Direct Detection of Rifampin-Resistant *Mycobacterium tuberculosis* in Respiratory Specimens by PCR-DNA Sequencing


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This study evaluated the feasibility of a molecular strategy based on identification of *Mycobacterium tuberculosis* by IS6110 PCR or Cobas Amplicor PCR, and rpoB PCR-DNA sequencing of the 81-bp rifampin resistance determining region (RRDR) for direct detection of rifampin resistance in respiratory specimens. A collection of 2,138 respiratory specimens and 352 nonduplicate *M. tuberculosis* isolates (including 233 isolates from the evaluated respiratory specimens and an additional collection of 119 stored isolates) from Southern China was investigated. Using culture as the reference test, the overall diagnostic sensitivities of an acid-fast bacillus (AFB) smear, Cobas Amplicor PCR, IS6110 PCR were 54.5% (156 of 286), 86.7% (248 of 286), and 89.2% (255 of 286), respectively. The sensitivities of the rpoB PCR for the specimens with positive AFB smears and with positive PCR results in the IS6110 PCR and/or Cobas Amplicor PCR were 100% (156 of 156) and 92.3% (239 of 259), respectively. Of the 352 nonduplicate *M. tuberculosis* isolates, the agar proportion method for rifampin reported 39 resistant strains. Full agreement (352 of 352) was found with the agar proportion method and the genotype inferred from the rpoB DNA sequencing data for rifampin. Thirty-nine mutations of nine distinct kinds, eight point mutations, and one deletion within the RRDR were found in the 39 resistant strains. For the direct DNA sequencing performed on rpoB PCR-positive respiratory specimens, the concordance with the agar proportion method and the subsequent PCR-sequencing for the culture isolate was 100%. This strategy has potential application for direct and rapid diagnosis of rifampin-resistant *M. tuberculosis* in IS6110 PCR or Cobas Amplicor PCR-positive respiratory specimens.

Tuberculosis remains an important disease worldwide. The emergence of multidrug resistance in strains of *Mycobacterium tuberculosis* (*M. tuberculosis*) is one of the most important threats to tuberculous control. Despite continued efforts directed to improve tuberculosis control programs at national and international levels, recent surveys reveal that drug-resistant tuberculosis is still ubiquitous and rates of infection are alarmingly high in several countries. According to Espinal et al. (9), multidrug-resistant tuberculosis (MDR-TB) defining as resistance to at least isoniazid (INH) and rifampin (RIF) was present among new cases in 54 of 58 surveyed geographic sites in 1996 to 1999. Among new cases, alarming rates were reported in Estonia (14.1%), Latvia (9.0%), Tomsk Oblast of Russian Federation (6.5%), Ivanovo Oblast of the Russian Federation (9.0%), and Henan of China (10.8%). Overall, in all 58 surveyed sites and among the previously treated cases (9), the median MDR-TB rate was 9.3%, and for 35 geographic sites it was present at rates of 5% or higher.

In *M. tuberculosis*, mono-resistance to RIF is rare, and at least 90% of all RIF-resistant clinical isolates are also resistant to isoniazid (7). Hence, a positive result for RIF resistance would be useful as a strong surrogate of MDR-TB. In resistant isolates, it has been shown that up to 95 to 98% RIF resistance is caused by mutations in the rpoB gene encoding the RNA polymerase β-subunit. In the great majority of the RIF-resistant isolates, mutations occurred within a 81-bp hotspot region (the rifampin resistance determining region [RRDR], encoding 27 amino acids and corresponding to codons 507 to 533 or cluster I according to *Escherichia coli* numbering) in the center of the 3,516-bp *rpoB* gene (24). Within the RRDR, more than 80 distinct variants of single-base mutations or in-frame deletions or insertions have been reported among RIF-resistant isolates. Although several different molecular techniques such as heteroduplex analysis, line probe assay, and PCR single-strand conformational length polymorphism have been used for analysis of *rpoB* gene mutations, these techniques were limited in that only the most frequent types of mutations were included.

In the present study we report a rapid assay based on PCR and sequencing of the *rpoB* gene for direct detection of RRDR-associated RIF-resistant *M. tuberculosis* in clinical isolates and respiratory specimens. A total of 2,138 respiratory specimens and 142 *M. tuberculosis* isolates were used to evaluate the specificity and sensitivity of this assay. The results were compared to commercial and in-house diagnostic assays for *M. tuberculosis* and phenotypic drug susceptibility testing.

