a molecular beacon-based real-time PCR assay for this purpose (14, 15) and later converted this method into a multicolor, single-tube assay format (4). The single-well molecular beacon assay used five molecular beacons, each hybridizing to a different target segment within the rpoB core region and each labeled with a differently colored fluorophore. Each molecular beacon was designed to be so specific that it could not bind to its target if the target sequence differed from the wild-type M. tuberculosis rpoB sequence by even a single nucleotide substitution (10). Because molecular beacons fluoresce only when they are bound to their targets (24), the absence of fluorescence from any fluorophore in the assay indicates the presence of a mutation and thus predicts rifampin resistance (4). The assay thus has the advantage that it can detect unknown mutations in the rpoB region. The assay was simple, rapid, specific, and highly sensitive in tests on isolates of M. tuberculosis from New York City and Madrid (15). It also correctly predicted that 11 clinical sputum samples collected in Rio de Janeiro (Brazil) were rifampin susceptible (4). However, the ability of the assay to detect rpoB mutations in countries with a high incidence of tuberculosis, where different mutations could cause rifampin resistance, had not been tested. Here we assess the suitability of the single-tube molecular beacon assay to detect mutations in the rpoB gene of...
clinical *M. tuberculosis* isolates from the high-incidence countries India and Mexico.

**MATERIALS AND METHODS**

*M. tuberculosis* isolates. A total of 243 isolates of *M. tuberculosis* from patients from north India and Mexico were tested for mutations associated with resistance to rifampin by the molecular beacon assay. Thirty-seven rifampin-susceptible and 16 rifampin-resistant isolates were obtained from 53 patients with tuberculosis in the outpatient department of Respiratory Medicine at the Vallabhbaib Patil Chest Institute in Delhi, India, between January 2001 and January 2002. The Vallabhbaib Patil Chest Institute serves as a referral center for patients with respiratory diseases in north India. A large number (35%) of these patients had histories of previous treatment at the time of collection of their sputum. The isolates were biochemically characterized with nitrate reduction, niacin production, catalase (7), and BACTEC NAP tests (Becton Dickinson Microbiology Systems, Sparks, Md.). All 53 isolates were also characterized by IS6110 fingerprinting (1). Another 190 isolates of *M. tuberculosis* were obtained from the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, in Mexico City, Mexico, which serves as a reference center for patients with tuberculosis. The Mexican isolates were obtained from three different geographical regions (Mexico City, Huauchinango, and Orizaba) between 1995 and 2002 and were identified by BACTEC (Becton Dickinson) and the NAP test (AccuProbe). Fifty-nine of the isolates had been characterized by IS6110 typing (25). Of the 190 Mexican isolates, 64 were rifampin resistant. No isolate from either country was rifampin monoresistant.

**Susceptibility testing.** The susceptibility of the Delhi *M. tuberculosis* isolates to isoniazid, rifampin, ethambutol, and streptomycin was tested by the standard proportion method (7). Resistance was defined as greater than 1% growth in the presence of 0.2 μg of isoniazid/ml, 1 μg of rifampin/ml, 5 μg of ethambutol/ml, and 2 μg of streptomycin/ml (7). The susceptibility of the Mexican isolates to the primary antituberculosis drugs was determined by the 460 TB BACTEC system (Becton Dickinson) at the Instituto Nacional Ciencias Médicas y Nutrición, as described previously (2).

**Sample preparation for PCR.** The *M. tuberculosis* reference strain H37Rv was used as a positive control in the molecular beacon assays. Genomic DNA was extracted from H37Rv and clinical *M. tuberculosis* isolates by treatment with cetyltrimethylammonium bromide (CTAB) (Sigma, St. Louis, Mo.) in the presence of 0.7 M sodium chloride, as described previously (25).

**Oligonucleotide sequences.** The nucleotide sequences of the molecular beacon probes and primers used in this study have been described previously (4). Five molecular beacon probes were designed so that they hybridized to different segments of the wild-type sequence of the *M. tuberculosis rpoB* core region. Each 15- to 20-bp-long probe sequence was selected so that the probe-target hybrid was at least 100 bases long, and the spacing between each of the two complementary oligonucleotides was 40 nucleotides. The target sequence was nucleotide 1321 to 1341, which contains the core region of the *rpoB* gene. The 15 probe sequences were designed to be unique with respect to one another, while maintaining a high degree of sequence conservation.