MATERIALS AND METHODS

**Specimens and isolates.** Between March 1999 and October 2002, 2,138 respiratory specimens (1,807 expectorated sputum and 331 bronchoalveolar lavage samples) were collected from 1,571 patients suffering from chest symptoms and/or chest radiographic infiltrates of undetermined origin, including 712 pa-
patients in Queen Mary Hospital, 642 patients in Grantham Hospital, and 217 outpatients in Polyclinics of the Department of Health in Hong Kong. An additional 119 nonduplicate clinical isolates of M. tuberculosis were collected from three major cities in Pearl River Delta of the South China region of the Peoples Republic of China: Hong Kong (84 isolates), Macau (25 isolates), and Guangzhou (12 isolates) (16). After a direct smear for acid-fast bacilli (AFB), the digested sediments were divided equally for AFB culture and subsequent PCR assays. Cultures positive for AFB were identified by using the AccuProbe hybridization assay (Gen-Probe, Inc., San Diego, Calif.) and conventional biochemical tests. Antimicrobial susceptibility testing for INH, RIF, streptomycin (STR), pyrazinamide (PZA), and ethambutol (EMB) was performed using the agar proportion method (20). In brief, growth from the primary isolation medium was subcultured onto Lowenstein-Jensen (LJ) medium. The bacterial inoculum was prepared by picking fresh colonies from the LJ medium and adjusted to a turbidity matching that for a McFarland 1 standard. A 0.1-ml aliquot of 100- and 10,000-fold dilution samples were placed onto drug-containing and -no drug sections of Middlebrook 7H10 quadrants. The following drug concentrations were used: INH (0.2 μg/ml), RIF (1 μg/ml), PZA (25 μg/ml), EMB (5 μg/ml), and STR (2 μg/ml). Inoculated plates were examined weekly for up to 3 weeks. Once the no-drug control quadrant of either dilution shows at least 50 to 150 colonies from 233 patients were culture positive for M. tuberculosis. Culture negative for NTM, nontuberculous mycobacteria, including M. avium-intracellular complex (n = 25), M. chelonii (n = 16), M. gordonae (n = 8), M. scrofulaceum (n = 8), and M. fortuitum (n = 9).

The six rpoB PCR products were sequenced and found to align with M. avium-intracellular complex (n = 5) and M. gordonae (n = 1) sequences (accession AY554889 and AY554919).

The four culture-negative samples were obtained from four different patients with tuberculosis as confirmed by positive culture results from previous respiratory or concurrent extrapulmonary specimens.

### TABLE 1. Comparative sensitivities and specificities of three PCR assays for detection of M. tuberculosis

<table>
<thead>
<tr>
<th>Sample (n)</th>
<th>IS6110 PCR (UE)</th>
<th>Cobas Amplicor PCR (UE)</th>
<th>rpoB PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear positive (156)</td>
<td>156/156 (100)</td>
<td>156/156 (100)</td>
<td>128/156 (82)</td>
</tr>
<tr>
<td>Smear negative (130)</td>
<td>99/130 (76)</td>
<td>92/130 (71)</td>
<td>54/130 (42)</td>
</tr>
<tr>
<td>NTM culture positive (66)</td>
<td>0/66 (0)</td>
<td>0/66 (0)</td>
<td>6/66 (9%)</td>
</tr>
<tr>
<td>Culture negative for Mycobacterium spp. (1,786)</td>
<td>4/1,786 (0.2)</td>
<td>4/1,786 (0.2)</td>
<td>4/1,786 (0.2)</td>
</tr>
</tbody>
</table>

**ND** = not done.

- **UE**, unextracted concentrate; **3×E**, threefold-concentrated extract.
- **MTB**, Mycobacterium tuberculosis; **NTM**, nontuberculous mycobacteria.
- **a**. The four culture-negative samples were obtained from four different patients with tuberculosis as confirmed by positive culture results from previous respiratory or concurrent extrapulmonary specimens.
- **b**. The 2,138 respiratory specimens were obtained from 1,571 patients. Overall, 286 specimens from 23 patients were culture positive for M. tuberculosis.
- **c**. NTM, nontuberculous mycobacteria, including M. avium-intracellular complex (n = 25), M. chelonii (n = 16), M. gordonae (n = 8), M. scrofulaceum (n = 8), and M. fortuitum (n = 9).

***RESULTS***

**Sensitivity and specificity of the PCR assays.** Of the 2,138 respiratory specimens shown in Table 1, 286 (13.4%) specimens from 233 patients were culture positive for *M. tuberculosis* standard strain H37Rv, serial dilutions of heated extract were used for determination of analytical sensitivity of IS6110, 16S rRNA, and rpoB PCR assays.

**PCR for M. tuberculosis 16S rRNA and IS6110.** The Cobas Amplicor M. tuberculosis test (Roche) uses Mycobacterium genus-specific biotinylated primers to amplify a sequence of 584-bp within a 1,500-bp region encoding the 16S rRNA of *M. tuberculosis*. An internal control was incorporated in each reaction for monitoring of PCR inhibitors. A manual one-tube nested PCR for IS6110 was performed as described previously (3, 33, 36). A 50-μl and a 10-μl aliquot of the undiluted DNA extract were used as the PCR templates in the Cobas Amplicor test and IS610 assay, respectively.

**PCR and DNA sequencing for rpoB.** Each PCR contained 10 μl of DNA template (undiluted or threefold-concentrated extract). For amplification of rpoB gene, PCR was performed in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl (Applied Biosystems); 1.25 mM MgCl2 (Applied Biosystems); 200 μM (each) deoxynucleoside triphosphate (Cobas Amplicor Diagnostics); 0.4 μM (each) primers (Life Technologies), and 2 μl of AmpliTaq Gold Polymerase (Applied Biosystems). The primers used to amplify the rpoB gene fragment were modified from the TR8 and TR9 primer pair reported by Tenti et al. (28): forward primer TR8s (5′-TGCGCGGATCAAGGAGTTCCGCCGC-3′; positions 76876 to 76811 under accession no. BX424574 [underlined bases represent the original primer sequence]) and reverse primer TR9s (5′-TGCGCCTGCGGAGCTCCGACGCACCGGCAC-3′; positions 76942 to 76915). AmpliTaq Gold polymerase in the master mix was first activated by incubation at 94°C for 12 min. The reaction mixture was then subjected to 40 cycles of amplification (denaturation at 94°C for 1 min and annealing and extension at 72°C for 2 min), followed by a final 7-min extension at 72°C. After PCR amplification, a 10-μl aliquot of the PCR product was electrophoresed for 1 h through 2% agarose gel, and the target band of 157-bp was visualized under UV illumination. This 157-bp DNA fragment included the complete cluster 1 region (positions 507 to 533) of rpoB gene. For positive samples, the 157-bp fragment was subjected to cycle sequencing by the ABI Prism dye terminator cycle sequencing ready reaction kit (version 2.0) at a quarter of the recommended reaction volume and an ABI 377 genetic analyzer (Applied Biosystems). For all samples, the sequences of both strands of the amplicons were determined. The generated sequences were assembled and edited by using EDITSEQ v.4.0 program in DNASTAR software (Lasergene). The edited sequence was further compared to published sequence of rpoB gene for H37Rv (GenBank accession BX424574) to identify RIF resistance in terms of any mutations, insertions, and deletions in RRDR (codons 507 to 533). The nucleotide and amino acid numbering was based on homologous mutations in *E. coli* (29).
**First-line drug resistance patterns.** The susceptibilities of the 352 *M. tuberculosis* isolates (233 isolates from respiratory specimens and 119 isolates from purified culture) to INH, RIF, STR, PZA, and EMB were determined by the agar proportion method. Overall, 88.9% (313 of 352) of the isolates were susceptible to rifampin and 39 were resistant. Only five showed resistance to only RIF, whereas 34 isolates were at least resistant to INH and RIF. Of these MDR-TB isolates, 29 strains showed further resistance to at least one other first-line drug and two were resistant to RIF, INH, STR, PZA, and EMB. The number of RIF-resistant *M. tuberculosis* strains from Hong Kong, Macau, and Guangzhou were 21, 12, and 6, respectively.