**PCR conditions.** DNA was amplified in a 20-μl reaction mixture containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.2 mM dNTPs, 0.2 μM of each primer, and 0.25 μl of Taq polymerase (Promega, Madison, Wis.). The reactions were performed in a 56% Mycoplasma mycoides strain (ATCC 16987) in a programmable thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The amplification protocol consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

**RESULTS**

**Molecular beacon assay results.** The results of the molecular beacon assays are summarized in Table 1. Typical assay results are shown in Fig. 1 and 2. Overall, the sensitivity of the assay in both populations was 89%, specificity was 99%, positive predictive value was 99%, and negative predictive value was 95%. Fluorescence signals in all five molecular beacons developed in all 37 of the rifampin-susceptible isolates from Delhi, India (specificity, 100%), including 5 rifampin-susceptible isolates that had previously been misidentified as being rifampin resistant by the standard proportion method. The rifampin susceptibility of these five isolates was confirmed by repeat susceptibility testing after the results of the molecular beacon assays were known. All 16 of the rifampin-resistant isolates from Delhi produced a flat signal for at least one of the molecular beacon probes (sensitivity, 100%) (Fig. 1). Probe E most commonly detected a mutation, followed by probes B, D, and A (Table 2). None of the isolates from Delhi appeared to contain a mutation in the region of probe C. Two molecular beacon probes failed to fluoresce in each of three isolates from Delhi (Fig. 2). In one isolate, both probe A and probe D failed to fluoresce; in a second isolate, probe B and probe E failed to fluoresce; and in a third isolate, probe A and probe B failed to fluoresce. These results suggested that the three isolates contained more than one mutation in the *rpoB* core region.

Fluorescence signals in all five molecular beacon assays developed in 124 of the 125 rifampin-susceptible Mexican isolates (specificity, 99%). A fluorescence signal failed to develop with probe E in one of the Mexican isolates identified as susceptible to rifampin by BACTEC analysis. Fifty-five of the 64 rifampin-resistant isolates from Mexico presented a flat signal for at least one of the molecular beacon probes, while fluorescence developed in all five molecular beacons in nine of the rifampin-resistant isolates (specificity, 86%). Probe E was again the most common molecular beacon with a flat signal, followed by probe D (Table 2). None of the isolates from Mexico gave a negative signal with probes A and C or a negative signal with more than one molecular beacon simultaneously.

**DNA sequencing.** The *rpoB* core region was sequenced in the three Indian isolates that gave a single negative signal with probe A. All three isolates were found to have the L511P (CTG→CCG) mutation (Table 3). The Delhi isolates with two
negative signals each were also sequenced. One contained a novel deletion between codons 511 and 514 (wild-type sequence TGAGCCAAT was deleted). The second isolate contained both the L511P (CTG→CCG) and the H526Q mutations. These mutations were detected using real-time PCR with molecular beacons, as shown in Fig. 1.

**FIG. 1.** Typical real-time PCR results for selected rifampin-susceptible and rifampin-resistant *M. tuberculosis* isolates. (A) A rifampin-susceptible isolate in which all five differently colored molecular beacons hybridized to the *rpoB* core region. (B to E) Rifampin-resistant isolates in which either (B) probe A, (C) probe B, (D) probe D, or (E) probe E failed to fluoresce. None of the isolates had a flat signal for probe C. The fluorescence of each molecular beacon is indicated as follows: ○, probe A; ▲, probe B; ■, probe C; ▲, probe D; and Δ, probe E.

**FIG. 2.** Detection of double mutations or deletions. No fluorescence increase was observed for two differently colored probes in three of the isolates, suggesting the presence of multiple mutations. (A) Probes A and D failed to fluoresce; (B) probes B and E failed to fluoresce; and (C) probes A and B failed to fluoresce. The fluorescence of each molecular beacon is indicated as follows: ○, probe A; ▲, probe B; ■, probe C; ▲, probe D; and Δ, probe E.

**TABLE 2.** Distribution of assay results obtained with *M. tuberculosis* clinical isolates in which at least one flat fluorescence signal was observed.

<table>
<thead>
<tr>
<th>Molecular beacon</th>
<th>No. of positive detections of fluorescence signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>India</td>
</tr>
<tr>
<td>A</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>9</td>
</tr>
</tbody>
</table>

*a* Double mutations were observed in two isolates (flat signals for probes A-D and A-B).

*b* Double mutations were observed in two isolates (flat signals for probes A-B and B-E).
lates as rifampin-resistant and two rifampin-susceptible Mexican isolates that were investigated in this study had previously been characterized by single-strand conformational polymorphism analysis. Twelve rifampin-resistant isolates from countries with a high incidence of tuberculosis (13) were identified by the molecular beacon assay. In contrast, one of the susceptible isolates as being rifampin susceptible could not be retested.

Comparison of the molecular beacon assay to single-strand conformational polymorphism analysis. Twelve rifampin-resistant and two rifampin-susceptible Mexican isolates that were investigated in this study had previously been characterized by single-strand conformational polymorphism PCR (2). The SSCP PCR assay identified 4 of the 12 rifampin-resistant isolates as rifampin-resistant rpoB mutants, while the molecular beacon assay correctly identified 11 of these isolates as rifampin resistant. One rifampin-resistant isolate was identified as susceptible by both SSCP PCR and by the molecular beacon assay. DNA sequencing of this isolate showed that an S531W mutation (normally strongly associated with rifampin resistance) was present. Unfortunately, this isolate had lost viability during freezing storage and rifampin susceptibility could not be retested.

Table 3. Mutations detected in the rpoB core region from isolates with flat fluorescence signals for probe A or E

<table>
<thead>
<tr>
<th>Probe</th>
<th>Mutant codon</th>
<th>Nucleic acid substitution</th>
<th>Amino acid substitution</th>
<th>No. of mutant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>India</td>
</tr>
<tr>
<td>A</td>
<td>511</td>
<td>CTG→CCG</td>
<td>L→P</td>
<td>3*</td>
</tr>
<tr>
<td>E</td>
<td>530</td>
<td>CTG→GCT</td>
<td>L→A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>531</td>
<td>TCG→TTG</td>
<td>S→L</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>532</td>
<td>TCG→TGG</td>
<td>S→W</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>533</td>
<td>CTG→TTC</td>
<td>S→F</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTG→CCG</td>
<td>L→P</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTG→GCC</td>
<td>L→A</td>
<td>0</td>
</tr>
</tbody>
</table>

* A fourth isolate with a negative signal for probe A had a deletion between codons 511 and 514.
to detect silent rpoB mutations when they were present and would falsely identify such mutants as rifampin resistant. However, silent mutations are exceedingly rare in M. tuberculosis (19), thus, this problem does not have an important effect on specificity. In fact, it is worth noting that in the present study, the assay correctly identified five rifampin-susceptible isolates from India that had been initially classified as rifampin resistant by conventional susceptibility testing (but later confirmed to be susceptible by repeat testing). This observation suggests that the specificity of the molecular beacon assay may sometimes be higher than that of conventional susceptibility testing.

We found that four isolates from Delhi gave negative signals with probe A. Three of these isolates had mutations in codon 511, and the fourth had a novel deletion spanning codons 511–514. Mutations at codon 511 have been found by other workers in India (8). No rifampin-resistant isolate from Mexico contained a mutation in this region. This difference could reflect regional strain variations or differences in host factors. None of the 243 isolates showed a negative fluorescence signal with probe C, which targets rpoB codons 518 to 522. Earlier studies from India have reported mutations in codon 518, but only when they were accompanied by mutations in codon 531 (8). Other studies have reported mutations at codons 518, 521, and 522 at frequencies of only 0.8, 1.5, and 3%, respectively (18). Thus, it is possible that probe C could be omitted from future assays without a major effect on assay sensitivity. We also compared the results from the molecular beacon assays to results obtained with SSCP PCR in 14 isolates from Mexico City. Our results show that the molecular beacon assays were much more sensitive in detecting rifampin resistance in this group of isolates.

In summary, the molecular beacon assay was as effective at detecting mutations associated with rifampin resistance in M. tuberculosis isolates from northern India and Mexico as has previously been reported for isolates from the United States and Spain (15). The assay also identified rifampin-susceptible isolates that had previously been misidentified as resistant, further supporting the utility of a genetic approach to susceptibility testing. The assay was also more effective than SSCP PCR at detecting rifampin-resistant isolates. The assay is not dependent on probes hybridizing to specific mutant codons; hence, new and unknown mutations arising in a population, such as those identified in this study, can be easily detected with the same set of probes. With real-time instruments becoming more affordable, we anticipate that the assay can become economically feasible for developing countries in the near future. Ultimately, this assay will enable more rapid diagnosis, earlier treatment, and prompt implementation of infection control procedures to reduce the morbidity, mortality, and the spread of drug-resistant tuberculosis.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants AI-46669 and EB-00277. M.V.-B. received an Overseas Associateship from the Department of Biotechnology of the Indian government. D.A. and F.K. are among a group of coinvestigators who hold patents in molecular beacon technology, and they receive income from licenses.

REFERENCES