**Correlation between susceptibility testing and rpoB mutations.** The 157-bp amplicons from the 239 rpoB PCR-positive respiratory samples and the 428 purified *M. tuberculosis* isolates were analyzed by direct sequencing. A comparison of the genotypic prediction with susceptibility results obtained by the agar proportion method is shown in Table 2. For the 235 rpoB PCR-positive and *M. tuberculosis* culture-positive respiratory samples, the rpoB DNA sequences were identical to those for the corresponding *M. tuberculosis* isolates. For the four rpoB PCR-positive and *M. tuberculosis* culture-negative respiratory samples, no rpoB mutations were found. For all 313 RIF-

**TABLE 2. Comparison of genotypic testing by rpoB PCR-sequencing to the agar proportion method for 286 M. tuberculosis culture-positive respiratory specimens and 352 purified isolates**

<table>
<thead>
<tr>
<th>Sample for genotypic testing and result (n)</th>
<th>No. of isolates (no. of individual patient strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>Resistant</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> culture-positive respiratory specimens (286a)</td>
<td></td>
</tr>
<tr>
<td>RRDR mutation absent (230)</td>
<td>230 (184)</td>
</tr>
<tr>
<td>RRDR mutation present (5)</td>
<td>0</td>
</tr>
<tr>
<td>PCR negative (51)</td>
<td>51 (45)</td>
</tr>
<tr>
<td>Purified nonduplicate isolates (352)</td>
<td></td>
</tr>
<tr>
<td>RRDR mutation absent (313)</td>
<td>313</td>
</tr>
<tr>
<td>RRDR mutation present (39)</td>
<td>0</td>
</tr>
</tbody>
</table>

a The 286 culture-positive specimens were obtained from 233 patients. The numbers of individual patient strains, as defined by a unique genotype, are shown in parentheses.

b The 39 RIF-resistant strains included four from the tested respiratory specimens and 35 from the 119 purified isolates.
susceptible isolates, there was no mutation in the RRDR. In contrast, RRDR mutations were found for all 39 RIF-resistant isolates. One had a double mutation (Gln513Arg and His526Asn). Of the remaining 38 mutations, one was a deletion (518Asn) and the remaining 37 had missense mutations which led to the following amino acid substitutions: Ser531Leu (n = 15), Ser531Tyr (n = 7), His526Arg (n = 5), His526Asp (n = 3), Asp516Val (n = 6), and Ser522Leu (n = 1).

**DISCUSSION**

In clinical laboratories, direct detection of *M. tuberculosis* in clinical specimens by PCR has become increasingly popular. The IS6110 PCR and Cobas Amplitarg PCR represent examples that have been extensively investigated in terms of their sensitivities and specificities. In accordance with previous studies (32, 36), we found here that both assays were 100% specific and had overall diagnostic sensitivities of ca. 90% for culture-positive respiratory specimens. Since 1999, one of the PCR assays has become routinely available for all of the regional and chest hospitals in Hong Kong. In our hands, the diagnostic sensitivities of these PCR assays were consistently higher than the 40 to 60% for AFB smear, although their sensitivities for smear-negative specimens are still limited at 60 to 70% (3, 33, 36), which is similar to those reported by other investigators (2, 17). In a cohort of 155 patients being investigated for tuberculosis, our team recently showed that the molecular diagnostic service is useful for making clinical decisions on the initiation of antituberculosis treatment, particularly among patients with equivocal presentations (5).

The present study extends these findings by evaluating the direct application of a PCR-sequencing assay in clinical specimens for genotypic prediction of rifampicin resistance. In combination with PCR assays for *M. tuberculosis* identification, our rpoB PCR was 100% specific. There was no false amplification of the rpoB for a variety of nonmycobacterial pathogens. Among the high level of rpoB sequence homology between *M. tuberculosis* and some NTM, the rpoB PCR was positive for six samples containing NTM but not *M. tuberculosis*. In a heminested rpoB PCR with sputum samples and heteroduplex analysis, Williams et al. have also reported false-positive result due to samples containing *M. avium* (31). The present investigation demonstrates that this potential confusion can be readily resolved by an analysis of the rpoB sequences and a negative result in the IS6110 or Cobas Amplitarg PCR. However, it should be noted that some populations have a significant number of IS6110-negative strains. In these populations, IS6110 amplification should not be relied on too much. Our 100% sensitivity for smear-positive respiratory specimens was better than the 82.8% (19) and 97.5% (25) sensitivities recently reported by others. In addition, our findings further extend this application to AFB-smear-negative respiratory specimens for both identification and rifampicin resistance determination with a short turnaround time, although the sensitivity is limited at ca. 60%.

As with other genotypic testing, a positive result is more useful than a negative result. In *M. tuberculosis*, mutations in the RRDR appear to be strongly if not always predictive of RIF resistance in susceptibility testing. DNA sequencing is most likely to yield the maximal benefit obtainable for a positive genotypic test result because the full spectrum of mutations can be accurately identified. This is particularly relevant in the prediction of RIF resistance since more than 80 distinct allelic variants, including missense mutations, either deletions or insertions alone or in combination, have been reported in the 81-bp core RRDR (19, 24, 26, 27, 30). All positive results in our genotypic assay were “true resistance” compared to concordances of 90% for the line probe assay (6), 87.2% for amplification refractory mutation system (10), and 82.8% for allele-specific PCR assay (19). If our specimens and strains were subjected to analysis by the line probe assay (INNO-LIPA Rif.TB; Immugenetics, Ghent, Belgium), it is likely that all strains with RRDR mutations can be detected by negative signals for one or more of the wild-type probes S1 to S5. However, the line probe assay is inferior to sequencing in that the ability to assign the specific resistance mutation is limited. Thus, of the nine unique resistance mutations identified, the line probe assay would only be able to assign Ser531Leu, His526Asp, and Asp516Val. The confidence to predict RIF susceptibility from a no-mutation result, however, is likely to differ depending on the populations and might vary as new resistance mechanism emerges. In the present sample drawn from South China, all of the negative (i.e., no mutation) results represented “true susceptibility” since all of the RIF-resistant isolates had at least one mutation in the RRDR, as previously described for isolates from Italy (22). In contrast, others have reported that some RIF-resistant strains had no mutations in the RRDR core: 2.6 to 10% in China (10, 35), 3.3 to 11% in other Asian countries (12, 23, 34), 3% in Australia (37), and 5% in Germany (11). In the present investigation, the most common substitutions among the RIF-resistant strains occurred at codons Ser-531 (59%) and His-526 (23.1%). This finding is similar to results compiled for 478 RIF-resistant *M. tuberculosis* isolates from various parts of the world, in which the frequencies of substitution at codons 531 and 526 were 41 and 36%, respectively (24).

A model for implementation of a suggested strategy of directly applying the rpoB PCR-sequencing on respiratory specimens or on culture isolates is depicted in Fig. 2. Due to the significant costs of PCR and sequencing, respiratory specimens should be prioritized rather than tested universally. High priority should be given to circumstances in which the risk of MDR-TB is high, such as recurrent tuberculosis (18), previously treated cases (8), treatment default (14), irregular prior treatment (15), and smear positivity after 2 months of therapy (4). At this time, the service should only be offered selectively at the reference laboratory level. As a cost reduction measure, our experience demonstrates that an adequate result could be obtained by using one-quarter reaction volumes for DNA sequencing. However, a potential caveat is that in *M. tuberculosis*, resistance to RIF can occur at low frequency with only a few percent of the population being resistant. In the agar proportion method, a strain is defined as resistant if there is any growth of >1% compared to no drug treatment. Thus, the cell population in a resistant strain is mixed with a small resistant subpopulation and a larger sensitive subpopulation. Depending on the population fractions sampled, sequencing-based or other molecule-based testing might yield a false-negative result if the resistant subpopulations are not included. Another limitation of the present method is that direct determination of RIF resistance was not possible for 40% of the 130 smear-
negative–culture-positive specimens. In a retrospective review of 29,057 tuberculosis cases, including 603 MDR-TB cases, it was found that 40% of patients with MDR-TB had negative smears in respiratory specimens at presentation (13). Hence, further studies to enhance the diagnostic performance of rpoB PCR-sequencing for this group of specimens are clearly warranted.

FIG. 2. Model for the implementation of genotypic detection of RIF resistance by rpoB PCR-sequencing. MTB, M. tuberculosis.

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